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Isolation and Molecular Characterization of *Lactobacillus johnsonii* Isolate for its Application as an Immunomodulatory Probiotic in Chicken

Mahmoud Abd El-Mongy[1], Aya Abd El-Moez Ahmed [2*] and Nasser A. Sherif [2]

[1] *Microbial Biotechnology Department, Genetic Engineering and Biotechnology Researches Institute, University of Sadat City, Egypt.*

[2] *Central Laboratory for Evaluation of Veterinary Biologics (CLEVB). Agricultural Research Center (ARC), Abbasia, Cairo, Egypt.*

Abstract

THIS investigation aimed to isolate and characterize particular microorganisms from the intestinal tract of chickens that could function as promising probiotic agents for poultry. Probiotics exert direct influences on the digestive system while indirectly affecting the immune response in chickens. Intestinal samples were obtained from 25 disease-free chickens aged 21 days at a facility located at the Central Laboratory for Evaluation of Veterinary Biologics (CLEVB), Cairo, Egypt. Following the isolation of 25 bacterial strains, their identity was confirmed through DNA analysis. One particular strain was identified as *Lactobacillus johnsonii* through its cultivation on MRS medium and biochemical profile analysis. Molecular identification using PCR methodology was performed to confirm the *Lactobacillus johnsonii* species, subsequently validated through 16S rRNA genetic sequencing and phylogenetic evaluation. The identified strain was registered in GenBank under accession code PV616976 *L. johnsonii.* The results of this investigation validate the effective isolation and identification of an indigenous *Lactobacillus johnsonii* strain from the chicken digestive tract, demonstrating its capability as an advantageous probiotic agent for improving poultry health and immune function.

Keywords: chickens, immunity, intestine, sequencing.

Introduction

The genus Lactobacillus comprises Gram-positive, non-sporulating, Rod-shaped bacteria that are catalase-negative. These organisms demonstrate optimal growth under microaerophilic conditions. This genus is classified within the Lactobacillaceae family, encompassing 170 distinct species and 17 subspecies [1]. Although *Bifidobacterium* and *Lactobacillus* are commonly identified as lactic acid bacteria (LAB) in commercial probiotic formulations [2], the spectrum of probiotic microorganisms is extensive, encompassing *Bacillus*, *Enterococcus*, *Pediococcus*, *Leuconostoc*, *Escherichia coli*, and *Streptococcus* [3, 4]. Probiotics constitute beneficial microorganisms for human and animal health, considered safe for consumption and recognized for their positive impact on intestinal microbiome balance while inhibiting pathogenic bacterial proliferation [5]. Creating probiotics capable of efficient intestinal establishment remains essential for supporting poultry health [6].

The designation "probiotic" originates from Greek terminology meaning "supporting life" and has evolved in meaning throughout history. Probiotics are characterized as viable microbial dietary additives [7] that enhance intestinal microbial harmony in animals through stimulating immunoglobulin A (IgA) production and antimicrobial compound synthesis [8]. Incorporating *Lactobacillus acidophilus* into poultry diets yields outcomes similar to antibiotic supplementation, notably enhanced weight gain and superior feed conversion [9]. Probiotic administration enhances growth metrics, increases antioxidant potential, favorably affects blood chemistry parameters, improves carcass characteristics, and strengthens lipid stability against oxidation [10].

Probiotics demonstrate beneficial impacts on immunoglobulin M and A concentrations. As poultry naturally lack genetic capacity for polysaccharide lyase production and glycosidic bond cleavage, both essential for polysaccharide metabolism, bacterial

*Corresponding authors: Aya A. Ahmed, E-mail: ayamoez9@gmail.com, Tel.: +0201000806938
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populations serve crucial functions in enabling this digestive process [11].

Within nations such as Egypt, poultry production represents a crucial component of animal agriculture. Implementing robust disease management and prevention strategies remains essential to minimize significant financial impacts. Utilizing probiotics for bacterial disease management in poultry, while showing their capacity to enhance growth metrics and immunological responses, ensures food safety and meat quality for consumer welfare [12].

