IN VITRO PROBIOTIC PROPERTIES OF A LACTIC ACID BACTERIA ISOLATED FROM A BROILER CHICKEN

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Abstract

This study was conducted in order to evaluate in vitro the probiotic properties of lactic acid bacteria (LAB) isolated from ileum intestinal content tract of a healthy broiler (45-d-old Cobb 500). The isolate was assayed morphologically, culturally, biochemically. The tolerance to low pH an11d bile salt were tested as well. Phenotypically, the isolate strain was identified to be Lactobacillus acidophilus. The new strain was conserved as Lactobacillus acidophilus IBNA 64 in the Collection of INCDBNA. It is Gram-positive Bacillus, thin, non-spore forming, appears isolated, rarely in the diploid form, in short chains or in small irregular piles in culture of 24h in Oxoid MRS broth and MRS agar medium. The strain is an aerotolerant bacteria. The identification and analysis of the biochemical characteristics was performed by catalase assay, API 50 CHL Biomerieux strips, apiweb API 50 CHL V 5.1 soft (Lactobacillus acidophilus 1, 74.4% ID and L. crispatus 19.0% ID) and ABIS online (Lactobacillus acidophilus ~ 88.3%). The strain showed good viability at pH 7±2 (8.32±0.330 log CFU/mL), respectively was able to resist at pH 3 (7.14±0.133 log CFU/mL) and 0.3% bile salts (7.56±0.19 log CFU/mL) during 3 h exposure. Results obtained provide some probiotic properties for L. acidophilus, but further studied will be done until to use for in vivo test in poultry feed.

Key words: lactic acid bacteria, probiotic, chicken

INTRODUCTION

Lactobacillus spp. are part of normal poultry intestinal microbiota [1], being the largest reservoir of bacteria from animals [2]. The strains from this genus are characterized as Gram positive, catalase-negative able to produce lactic acid [3] as the main end product of carbohydrate fermentation [1, 4].

There is a balance between beneficial and non-beneficial bacteria in the gastrointestinal tract (GIT) of healthy and non-stressed broilers [2]. The lactobacilli are implied in normal microflora of animal status health [5] and could be considering probiotics with high benefits by improving intestinal microbial stability [3].

In general, probiotics based on lactic acid bacteria (LAB), used in poultry diets improve feed intake and digestion process [6], inhibit gastrointestinal pathogens and in the same time, diminished susceptibility to diseases [7] by maintaining a healthy gut, maximize growth efficiency with beneficial effects on broiler performance [8]. Also, to obtain beneficial results, it is necessary that the probiotic candidate used as feed additive, to be removed some *in vitro* tests: resistance to low pH value from stomach, bile salts from intestine and their percentage of survival at these *in vitro* gastrointestinal conditions [9]; capacity to adhere to the host intestinal epithelium, to present antagonistic activity against pathogenic bacteria, to keep its viability during processing and storage of feed [9] etc.

According to the definition [10] "probiotics are live microorganisms when are

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administered in sufficient amounts, confer a health benefit on the host" [11].

Criteria for probiotic selection was to isolate, identify and phenotypically characterize a *Lactobacillus* spp. strain present, naturally, in the broiler GIT and to investigate their ability to colonize the chicken's gut (resistance to low pH, bile salts and percentage of survivability in these conditions).

MATERIAL AND METHOD

Bacterial strain media, growth conditions, isolation and identification

LABs present in GIT of a broiler chicken (45-d-old) was morphologically, culturally, and biochemically investigated. One g of ileum content from a healthy broiler was homogenized with 7 ml Oxoid BHI (Brain Heart Infusion) broth and 2 ml glycerol, and instantly frozen at – 20ºC until testing (no more than three months) [12]. After defrost, the sample was supposed to decimal dilution in Oxoid PBS (Phosphate Buffered Saline) from 10-4 to 10-8 and from every dilution tube there were inoculated three Petri dishes with Oxoid MRS (de Man, Rogosa and Sharpe) agar. The culture was incubated overnight in microaerobic conditions (Jar with Anaerogen 2.5L from Oxoid). After 24 h incubation, the colonies were randomly selected from the plates and subcultured two times on a new MRS agar Petri dishes.

