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Probiotic Potential of Thermotolerants Lactobacilli Isolated from Chicken Gastrointestinal Digestive and Their Use as Poultry Feed

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Abstract: This research was conducted in order to evaluate probiotic properties of lactobacilli species isolated from intestinal tract of indigenous Algerian chickens. Thirteen isolates thermotolerant lactobacilli were isolated from the crops and small intestines of domestic checkens (*Gallus domessticus*). The probiotic are a live microbial feed supplements which positively affects the health of the host animal by improving its intestinal balance. These isolates were characterized and identified. They were screened by use main criteria's of probiotic selection *in vitro*, only the isolate survived and grown in acidic medium and resisted to bile salts were retained. The results showed that among thirteen isolates five isolates were responded to these criteria and were identified as *Lactobacillus gallinarum*, *Lactobacillus crispatus*, *Lactobacillus dulbreuki*i, *Lactobacillus acidophilus* and *Lactobacillus jonhsonni*. These strains were homofermentatives and produced more than 0.6 % lactic acid (w/v) after 24 h of incubation and the nature of the isomeres were confirmed by the High Performance Liquid Chromatographic. All the strains were proteolytic. An anti-salmonella activity was detected *in vitro* against the species of *Salmonella enteriditis* and *Salmonella infantis.*

Key words: Thermotolerant lactobacilli · Poultry · Probiotic · *Gallus domessticus*

INTRODUCTION

In recent years considerable interest has been shown in using some probiotic microorganisms and organic acids as an alternative to the use of antibiotics in feeds [1]. Probiotics are a live microbial feed supplements which positively affects the health of the host animal by improving its intestinal balance [2, 3]. The basic requirements for lactic acid bacteria strain which can be used as probiotic have been described as follows. These strains should be tolerant to acid and bile salts and be able to: adhere to the intestinal epithelium of the hosts; show an antagonistic activity against pathogenic bacteria and keep their viability during processing and storage [4]. The use of probiotics to promote health and nutrition has been attracting a great deal of attention for a long time [5, 6]. The term "probiotic" is defined as a live microbial feed supplement, which beneficially affects the most animal by improving the intestinal microbial balance [2]. The effect of probiotics on animal production has been widely studied, but the results are often contradictory [7].

The progressive reduction of the use of the antibiotics as promoters of growth caused a renewal of interest for the incorporation of probiotics in the feed, in order to maintain the beneficial effect with the antibiotics.

Since 1994, the European guideline (guideline 94/40) governing the use of the additives in animal feed has been modified to include the probiotics and the enzymes. The microbial flora of the birds is characterized by the high number of lactobacilli in the jowl, the lactobacilli, responsible for sugars fermentation, dominates, adhere to the mucous membrane and can reach sizes of populations of one billion of bacteria by gram of content [8].

In poultry, the lactobacilli genus is sometimes represented by *Lactobacillus acidophilus*, *Lactobacilus fermentum*, *Lactobacilllus buchneri* and *Lactobacillus ruminis*, *Lactobacillus delbrueckii*, *Lactobacillus coryniformis* and *Lactobacillus viridescens* [9]. The majorities of the species of this genus were isolated from the digestive tract of poultry and can grow at 45 and 50°C [10-13].

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However, a little knowledge about the biodiversity of the thermotolerant lactobacilli in nature, because few studies have been done and focused on lactobacilli from the standpoint of the thermotolerant.

The aim of the present work was to identify thermotolerant lactobacilli strains isolated from gastrointestinal digestive tracts of Algerian indigenous chickens and study their responses to the criteria's selection probiotic in order to select strains to be used as probiotic in the poultry feed.

MATERIALS AND METHODS

Lactic Acid Bacteria Isolation: The microbial strains were isolated from contents of crop, proventriculus, gizzard, ileum and ceca of an adult domestic chicken (*Gallus domesticus*), fed without antibiotics. The sample was mixed with sterile saline buffer (0.85%, pH 7.0) and homogenized using a stomacher. Decimal dilution of these samples were mixed with MRS broth (Difco) and incubated at 45°C for 48 h under anaerobic conditions. The colonies were randomly selected from the highest dilutions of each MRS agar plate. Colonies of different morphological appearance were purified by re-streaking on MRS agar 2-3 times. The pure cultures were characterized using Gram stain, cell morphology and catalase reaction tests. Gram positive and catalase-negative isolates were stored at-20°C in MRS broth supplemented with 30% (v/v) glycerol. Gram positive rods with a negative catalase reaction were retained as presumptive *Lactobacillus* species [6]. Thereafter, strains were routinely cultured on MRS agar (Difco) and MRS broth and incubated at 45°C.