Probiotics were defined as microbial dietary additives providing beneficial impacts on host intestinal function [13]. Based on earlier research [14], individual bacterial strains require specific characteristics for consideration as viable probiotics. Lactobacilli represent frequently employed probiotic organisms with extensive historical application in food production, and *Lactobacillus* varieties are widely accepted as safe [15].

Lactobacilli naturally occur throughout various environments, such as soil systems, aquatic habitats, decomposing vegetation, and within standard intestinal microbiomes of animal species [16]. Appropriate bacterial strains for poultry probiotics must originate from indigenous intestinal microbiomes [17].

This study's purpose involved isolating and characterizing a contemporary *Lactobacillus johnsonii* strain suitable for probiotic application in poultry production.

Material and Methods

Lactobacillus Strain:

Intestinal material specimens were obtained from 25 disease-free chickens aged 21 days at the Central Laboratory for Evaluation of Veterinary Biologics (CLEVB) facility, Abbasia, Cairo, Egypt.

Isolation of the Lactobacillus Strains:

Chicken intestines were collected aseptically immediately after the chickens were euthanized .

Intestinal tissue specimens from 25 disease-free chickens (21 days old) underwent washing with sterile phosphate-buffered saline (PBS) for removing intestinal material and surface mucous, subsequently collecting adherent bacterial populations. Tissue samples were inoculated into MRS liquid medium, maintained at 37°C for 24 hours, then transferred onto MRS agar media. Following 72-hour incubation at 37°C, individual colonies were selected and re-streaked on fresh MRS plates. Bacterial isolates underwent triple subculturing on MRS agar for purification. Purified isolate stocks were preserved in 20% glycerol solution at -80°C [23]. Bacterial cell viability determination involved colony enumeration (CFU/ml) on culture plates [24].

Strain selection criteria included biosafety considerations, morphological attributes (light microscopy examination), Gram reaction, preservation stability at 4°C, and antimicrobial characteristics [25]. Culture identification involved comparing observed features with lactobacilli descriptions in Bergey's Manual of Determinative Bacteriology [26].

Identification and Preliminary Screening of Isolates:

Individual isolates cultivated overnight on MRS agar underwent Gram staining and microscopic analysis for morphological assessment. Catalase testing followed. Gram-positive, catalase-negative isolates were selected for additional investigation [23, 27]. Master mixture preparation followed Table (2) specifications.

Materials used for identification of strain:

By VITEK 2

Bacterial strain identification for lactic acid bacteria (LAB) employed the VITEK 2 automated system version 9.02 (BioMerieux, USA).

By PCR using 16S gene-

Emerald Amp GT PCR master mixture (Takara) Reference No. RR310A and Gene ruler 100 base pair DNA marker (Catalog No. SM0243) obtained from Fermentas, containing 10 distinct bands ranging 100-1000 bp, with 1.5% agarose utilized for gel electrophoresis procedures [18].

Materials used for PCR product purification:

QIAquick PCR Product Extraction system (Qiagen Inc. Valencia CA), Reference No. 28104 served for direct PCR product purification.

Material used for sequencing of the purified PCR product:

BigDye Terminator V3.1 cycle sequencing system (Perkin-Elmer, Foster City, CA) Reference No. 4336817 facilitated DNA sequencing procedures.

Centrisep purification columns, Reference No. CS-901 (100 reactions) were employed for sequence reaction cleanup. Applied Biosystems 3130 genetic analyzer (ABI, 3130, USA) utilizing BigDye Terminator V3.1 cycle sequencing system (Perkin-Elmer/Applied Biosystems, Foster City, CA), Reference No. 4336817. BLAST® (Basic Local Alignment Search Tool) analysis [20] established initial sequence similarities with GenBank entries.

Material for phylogenetic tree analysis

Sequence comparison employed CLUSTAL W multiple alignment software, version 12.1 within MegAlign component of Lasergene DNA Star package (Madison, Wisconsin, USA) [21]. Phylogenetic evaluation utilized maximum

likelihood, neighbor-joining, and maximum parsimony approaches through MEGA7 software [22].