The cultural examination of the isolate was performed according to Bergey's Manual of Determinative Bacteriology by using the methods and criteria of Sharpe [12].

After morphological evaluation by Gram staining, the isolate strain from MRS agar was identified by biochemical tests (catalase assay, API 50 CHL Biomerieux strips), API 50 CHL V 5.1 and ABIS online soft [3, 13], according to manufacturer's instructions.

Preservation of bacterial strains

The pure culture was stored at room temperature and 4°C in MRS broth medium, respectively at -80°C with 20% (v/v) sterile glycerol, until the moment when the preservation viability will be tested. The strain can be found in the Collection of National Research Development Institute for Biology and Animal Nutrition Balotești (INCDBNA), Romania, under the code IBNA 64.

Determination of colony forming units (CFU/g intestinal content)

To determine the growth rate, the culture was cultivated on MRS medium (broth and agar), at 37°C, for 48h, in anaerobic conditions [3].

The catalase test

The catalase test was performed according to the method described [3].

Acid tolerance

The overnight culture of *Lactobacillus* spp. [14] (7-8 log UFC/ml in PBS, pH 7.2), which was grown in anaerobiosis, was inoculated (1:10, v/v) in MRS broth adjusted to pH 3, with 1N HCl 37%. The inoculated tube at pH 3, was incubated anaerobically at 37°C for 0 h, 1h:30 min. and 3 h. After each incubation time, serial dilutions were performed (10-7) in sterile PBS. To determine the CFU/ml, 100 µl from 10-4-10-7 were dispersed on MRS agar plates (3 plates/dilution) and incubated at 37°C, 24 h, in anaerobiosis. Tolerance to low pH condition was estimated by comparing the CFU/ml after exposure to pH 3 with normal MRS broth (control, pH = 6.2±0.2), in the same growing conditions (37°C, 24 h, in anaerobiosis).

Bile salts tolerance

The overnight culture of *Lactobacillus* spp. was assayed according to the method [14], with minor modification. The isolate strain with a concentration of 7-8 log CFU/ml, was inoculated (1:10, v/v) in MRS broth with 0.3% (w/v) bile salts (oxgall, Sigma) at 37°C, for 0 h, 1:30 min. and 3 h, in anaerobiosis.

The viability of *Lactobacillus* spp. strain was determined by estimating the number of colonies, by successive dilutions in sterile PBS (10-4 to 10-7), on MRS agar plates (3 plates/dilution), incubated at 37°C, 24 h, in anaerobic conditions.

The control sample was represented by the culture developed in MRS Oxoid broth (pH=6.2 ± 0.2), without bile supplementation.

The survival percentage was calculated using the method presented [15]:

$$\text{Survival (\%)} = \frac{\text{Log}\, number\ of\ cells\ survived\ \left(\frac{CFU}{ml}\right) x\ 100}{\text{Log}\, number\ of\ initial\ cells\ inoculated\ \left(\frac{CFU}{ml}\right)}$$

Statistical Analysis

The analytical data were compared using variance analysis (ANOVA) with STATVIEW for Windows (SAS, version 6.0). The results were expressed as mean values and standard error of the mean (SEM), the differences between means considered statistically significant at $P<0.05$, using Tukey LSD test for untitled compact variable

RESULTS AND DISCUSSIONS

Bacterial strain media, growth conditions, isolation and identification

The taxonomic classification of bacterial strain in *Lactobacillus* spp. was performed by morphological (Gram positive bacilli, thin, non-spore forming rods, appears isolated, rarely in the diploid form, in short chains or in small irregular piles in culture of 24h in Oxoid MRS broth and MRS agar medium – Fig. 1), cultural (anaerobic growth) and biochemical characters (negative catalase test, [16]).

The isolate strain was identified by its ability to ferment different carbohydrates from API 50 CHL test kit (BioMerieux, S.A., France). The results are presented in Table 1. The strain was identified as *Lactobacillus acidophilus* as follow: *L. acidophilus* 1, - 74.4% ID (% percentage of identification) by API 50 CHL V5.1, respectively *L. acidophilus*, - 88.3% (% of similarity) by ABIS online. The fermentation capacity of our isolate was observed by a discoloration of the basal medium from purple to yellow, according to the manufacteur protocol.

