Evaluation of *Lactobacillus* spp. based on phenotypical profile as direct-fed microbial candidate for poultry nutrition

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ABSTRACT

The present study was conducted to isolate, identify and characterize a lactic acid bacteria strain from turkey ileum content (46-day-old). The new strain was phenotypically confirmed as *Lactobacillus acidophilus* (*L. acidophilus*) and conserved under the code IBNA 09. Bacterial profile of *L. acidophilus* was compared with other strains known as *L. paracasei* CCM 1837 and *L. plantarum* ATCC 8014, based on cultural, morphological, biochemical and enzymatic activity (amylase and cellulase). The strains appear as Gram positive bacilli, thin, non-spore-forming, isolated, diplo form, in short chains or in small irregular piles on Man Rogosa and Sharp (MRS) broth and agar medium. The identification and biochemical traits were performed by catalase assay, API 50 CHL V 5.1 soft (*L. acidophilus* biotype 2, 99.9% ID; good identification to the genus *L. paracasei* spp. *paracasei* 1 or 3, 48-51% ID; *L. plantarum* 1, 99.9% ID) and ABIS online (*L. acidophilus* ~ 88%; *L. paracasei* spp. *paracasei*, ~ 90%; *L. plantarum*, ~91%). The highest total score of extracellular amylase activity was recorded by *L. acidophilus* IBNA 09 at 24-48 h (5.10 ± 0.176 U/mL, 4.99 ± 0.409 U/mL), followed by *L. paracasei* CCM 1837 (0.12 ± 0.002 U/mL, 0.15 ± 0.001 U/mL). During entire period, cellulase production was observed only for *L. acidophilus* (0.28 ± 0.019 U/mL), comparative with *L. paracasei* where the activity was observed in the first 24 h, respectively at 72 h for *L. plantarum*. These results suggest that *L. acidophilus* IBNA 09 possesses potential probiotic traits as a suitable candidate for amylase and cellulase production, and starter culture can improve cereal fermentation and the process of digestion in poultry nutrition.

Keywords: *Lactobacillus* spp., identification, enzymatic activity, probiotic, poultry

INTRODUCTION

Addition as live microorganisms in animal feed, probiotics determine an increase of nutritional value of material raw, with inhibition of antinutritional factors from forages, maximizing the enzymatic profile of nutrients for improving the zootechnical efficiency (Bedford, 2001).

The GI microbiota of monogastric animal has an essential role in health, nutrition, and animal performance (Pieper et al., 2006). Probiotics represent a high number of multiple beneficial effects between intestinal bacteria and effects on the body host (Kizerwetter –Swida and Bineak, 2016). Some beneficial effects can be associated with another organic acid, hydrogen peroxide, competition for nutrients, capability to reduce adherence, and colonization of pathogenic bacteria in GI (Kizerwetter –Swida and Bineak, 2016; Walter, 2008). Microbiota is composed essentially from bacteria, generally Gram-positive (aerobic and facultative anaerobes as lactobacilli, streptococci); the numbers of bacteria range from 400 to 500 with an increase from proximal to distal GI (Richards et al., 2005). Colon and faeces content can present up to 10^{10} - 10^{12} bacterial cells (Richard et al., 2005). For example, the density and diversity of the lactobacilli in the human body increases from the proximal level of the small intestine (10^3 CFU/mL luminal content in the duodenum) to the colon (up to 10^8 CFU/mL) compared to some animal species which has an abundant spectrum of bacteria (especially lactobacilli), starting with the proximal intestine (Walter, 2008). The addition of probiotic bacteria occurs an important place in the animal body due to their implication to inhibit and control enteric pathogens along with improving the functioning and production capacity of animals (Anee et al., 2018).