Isolation of the Lactobacillus Strains

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Identification and Preliminary Screening of Isolates:

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Sequencing reaction:

Purified PCR products underwent bidirectional sequencing using Applied Biosystems 3130 genetic analyzer (ABI, 3130, USA) employing BigDye Terminator V3.1 cycle sequencing system (Perkin-Elmer/Applied Biosystems, Foster City, CA), Reference No. 4336817. BLAST® analysis [20] established initial sequence correspondence with GenBank records. Sequencing reactions followed manufacturer's protocols as detailed in Table (4).

Loading the sequencer machine:

Following Centrisep purification, samples received 10µl Hi-Di formamide addition, mixed thoroughly, loaded into plate wells, then subjected to thermal cycling at 95°C for 3 minutes (denaturation), followed by immediate ice cooling (preventing re-annealing). Prepared plates were analyzed using Applied Biosystems 3130 genetic analyzer (USA) with appropriate parameter settings.

Sequence Alignments and Phylogenetic analysis:

DNA sequence datasets were assembled using BioEdit sequence alignment software, version 7.0.9.0 [28]. Sequence distances were calculated through MegAlign component of Lasergene DNA Star.

Results

Isolation of Lactobacillus

Intestinal content specimens from 25 disease-free chickens (21 days old) cultivated on MRS agar exhibited Lactobacillus-characteristic morphology. Specimens produced white-colored colonies varying from small to large dimensions with circular boundaries and smooth peripheries (Figure 1). Specimens showing positive Gram reactions and displaying rod-shaped bacteria lacking non spores formes under microscopic examination (Figure 2) were provisionally classified as *Lactobacillus.*

Biochemical and Molecular Identification:

Biochemical analysis performed on catalase-negative specimens using VITEK-2 Diagnostic System yielded results presented in Table (5). Using PCR methodology and 16S rDNA sequencing with DNA sequence alignment evaluation (Figures 3, 5), one isolate was confirmed as *Lactobacillus johnsonii*. Phylogenetic reconstruction was completed (Figure 4), and the strain received GenBank registration under Accession No. PV616976 *L. johnsonii.*

Discussion

Isolating and characterizing Lactobacillus johnsonii represents a significant advancement in developing poultry probiotics, since these microorganisms directly influence digestive function and indirectly regulate avian immune responses. Probiotics additionally enhance growth metrics, elevate antioxidant status, and improve meat characteristics. Essential requirements for chicken probiotic strains include isolation from indigenous gastrointestinal microbiomes, ensuring efficient colonization capabilities crucial for supporting poultry wellness.

Probiotics reduce the need for antibiotics in poultry by improving gut health and immune function, which leads to better growth and less diseasea by strengthening the gut barrier, and modulating the gut microbiome to favor beneficial bacteria. This improves digestion, nutrient absorption, and overall flock health.

This investigation focused on isolating and characterizing a contemporary Lactobacillus johnsonii strain from chicken intestinal sources for immunomodulatory probiotic applications in poultry. Lactic acid bacteria (LAB) identification relied on morphological features and biochemical profiling. Lactobacillus species produced white-colored colonies varying in size with circular boundaries on MRS medium (Figs. 1, 2), consistent with previously reported findings [15, 29].

Isolate characterization employed phenotypic and genotypic methods following FAO/WHO (2001) guidelines. Identification testing using VITEK 2 automated system 9.02 (BioMerieux, USA) revealed the Lactobacillus isolate's fermentation capabilities for galactose, glucose, fructose, maltose, rhamnose, xylose, sucrose, and raffinose (Table 5). Molecular approaches, particularly 16S rRNA genetic sequencing, offer reliable and accurate microbial identification methods, supported by comprehensive research evidence. Genus-specific primer application for 16S rDNA amplification verified bacterial classification within the *Lactobacillus* genus [19].

The 16S rDNA PCR product underwent direct purification using QIAquick PCR Product Extraction Kit before sequencing. The 16S rRNA genetic sequencing revealed one isolate showing 99% similarity to L. johnsonii JNO12221 (GenBank reference) assigned accession number PV616976. Sequence comparison was done by CLUSTAL W multiple sequence alignment software, phylogenetic analysis was done by maximum likelihood, neighbor-joining, and maximum parsimony methods in MEGA7 [21, 22]. These results are in accordance with previous studies showing isolation of Lactobacillus johnsonii from avian gastrointestinal tract, in line with previous findings [30].

