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Original Article

Antimicrobial role of *Lactobacillus* species as potential probiotics against enteropathogenic bacteria in chickens

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Abstract

Introduction: The emergence of antimicrobial resistance among bacterial community resulted in a ban on drugs as the growth promoter in poultry feed. This situation demands to explore alternatives as food supplements with health benefit to poultry. Therefore, probiotic microorganisms, which are considered as safe and possess various health benefits can be a choice. Present study was designed to explore the probiotic potential of the isolated *lactobacillus* species in chickens.

Methodology: Out of 220 samples, 100 *Lactobacillus* species were isolated from various regions of chicken intestine. They were further characterized on the basis of morphology, staining and catalase test. Species-level identification was made by amplifying *Lactobacillus* specific 16S rRNA gene. Out of 100 isolates, 21 were selected for sequencing on the basis of band intensity.

Results: Among 21 sequences, 16 were identified as *L. paracasei* (n = 6), *L. salivarius* (n = 3), *L. johnsonii* (n = 3), and *L. agilis, L. fermentum, L. sakei,* and *L. curvatus* (n = 1 each). These strains were found to be significantly acid-tolerant with 81.68 - 85.01% survival rate at pH 2)and bile-tolerant with 81.96 -84.65% survival rate at 0.3% bile. Except three; all strains showed salt tolerance to 2% and 4% NaCl. Among 21 *Lactobacillus* strains, 6 showed good antimicrobial activities against *S. aureus*, *Salmonella* Typhimurium *and E. coli.*

Conclusion: Lactobacillus species with probiotic property can be used in poultry feed formulation for their health benefit to combat gastrointestinal infections.

Key words: Probiotics; *Lactobacillus*; DNA sequencing; antimicrobial activity; chickens.

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Introduction

Poultry industry plays a crucial role to provide protein-rich food for human consumption in terms of eggs and meat [1]. With the continuous expansion of poultry industry incidence of bacterial infections are widely increased posing health concerns to human population with huge economic losses to the poultry industry from production to marketing [2]. The most common causative agents of bacterial infections responsible for diarrhea and low poultry productivity are the serotypes of *Salmonella* and *Escherichia coli* [3]. The transmission of *Salmonella* occurs through oral-fecal route via contaminated poultry products causing typhoid, food poisoning, gastroenteritis and enteric fever [4,5,6]. The main reservoir of *Salmonella enterica* includes chickens, turkeys, ducks, parrots and coastal species. *Salmonella* infections are recognized as zoonotic infections transmitted through contaminated meat and processed poultry products [7,8].

To inhibit bacterial infections in poultry, antibiotics have been used for decades as feed supplements [9]. Thirty different classes of antibiotics (broad and short spectrum antibiotics) were used at sub-therapeutic level as feed additives in poultry industry to enhance poultry production [10]. This excessive use of antimicrobial drugs in poultry industry is one of the main causes for emerging antimicrobial resistant superbugs as well as for the hypersensitivity reactions in humans [2]. Beside responsible for antimicrobial resistance, the use of antibiotics also destroys the normal microbiota of chicken making them vulnerable to various other diseases [11].

Considering the evolving problem of antimicrobial resistant bacteria, it is important to explore an alternate having growth promoting effects in animal feed. Probiotics, a name coined by Nobel laureate Élie Metchnikoff are the microorganisms that promote health benefits upon ingestion. Probiotics have a

number of beneficial effects on the host; they can help to maintain intestinal microbial flora and aid in digestion and stimulate immune system. According to FAO/WHO, probiotics are live microorganisms which when administered in adequate amounts confer a health benefit for the host [12]. Among the most commonly used probiotics in poultry production; lactic acid bacteria (LAB) inhibit the growth of pathogens through competitive exclusion in the gastrointestinal tract therefore enhance the health of the chickens. Out of many species of Lactobacillus, some species such as *Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus casei, Lactobacillus paracasei, Lactobacillus johnsonii, Lactobacillus reuteri,* and *Lactobacillus rhamnosus*, have been used as probiotics [13].

The purpose of this study is to identify indigenous *Lactobacilli* species by molecular methods and assess their role as potential probiotic.

Methodology

Samples

This research was conducted at the Poultry Research Laboratory (PRL) Department of Physiology, University of Karachi (UoK), Karachi. A total of 220 samples were collected from intestine, caecum and cloaca of chickens from various market places and poultry farms located in the vicinity of Karachi. Sterile cotton swabs were used to collect the samples and placed in the sterile vials containing De Man, Rogosa and Sharp (MRS) broth (Oxoid, Basingstoke , UK). The samples were transported aseptically to PRL for further processes.