The administration of probiotics in animal feed can prevent GI disorders (changes in composition of intestinal microbiota) by GIT colonization, preservation of microbiota stability and prevention of pathogens (Vamanu et al., 2015). Probiotics addition increases the resistance to *Salmonella* spp. infections (Menconi, 2013a; Dumitru et al., 2021a,b). The most common bacteria used as probiotic supplements are lactic acid bacteria (LAB) from the genus *Lactobacillus* and *Pediococcus* (Latorre et al., 2016), and the species from the present genus are known as non pathogenic bacteria with capacity to form lactic acid as the principal metabolic end product of sugar process (Walter, 2008).

LAB can be found in plants or material of vegetable origin, silage, fermented food (yogurt, cheese, olives, pickles, salami, etc.), as well as in the oral cavities, GIT, and vaginas of humans and animals (Walter, 2008). LABs are bacteria with implications in terms of animal nutrition, improving nutritional's value of the GI microbial ecosystem in monogastric animals (poultry, pigs etc.) and some biological functions can be improved for the host (Yelnetty et al., 2014; Bakari et al., 2011). *Lactobacillus* spp. develop a low

proteolytic and lipolytic enzymatic activity, but can release significant amounts of exogenous enzymes (amylase and cellulase) in the culture medium, favouring the higher degradation of starch and cellulose found in animal forages (Padmavathi et al., 2018; Dumitru et al., 2016). The use of enzymes produced from *Lactobacillus* is known as safe because, as affirmed Singh et al., (2006), they are non-pathogenic bacteria.

The objective of this work was to isolate, identify and evaluate a *Lactobacillus* strain present in the broiler's turkey GIT (ileum content) and to determine the enzymatic activity compared with two reference strains, as preliminary investigation for use as probiotic candidate in poultry nutrition

MATERIALS AND METHODS

Statement of Ethics

A total of 288 one-d-old Hybrid Grade maker male turkey (0-70 feeding day trial), housed in the Experimental Biobase of National Research Development Institute for Biology and Animal Nutrition (IBNA) Balotesti were randomly selected for this topic. The bird's care and use protocol were approved by the Animal Ethics Committee at the IBNA, following the principles of EU Directive 2010/63/EU as transposed to Romanian legislation on Animal Protection used for Scientific Purposes (Law no. 199/2018).

Lactobacillus Strain

Lactobacillus strain isolated from turkey GIT (46-day old) was phenotypically and enzymatically (amylase and cellulase activity) investigated. The results were compared with other two reference strains: *L. paracasei* CCM 1837 (LC) and *L. plantarum* ATCC 8014 (LP). The identification of all strains was done by biochemical assay (catalase, API 50 CHL Biomerieux strips, and ABIS online soft).

All strains were evaluated for enzymatic activity by Hostettler's method for amylase enzyme and Petterson's and Porath's method for cellulase enzyme at 24, 48, and 72 h, at 37°C. The research was carried out at the Laboratory of Biotechnology of National Research Development Institute for Biology and Animal Nutrition Balotești (INCDBNA), Romania.

Samples Collection for Bacterial Isolation

The sample of broiler turkey intestinal content (ileum) was collected by cervical dislocation after which their crop and intestine was aseptically removed, placed in sterile plastic bags, and immediately carried to the laboratory in box with ice for microbiological analysis.

Isolation of LAB. Morphological and cultural characters

After collected of the intestinal content, 1 gram from ileum was aseptically removed and enriched in MRS broth (Man, Rogosa, Sharpe from Oxoid CM0361), homogenized and incubated at 37°C, for 24, 48 and 72 h, in anaerobic atmosphere (Oxoid Jar with Anaerogen 2.5 L). In the first 24 h, a slight turbidity was observed in the culture tube. In the following hours, the lactic acid bacteria culture involved a concentrate turbidity, with a rich deposits, without surface formations.

The morphological characteristics were examined, following ahead the Gram strain protocol according to Bergey's Manual (1984). MRS agar plates display white and creamy colonies (presumptive for LAB). Carefully, the colonies were chosen, separated and purified through consecutive transfers on MRS agar until was obtained a pure culture. The strains were stored at 4°C as a relevant technical character, until utilisation (no more two week, Sorescu et al., 2019). For the strains already known (*L. paracasei* CCM 1837 and *L. plantarum* ATCC 8014) was followed the same protocol.