Identification of the Isolates: Gram stains and morphology of isolated lactic acid bacteria were examined after 24 h of incubation on MRS agar for catalase activity and gas production. Arginine hydrolysis was also tested on MRS broth. Sugar fermentation was determined in MRS broth containing bromocresol purple ($0.04 g l^{-1}$) as a pH indicator and supplemented with 1% sugar. Sixteen sugars (glucose, galactose, mannose, mannitol, sorbitol, arabinose, xylose, rahmnose, saccharose, lactose, maltose, cellobiose, raffinose, treahalose, esculine, salicine) were subjected to a fermentation test in MRS broth under anaerobic condition; each tube was topped up with two drops of sterile liquid paraffin after inoculation [14].

Acidifying activity was measured according to the International Dairy Federation (IDF) standard 306 [15], Kihal *et al.* [16]: Alonso-Calleja *et al.* [17]: Herrero *et al.* [18]. The results were expressed as percentage of lactic acid. Milk medium was prepared from reconstituted skim milk powder 10%(w/v) and sterilized at 110 °C for 10 min. Sterilized milk was inoculated with 0.2% with each strain pre-cultured in MRS broth at 45°C for 24 h, to obtain approximately 10^6 cfu ml^{-1} and then incubated at 45°C for 24 h.

Lactic Acid Isomer Determination: The isomer of lactic acid produce by *Lactobacillus* species was determined by HPLC according to Gonzàlez *et al.,* [19] and Fitisimones *et al.*, [20]. The extraction of organic acid from sample: 25 ml sulphuric acid (4.5 mM) added to 5 ml of 24h culture at 45°C in milk, after 1h stirring and centrifuge (2000 g, 10min), the supernatant is filtered through Wathman (n°1) paper filter.

An aliquot 50µl of the lactic acid (L+) 90 % of purity (Merck) was injected as standard and equal volume were injected of samples using auto-sampler Waters 717 plus, shrisorb C8 HPLC column and separated by isocratic elution (solvent: 20mM H_3PO_4). Column temperature was set at 30°C and the flow rate was set 1ml min^{-1}. Analysis were measured by UV detection at 210 nm using UV/VIS Detector Water 484. The acquirement of the data is done by millennium 32 software.

Probiotic Characteristic: The probiotic characteristic was tested using the sensitivity or resistance to low pH and bile salt. The screening probiotic strain was prepared according Haller *et al*., [21]. Intestinal conditions were simulated with bile salts 2% and the pH of bile salts solution was adjusted with 50 mM $NaHCO_3$ at pH 8.0 ± 0.2. Bacterial cells were harvested by centrifugation (3000 g, 15 min) at the stationary growth phase and washed tree times with sterile saline phosphate buffer (NaCl, 8 g l^{-1}; KCl, 0.2 g l^{-1}; KH_2PO_4, 0.12 g l^{-1}; anhydrous Na_2HPO_4, 0.9 g l^{-1}).

The tolerance of strains to gastric and intestinal conditions was investigated in a two-step procedure. An aliquot of 100 µl of bacterial suspension was initially added to 900 µl HCl and incubated at 37°C for 1h. From this mixture 100µl were added to either 900 µl of solution bile salts at 0.3% (w/v) in water for 1h at 37°C. Resistance was assessed in terms of the viable bacteria count and enumerated after incubation at 45°C. Samples were taken and the viable bacteria were determined by surface plating.

Proteolytic Activity: The proteolytic activity was determined on YMA agar (Yeast Milk Agar). The strains were inoculated by a sterile multipoint and incubated for 48h at 45°C. The proteolysis activity is characterized by the observation of a clear zone surrounding the colonies [22].