Conclusion

The study was able to isolate and identify a strain of lactic acid bacteria (LAB) of healthy chicken intestines and was identified as *Lactobacillus johnsonii*. Characterization was achieved by morphological and biochemical analysis coupled with advanced molecular techniques such as 16S rRNA genetic sequence. GenBank analysis of the DNA sequences revealed a 99 percent similarity of the *Lactobacillus johnsonii* present in the GenBank databases with an accession number of PV616976 L. *johnsonii*. The discovery is quite relevant as probiotics that have been created using intestinal microbiomes of native host animals have better colonization success rates. The use of probiotics as alternatives to antibiotics is a critical measure to deal with bacteria diseases and avoid severe economic consequences in poultry farming. This newly described strain provides a robust foundation upon which further research can be undertaken to determine its potential as an immunomodulatory probiotic with the potential to enhance poultry wellness and productivity, and safeguard consumer wellbeing.

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Funding statement

This study didn't receive any funding support

Declaration of Conflict of Interest

The authors declared that present study was performed in absence of any conflict of interest.

Ethical of approval

Not applicable.

TABLE 1. Oligonucleotide primers sequences Midland Certified Reagent Company oilgos (USA) which used in mastermix kit

Gene	Primer Sequence 5'-3'	Amplified product (bp)	Reference
Lactic acid bacteria 16S rRNA	F :TCCGGATTTATTGGGCGTAAAGCGA R : TCGAATTAAACCACATGCTCCA	411	[19]

TABLE 2. Preparation of Master Mix

Component	Volume/reaction
Emerald Amp GT PCR Mastermix (2x premix)	12.5 µl
PCR grade water	5.5 µl
Forward primer (20 pmol)	1 µl
Reverse primer (20 pmol)	1 µl
Template DNA	5 µl
Total	25 µl

TABLE 3. Cycling conditions of the different primers during cPCR according to specific Emerald Amp GT PCR mastermix (Takara) kit

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
Lactic acid bacteria 16S rRNA	94˚C 5 min.	94˚C 30 sec.	60˚C 40 sec.	72˚C 45 sec.	35	72˚C 10 min.

TABLE 4. Preparation of master mix using Big dye Terminator V3.1 cycle sequencing kit

Amount	Reagent
2µl	Big dye terminator v.3.1
1µl	Primer
From 1 to 10 µl	Template according to quality of band and concentration of DNA
Complete till to total volume become 20µl	Deionized water or PCR grade water
20 µl (Mix well, spin briefly)	Total volume

TABLE 5. Physiological and biochemical characteristics of *Lactobacillus johnsonii*

Items	Result	Items	Result
Glucose	+	Sorbitol	-
Cellobiose	-	Xylose	+
Galactose	+	Methyl Red test	-
Maltose	+	Raffinose	+
Fructose	+	Salicin	-
Aesculin	-	Sucrose	+
Mannitol	-	Inulin	-
Sorbitol	-	Hydrogen sulfide	-
Rhamnose	+	Mannitol	-

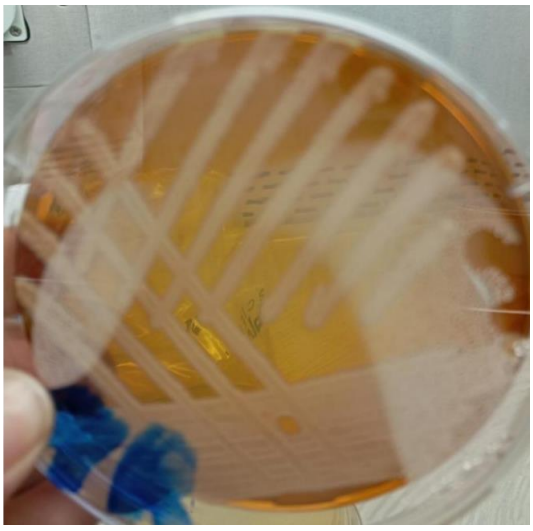

Fig. 1. Typical isolated colonies of *Lactobacillus* spp whitish colonies with smooth edges.