LAB Phenotypical Identification

The Catalase Assay

The presence of catalase from a bacterial isolate is obvious when a small amount of bacterial culture from their agar selective medium developed bubbles (effervescence) at addition of 3% hydrogen peroxide (3%, v/v) (MacFaddin, 2000).

The API 50 CHL

All strains were identified (genus and species) and characterized based on biochemical evaluation tests using a bioMerieux API 50 CHL test kit according to the manufacturer's instructions (BioMerieux, Marcy l'Etoile, France). The test API 50 CHL put in evidence the enzymatic equipment bacteria, which differs from one species to another. The API 50 CHL consists of 50 microtubes used to study carbohydrates fermentation. The fermentation tests are inoculated with bacterial strain suspension in API 50 CHL medium which rehydrates the substrates. During incubation, the ability of isolate to ferment carbohydrates is indicated by the colour change of the basal medium used, caused by the anaerobic production of acid and detected by the pH indicator present in the selective medium. The strips are visually examined after 24–72 h. A positive or negative result was determined from the colour change from purple to yellow. The results were then introduced to the API databases using APIweb™ 50 CHL V5.1 and ABIS online software (Stoica and Sorescu, 2017).

Preservation of Stock Culture

The LABs were conserved in MRS broth medium containing 20% (v/v) glycerol as frozen cryoprotectant at -80°C. The glycerol were done by homogenized 10 mL overnight culture (1:10) including 2 mL sterile glycerol.

LAB Haemolytic Activity

The overnight pure cultures of LAB isolate, respectively *L. paracasei* CCM 1837 and *L. plantarum* ATCC 8014, were streaked onto TSA agar plates (Oxoid, P00163) with 5% of sheep blood (commercial) followed by incubation at 37°C, for 24 h. Haemolytic activity was verified based on the ability of strains to lyse blood cells from culture medium. The interpretation was done according to Reuben et al. (2019). Furthermore, Dumitru et al. (2020) present that a clear zone around colonies on TSA medium indicates complete beta hydrolysis (β -haemolysis) and the strain must be eliminated for utilization as a probiotic in animal nutrition. According to Prieto et al. (2012) and Seker (2010), non-haemolysis (γ -haemolysis with non-change registered in the media) and α -haemolysis (a small green zone discoloration around colonies) are considered to be safe.

Crude Enzymes Preparation

The crude enzyme secreted by LABs (LAB isolate, *L. paracasei* CCM 1837 and *L. plantarum* ATCC 8014) was prepared freshly until use. In relation to 1:10 (v/v) of 24-h-old culture, LAB was inoculated in MRS broth medium and incubated at 37°C, for 24 h in aerobic conditions. LABs cell-free supernatant containing crude enzymes was collected by centrifugation at 5000 rpm, 20 min at 4°C. Until use, the supernatant was kept at 4°C (no more than one week, Ho and Sze, 2018).

Evaluating the Enzymatic Activity

All *Lactobacillus* spp. were grown in MRS selective medium according to Bergey's Manual (1984). The enzymatic activity was evaluated at 24, 48 and 72 h, at 37°C in aerobic conditions, by next methods:

1. *Hostettler's method for amylase activity.* It is based on the action of α -amylase on starch and production of maltose after the hydrolysis process, with 3,5 - dinitrosalicylic acid (DNS), at pH 6.9, 30°C, as a colorimetric method. An amylase unit activity (U) corresponds to a quantity of maltose (μ mol) released under the action of 1 mL of enzyme preparation in one minute, at 30°C.

2. *Petterson's and Porath's method for cellulase activity.* It is based on the enzymatic hydrolysis of carboxymethylcellulose (CMC, Roth) and the dosing of reducing sugars released with 3,5-dinitrosalicylic acid (DNS) colorimetric method. A cellulolytic unit activity (U) represents the amount of enzyme able

to release from a CMC solution, an amount of reducing sugars, which form with the DNS reagent, the same optical density, equivalent to a milligram of glucose (Jurcoane et al., 2006).

