



IDENTIFICATION OF *Lactobacillus* sp. ISOLATED FROM CHICKEN GASTROINTESTINAL TRACT WITH ANTAGONISM AGAINST PATHOGENS

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Abstract- Antibiotics use had its limitations due to the emergence of new strains caused by multi-resistant misuse of these compounds in the poultry sector. The use of probiotics is necessary to promote healthy microflora antagonistic to pathogens and screws can register as an alternative strategy proposed to protect poultry pathogens and to replace antibiotics as growth factor. The digestive microflora of two Algerian farmer's cocks were studied to isolate and identify lactic acid bacteria for use as potential probiotic in food animal production in order to protect against animal diseases as alternative to antibiotics. A total of 22 strains were isolated from different compartments as crop, caecum, ileum, jejunum, gizzard and colon to evaluate their probiotic proprieties. A use of specific primers targeting partial regions of 16S rDNA gene and sequencing of the amplified fragments preconized to 480 pb were used to identify isolates belonging to the *Lactobacillus* genus. The most diagnostic strains by analysis BLAST were *Lactobacillus acidophilus*, *Lactobacillus gallinarum*, *Lactobacillus crispatus* and *Lactobacillus johnsonii*, about 99% of homology for all strains. The study on antagonism cultures of *Lactobacillus* strains using the disc diffusion method showed inhibitory activity during interactions opposite pathogens including *Staphylococcus aureus*, *Escherichia coli* and *Salmonella typhi* by appearance of clear halos in vitro and twelve of them were selected due to their strong inhibitory action. Furthermore, the inhibitory effect of neutralized cell free supernatants of these isolates was studied and confirmed that the selected strains secreted substances witch are responsible for the antagonistic action observed on the pathogens. In addition, the acidifying power strains were determined by titratable acidity. In long term, these strains, once best characterized, could be proposed as probiotics in poultry feed.

Keywords- poultry sector, probiotics, pathogens, BLAST

Introduction

In 2006, the European Union has permanently banned the use of antibiotics as growth promoters in poultry production. Recently new prevention strategies have been proposed as alternatives to antibiotics to reduce the incidence of enteric pathogens in poultry. These strategies include the use of probiotic lactobacilli [1-3] seem to offer the most promising results. Microorganisms authorized in Europe today feed belonging to bacteria of the genus *Lactobacillus*, *Enterococcus*, *Streptococcus*, *Bifidobacterium*, *Pediococcus*, *Bacillus* and yeasts, probiotics additives are mainly used in mono- or multi-strains [4]. Probiotics has been defined by WHO as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance" [5]. However, lactic acid bacteria (LAB) have attained major attention for probiotic activity and have generally been considered as good probiotic organisms [6,7]. Among of them, lactobacilli are the most important [8]. The crop and ileum flora are mainly composed of lactobacilli in poultry [9]. LAB constitutes important members of the microbial population in chicken intestine, crop, and feces and plays an essential role in maintaining the ecological equilibrium between the different species of microorganisms inhabiting these environments. Recently, Nazef, et al [10] showed that most LAB found in poultry feces were *Streptococcus* and *Lactobacillus* species, while Souza, et al [11] reported the presence of different lactobacilli species in chicken cecum. Attempts to use LAB as a probiotic for poultry have been described. Some probiotics as *Lb. johnsonii* reduced salmonella [12], *Bifidobac-*

