B-5176
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RESEARCH ARTICLE Indian Journal of Animal Research

Probiotic Potential of Autochthonous *Lactobacillus* against Avian Pathogenic *Escherichia coli*

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10.18805/IJAR.B-5176

ABSTRACT

Background: The aim of current study was to investigate the probiotic potential of autochthonous *Lactobacillus* species against pathogenic *Escherichia coli* in poultry.

Method: A total of 73 samples were collected from cloacal swabs and tissues of desi and commercial chicken. The *Lactobacillus* were isolated based on cultural, biochemical and molecular teats. The probiotic potency was tested *in vitro* and antimicrobial activity and ABST was carried by Agar well diffusion and disc diffusion methods, respectively. Nucleotide sequencing was done by Sanger sequencing. Haemolysis and gelatin hydrolysis assays were used as safety testes.

Result: From 73 cloacal swabs and tissues of desi and commercial chicken, 56 *Lactobacillus* were isolated of which 22 showed high autoaggregation and hydrophobicity potential. Based on survivability at acidic pH (2.5) and bile concentrations (0.5%), 16 isolates were selected and were subjected to well diffusion assay against pathogenic *E. coli.* All the isolates showed zone of inhibition against *E. coli* ranging from 10 -18 mm. ABST revealed that all the isolates are sensitive to chloramphenicol, ampicillin and erythromycin and 75% of the isolates were resistant to streptomycin, gentamicin and vancomycin. Only one isolate out of 16 tested was non hemolytic and none of the isolates tested positive for gelatine hydrolysis. Sequencing result of selected isolate revealed 96.82% of its similarity to *Lactobacillus fermentum*.

Key words: Autochthonous *Lactobacillus*, Pathogenic *E. coli,* Probiotic potency.

INTRODUCTION

Colibacillosis is a major bacterial disease in chicken caused by *Escherichia coli* (APEC). It affects gut of poultry causing reduced weight gains and mortality, resulting in economic loss to the poultry producers. In order to prevent and control this disease, antibiotics are frequently used, which has led to an increase in antimicrobial resistance. Probiotics are a potent substitute for antimicrobial drugs, which are defined as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” (WHO, 2002). Among a variety of probiotic organisms, *Lactobacilli* are the most significant bacterial group having biological, medicinal and host immune-modulating characteristics that are generally recognized as safe. Supplementing *Lactobacillus* species in poultry improved the birds’ resistance to infectious agents like *Escherichia coli.* (Jin *et al*., 1998).

An organism to be selected as a probiotic species should have the ability to attach and colonise the intestinal lining and must be able to survive the harsh conditions of bile salt and stomach acid (Mahasneh and Abbas, 2014). choosing species-specific probiotic species from the gut microbiota is beneficial since autochthonous probiotic species are more resilient and stable in the gut of host from which they originate (Soto *et al*., 2010). Therefore, the present research was aimed to investigate the antimicrobial ability of autochthonous poultry-specific *Lactobacillus* species against pathogenic *E. coli* in order to include them in probiotic or synbiotic formulations for poultry.

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How to cite this article: Pooja, M., Srivani, M., Ramanipushpa, R.N., Aswanikumar, K. and Srilakshmi, J. (2024). Probiotic Potential of Autochthonous *Lactobacillus* against Avian Pathogenic *Escherichia coli.* Indian Journal of Animal Research. doi: 10.18805/IJAR.B-5176.

Submitted: 14-06-2023 **Accepted:** 23-01-2024 **Online:** 16-03-2024

MATERIALS AND METHODS

Collection of samples

A total of 73 samples (50 cloacal swabs and 23 tissues) from desi and commercial chicken were collected from different areas of Krishna district, Andhra Pradesh during August 2021 to February 2022 using sterile cotton swabs. The swabs were then immediately placed in LMB (L-

cystiene MRS Bromocresol green) broth and were transported to the Microbiology Laboratory of the Department of Veterinary Microbiology, NTR College of Veterinary Science, Gannavaram in anaerobic candle jar. These samples were streaked onto LMB agar plates and incubated in CO_2 incubator at 37°C for 48 h. Characteristic green centered colonies with transparent halo around colonies were tested for Gram's staining and streaked onto MRS agar and incubated in CO_2 incubator at 37°C for 48 h.

