In vitro Evaluation of Some Probiotic Properties of *Lactobacillus* Strains Isolated from Chickens' Gut

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Abstract

The objective of this study was to isolate, identify, and characterize new *Lactobacillus* strains with high probiotic potentials from the gastrointestinal tract of broiler chickens. The selected lactobacilli include five species: *Lactobacillus acidophilus*, *Lactobacillus brevis*, *Lactobacillus fermentum*, *Lactobacillus salivarius* and *Lactobacillus plantarum.* All strains were evaluated for their viability on selective medium, pH 3.0 tolerance and 0.3% bile salts for 3h. The strains IBNA 29 and IBNA 48 were the most resistant following exposure to gut conditions. After 3h of incubation, IBNA 29 registered a hight survival rate around 85% at low pH (8.50±0.176 log CFU/mL), respectively 90% (9.01±0.045 log CFU/mL) in the presence of bile salts*;* IBNA 48 showed a growth with 15.32% (8.027±0.04 CFU/mL), and 34.32% (6.22±0.242 CFU/mL), less at these useful traits. Taken together, the strain *Lactobacillus salivarius* IBNA 29 manifested the best resistance with the potential for use in the production of probiotic and can be a possible candidate for application as a feed additive in poultry nutrition.

Keywords: gastrointestinal resistance, *in vitro* properties, probiotic

1. Introduction

The term probiotics was used in animal production as well in humans. Probiotics are defined as live microbial feed supplements which beneficially affects the host animal by improving intestinal microbial balance [1]. The use of probiotics in poultry diets has received increasing attention improving growth performance, health benefits [2], by treating or preventing diarrhea incidence and animal's illness [3]. Generally, the gut is a source of lactic acid bacteria (LAB) with notable functional and technologies assets as a potential source of probiotics [4]; LAB can be found also in fermented food products (dairy, fruits, vegetables, meat and beverages) [5].

A probiotic bacterium is necessary to present properties for balance and improving the host gut microbiom [4, 6]. Interaction between intestinal bacteria and probiotic effects on the host [7] must confer beneficial effects as follow: should be recognized as safe (GRAS) [8], production of antimicrobial compounds, such as organic acids (lactic and acetic acid), inhibitors such as bacteriocins [9], hydrogen peroxide, compete for nutrients, have ability to diminish adherence and colonization of pathogenic bacteria in gastrointestinal tract (GIT), to survive and function in the digestive system, tolerance and survival in gastric acidity and bile salts contents, adhesion to the intestinal epithelium of the host [10, 11] and colonize the lumen of the intestinal tract [5]. Strains of LAB belonging to the genera *Lactobacillus* (LAB) and *Bifidobacterium* represent the principal source of probiotics [12]. Lactate production is the main metabolite of *Lactobacillus* genera which diminishes the percentage of infections, controls and adjusts the gut microbiota, by protecting as well, against pathogenic bacteria [13].

With an attempt to obtain probiotic based on LAB from poultry origins, we isolated new *Lactobacillus* strains from GIT content (ileum and

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cecum), which were identified and characterized for some of the properties previously presented.

2. Materials and methods

The birds' care and protocol used in this study was approved by the Animal Care and Use Committee at National Research-Development Institute for Biology and Animal Nutrition (INCDBNA-IBNA) Balotești, Romania and consistent with the principles of EU Directive 2010/63/EU and Romanian Law on Animal Protection.

Isolation, characterization and identification of Lactobacillus spp.

Gut microflora sampling. Bacteria were isolated from GIT content (ileum and cecum) of 26 and 45 days old healthy broiler chickens, that were randomly selected. Broilers were euthanized by cervical dislocation and intestinal content (ileum from 1 cm distal to Meckel's diverticulum to the ileo-caecal junction, and caecum) was aseptically removed and stored in individual sterile plastic containers, on ice and immediately processed at the laboratory.