terium, *Enterococcus* and *pediococcus* increase animal performance parameters and modulate the composition of the microflora of the cecum [13]. Other of them, such as *Pediococcus acidilactici* or *Saccharomyces boulardii* improves resistance to coccidiosis [14]. Moreover, the authors reported that the benefits due to lactobacilli as probiotics are multiples such inhibition of *Campylobacter* [15], *Eimeria tenella* inhibition in vitro [16] and the action of *Lactobacillus johnsonii* F19785 in endemic control necrotic enteritis caused by *Clostridium perfringens* reducing economic losses and the use of antibiotics [17]. Under the use of probiotics, a legal framework has been established in 2002 by the EFSA (European Food Safety Agency) where three principal chapters main spring; identity, safety and efficacy of strain. The identification of microorganisms by DNA methods has become an essential method [18,19]; it is more discriminating compared to traditional methods of identification and classification. However, recent molecular techniques indicate that only 20 to 50% of the bacterial species present in the intestinal tract have been cultured [20]. The aim of this work was to isolate lactobacilli strains from avian microbiota and to identify those with DNA methods using genus-specific primers to an internal region of the 16S rDNA gene. In addition, probiotic properties of these isolated *Lactobacillus* sp. strains were studied to evaluate the antagonistic activity of the isolates against enteric pathogens and partially characterize substances of the cell-free supernatant responsible for antibacterial activity. Finally, the production of acid by the selected lactobacilli was estimated in degrees Dornic.

Materials and Methods

Sampling

Two native's cocks (24 weeks, weight 1.5 -1.8 kg, fed without antibiotics) were collected; they had been raised under natural farming and feeding conditions in different arid areas. Once sacrificed cocks, their gut recovered and transported to the laboratory in sterile conditions at 4°C.

Bacterial Isolation and Culture Media

Fresh intestinal contents weighing 1g, obtained after dissection of each digestive tract compartment from crop, cecum, gizzard, duodenum, jejunum and ileum was collected and homogenized with 0.9% NaCl in sterilized dionized water. Suitable dilutions of 100ul were spread in MRS Agar adjusted to pH 6.5 (De Man Rogosa and Sharp) [21] and incubated at 30°C and 45°C in anaerobic conditions for 48h to 72h for isolation of mesophilic and thermophilic lactobacilli. In order to obtain pure isolation, re-streaking on agar of the same media and conditions was done. Two references strains were used as negative and positive control successively *Streptococcus thermophilus* CNRZ1066 and *Lactobacillus delbrückii* ATCC11842. Indicator strains were collected from hospital (UHU Oran), namely *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 and *Salmonella typhi*.

Bacterial Morphology and Catalase Assay

Gram staining of each isolate was done in order to separate Gram positive, Gram negative, rod and cocci forms. Catalase reaction by 3% hydrogen peroxide (H₂O₂) and also cells morphology using phase contrast microscopy were used for primary investigation of the isolates [22,23]. Selected strains were stored at -80°C in MRS broth supplemented with 20% glycerol until further study.

Genomic DNA Extraction of Selected Bacterial Strains

The genomic DNA of selected bacteria was extracted using the modified method of Gevers, et al [18]; cells from one plate were harvested into 1 ml of sterile distilled water, centrifuged and frozen for at least 1 hr. at -20°C. The thawed pellet was washed in 1 ml TES buffer (6.7% sucrose, 50 mM Tris-HCl, pH 8.0, 1 mM EDTA) and re-suspended in 300µl STET buffer (8% sucrose, 5% Triton X-100, 50 mM Tris-HCl, pH 8.0, 50 mM EDTA). Lysis buffer 75µl (TES containing 1330 U/ml mutanolysin and 40 mg/ml lysozyme) was added and the suspension was incubated at 37°C. After addition of 40µl preheated (37°C) 20% SDS in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and glass beads, cells were vortexed for 60 s and incubated at 37°C for 10 min, followed by 10 min incubation at 65°C. TE buffer 100 µl was added and the lysate was extracted with 1v phenol/chloroform/isoamylalcohol. The phases were separated by centrifugation (12,000x g, 10 min). The aqueous phase was carefully mixed with 70 µl 5 M NaCl and 1 ml isopropanol, and DNA was precipitated on ice for at least 15 min. DNA was collected by centrifugation (12,000x g, 10 min) and the pellet washed in cold 70% ethanol. The pellet was re-suspended in 250 µl of distilled water. The DNA was verified by a 1% agarose gel electrophoresis in Tris-acetate-EDTA buffer (TAE). A DNA Smart Ladder 200 pb (Bio-Rad, Hercules, CA) was used as a size marker.