Identification of *Lactobacillus*

A series of biochemical tests which included Catalase, Indole, Methyl red, Voges-Proskauer, Citrate and Sugar fermentation were performed for identification of *Lactobacillus spp.* and the results were interpreted according to Bergey's Manual of determinative bacteriology. *Lactobacillus spp*. were further confirmed using genus specific PCR targeting 16S rRNA (Sharif *et al*., 2018). The primers used and the amplicon size was given in Table 1. The assay was performed in thermal cycler under standardized cycling conditions which include initial denaturation 95°C for 3 min, 1 cycle; denaturation 95°C for 30 sec, primer annealing 60°C for 1 min, extension 72°C for 1 min for 35 cycles; final extension 72°C for 7 min 1 cycle; hold/stand by 4°C for 10 min.

Aggregation test

Carried out as per the method of Jankoviæ *et al*. (2012) with some modifications. Overnight cultures of *Lactobacillus* were taken in MRS broth. Bacteria grown in broth were harvested by centrifugation at 3000 rotation per minute (rpm) for 5 min, then washed and resuspended in PBS to give a final optical density of 1 (about 1×10^9 CFU/mL) at 600 nm. The OD values were measured for 4 h and 24 h. Percentage of aggregation was calculated according to the following equation.

$$\%A = 1 - (A/A0) \times 100$$

Where:
A= Absorbance after 4 h incubation and 24 h of incubation.
A0= Absorbance before incubation.

Cell surface hydrophobicity

Tested as per the method of Del *et al.* (2000) with some modifications. Two ml of an overnight culture of *Lactobacillus* cultured in MRS broth were used for the test. The cultures were centrifuged and the pellet was suspended in PBS to give a final optical density of 1 (about 1×10^9 CFU/mL) at 600 nm. 500 µL of bacterial suspension was transferred into another eppendorf tube and 200 µL of xylene was added. 200 µL of n-hexadecane was added to remaining 500 µL of bacterial suspension. Hydrophobicity was calculated as the percentage decrease in the OD_{600} of the bacterial suspension due to partitioning of cells into the hydrocarbon layer.

$$\%H = \frac{(A_0\text{-}A)}{A_0} \times 100$$

Where:
A_0 and A= Absorbance before and after hydrocarbon extraction respectively.

Acid and bile tolerance tests

Carried out as per the method of Torshizi *et al.* (2008) with some modifications. Cell suspensions of *Lactobacillus* were prepared in MRS at pH 2, 3 and 6.5, which were incubated for 3 h in shaker incubator at 37°C. After 3 h each sample was streaked onto MRS agar and incubated at 37°C for 48 h anaerobically to determine the presence or absence of growth, which was used to confirm livability of the strains. The OD values of the bacterial suspensions were taken at 600 nm after 3 h.

Overnight cultures of the isolates were centrifuged for 10 min at 10,000 rpm. The pellet was suspended in MRS broth with different concentrations of ox-bile (0.1%, 0.3% and 0.5%) and incubated in shaker incubator at 37°C. Test cultures were evaluated at 2, 4 and 6 h for the presence or absence of growth by streaking samples onto MRS agar and the OD values of the bacterial suspensions were taken at 600nm for 2, 4 and 6 h.

Agar well diffusion assay

Carried out as per the method of Chen *et al.* (2018). The MH agar plates were swabbed on the surface with pathogenic *E. coli,* which was isolated from an outbreak and serotyped (O101) by National Salmonella and Escherichia Centre, Central Reasearch Institute, Kasauli. Wells of 6 mm diameter were prepared and sealed with MRS agar and cellfree supernatants from isolated *Lactobacilli* were loaded in the wells (100 µl/well). Following 24 h incubation at 37°C, inhibition zones were recorded. DMSO was used as negative control.

Antibiotic susceptibility test

ABST was carried as per the method of (Bauer *et al.,* 1966). The density of bacteria suspension was adjusted until the visible turbidity was equal to 0.5 McFarland standard. The inoculum was spread evenly over the entire surface of the plates. Subsequently, paper discs of 15 commonly used antibiotics were laid on the plates and incubated anaerobically at 37°C. The inhibition zone diameters were measured and the results were expressed in terms of

Table 1: *Lactobacillus spp.* genus specific PCR primers and sequences.

Primers	Targetgene	Sequence (5′-3′)	Amplicon size (bp)	Reference
Lac 1F	16S rRNAgene	5′-AGCAGTAGGGAATCTTCCA-3′	341 bp	Sharif *et al.* (2018)
Lab-0677R		5′-CACCGCTACACATGGAG-3′		

resistance, intermediate or susceptible, according to interpretative standards (Charteris *et al*., 1998).