Culturing conditions. One grame of intestinal content (ileum and cecum) from a healthy broiler chicken was aseptically transferred into a sterile conical tube containing 7 mL Oxoid BHI (Brain Heart Infusion) broth and 2 mL glycerol, and instantly frozen at –20˚C until assay [14].

Isolation of *Lactobacillus.* After defrost, the sample was subject to 10-fold serially diluted in Oxoid PBS (Phosphate Buffered Saline) and appropriate dilutions were manual spread-on three Petri dishes with Oxoid MRS (de Man, Rogosa and Sharpe) agar. The culture was incubated overnight in anaerobic conditions (Jar with Anaerogen 2.5L from Oxoid). After 24h incubation, the colonies were randomly selected and subcultured two times on a new MRS agar plate.

Characterization of Lactobacillus isolates. After incubation, the isolates were evaluated by colony morphological and Gram staining characteristics.

Biochemical identification

After morphological evaluation by Gram staining, the isolates were identified by biochemical tests (catalase assay, API 50 CHL Biomerieux strips), API 50 CHL V 5.1 and ABIS online soft [15], according to manufacturer's instructions.

The catalase test was performed according to the method described by the Dumitru et al. [15].

Preservaion of bacterial strains

Lactobacillus isolates were stored at room temperature and 4°C in MRS broth, respectively at -80°C with 20% (v/v) sterile glycerol, according to Sorescu et al. [14].

Characterization of some probiotic properties

The isolated strains that were identified as belonging to genus *Lactobacillus* underwent some of the probiotic properties.

Determination of colony forming units (CFU/g intestinal content). To determine the growth rate, the isolates were cultivated on selective medium, at 37°C, for 48h, in anaerobic conditions [3] and the results were expressed as $\log_{10}$ CFU per gram.

Acid tolerance. The overnight isolates of *Lactobacillus* spp. (7-8 log UFC/mL in PBS, pH 7.2±2.0) were grown in anaerobiosis, inoculated (1:10, v/v) in MRS broth adjusted to pH 3.0 with 1N HCl 37% and tested for their ability to tolerate stomach acid using a previous method, describe by Shokryazdan et al. [16, 29].

Bile salts tolerance

The overnight cultures of *Lactobacillus* spp. in the stationary growth phase (previous incubation in MRS broth at 37°C, for 18 h), were assayed according to the method Shokryazdan et al. [16, 29]. The isolated 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 0h, 1:30 min. and 3h, in anaerobiosis. The viability of *Lactobacillus* spp. strain was determined by estimating the number of colonies, by successive dilutions in sterile PBS, on MRS agar plates, incubated at 37°C, for 24h, in anaerobic conditions.

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

The survival percentage for pH and bile salt resistance was determined using the method presented by Ritter et al. [17], following the formula:

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

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.

3. Results and discussion

Isolation, characterization and identification of Lactobacillus spp.

All five *Lactobacillus* isolates from healthy indigenous broiler chickens presented positive results in phenotype assay as fallow: positive in the Gram-staining test, non-spore forming, arranged in pairs or in short chains. The predominant species from lactobacilli group are *L. acidophilus, L. fermentum, L. salivarius, L. plantarum, L. crispatus* [14], *L. johnsonii, L. aviaries* [18, 19].

In our study, the lactobacilli presented in the intestinal content of broiler chickens were identified as *L. fermentum, L. acidophilus, L. salivarius, L. plantarum* and *L. brevis.* The strain's ability to ferment and degrade the carbohydrates from API 50 CHL galleries are confirmed in Table 1. The percentage of identification (ID), respectively their similarity (SIM for ABIS online) is shown in Table 2. Furthermore, after taxonomical identification, according to the manufacture protocol API Biomeriuex (France), strains were registered in the IBNA Bacterial Collection under a code of reference. All *Lactobacillus* strains were registered catalase-negative test.