Detection of Antimicrobial Activity: To measure the antibacterial activity, lactobacilli isolates were cultivated in MRS broth at 45°C for 18 h. The culture containing 10^8 cfu ml^{-1} was dropped on MRS agar and incubated at 45°C under anaerobic condition for 24 h. Two strains of *Salmonella* sp: *Salmonella infantis* and *Salmonella enteritidis* obtained from the Laboratory of Food Microbiology (Collection Institute Pasteur, Algiers, Algeria) were used as indicator strains. The colonies of lactobacilli on MRS agar plate were overlaid with 7 ml of soft nutrient agar with 1 ml of activated overnight indicator strains culture (50 x 10^6 cfu ml^{-1}). The agar plates were incubated at 37°C for 18 h and diameters of inhibition zone on the agar plate were measured [6, 23, 24]. Each assay was performed in triplicate. The antibacterial activity was calculated as follows:

The antibacterial activity = *Diameter of inhibition zone -Diameter of colony*

Statistical Analysis: Treatment effects were compared using analysis of variance and treatment means were separated using the least significant difference. The computation was done by using the SAS program [25].

RESULTS AND DISCUSSION

Identification of Isolates

Phenotypic Characterization: Thirteen thermotolerant lactobacilli isolates were obtained from the crops and small intestines of domestic chickens. All the isolates were rods, arranged in chain or in palisade, gram positive, catalase negative, homofermentatives, ADH negative, growth at 45°C and survival 30 min at 63°C. These isolates cannot grow to 6.5% NaCl. In Table 1 all phenotypic tests were showed. These results were compared to those obtained by Kovalenko [10] and Dashkevick and Feignhner [26] which were similar. The phenotypic and biochemical characters, compared with the data of Carr *et al.,* [27], allow identifying the five following strains, Lb.7 as *Lactobacillus crispatus;* Lb.8 as *Lactobacillus delbruecki* subsp. *lactis;* Lb.9 as *Lactobacillus acidophilus*; Lb.11 as *Lactobacillus gallinarum and* Lb.12 as *Lactobacillus jonhsoni*.

Probiotic Characteristic: To exert beneficial effects adequate numbers of viable cells of probiotic viable strains should reach the intestinal tract. In order to survive the passage through the gastrointestinal tract, resistance to low pH, bile salt and proteolytic enzymes is needed. Acid and bile salt resistance can be easily monitored and are considered important properties of probiotic lactic acid bacteria [28, 29]. From thirteen isolates only five strains were corresponding to the probiotic selection criteria's, tolerance to the low pH and bile salts. Among strains isolated from crop only one strain: *Lactobacillus crispatus* (Lb.7) was resisted both to acidic medium and bile salts, three isolates (Lb.2, Lb.5, Lb.6) were survived in acidic medium but inhibited by bile salts. The other isolates (Lb.1, Lb.3, Lb.4) were inhibited both in acidic medium and bile salts.

Little information is available on the bile tolerance of Gram-negative bacteria, but it is believed that they are naturally more resistant to bile salt than Gram-positive bacteria. The bile salts are often used in their selective enrichment medium for gram negative bacteria (Mac Conkey agar, *Salmonella-Shigella* agar, violet red bile agar and bile esculin agar) Bridson [30].

Gusils *et al*. [13] showed that two species isolated from intestinal tract of poultry (*Lactobacillus fermentum* and *Lactobacillus animalis*) were able *in vivo* to inhibit and avoid the attachment of *Salmonella gallinarum, Salmonella enteriditis* and *salmonella pullorum.* Two isolates (Lb.10 and Lb.13) from intestinal tract and three isolates from crop (Lb.1, Lb.3, Lb.4) were inhibited both in the acidic medium and bile salts. Probably theses isolates were not autochthones to the digestive tract, except of *Lactobacillus delbruecki* subsp. *lactis* and the others species are belonging to the natural habitat of poultry digestive tract [31]. In a similarly study, Jil *et al*. [7] demonstrated that twelve *Lactobacillus* strains isolated from chicken was slightly affected by bile salts (0.3%). In our study the strains resisted to 2% bile salts, certainly they have an important enzymatic activity of bile salt hydrolase. Dashkeviez and Feighner [26] confirmed that sub-therapeutic levels of feed additive antibiotics decrease bile salt hydrolase activity.