The bacterial strain has been registered as *Lactobacillus acidophilus* IBNA 64 in IBNA Bacterial Collection.

In our study, the positive results were obtained for fermentation of amygdalin, D-melibiose, D-trehalose, starch and gentibiose, comparative with the literature [12] where these substrates were not fermented by another *L. acidophilus* strain.

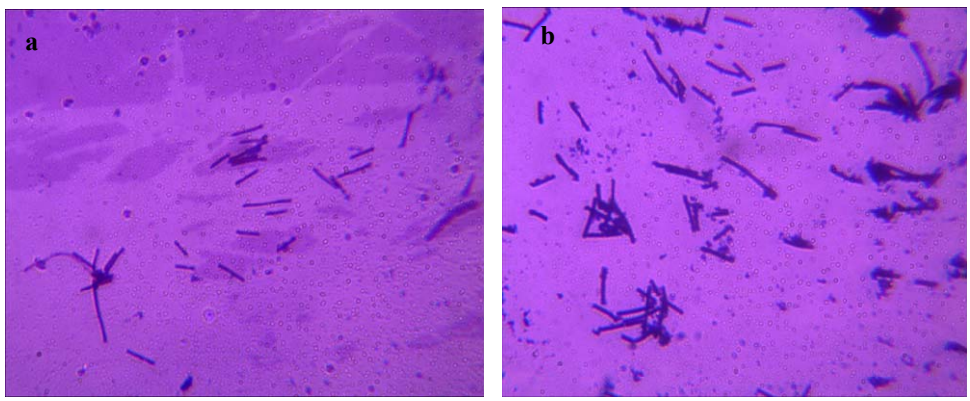

Fig. 1. *L. acidophilus* IBNA 64 anaerobe culture on MRS medium (Gram staining x 1000)
a) broth b) agar

Tab. 1 Biochemical characteristics of the *Lactobacillus* strain isolated from intestinal content of broiler chicken

Biochemical tests	Interpretation				
	24 h	48 h		24h	48h
Control	-	-	Esculin	+	+
Glycerol	-	-	Salicin	+	+
Erythritol	-	-	D-cellobiose	+	+
D-arabinose	-	-	D-maltose	+	+
L-arabinose	-	-	D-lactose	+	+
D-ribose	-	-	D-melibiose	-	+
D-xylose	-	-	D-saccharose (sucrose)	+	+
L-xylose	-	-	D-trehalose	+	+
D-adonitol	-	-	Inulin	-	-
Methyl-βD-xylopyranoside	-	-	D-melezitose	-	-
D-galactose	+	+	D-raffinose	+	+
D-glucose	+	+	Starch	?	+
D-fructose	+	+	Glycogen	-	-
D-mannose	-	+	Xylitol	-	-
L-sorbose	-	-	Gentibiose	?	+
L-rhamnose	-	-	D-turanose	-	-
Dulcitol	-	-	D-lyxose	-	-
Inositol	-	-	D-tagatose	-	-
D-mannitol	+	+	D-fucose	-	-
D-sorbitol	-	-	L-fucose	-	-
Methyl-αD-mannopyranoside	-	-	D-arabitol	-	-
Methyl-αD-glucopyranoside	-	-	L-arabitol	-	-
N-acetylglucosamine	-	-	Potassium gluconate	-	-
Amygdalin	+	+	Potassium 2-ketogluconate	-	-
Arbutin	+	+	Potassium 5-ketogluconate	-	-

,,–" Negative test; ,,+" Positive test; ,,?" Weakly positive.

Preservation of bacterial strains

The results of viability test for *Lactobacillus acidophilus* strain which are preserved at 4°C and room temperature are exposed in Table 2.

Tab. 2. The viability of *Lactobacillus acidophilus* preserved at 4°C and room temperature

4°C	Room temperature
-/66 day	-/45 days

L. acidophilus present only 66 days viability at 4°C *vs.* room temperature where the viability does not exceed 45 days. The strain isolated for intestinal content of broiler and its utilization as possible probiotic candidate must pass some in vitro tests for analyze its resistance under gastrointestinal conditions. A longer resistance of strains is an important probiotic trait for make it a good selection.