Sequencing

The 16s rRNA PCR amplified products of the different *Lactobacillus* isolates were sequenced using Sanger sequencing on a 3500 genetic analyser (Applied biosystems, California, USA). To identify the species of the isolate, a similarity search was performed using BLAST in the NCBI database.

Phylogenetic analysis

The evolutionary history was inferred using the UPGMA method (Sneath and Sokal, 1973). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein ,1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. This analysis involved 16 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 347 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura *et al*., 2021).

Haemolytic activity

Columbia agar plates containing 5% (w/v) sheep blood were incubated at 37°C for 48 h and evaluated based on green zones around colonies (α-haemolysis), clear zones around colonies (β-haemolysis) and no zones around colonies (γ-haemolysis) on Columbia blood agar plates (Mangia *et al*., 2019).

Gelatine liquefaction test

A gelatine medium containing 12% gelatine (Sigma-Aldrich, St. Louis, MO, USA) was inoculated with *Lactobacillus* strains at a concentration of 1% and incubated for 48 h at 37°C. Gelatine liquefaction of strains was assessed by storing medium in a refrigerator for 24 h and checking whether gelatine was hydrolyzed or not.

RESULTS AND DISCUSSION

A total of 73 samples (50 cloacal swabs and 23 tissues) were collected from desi and commercial chicken, of which 56 (76.71%) were found positive for *Lactobacillus* by cultural methods. All these isolates exhibited change in colour of LMB broth from copper blue to green colour, green centered colonies with transparent halo around colonies on LMB agar and pale white/cream coloured colonies on MRS agar. These isolates were found to be Gram positive bacilli, negative for catalase test, IMViC tests and gelatin liquefaction test. These results were consistent with earlier reports that the isolated *Lactobacillus spp*. from the G.I. tract of chicken (Niamsup *et al.*, 2003). All the *Lactobacillus* isolates which were confirmed by cultural and biochemical tests were found to be positive for 16S rRNA gene with 341 bp.

To be efficacious, a probiotic strain must be viable at the site of action and adhere to epithelial cells and mucosal surfaces (Aziz *et al*., 2019). We found that 37 isolates had autoaggregation ≥50%, 17 showed autoaggregation potential 20-50% whereas 2 showed little autoaggregation potential ≤20% after 24 h of incubation. Strains with ≥50% autoaggregation were considered to have high autoaggregation potential (Aziz *et al*., 2019).

Cell surface hydrophobicity (CSH) is amongst the most important surface attributes controlling cell adherence to abiotic and biotic surfaces and biofilm formation (Chen *et al.,* 2018). In this study 5 isolates showed CSH values higher than 93% (strong hydrophobic) and 33 isolates showed between 66 to 93% (hydrophobic) with N-hexadecane whereas, 25 were hydrophobic and none of the isolates showed strong hydrophobicity when tested with xylene (Fig 1). Out of the 56 isolates, 22 isolates with high

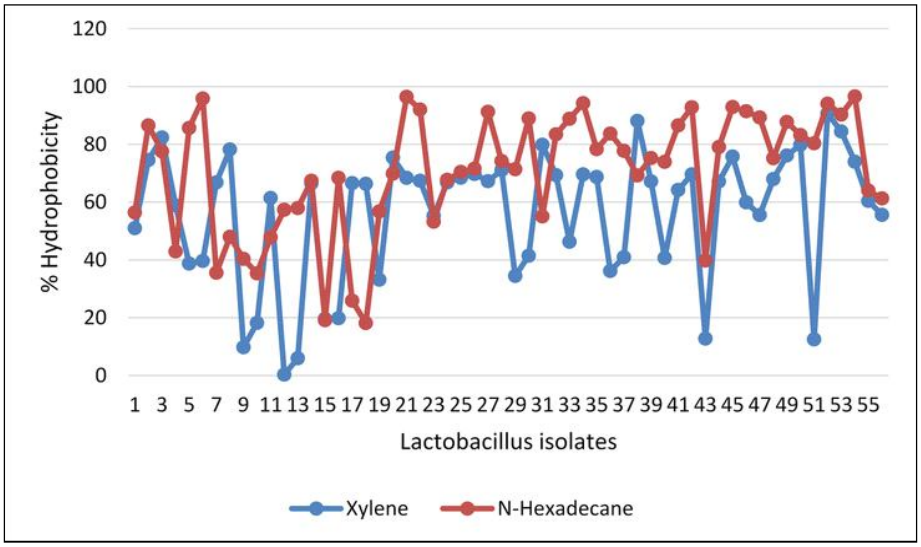


Fig 1: Hydrophobicity assay for *Lactobacillus* isolates.

autoaggregation and hydrophobicity potential were selected and subjected to acid and bile tolerance tests.