Antimicrobial Activity: The results (Table 3) showed that all the five strains were able to inhibit the growth of both *Salmonella enteriditis* and *Salmonella infantis*. The inhibition of *Salmonella infantis* is slightly higher to *Salmonella enteriditis*. Jin *et al*. [7] were found that 12 *Lactobacillus* isolated from chicken intestinal tract were able to inhibit five strains of *Salmonella.*

Table 1: Phenotypic and biochemical characteristics of selected lactobacilli

	Species				
Tests	7	8	9	11	13
Growth at 15°C	-	-	-	-	-
Growth at 37°C	+	+	+	+	+
Growth at 45°C	+	+	+	+	+
Growth at 6.5% NaCl	-	-	-	-	-
Hydrolysis of Arginin	-	-	-	-	-
Citrate	-	-	-	-	-
Production of CO_2 from glucose	-	-	-	-	-
Acetoin	-	-			
Lactic acid isomer	L(+)	L(+)	L(+)	L(+)	L(+)
Survival at 63.5°C for 30 min	+	+	+	+	+
Acid from lactose	+	+	+	+	+
Glucose	+	+	+	+	+
Galactose	+	+	-	+	+
Saccharose	+	+	+	-	-
Fructose	-	-	-	+	+
Sorbitol	+	-	-	-	-
Mannitol	+	-	-	-	-
Mannose	+	-	-	-	-
Rahmnose	+	-	-	-	-
Maltose	+	-	-	+	+
Xylose	-	-	-	-	-
Cellobiose	+	-	+	+	+
D-Raffinose	+	+	+	+	+
L-Arabinose	+	-	-	-	-
Trehalose	+	+	+	+	+
Esculine	+	-	-	+	-
Salicine	+	-	-	-	-

Species : 7 = *Lb. crispatus*, 8 = *Lb.dulbruecki* subsp. *lactis*, 9 = *Lb. acidophilus*, 11 = *Lb. gallinarum*, 13 = *Lb. johnsonii*

Table 2: Tolerance of the lactobacilli isolates to the consecutive exposure to hydrochloric acid and bile salts

	Crop							Small intestines					
Origin													
Isolates	Lb.1	Lb.2	Lb.3	Lb.4	Lb.5	Lb.6	Lb.7	Lb.8	Lb.9	Lb.10	Lb.11	Lb.12	Lb.13
Hcl pH 2.5	S	R	S	S	R	R	R	R	R	S	R	R	S
Bile salts 2%	S	S	S	S	S	S	R	R	R	S	R	R	S

S : Sensitive, R : Resistant

Table 3: Antagonism activity of selected lactobacilli against the species of *Salmonella enteriditis* and *Salmonella infantis* (diameter of inhibition zone in mm)

Strains	*Salmonella enteriditis*	*Salmonella infantis*
Lb. crispatus	20	25
Lb. dulbreucki subsp. *lactis*	19	23
Lb. acidophilus	21	22
Lb. gallinarum	22	22
Lb. johnsonii	20	24

Table 4: Growth of the selected thermotolerants lactobacilli strains (ufc/g)

Strains	*Lb. crispatus*	*Lb. delbruecki* subsp. *lactis*	*Lb. acidophilus*	*Lb. gallinarum*	*Lb. johnsonii*
Specific Growth rate μ	2.13	0.66	1.45	1.53	0.83
Final ufc/ml	80×10^9	35×10^9	53×10^9	140×10^9	150×10^8

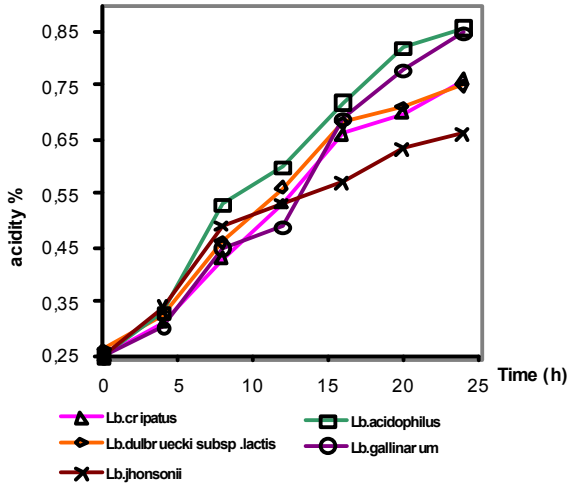

Fig. 1: Kinetic acidity evolution in pure culture of thermotolerants lactobacilli strains growth in skim milk