Isolation, preliminary screening and preservation of bacterial culture

Lactobacillus-specific De Man, Ragosa and Sharpe (MRS; Oxoid, city, UK) broth and agar were used to enrich and isolate *Lactobacillus* species by incubating at 37 ˚C for 18 to 24 hours. Isolated colonies were picked and characterized on the basis of Gram staining and catalase test [14] followed by preservation in glycerol (50:50) at -80°C for further investigations.

Acid and bile salt tolerance test

Tolerance tests were carried out as described previously [15]. In brief, acid and bile tolerance was carried out at pH 2.0, pH 3.0, pH 6.2 and bile salts (Oxoid, city, UK) of 0.2%, 0.3% respectively. Cell viability was calculated post 3-hrs incubation at 37°C by CFU estimation.

Effect of NaCl

Osmotic resistance to NaCl was evaluated according to Kobierecka *et al*., [16]. Briefly fresh culture of *Lactobacillus* was inoculated in MRS broth containing 2.0%, 4.0% and 6.5% NaCl and incubated overnight at 37 °C. Growth was evaluated on visual inspection of turbidity and results were recorded.

Polymerase Chain Reaction (PCR) and sequencing

DNA from Gram-positive and catalase-negative isolates was extracted using Promega DNA purification kit (Promega, city, USA) as per manufacturer protocol, DNA purity and integrity was measured through UV spectrophotometer and gel electrophoresis. The extracted DNA was amplified using *Lactobacillus*-specific primers (Table 1) designed in this study. PCR was carried out using GoTaq Green Master Mix (Promega, city, USA) containing 50 to 100ng of genomic using conventional thermocycler (Veriti, Applied Biosystem, city, USA) under following temperature conditions; initial denaturation 95 ˚C for 5 minutes, 35 cycles of denaturation at 95 ˚C for 30 seconds, annealing at 57 ˚C for 30 seconds and extension at 72 ˚C for 2 minutes and a final extension 72 ˚C for 7 minutes. The amplified PCR products were electrophoresed on 1.5 % agarose gel and visualized by using UV trans-illuminator ChemiDoc-It2 (UVP, Cambridge, UK) imager and vision works LS software (version 7.1).

For sequencing the PCR products were purified by using Gel purification kit (Bioline, UK), quantified and sequenced from Macrogen Inc. (Seoul, Rep. of Korea). Obtained sequences were assessed with Bioedit software (version 7.0). Forward and reverse sequences

Table 1. Primers used for 16S rRNA gene of *Lactobacillus* species.

Primer	Primer Sequence (5' → 3')	Target gene
ZF1	F- 5'GAGTGGCGGACGGGTGAGTAACACGTG3' R -5'GTTACGACTTCACCCTAATCATCTGTC3'	16S rRNA
ZF2	F- 5'CCGAACTGAGAGGTTGATC3' R-5'GTTACGACTTCACCCTAATCATCTGTC3'	16S rRNA
ZF3	F- 5'CCGAACTGAGAGGTTGA3' R- 5'GTTACGACTTCACCCTAATCATCTGTC3'	16S rRNA

aligned together with ClustalW and DNA contigs were prepared. These consensus sequences of DNA were further evaluated using BLAST of the GenBank (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and species identification were confirmed after comparison of similarity with the processed DNA sequences.

Antimicrobial activity

Antimicrobial activity of *Lactobacillus* strains was assessed by agar well diffusion method [17]. Cell free supernatant (CFS) of *Lactobacillus* strains was filter-sterilized (0.22 µm) and evaluated for antimicrobial potential against *S. aureus* (NCTC-6571)*, Salmonella* Typhimurium (ATCC-14028) and *E. coli* (ATCC-25922). Precisely a suspension of 10^8 cells of respective cultures was spread on Luria-Bertani (LB) agar; 6 mm diameter wells were punched and filled with 50 µL of *Lactobacillus* CFS. Plates were incubated at 37 ˚C; zone of inhibition was measured and characterized as potent (> 20 mm), moderate (10 to 20 mm) or no activity (< 10 mm).

Statistical analysis

One-way analysis of variance (ANOVA) was performed at significance level $p < 0.05$ for the acid and bile tolerance test using SPSS (Statistics 22, IBM). Multiple comparisons of means were assessed by Tukey's HSD post hoc test at significance level $p < 0.05$.