Table 1. API 50 CHL - Biochemical characteristics of *Lactobacillus* isolates from intestinal content of broiler chickens

Biochemical tests	Interpretation									
	1		2		3		4		5	
	24h	48h	24h	48h	24h	48h	24h	48h	24h	48h
Control	-	-	-	-	-	-	-	-	-	-
Glycerol	-	-	-	-	-	-	+	+	-	-
Erythritol	-	-	-	-	-	-	-	-	-	-
D-arabinose	-	-	-	-	-	-	-	-	-	-
L-arabinose	+	+	-	-	-	-	-	-	+	+
D-ribose	+	+	-	-	-	-	+	+	+	+
D-xylose	-	-	-	-	-	-	-	-	+	+
L-xylose	-	-	-	-	-	-	-	-	-	-
D-adonitol	-	-	-	-	-	-	-	-	-	-
Methyl-βD-xylopyranoside	-	-	-	-	-	-	-	-	-	-
D-galactose	+	+	-	-	+	+	+	+	+	+
D-glucose	+	+	+	+	+	+	+	-	+	+
D-fructose	-	-	+	+	+	+	+	+	+	+
D-mannose	-	-	+	+	+	+	+	+	-	-
L-sorbose	-	-	-	-	-	-	-	-	-	-
L-rhamnose	-	-	-	-	-	-	-	-	-	-
Dulcitol	-	-	-	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-	-	-	-
D-mannitol	-	-	-	-	+	+	+	+	-	-
D-sorbitol	-	-	-	-	-	-	+	+	-	-
Methyl-αD-mannopyranoside	-	-	-	-	-	-	+	+	-	-
Methyl-αD-glucopyranoside	-	-	-	-	-	-	-	-	-	-
N-acetylglucosamine	-	-	+	+	+	+	+	+	-	-

1= *Lactobacillus fermentum*; 2= *Lactobacillus acidophilus;* 3= *Lactobacillus salivarius;* 4= *Lactobacillus plantarum;* 5= *Lactobacillus brevis*"; –" Negative test; "+" Positive test;

Table 1. API 50 CHL - Biochemical characteristics of *Lactobacillus* isolates from intestinal content of broiler chickens (continued)

Biochemical tests	Interpretation									
	1		2		3		4		5	
	24h	48h	24h	48h	24h	48h	24h	48h	24h	48h
Amygdalin	-	-	-	-	-	-	+	+	-	-
Arbutin	-	-	-	-	-	-	+	+	-	-
Esculin	-	-	+	+	+	+	+	+	-	-
Salicin	-	-	-	-	-	-	+	+	-	-
D-cellobiose	-	-	-	-	-	-	+	+	-	-
D-maltose	+	+	-	-	+	+	+	+	+	+
D-lactose	+	+	-	-	+	+	+	+	+	+
D-melibiose	+	+	-	-	+	+	+	+	+	+
D-saccharose (sucrose)	+	+	+	+	+	+	+	+	+	+
D-trehalose	-	-	-	-	+	+	+	+	-	-
Inulin	-	-	-	-	-	-	-	-	-	-
D-melezitose	-	-	-	-	-	-	+	+	-	-
D-raffinose	+	+	+	+	+	+	+	+	+	+
Starch	-	-	-	-	-	-	-	-	-	-
Glycogen	-	-	-	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-	-	-	-
Gentibiose	-	-	-	-	-	-	+	+	-	-
D-turanose	-	-	-	-	-	-	-	-	-	-
D-lyxose	-	-	-	-	-	-	-	-	-	-
D-tagatose	-	-	-	-	-	-	+	+	-	-
D-fucose	-	-	-	-	-	-	-	-	-	-
L-fucose	-	-	-	-	-	-	-	-	-	-
D-arabitol	-	-	-	-	-	-	-	-	+	+
L-arabitol	-	-	-	-	-	-	-	-	-	-
Potassium gluconate	+	+	-	-	-	-	+	+	+	+
Potassium 2-ketogluconate	-	-	-	-	-	-	-	-	-	-
Potassium 5-ketogluconate	-	-	-	-	-	-	-	-	-	-