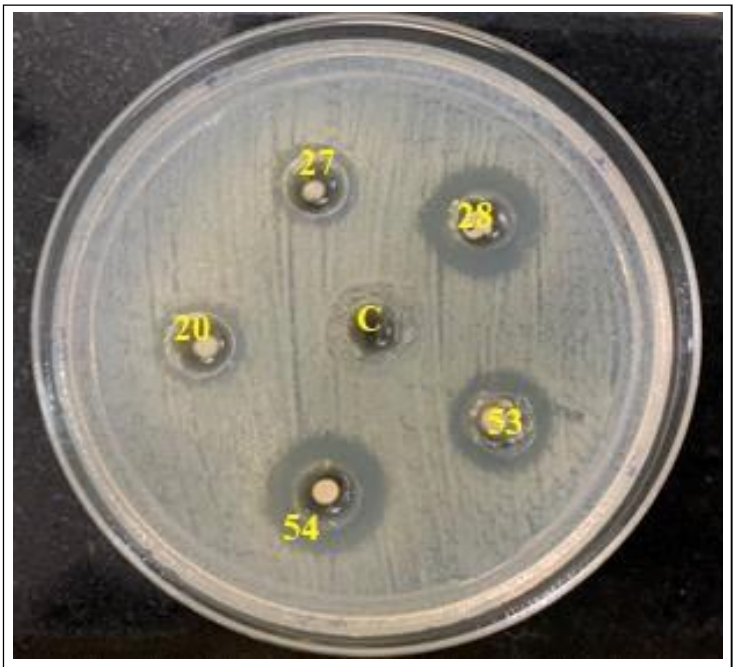
Probiotic bacteria must be able to endure the acidic and bile environment of the intestine. The pH in GIT of chicken ranges from 2.6 in proventriculus to 6.3 in large intestine (Church and Pond, 1974). We found that 16 of the tested isolates withstand pH 2.5, whereas all isolates showed high viability at pH 6.5. Bile salt tolerance is required for strains to establish and survive in the chicken intestine (Du *et al.,* 1998). In this study, all the tested 22 isolates showed viable colonies at all concentrations of ox-bile (0.1%, 0.3% and 0.5%) after 2, 4 and 6 h of incubation.

The ability of probiotic *Lactobacilli* to suppress the growth of pathogenic bacteria is one of their most essential characteristics (Ben *et al*., 2012). Sixteen isolates were selected based on viability at acidic pH and all bile concentrations and were subjected to well diffusion assay against pathogenic *E. coli*. All the tested isolates showed inhibitory activity against *E. coli* with zone of inhibition ranging from 10-18 mm (Fig 2). The inhibitory activity may be due to production of organic acids primarily lactic acid, which lowers the pH, making it unsuitable for bacterial development.

Probiotics must be tested for antibiotic sensitivity to make sure they are free of antibiotic resistance genes. (Nallala *et al*., 2017). All the 16 isolates showed sensitivity to chloramphenicol, ampicillin and erythromycin. *Lactobacillus* were sensitive to antibiotics inhibiting protein synthesis, such as clindamycin, chloramphenicol and erythromycin (Charteris *et al*., 1998). We found that 75% of the isolates exhibited resistance to nalidixic acid, vancomycin, tetracycline and streptomycin but the selected isolate is sensitive to tetracycline. High intrinsic resistance of *Lactobacillus* has been reported against streptomycin, gentamicin and vancomycin which are aminoglycosides and glycopeptide (Jose *et al*., 2015). *Lactobacillus* to be used as a feed additive must be susceptible to ampicillin, gentamicin, streptomycin, erythromycin, clindamycin, tetracycline and chloramphenicol (Marchwińska and Gwiazdowska, 2022). But, the isolates in this study exhibited resistance to vancomycin, gentamicin and streptomycin which may be chromosomally encoded and is an intrinsic feature of *Lactobacillus*, hence may not be transferable and such isolates may be used as a feed additive (Casarotti *et al*., 2017).

One of the key unfavourable metabolic activities of probiotic bacteria that contribute to increased pathogenicity is hemolytic activity and hence non-haemolytic strains should be chosen because they are considered safe (Ambalam *et al.,* 2013). One isolate among the 16 selected *Lactobacillus* isolates was non-haemolytic (γ-haemolysis) and the remaining showed haemolysis (-haemolysis). Gelatinase enzyme is considered a virulence factor as it may hydrolyze collagens that initiate an inflammatory response hence, strains which are negative for gelatinase activity must be selected (Da Silva *et al*., 2019). All the isolates in our study were negative for gelatin hydrolysis assay.