Results

Prevalence of Lactobacillus species, DNA sequencing and identification

Out of 21 samples sequenced, 16 were identified as *Lactobacillus* species. The prevalence of *Lactobacillus* species in intestine of chicken 56% was positive in cloaca, 25% in caeca and 19% in small intestine. Moreover the specie level differentiation was achieved through amplification and sequencing of 16S rRNA gene followed by BLAST that suggest the identification of *L. paracasei* (38%), *L. salivarius* (19%), *L. johnsonii* (19%), *L. fermentum* (6%), *L. sakei* (6%), *L. curvatus* (6%) and *L. agilis* (6%).

Acid tolerance of Lactobacillus species

The mean log CFU / mL of *Lactobacillus* strains on control, pH 2 and pH 3 groups were ranged between 8.33 ± 0.02 to 8.57 ± 0.01, 6.92 ± 0.02 to 7.10 ± 0.05 and 7.37 ± 0.47 to 7.76 ± 0.51 respectively that indicate significant difference in acid tolerance ($p < 0.05$) (Table 2). Higher survival rate was observed at pH 3, which ranged between 86.07% (ZA78Cl) to 92.24% (ZA81Cl) compared to their lower survival rate at pH 2, between 81.68% (ZA78Cl) to 85.01% (ZA62Cl) (Table 2).

Bile salt tolerance Lactobacillus species

A significant ($p < 0.05$) difference between control and bile salt group (0.2% and 0.3%) was observed among various *Lactobacillus* strains. However, mean log cfu/mL ranged from 8.33 ± 0.02 to 8.57 ± 0.02, 7.86 ± 0.02 to 8.20 ± 0.01 and 6.94 ± 0.07 to 7.16 ± 0.08, for control, 0.2% and 0.3% bile salt respectively (Table 3).

Table 2. Acid tolerance of various *Lactobacillus* strains at low pH.

S. No.	Strain ID	Log cfu / mL mean ± SD			Survival (%)		p - value
		Control	pH 2	pH 3	pH 2	pH 3	
1	ZA15SI	8.45 ± 0.03	6.95 ± 0.06	7.74 ± 0.55	82.30	91.54	0.004
2	ZA16C	8.51 ± 0.04	6.97 ± 0.08	7.37 ± 0.47	81.87	86.68	0.001
3	ZA27SI	8.49 ± 0.02	7.08 ± 0.09	7.69 ± 0.46	83.45	90.60	0.002
4	ZA30SI	8.52 ± 0.06	7.10 ± 0.05	7.76 ± 0.51	83.36	91.10	0.004
5	ZA32Cl	8.50 ± 0.02	6.97 ± 0.08	7.74 ± 0.55	81.95	91.05	0.003
6	ZA78Cl	8.57 ± 0.02	7.00 ± 0.09	7.37 ± 0.47	81.68	86.07	0.001
7	ZA67C	8.51 ± 0.04	7.00 ± 0.09	7.37 ± 0.47	82.29	86.70	0.001
8	ZA68Cl	8.50 ± 0.06	7.07 ± 0.11	7.73 ± 0.56	83.20	90.93	0.006
9	ZA74Cl	8.44 ± 0.06	6.92 ± 0.02	7.74 ± 0.55	81.97	91.71	0.003
10	ZA61C	8.55 ± 0.04	6.99 ± 0.08	7.38 ± 0.47	81.74	86.33	0.001
11	ZA64Cl	8.47 ± 0.08	7.00 ± 0.04	7.37 ± 0.47	82.66	87.03	0.002
12	ZA79Cl	8.33 ± 0.02	7.03 ± 0.08	7.41 ± 0.46	84.46	88.98	0.003
13	ZA62Cl	8.36 ± 0.09	7.10 ± 0.05	7.41 ± 0.46	85.01	88.66	0.003
14	ZA66Cl	8.51 ± 0.05	7.10 ± 0.08	7.74 ± 0.55	83.49	90.94	0.005
15	ZA80C	8.37 ± 0.11	6.92 ± 0.02	7.45 ± 0.58	82.60	88.98	0.005
16	ZA81Cl	8.34 ± 0.13	6.95 ± 0.06	7.69 ± 0.46	83.42	92.24	0.003

Cfu = colony forming unit; Data represented as Mean ± SD, each in triplicate. All parameters were calculated using one-way ANOVA; P value < 0.05 taken as significant.