Statistical Analysis

The graphics for the present study were generated using SigmaPlot V.14.5 software (San Jose, CA, USA). and some of the parameters were determined using MS Excel 19.

RESULTS AND DISCUSSION

Characterization and isolation of lactic acid bacteria

Species of LAB are probiotics sources in animal feed due to their properties to enhance the GIT health and performance. Furthermore, LAB are known as producer of lactic acid from carbohydrate fermentation, and the extracellular metabolites have been demonstrated to be a potential substitute for antibiotics to improve meat quality and broiler chicken, laying hens, and piglets performance (Lim et al., 2019; Kareem et al., 2016; Loh et al., 2013). The strain isolated from ileum content of turkey appears as punctiform colonies at 24 h with a Gram-positive staining. In MRS broth, after 24 - 48 h in aerobiosis, intense turbidity was noticed, with abundant deposits without surface formations, which phenotypically correspond to the genus *Lactobacillus*. A summary of morphology characteristics of our isolate identified as *L. acidophilus* IBNA 09 (LA), comparatively with LC and LP strains can be observed in Table 1.

Table 1. Colonies and cell morphology of *Lactobacillus* strains

LAB	Colony morphology	Gram stain	Shape
LA	round, opaque, whitish, with irregular margins, 1.0 mm up to 1.5 mm Ø after 48 h, in anaerobiosis	+	Bacilli, thin, rods, isolated, short chains or in small irregular piles, immobile, non-spore forming
LC	small colonies, 0.2-0.5 mm Ø after 48 h, anaerobiosis/microaerophile, regular margins, with white-beige pigment, sediment formation	+	Isolated, arranged in short chains, non-spore forming
LP	Small colonies with smooth aspect (round, regular margins), 0.5-2.0 mm Ø, microaerophile, white-beige pigment, sediment formation	+	rods, isolated, short chains or in small irregular piles, immobile, non-spore forming

+: positive; LA- *L. acidophilus* IBNA 09; LC- *L. paracasei* CCM 1837 (IBNA 04); LP- *L. plantarum* ATCC 8014 (IBNA 03).

Aerobiosis was done in Oxoid Jar with Anaerogen 2.5 L; colonies size were measured with a ruler as millimeter (mm).

Our results based on morphological data and presented in Table 1 are similar with Hoque et al. (2010) report which affirmed that *Lactobacillus* genus is genetically and physiologically a diverse group of rod-shaped, Gram-positive, with catalase-negative and non-spore-forming bacteria.

The colonies characteristics found after Gram staining technique included our isolate as a strain from *Lactobacillus* group. The isolate presented similar traits to the finding of Kabir et al. (2016), morphological traits which are supported by another researcher (Dumitru et al., 2020; Sorescu et al., 2021; Mannan et al., 2017).

LAB Phenotypical Identification

All strains were catalase-negative. The identity of the new strain was confirmed by sugars fermentation and biochemical test API 50 CHL V5.1 („excellent identification”, *L. acidophilus* 2, 99.9% ID) and ABIS online (*L. acidophilus* ~ 88%) following the manufacturer instructions and used in several reports (Dumitru et al., 2021; Sorescu et al., 2019). Furthermore, the phenotypical profile of *L. paracasei* CCM 1837 (LC) and *L. plantarum* ATCC 8014 (LP), strains that were purchased in the form of freeze-dried, were used as reference strains. In addition, after revitalized in MRS broth medium and identified by API 50CHL Biomerieux, all three strains of *Lactobacillus* involve different fermentation of monosaccharide and disaccharide. The strains capability in fermenting carbohydrates was observed by the discoloration of the basal medium from purple to yellow (Figure 1).

Figure 1. API 50 CHL strips inoculated with *Lactobacillus* species
(a – *L. acidophilus* IBNA 09; b – *L. paracasei* CCM 1837; c – *L. plantarum* ATCC 8014)

In this study, the results of sugar fermentation were presented in Table 2.