Amplification PCR

Lactobacillus genus-specific primers V3-357f (5'-CTCCTACGGG AGGCAGCAG-3'), V4-R806 (3'-GGACTACCAGGTATCTAAT-5'),

targeting 16S rRNA gene was used for the confirmation of *Lactobacillus* genus as previously described [24].

The PCR mixture (50µl) contained 100ng template DNA polymerase, 0.2 mM of dNTP, 1.0 µM of each primer and 2.0 mM MgCl₂. 100ng template DNA polymerase, 0.2 mM of dNTP, 1.0 µM of each primer and 2.0 mM MgCl₂. Amplification was performed on a PCR TECHNE TC-312 (Barloworld Scientific Ltd, stone ST 15 OSA, UK) programmed as follows: 5 min at 95°C for initial heat activation and 35 cycles of 30 sec at 94°C for denaturation, 30 s at 60°C for annealing and 1 min at 72°C for extension and 7 min at 72°C for a final extension. Amplification was verified by electrophoresis on 1.5% (w/v) agarose gel in 1 X TAE buffer using a 200 pb Smart Ladder (Bio-Rad, Hercules, CA) as a molecular weight marker. Gels were stained with ethidium bromide (1 mg·mL⁻¹) and visualized under UV light.

Sequencing of the PCR Product

Sequences were performed from PCR product in PCR TECHNE TC-312 (Barloworld Scientific Ltd, stone ST 15 OSA, UK). Sequencing reaction of 16S rDNA (V3/V4) were done using the big dye terminator sequencing Kit on the 370A DNA analyzer (Applied Biosystem).

Inhibition Spectra of *Lactobacillus* Strains against Indicator Microorganisms

Inhibitory activity was carried out according to the standard Disc diffusion method by Tadesse, et al [25] to assess the ability of strains to produce antimicrobial substances. An indicator bacteria culture (which has been adjusted to 0.5 McFarland standard), was used to lawn agar MRS plates evenly using a sterile swab. The plates were dried for 15 minutes and then used for the sensitivity test. 30 µl of lactobacilli cultures from 24-72 h were added to paper sterile discs (diameter 6mm) and placed on the MRS agar surface. The plate was then incubated at 37°C for 18 to 24 hours depending on the species of bacteria used in the test. After incubation the plates were examined for inhibition zone formation around the discs.

Preparation of cell-free filtrate

Lactobacilli strains were cultured at appropriate temperature incubated anaerobically for 48-72h in MRS broth. The cultures were then centrifuged at 3000 rpm for 15 min and supernatants were collected in conical flasks and the pH was adjusted to 6.8. The cell-free supernatant was sterilized by membrane filtration (0.22µm pore size) [26] and used for the antagonistic activity assay by means of disc diffusion method described above.

Determination of the Acidity

The acidifying power strains on MRS medium liquid was estimated by titration in a burette with sodium hydroxide using phenolphthalein as an indicator [27]. Each strain was inoculated into 10 ml of sterile skim milk (10% w/v). Pre-cultures were prepared by incubation at 30°C and 45°C until coagulation. 3% of the pre-culture was transferred aseptically in 100 ml of skim milk and 5 drops of phenolphthalein indicator were added and determination of pH was done by dipping a digital pH meter. Under magnetic stirring, the acidity was neutralized by NaOH (1/9N) until the appearance of a persistent pink color. The volume of the titrating solution was measured. The acidification kinetics was performed with regular intervals at 0, 2, 4, 6, 8, 10, and 24 hrs. The results were expressed in degrees Dornic.

Results

Morphological Characteristics of the Selected Strains

A total of 48 strains were isolated from 2 avian digestive tract compartments. Microscopic identification could determine the rod shaped cells. The gram's staining and catalase test could support the characterization of lactobacilli. After taking these criteria into account, 22 strains were found to be gram positive rod shaped, non-spore forming, and catalase negative which indicated the typical basic characteristics of lactobacilli.

Molecular identification of lactobacilli strains

A DNA band of 480 bp was amplified for 22 lactobacilli strains and for the reference strain *Lactobacillus delbruekii* ATCC 11842 used as positive control, the presence of any significant band was observed for the strain *Streptococcus thermophilus* CNRZ 1066 reference used as negative control [Fig-1].