Determination of colony forming units (CFU/g intestinal content)

Lactobacillus acidophilus IBNA 64 present a good capacity for growth in MRS broth, $4.6x10^8$ CFU/g was registered by incubation at 37°C, 24 h, in anaerobic conditions.

Acid tolerance

To investigate the resistance of *Lactobacillus acidophilus* IBNA 64 in the presence of acid, the strain was exposed to low pH. The results obtained were presented in Table 3. The strain registered a good survival rate which differ significantly in comparison with the control ($P \leq 0.05$). However, with increase of incubation time at pH 3, the growth rate of *Lactobacillus acidophilus* IBNA 64 decreased.

Since, entering into the animal mouth [16], the lactobacilli must survive to difficult conditions as acidic environment from gastrointestinal tract.

The stomach has a low pH between 1.5 - 3.5, due to gastric juice secretion, and the intestine incline to alkaline pH values between 8 - 8.5 [16, 17]. Also, the pH of gastric juice depends on the animal feeding time, growing stage, between 2.0 to 3.5 [13]. In the present study, the results obtained confirm that the isolate strain presented a survival rate after 3 h (85.81%) at pH 3. One of the critical properties of a bacterial probiotic is the ability to tolerate the low pH from the stomach and in the same time, to survive to the high concentration of bile salts from GIT. These traits are in generally, evaluated as preliminary tests for selected a possible probiotic strain [19].

The lactobacilli have the properties to ferment the carbohydrates group to lactic acid [3]; by their development, lactobacilli determine acidification of raw materials from feed.

Tab. 3 The resistance of *Lactobacillus acidophilus* IBNA 64 to low pH

Strain	Initial log_{10} CFU/ml	pH 3				
		0 min.	1h:30 min.	3 h	*SEM*	*P value*
L. acidophilus IBNA 64	8.32[a]	8.41[b]	8.07[c]	7.14[d]	0.162	0.0007
	% of viability	101.08%	96.99%	85.81%	na	na

*Values are the means of three independent experiments (n=3). [abcd] Means in the same row differ significantly at P<0.05. na= not applied

Bile salts tolerance

The results from Table 4 showed that the isolate strain resist to 0.3% oxgall bile salts concentration. The value obtained after 3 h exposure to bile salts was 10.05% less than initial strain concentration.

Tab. 4 The resistance of *Lactobacillus acidophilus* IBNA 64 to bile salts

Strain	Initial log_{10} CFU/ml	0.3% bile salts				
		0 min.	1h:30 min.	3 h	*SEM*	*P value*
L. acidophilus IBNA 64	8.32[d]	7.43[a]	7.59[b]	7.56[c]	0.117	0.0029
	% of viability	89.30%	91.22%	90.86%	na	na

*Values are the means of three independent experiments (n=3). [abcd]Means in the same row differ significantly at P<0.05. na= not applied

The maximum survival rate was showed after 1h:30 min a good viability percentage 91.22%.

The ability to survive under high bile salts concentration and low pH, are the important characteristics for the successful passage through the gastrointestinal tract [20]. The strain was exposed to artificial simulated conditions and their viability was higher than 85% both to low pH and to bile salts. The assay on bile salts during 3 h of incubation at 37°C, in anaerobiosis conditions, showed that the isolate strain differ significantly ($P \leq 0.05$) between all times of incubation. The viability in our research was similar to literature data [21].

CONCLUSIONS

Gastrointestinal tract is a good source of lactic acid bacteria. From the result of our study, *Lactobacillus acidophilus* IBNA 64 presents high potential properties with a good viability at pH 7±2 (8.32 log CFU/ml), respectively was able to resist at pH 3 (7.14 log CFU/ml) and 0.3% bile salts (7.56 log CFU/ml) during 3 h exposure.

The obtained results, indicated that poultry intestine is a good resource to isolate lactic acid bacteria.

Our isolate strain provides some probiotic properties, but furthermore *in vitro* and *in vivo* studies must be performed until its usage as feed additive in poultry feed.

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