1= *Lactobacillus fermentum*; 2= *Lactobacillus acidophilus;* 3= *Lactobacillus salivarius;* 4= *Lactobacillus plantarum;* 5= *Lactobacillus brevis*“; –” Negative test; “+” Positive test;

Table 2. *Lactobacillus* strains identification by API 50 CHL and ABIS online

No	Species	Strains	Source	Identification	
				API 50 CHL (ID)	ABIS online (SM)
1	*L. fermentum*	IBNA 25	ileum	*L. fermentum 1*, 96.9% (GI)	*L. fermentum* ~95%
2	*L. acidophilus*	IBNA 26	ileum	*L. acidophilus 3*, 95.6% (GI)	*L. delbrueckii* ~92%, target taxon *L. acidophilus* ~78%
3	*L. salivarius*	IBNA 29	cecum	*L. salivarius*, 98.9% (GI)	*L. salivarius* ~94%
4	*L. plantarum*	IBNA 48	ileum	*L. plantarum* 1, 99.7% (GI)	*L. plantarum* ~ 86%
5	*L. brevis*	IBNA 50	ileum	*L. brevis 2,* 98.5% (GI)	*L. brevis* ~ 94%

ID-identification; GI- good identification; SM-similarity

Preservation of bacterial strains

In Table 3 are presented the results of the viability test for *Lactobacillus* isolates which are in accordance with Sorescu et al. [14]. The resistance at 4ºC and room temperature are relevant technical features for evaluating the strains. A probiotic bacteria for use as source of feed additive in animal nutrition generally is very important to maintain viability during storage without significant loss of living cells [14].

Table 3. The viability of *Lactobacillus* isolates at 4°C and room temperature

Strains	**4°C viability**	**Room temperature viability**
L. fermentum IBNA 25	≤ 7 months	< 3 months
L. acidophilus IBNA 26	< 48 days	
L. salivarius IBNA 29	< 48 days	
L. plantarum IBNA 48	≤ 9 months	< 3 months
L. brevis IBNA 50	< 3 months	< 45 days

Acid tolerance

The resistance of the new *Lactobacillus* isolates under pH 3.0 is shown in Table 4. All tested strains grown under pH 3.0, at which the number of survivability exceeded 10^8 CFU/mL after 3h of incubation for the first four strains, and only 10^6 CFU/mL for *L. brevis* IBNA 50. The strains IBNA 25, IBNA 26, IBNA 29 and IBNA 48 possessed excellent tolerance at pH 3.0 compared with the reference IBNA 50, where the colonies involve were affected by pH. The mean log CFU/mL of *Lactobacillus* isolates at pH 3.0 were ranged between 9.41 to 8.72 for *L. fermentum*, 9.46 to 6.56 for *L. acidophilus*, 9.98 to 8.50 for *L. salivarius*, 9.47 to 8.59 for *L. plantarum* and 8.44 to 5.86 for *L. brevis* respectively, that indicate significant difference in acid tolerance (P<0.05). Higher survival rate was observed at pH 3.0, as folow: 93.28% to 80.58% (*L. salivarius*), 92.67% to 90.70% (*L. fermentum*), 90.87% to 84.68% (*L. plantarum*), compared to their lower survival *L. acidophilus* (86.89% to 69.34%) respectively *L. brevis* (77.73% to 64.37%), during 3h of incubation (Figure 1).