Sequencing results revealed that the selected isolate showed 96.82% of its similarity to *Lactobacillus fermentum* which was the major *Lactobacillus* species in the gastrointestinal tracts of swine and poultry and



20- 11 mm, 27-10 mm, 28-16 mm, 53-15 mm, 54-17 mm, C-Control.

Fig 2: Agar Well diffusion of *Lactobacillus* against *E. coli* Zones of inhibition.

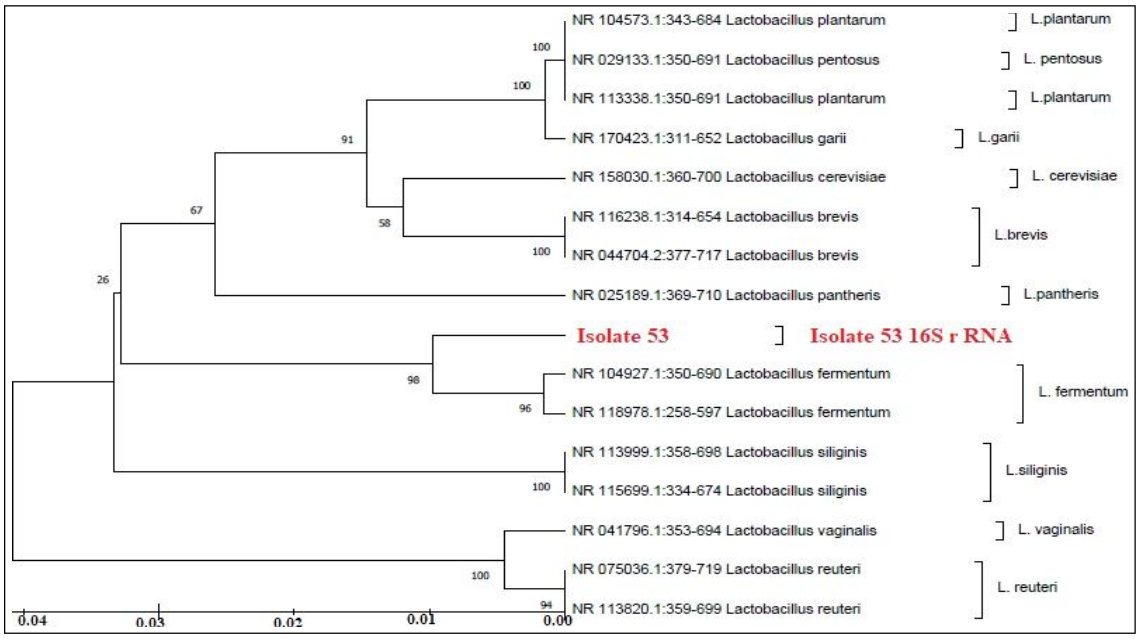


Fig 3: Evolutionary relationships of taxa-UPGMA tree.

exhibited good adherence to the intestinal epithelium, resistance to the gastric juice, bile tolerance and antagonistic effects against enteric pathogenic bacteria (Lin *et al*., 2007).

Phylogenetic tree revealed that the similarity of the selected isolate to different strains of *Lactobacillus* in descending order is as follows: 99.83% similar to *Lactobacillus fermentum* NR 104927.1:350-690, 99.79% to *Lactobacillus fermentum* NR 118978.1:258-597, 99.48% to *Lactobacillus cerevisiae* NR 158030.1:360-700, 99.45% to *Lactobacillus garii* NR 170423.1:311-652, 99.42% to *Lactobacillus reuteri* and *Lactobacillus pantheris*, 99.415% to *Lactobacillus plantarum* and *Lactobacillus pentosus*, 99.41% to *Lactobacillus brevis,* 99.39% to *Lactobacillus vaginalis* NR 041796.1:353-694, 99.32% to *Lactobacillus siliginis* (Fig 3).

CONCLUSION

We conclude that the autochthonous probiotic *Lactobacillus fermentum* species successfully suppressed pathogenic *E. coli*. Therefore, it may further be tested *in vivo* before its inclusion in probiotic/synbiotic feed additives as alternate to antibiotic growth promotor in poultry.

Conflict of interest

The authors declare that they have no conflict of interest.

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