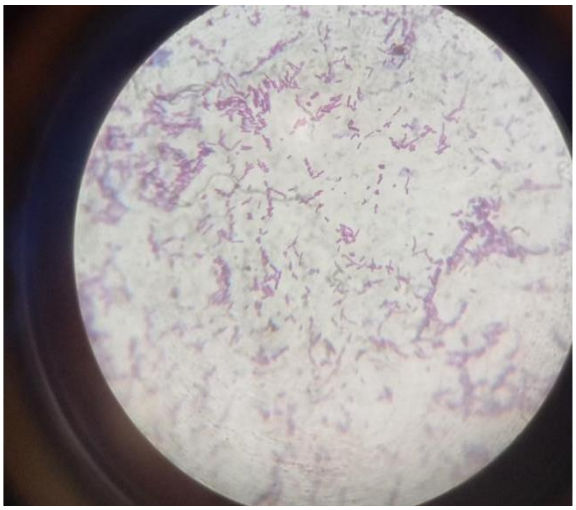

Fig. 2. Gram stain characteristics of the bacterium revealed that all of the bacteria were Gram-positive, bacilli shaped

Fig. 3. PCR products by specific genus primer

Lane 1 (L): Ladder
Lane 2 (P): Positive Control
Lane 3 (N): Negative Control
Lane 4 (S): Isolate of Lactobaciilus

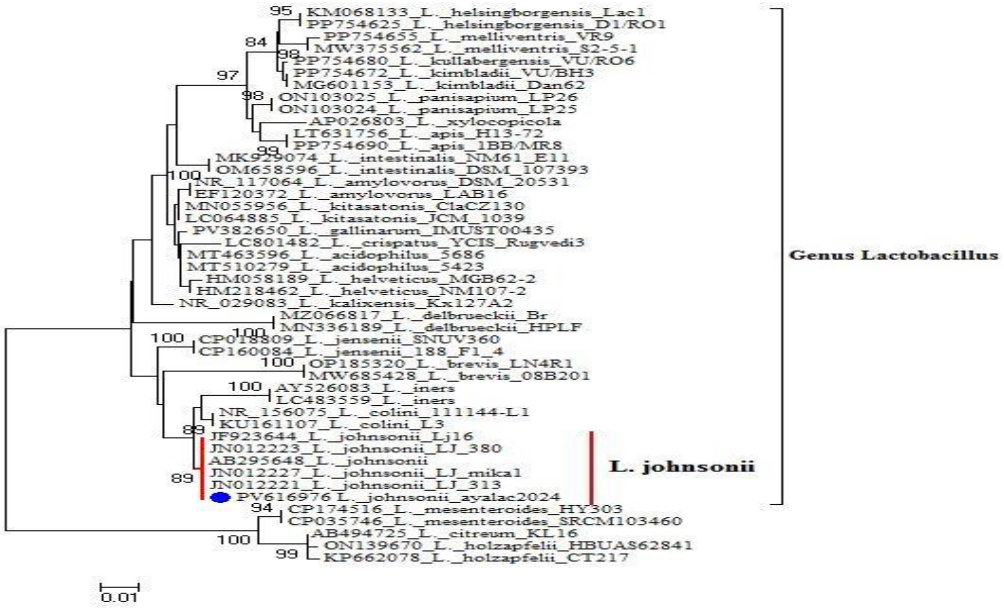

Fig. 4. Phylogenetic tree showing relationships among 16S rRNA gene sequences of species in the *Lactobacillus johnsonii group*