Table 2. The results obtained with API 50 CHL for *Lactobacillus* strains

Biochemical Test	Strains		
	<i>L. acidophilus</i> IBNA 09	<i>L. paracasei</i> CCM 1837	<i>L. plantarum</i> ATCC 8014
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	+	+ 3/6 days	+
D-sorbitol	-	-	+
Methyl-αD-mannopyranoside	-	-	+
Methyl-αD-glucopyranoside	-	+	+
N-acetylglucosamine	+	-	+
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 / 72 h	+	+	+ 3-6 days
D-lyxose	-	-;? 3-6 days	-
D-tagatose	-	+	-
D-fucose	-	-	-
L-fucose	-	-	-
D-arabitol	-	-	-
L-arabitol	-	-	-
Potassium gluconate	?	?	?
Potassium 2-ketogluconate	-	-	-
Potassium 5-ketogluconate	-	-	-

+: positive test; -: negative test; ?: dubious, weekly

It was obtained a good percentage of identification for *L. paracasei* CCM 1837 and *L. plantarum* ATCC 8014 by API 50 CHL (*L. paracasei* spp. *paracasei* 1, good identification to the genus 48-51% ID; *L. plantarum* 1, very good identification, ID 99.9%) and ABIS online (*L. paracasei* spp. *paracasei*, ~ 90%; *L. plantarum*, ~91%). Mannan et al. (2017) and Na-Kyoung et al. (2008) present similar results of *Lactobacillus* bacteria fermentation. All the isolates fermented galactose, glucose, fructose, mannose, esculin, cellobiose, lactose, and turanose. The difference of our isolate compared with LC and LP consist in their capacity to ferment D-raffinose substrate (positive).

Preservation of Stock Culture

Regarding the storage conditions, all strains were conserved at -80°C, with the addition of 20% glycerol as cryoprotectant agent and can be found in the intern Bacterial Collection of IBNA Balotesti, Department of Animal Nutrition and Biotechnology as *L. acidophilus* IBNA 09, *L. paracasei* CCM 1837 (IBNA 04), and *L. plantarum* ATCC 8014 (IBNA 03).

LAB Haemolytic Activity

The present *Lactobacillus* strain identified as *L. acidophilus* was found nonhemolytic on a TSA agar after 24 h, at 37°C. In addition, LC and LP reference strains, involved similar results as alpha haemolytic activity. All strains are considered a safe prerequisite for application as a probiotics source in poultry. Previously report (Olufemi et al., 2018), affirmed that popular of LAB strains are non-haemolytic. Therefore, apart from other advantageous properties, sourcing of probiotic strains isolated from their natural niche, is most ideal, as such bacterial strains are familiar with the gastrointestinal tract of the host, and can naturally multiply and express the desired beneficial effects better than strains isolated from other sources (Reuben et al., 2019). The results from our research correspond with FAO (Food and Agriculture Organization of the United Nations) guidelines which affirmed that until to use a bacterial strain as probiotic supplementation is recommended to be safe without any negative effect in the host.

Evaluating the Amylase Activity

Extracellular amylase of LA, LC, and LP that grown in selective medium was subsequently determined in this study. The calibration maltose curve was used to estimate the amylase activity values (Figure 1) and the optical density (OD) at 546 nm was measured.

Figure 1. Standard curve of maltose

The results obtained demonstrated that the specific extracellular enzymatic activity was varied considerably. In the first 24 h of incubation, from all *Lactobacillus* strains, only LA ranked highest amylase activity (5.10 ± 0.179 U/mL), followed by LC (0.12 ± 0.002 U/mL). This finding was very interesting because, at 48 h, our isolate involved a slow decrease of amylase level activity (4.99 ± 0.409 U/mL), while LC registered a slight increase of amylase production (0.15 ± 0.001 U/mL). Based on a standard curve of maltose, LP strain during 72 h did not show any amylase activity (Figure 2).