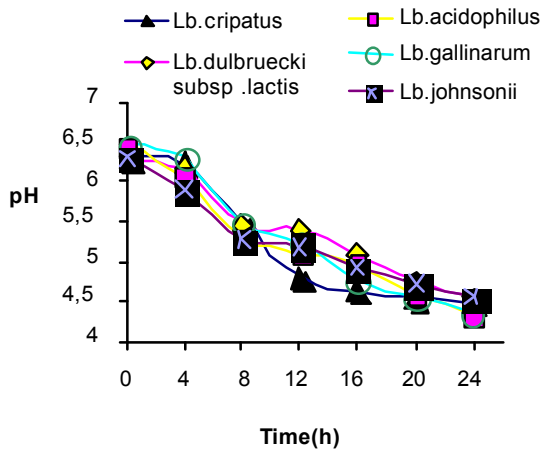

Fig. 2: pH evolution of the culture of thermotolerants lactobacilli strains in skim milk

Figure 1, shows the acidifying activities of the selected isolates and the Figure 2 shows the pH evolution of selected strains. The HPLC analysis was confirmed that the organic acid produced is lactic acid (L+). The production of lactic acid by *Lactobacillus* species in crop of the bird contributed to inhibit pathogenic bacteria and digestion of nutriments. The selected strains produced between 0.66-0.86 % of lactic acid in skim milk medium after 24 h to 45°C. The lactic acid accumulated in the medium at pH 6 seemed to be the key factor for the early beginning of the stationary phase and the low cell viability [32]. Growth of selected strains in skim milk medium was reported in Table 4 and the kinetic of growth in Figure 3. All strains growth well in skim milk and give a high density of viable cell which reach 10^{10} ufc ml^{-1}.

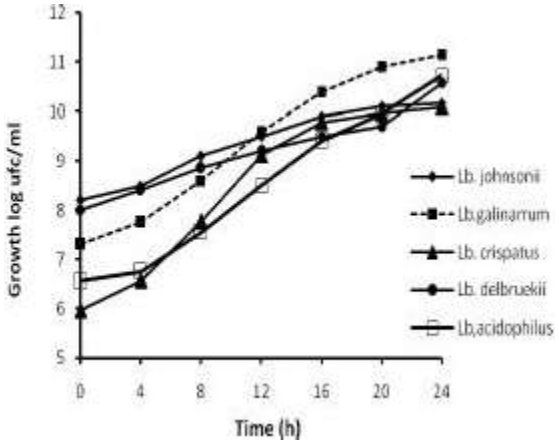

Fig. 3: Kinetic of growth of the selected thermotolerant lactobacilli strains (ufc/g) in skim milk at 45°C

In addition to resistance to low pH, adhesion to gut epithelial tissue and production of antimicrobial substances, resistance to bile toxicity is one of the criteria used to select probiotic strains that would potentially be capable of performing e?ectively in the gastrointestinal tract [33]. Therefore, to date, most of the work

These results revealed that all strains are protease positive. The proteolyse activity of these bacteria is benefit for host, by the liberation of the amino acids from feed or endogenous proteins.

The results indicate that the natural gut microflora of poultry serves as an excellent source for thermotolerants lactobacilli as potential probiotic. These strains selected on the basis of their tolerance to the consecutive exposure to hydrochloric acid and bile salts such as *Lactobacillus crispatus*, *Lactobacillus dulbruecki* subsp. *lactis*, *Lactobacillus acidophilus*, *Lactobacillus gallinarum* and *Lactobacillus johnsonii*, could be used as probiotics in the feed poultry. The optimal exploitation of them requires specific conditions of use and further, more detailed studies of their probiotic properties. Such probiotics will open a new way of progress in the field of the animal health. In recent years, the approach of using innovative strategies such as probiotics or bacteriocins for the prevention or treatment of bacterial infections has come into focus

CONCLUSION

As far as we know, this is the first report that describes the isolation and characterization of strains of thermotolerants lactobacilli isolated from Algerian chickens. The results showed that among thirteen isolates

five isolates were responded to these criteria and were identified as *Lactobacillus gallinarum*, *Lactobacillus crispatus*, *Lactobacillus dulbreuki*i, *Lactobacillus acidophilus* and *Lactobacillus jonhsonni*. These strains were homofermentatives and produced more than 0.6 % lactic acid (w/v) after 24 h of incubation and the nature of the isomers were confirmed by the High Performance Liquid Chromatographic. Some isolated strains were able to inhibit the growth of pathogenic bacteria as *Salmonella* species. Strains isolated showed also some probiotic proprieties which suggests their possible use in the food industry. However, more studies are needed to complete the isolation and the characterization of new strains of thermotolerants lactobacilli that could be beneficial for the chicken health.

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