Table 4. The resistance of *Lactobacillus* isolates incubated at pH 3

No	**Species**	**Viable counts (log $_{10}$ CFU/mL)**					
		Initial	**pH 3.0**				
			0 min.	**1:30 min.**	**3 h**	***SEM***	***P value***
1	*L. fermentum* IBNA 25	9.41^{a}	8.55^{bc}	8.07^{cd}	8.72^{d}	*0.547*	*0.0015*
2	*L. acidophilus* IBNA 26	9.46^{a}	8.16^{bc}	8.22^{c}	6.56^{d}	*0.321*	*0.0001*
3	*L. salivarius* IBNA 29	9.98^{a}	9.31^{b}	9.09^{c}	8.50^{d}	*0.161*	*0.0001*
4	*L. plantarum* IBNA 48	9.47^{a}	8.02^{bc}	8.07^{c}	8.59^{d}	*0.174*	*0.0001*
5	*L. brevis* IBNA 50	8.44^{abc}	6.56^{b}	5.43^{c}	5.86^{d}	*0.363*	*0.0001*

Values are the means of three independent experiments (n=3). ^{abcd} Means in the same row with different superscript significantly differ at P<0.05.

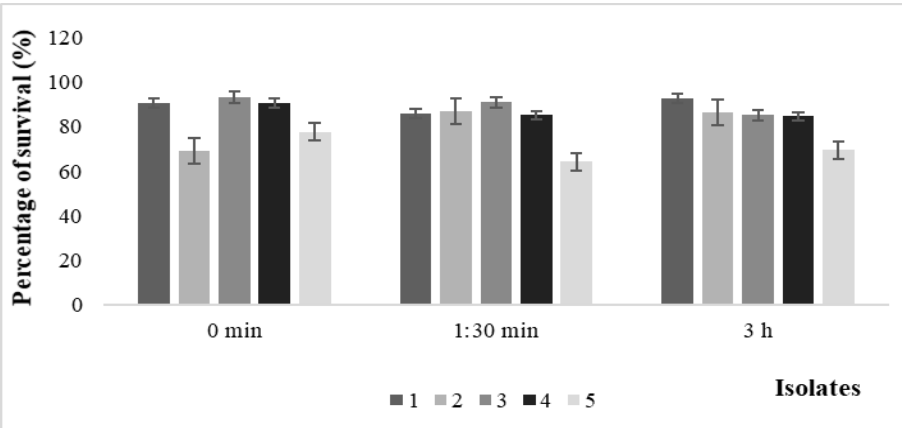

Figure 1. The survival percentage of *Lactobacillus* isolates in pH 3.0
1= *L. fermentum* IBNA 25; 2= *L. acidophilus* IBNA 26*;* 3= *L. salivarius* IBNA 29*;* 4= *L. plantarum* IBNA 48*;* 5= *L. brevis* IBNA 50

In this study, *Lactobacilli* isolates obtained from broilers intestinal content were evaluated according to their survivability under the artificially simulated conditions in digestive systems. The ability to survive under low pH is important feature for successful passage through the gastrointestinal tract [20], and furthermore to be considered a strong probiotic [21]. Hilmi et al.

[22] reported that the gastric juice pH in chickens is very low and the strain survival must exceed 70%. The optimal pH for the growth of *Lactobacillus* was reported to range between 6.2-8.8 [26]. In this study, the low pH is considered a barrier for the survivability. *L. acidophilus* and *L. brevis* exhibited poor growth at low pH. Our data are in accordance with Singh et al. [23] which reported that the maximum acid tolerance of lactobacilli was ranged between 46.70% to 102.48% at pH 3.0. The GIT pH of chickens ranges from 2.5 to 4.7 and ingestion can take up to 1 to 3 h depending on the size of the animal feed [24].

Bile salts tolerance

The potential of *Lactobacillus* to resist of the bile salts are shown in Table 5. Only *L. fermentum*, *L. salivarius* and *L. plantarum* survived at 0.3% bile salts addition in the basal medium, and between them a high growth present *L. salivarius* cells with more than 10^9 UFC/mL after 3 h of incubation. In the case of *L. acidophilus* and *L. brevis* the bile salts assay was not favorable, and after 3 h was not observed any involvement of the colonies. Figure 2 showed the survival rate of *Lactobacillus* isolates in MRS medium with 0.3% bile salts addition.