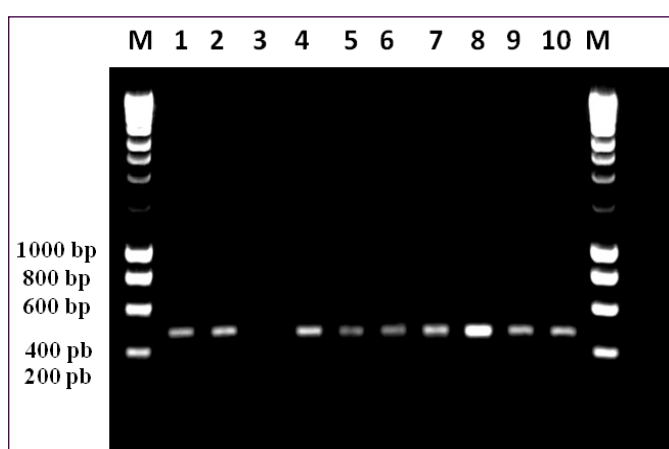


Fig. 1- 16S rDNA PCR product of 480 pb fragment on 1.5 % agarose gel

Lane M: Smart Ladder 200 bp DNA; Lane 1: LGM A14; Lane 2: LGM A15; Lane 3: *Streptococcus thermophilus* CNRZ 1066 (negative control); Lane 4: *Lactobacillus delbruekii* ATCC 11842 (positive control); Lane 5: LGM A62; Lane 6: LGM A63 strain; Lane 7: LGM A71 strain, Lane 8: LGM A72 strain; Lane 9: LGM A73; Lane 10: LGM A74, (LGM's: lactobacilli strains).

Analysis of the 16S rDNA Gene Sequence

Sequencing of different amplification product obtained was done and the scores displayed when processing sequences by BLAST via internet showed high homology with sequences deposited in the database. This high homology around 99% similarity between sequences notified the presence of *Lb. acidophilus*, *Lb. gallinarum*, *Lb. crispatus*, *Lb. johnnossi*, *Lb. fermentum* and *Lb. helveticus* for the majority of isolates examined. At this level of investigation, the selected strains cannot be assigned to the species level and are grouped only in the genus *Lactobacillus* sp.

Inhibitory Activity and Spectrum of *Lactobacillus* sp. Strains

The isolated *Lactobacillus* sp. strains were found to possess antibacterial activity. From 22 isolates, 11 strains showed an inhibitory activity against several pathogenic bacteria including *Escherichia coli* ATCC25922 [Fig-2A], *Staphylococcus aureus* ATCC25923 and *Salmonella typhi* (data not shown). The inhibition zones were estimated and the results indicated that the inhibition intensity and range varied depending on the *Lactobacillus* species assayed.

The average diameters of *Lactobacillus* sp. strains LGM A62, LGM

A63 and LGM A14 isolated respectively from cecum, jejunum and ileum against *E. coli* ATCC25922 revealed various diameters but highest than the other LGM strains [Fig-2A].

In other hand, activity of neutralized cell-free supernatant was evaluated against enteric pathogens. The strains tested showed remarkable inhibition spectra against the latter [Fig-2B].