Figure 2. The amylase activity of *Lactobacillus* strains by Hostettler's method
Data are represented as means $\pm$ standard error of means (SEM) (n=3)

Rapid hydrolysis of starch is an important aspect in fermentation processes (Oguntoyinbo and Narbad, 2015). During exponential stage of growth, a good producer of amylase was seen in the first 24 h for LA followed by LC. The secretion of amylase by LA continued to be visible at 48 h under the assay conditions, confirming thus the statement of Fossi and Tavea (2013) who specified a solid relationships between cell growth and amylase production. No amylase activity was detected for LP. *Lactobacillus* spp. involve different extracellular amylase production during fermentation process. Similar data were presented in Lee et al. (2019) report where seven *L. plantarum* strains were compared based on enzymatic activity in MRS medium. Additionally, the amylase production of LP is in accordance with *L. plantarum* strain of Dumitru et al. (2018) study, where the amylase activity registered was total absent in MRS broth medium. In addition, the fermentation capacity of amylase can be observed in API 50 CHL test, where from all LAB, LA and LC have the ability to hydrolyze starch substrate. This result suggests that LA is efficient to degrading starch and with a minimum activity for LC.

Amylolytic LAB uses starch biomass and converts into lactic acid in a single stage of fermentation which represents the primary step of process with a rapidly action of sugars degradation in substances as acids and alchols (Fossi and Tavea, 2013). The decline of strains growth and amylase secretion was observed at LP and LC and could be attributed as reported Singh et al. (2006) to the rise of lactic acid concentration as the end product of lactic acid bacteria capable of fermenting different sugars (Klaenhammer and De Vos, 2011).

Evaluating the Cellulase Activity

Extracellular cellulase by DNS method of *Lactobacillus* strains in MRS broth medium was measured during 24 - 72 h. For evaluation of cellulase activity, the absorbance was measured at 640 nm. The cellulase activity of LC at 24 h was ranked highest (0.63 ± 0.181 U/mL), followed by LA (0.59 ± 0.021 U/mL). Regarding the LP strain, the production of cellulase could not be determined in first 48 h. However, certain bacterial species present different responses to diverse environmental conditions, pH values, and their fermentation capacity can improve the fermentation efficiency, quality, aerobic stability, and antimicrobial capacity by mixing them as a probiotic source in animal feed (Sun et al., 2012; Zhao et al., 2021). Based on their metabolism, LAB can metabolise several monosaccharides (e.g. fructose, glucose, galactose, and xylose) which are lignocellulosic components from diverse raw materials (Tarran and Mazzoli, 2018).

Figure 3. Standard curve of glucose

The addition of LAB cultures with cellulase capacity of production in feedstuffs can degrade cellulose to monosaccharides or oligosaccharides, thus improving LAB proliferation to obtain higher fermentation quality. Experimentally, lactic acid bacteria culture based on cellulase activity was performed to determine their ability to consume cellulose by using optical density as a growth parameter.

Figure 4. The cellulase activity of *Lactobacillus* strains by DNS method
Data are represented as means $\pm$ standard error of means (SEM) (n=3)

In Figure 4 can be observed that, at 48 h, the cellulase LA secretion decrease slowly (0.2 ± 0.016 U/mL) vs. LP and LC strain where the enzyme production was totally absent. Therefore, at 72 h, the enzymatic capacity of LP strain was activated, followed by a cellulase secretion (0.32 ± 0.036 U/mL). For bacteria to hydrolyze and degrade insoluble cellulose from agriculture lignocellulosic biomass, the production of extracellular cellulase is essential (Deutscher, 2008). The secretion of enzymes can be affected by several factors as temperature, pH, and other extreme conditions found by bacteria cells in animal body.

CONCLUSION

In conclusion, from all LAB, *L. acidophilus* IBNA 09 exhibited notably extracellular enzymatic activity. Amylase production was registered at 24-48 h, comparatively with cellulase where the activity was observed during the entire period. These results suggest that *L. acidophilus* IBNA 09 isolated for turkey ileum content have potential probiotic traits as a suitable candidate for amylase and cellulase production, and can be used to improve raw materials fermentation and process of digestion in animal nutrition.

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