The bile salts test is an essential probiotic criteria for testing the survivability of any bacterial strain and evaluated its capacity to damage the structure of the cell membrane [4]. Lin et al. [27] reported that the chicken GIT (duodenum and cecum) presents a total bile salt concentration of 0.175 and 0.008%. The level 0.3% bile salts has been considered an optimal value for choosing a probiotic LAB [28].

Table 5. The resistance of *Lactobacillus* isolates to 0.3% bile salts

No	Species	Viable counts (log $_{10}$ CFU/mL)					
		Initial	pH 3				
			0 min.	1:30 min.	3 h	*SEM*	*P value*
1	*L. fermentum* IBNA 25	9.41^{a}	5.98^{bd}	5.36^{c}	6.09^{d}	*0.483*	*0.0001*
2	*L. acidophilus* IBNA 26	9.46	8.04	-	-	*na*	*na*
3	*L. salivarius* IBNA 29	9.98^{a}	9.04^{bc}	9.073^{c}	9.01^{d}	*0.123*	*0.0001*
4	*L. plantarum* IBNA 48	9.47^{a}	7.99^{b}	7.34^{c}	6.22^{d}	*0.356*	*0.0001*
5	*L. brevis* IBNA 50	8.44	-	-	-	*na*	*na*

Values are the means of three independent experiments (n=3). ^{abcd} Means in the same row with different superscript significantly differ at P<0.05. na= not applied

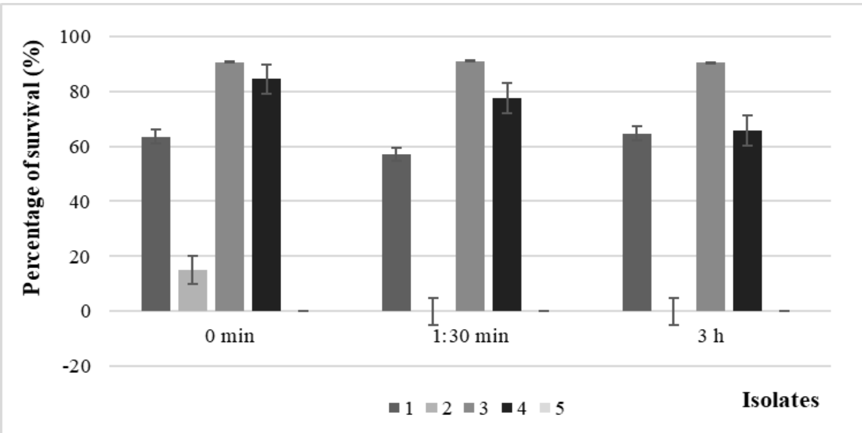

Figure 2. The survival percentage of *Lactobacillus* isolates in 0.3% bile salts concentration

Where: 1= *L. fermentum* IBNA 25; 2= *L. acidophilus* IBNA 26; 3= *L. salivarius* IBNA 29; 4= *L.plantarum* IBNA 48; 5= *L. brevis* IBNA 50

In present study, the results showed that only IBNA 25, IBNA 29 and IBNA 48 resisted to bile salts concentrations, with a special rate of loss for IBNA 26 and IBNA 50. For all isolates, *L. salivarius* showed the better growth in bile salts, following by *L. plantarum,* than other bacterial strains. The presence of bile salts in animal GIT is an important factor for lactobacilli viability [25].

4. Conclusions

The digestive tract of broilers chickens may offer a potential source for isolation of lactobacilli as probiotics candidates; *L. salivarius* IBNA 29 and *L. plantarum* IBNA 48 were able to tolerate low pH and bile salts, showed good survival during 3 h, thus they can be considered possible probiotics supplement for poultry.

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