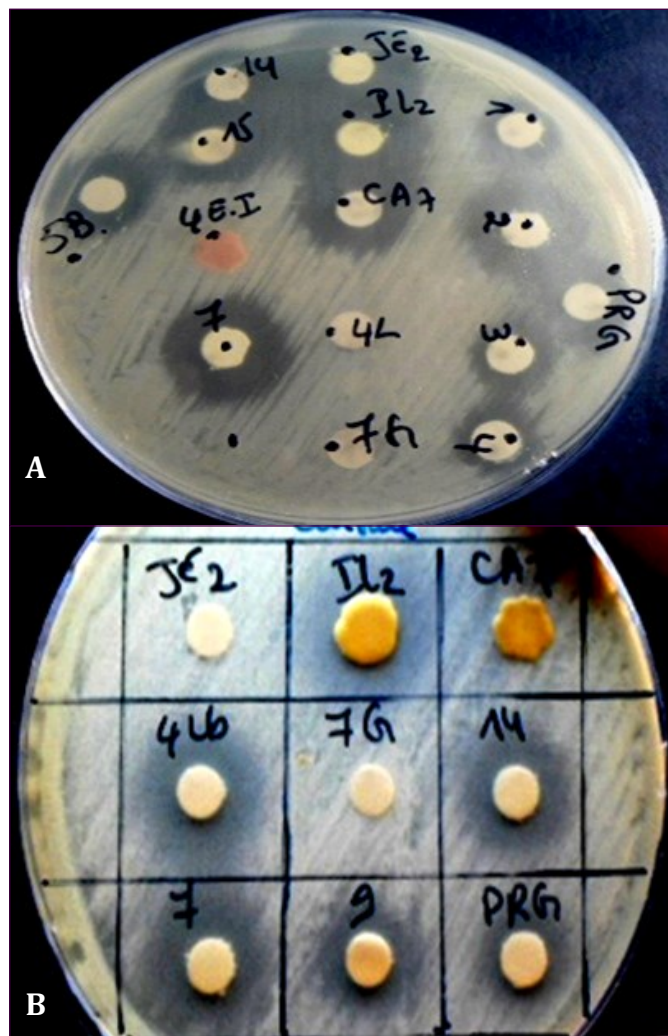


Fig. 2- Inhibition effect of *Lactobacillus* sp. strain on enteric pathogenic *Escherichia coli* ATCC25922; (A) effect of lactobacilli cultures; (B) effect of neutralized cell free supernatant.

Furthermore, to investigate whether the cause of the inhibition was due to the protein substance, buffered supernatants adjusted to pH 6.8 were used, the results obtained did not show the complete loss of antibacterial activity of the extracts which indicated that the causative inhibitory agent of some *Lactobacillus* sp. strain was the acid lactic produced. The remarkable inhibition spectra of the other strains obtained against the pathogens was may be caused by bacteriocin activity. These results agree with previously reported results [28,29]. The inhibitory capacity of LAB in vitro against pathogens seems to be a good probiotic property.

Kinetics of Acidification

Results obtained for the acidity of different samples are represented in Fig-3 & Fig-4. It can be deduced from these results that the pH vary from 6.54 to 3.77, LGM A62 was most acidifying performance

strain. It can equally be deduced from the results that the values of titratable acidity vary from 115 D°, for strain LGM A62 to 100.5 D° for strain LGMA63 and 99 D° for strain LGMA14. The samples showed an inverse relationship between titratable acidity and pH. All the species of *Lactobacillus* retained showed a high acidifying

activity, and LGMA62 strain produced the highest level of lactic acid in 24 hrs. compared to the commercial strain A74. All results are shown in Table-1. Otherwise, the work done on the acidifying power on *Lb. bulgaricus* after 6 hrs., were less efficient compared to present results [30].

Table 1- Results showing the acidifying power of lactobacilli strains

Souche	0H		2H		4H		6H		8H		24H	
	Ph	D°	Ph	D°	Ph	D°	Ph	D°	Ph	D°	Ph	D°
LGM A14	6,59	21	6,32	29	5,50	50	4,88	62	4,57	83	3,98	99
LGM A15	6,69	12	6,39	23	5,69	44	5,09	63	4,77	77	4,02	94
LGM A62	6,54	22	6,16	36	5,59	57	5,12	70	4,50	87	3,77	115
LGM A63	6,60	19	6,20	30,5	5,56	53	5,11	66	4,55	85	3,86	100,5
LGM A71	6,66	15	6,34	25	5,81	47	5,48	49	4,90	72	4,15	92
LGM A72	6,68	16	6,40	23	5,70	52	5,13	61	4,68	80	4,00	97
A74 commercial	6,61	17	6,35	24	5,60	50	5,04	65	4,60	82	4,10	96