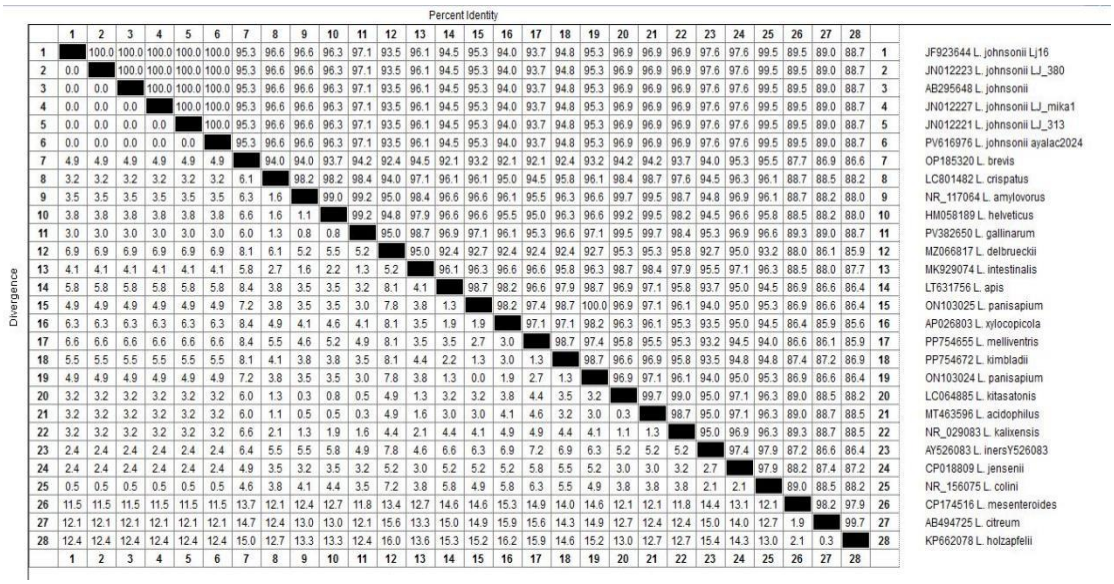

Fig. 5. Sequence distance created by the MegAlign module of Lasergene DNA Star

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عزل وتوصيف جزيئي لمعزولة لاكتوباسيلاس جونسونى لاستخدامها كبروبيوتيك محفز للمناعة في الدجاج

محمود عبد المنجي اسماعيل [1]، ايه عبد المعز عبد الفتاح احمد*[2] و ناصر عباس شريف[2]

[1] قسم التكنولوجيا الحيوية الميكروبية، معهد بحوث الهندسة الوراثية والتكنولوجيا الحيوية، جامعة مدينة السادات، مصر.
[2] المعمل المركزي للرقابه علي المستحضرات الحيويه البيطرية، مركز البحوث الزراعية، العباسية، القاهرة، مصر.

الملخص

هدفت هذه الدراسة إلى عزل وتوصيف بعض الكائنات الحية الدقيقة من القناة الهضمية للدجاج، والتي يمكن أن تُستخدم كعوامل بروبيوتيك واعدة في مجال الدواجن .تؤثر البروبيوتيك تأثيرًا مباشرًا على الجهاز الهضمي، كما تساهم بشكل غير مباشر في تعزيز الاستجابة المناعية للدجاج .تم جمع عينات معوية من 25دجاجة سليمة بعمر 21يومًا من مزرعة تابعة للمعمل المركزي لتقييم المستحضرات الحيوية البيطرية (CLEVB)بالقاهرة، مصر .تم الحصول على 25عزلة بكتيرية، وتم تحديد هويتها باستخدام التحليل الجزيئي القائم على الحمض النووي .أظهرت النتائج أن إحدى العزلات تعود لبكتيريا *Lactobacillus johnsonii*، وذلك بناءً على نموها على وسط MRSوتحليل خصائصها الكيميائية الحيوية .تم تأكيد التعريف الجزيئي باستخدام تقنية تفاعل البلمرة المتسلسل (PCR)، ثم تم التحقق من الهوية من خلال تسلسل جين 16S rRNAوالتحليل الوراثي التطوري .وقد تم تسجيل العزلة المحددة في قاعدة بيانات GenBankتحت رقم التسجيل (*Lactobacillus johnsonii*) PV616976تؤكد هذه النتائج نجاح عملية العزل والتعريف الجزيئي لعزلة وتعزيزكفاءتها *L. johnsonii*من أمعاء الدجاج، مما يبرز إمكاناتها كعامل بروبيوتيك فعال لتحسين صحة المناعية.

الكلمات الدالة : دجاج ، مناعة ، امعاء ، التسلسل.