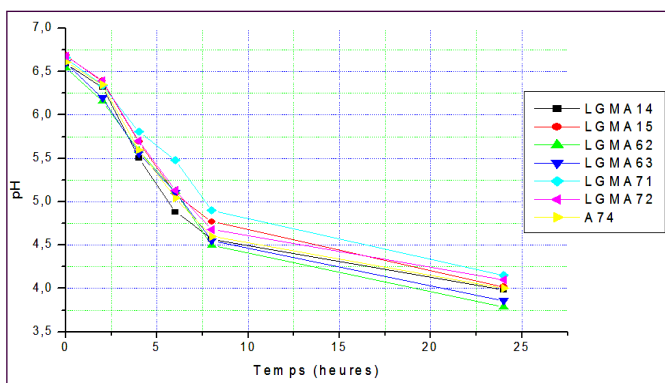


Fig. 3- Kinetics of pH evolution by different *Lactobacillus* sp. strains

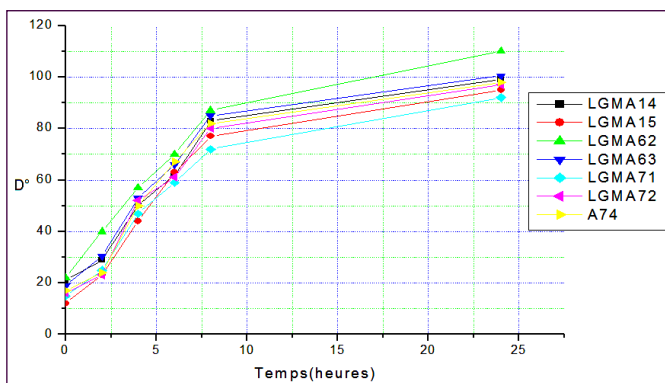


Fig. 4- Acidification kinetics of different *Lactobacillus* sp. Strains

Discussion

Understanding the microbial ecology of chicken gut is an important issue in the development of exclusive culture of probiotic and lactobacilli Microflora present in the gut microbiota of Algerian arid area farmer's coq shows an enriching diversity of microorganisms whose phenotypic profile can be exploited in the field of fight against pathogens. They represent 49,9% from the total flora, a review dedicated to the bacterial population in the digestive tract of chicken [31] reported a predominance of *Lactobacillus* strains (68.7%) in the ileum and jejunum. The strains isolated in this work were identified to *Lactobacillus* sp. by amplifying a 480 bp fragment from partial region of ADNr16S gene, these results are consistent with those of [32], this by amplifying a partial region of 250 bp between ADNr16S

regions and the 23S rDNA showed their strains belonging to the genus *Lactobacillus* sp. In the other hand, the work done in this study demonstrated that inhibition of the digestive tract lactobacilli are characterized by a wide spectrum of antagonistic action against pathogens used in this instance *Staphylococcus aureus*, *Escherichia coli* and *Salmonella typhi*. While Zang, et al [33] isolated 41 strains of *Lb. salivarius* with strong antagonism against *Salmonella* and *Campylobacter*; they did not demonstrate the nature of this antagonism. Makras, et al [34] studies have confirmed the antagonistic activity of LAB against pathogenic bacteria. The antibacterial properties of lactic acid bacteria depend on several criteria: the pH, the growth medium and the production of antibacterial substances as bacteriocins, organic acids, fatty acids and hydrogen peroxide [35]. Lactic acid plays an important role in the inhibitory capacity of LAB against several pathogenic bacteria acidification of the medium by the acid. Our findings on the production of acidity is consistent with those of Goepfert and Hicks [36] which have confirmed that although lactic acid and acetic acid produced by lactic acid bacteria are involved in the inhibition of *Staphylococcus aureus*. Sakaridis, et al [37] work showed that the LAB are characterized by a strong inhibitor against *Salmonella* sp. and *Listeria monocytogenes*. As a result, supernatant of lactobacilli were used and anaerobic condition were imposed to decrease H₂O₂ inhibitory activity, and the pH was adjusted to 6.8. So, the inhibition zone that was seen around the discs may be dependent on bacteriocin activity. According to several researchers, probiotics are well placed to take over antibiotics because of their nutritional and antimicrobial compounds very interesting abilities.

Conflicts of Interest: None declared.

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