Table 3. Bile salt tolerance of various *Lactobacillus* strains at different bile salt concentration.

S.No.	Strain ID	Log cfu/mL Mean ± SD			Survival (%)		P - value
		Control	BS (0.2%)	BS (0.3%)	BS (0.2%)	BS (0.3%)	
1	ZA15SI	8.45 ± 0.03	8.00 ± 0.05	7.05 ± 0.01	94.68	83.46	0.000
2	ZA16C	8.51 ± 0.04	7.95 ± 0.04	7.10 ± 0.05	93.39	83.51	0.000
3	ZA27SI	8.49 ± 0.02	7.91 ± 0.02	7.02 ± 0.12	93.18	82.68	0.000
4	ZA30SI	8.52 ± 0.06	8.01 ± 0.01	7.09 ± 0.10	94.03	83.19	0.000
5	ZA32Cl	8.50 ± 0.02	7.86 ± 0.02	7.09 ± 0.17	92.51	83.46	0.000
6	ZA78Cl	8.57 ± 0.02	7.86 ± 0.08	7.02 ± 0.08	91.72	81.96	0.000
7	ZA67C	8.51 ± 0.04	7.96 ± 0.03	7.08 ± 0.17	93.56	83.19	0.000
8	ZA68Cl	8.50 ± 0.06	8.04 ± 0.03	7.14 ± 0.10	94.61	84.06	0.000
9	ZA74Cl	8.44 ± 0.06	8.11 ± 0.02	7.10 ± 0.12	96.13	84.19	0.000
10	ZA61C	8.55 ± 0.04	7.94 ± 0.01	7.16 ± 0.08	92.80	83.75	0.000
11	ZA64Cl	8.47 ± 0.08	8.11 ± 0.03	7.13 ± 0.20	95.68	84.13	0.000
12	ZA79Cl	8.33 ± 0.02	7.96 ± 0.04	6.98 ± 0.09	95.54	83.77	0.000
13	ZA62Cl	8.36 ± 0.09	8.20 ± 0.01	7.07 ± 0.13	94.91	84.65	0.000
14	ZA66Cl	8.51 ± 0.05	7.92 ± 0.02	7.10 ± 0.15	96.36	83.45	0.000
15	ZA80C	8.37 ± 0.11	7.92 ± 0.01	6.97 ± 0.08	94.55	83.18	0.000
16	ZA81Cl	8.34 ± 0.13	7.93 ± 0.01	6.94 ± 0.07	94.96	83.25	0.000

BS = Bile salt; cfu = colony forming unit; Data represented as Mean ± SD, each in triplicate; All parameters were calculated using one-way ANOVA. P value < 0.05 taken as significant.

Effect of NaCl concentration on survival of Lactobacillus species

In the present study, NaCl resistance were variable as all strains survived in 0.34 mol/L (2.0% NaCl) and most of the strains survived in 0.68 mol/L (4.0% NaCl). However, two strains such as *L. paracasei* (ZA32Cl) and *L. johnsonii* (ZA79Cl) did not survive n 0.68 mol/L (4.0% NaCl). None of *Lactobacillus* strains survived in 1.11 mol/L (6.5% NaCl) (Table 4).

Antimicrobial activity of Lactobacillus species against enteric pathogens

All 16 isolates showed antimicrobial activity against *Staphylococcus aureus, Escherichia coli* and *Salmonella* Typhimurium. Zone of inhibition ranged from 11.5 – 22.8, 10.2 – 12.8, 10.5 – 14.3 mm for *S. aureus, S.* Typhimurium and *E. coli* respectively (Table 5). Out of 16, *L. paracasei* (ZA30SI), *L. salivarius* (ZA67C & ZA68C), *L. johnsonii* (ZA61C), *L. fermentum* (ZA66Cl) and *L. curvatus* (ZA81Cl) showed significant inhibition against Gram-positive pathogen compared to Gram-negative pathogens ($p < 0.05$).

Table 4. Resistance of *Lactobacillus* strains to NaCl

S.No.	Strain	Strain ID	Effect of NaCl		
			2.0%	4.0%	6.5%
1	*L. paracasei*	ZA15SI	++	+	-
2	*L. paracasei*	ZA16C	+++	++	-
3	*L. paracasei*	ZA27SI	++	++	-
4	*L. paracasei*	ZA30SI	+++	++	-
5	*L. paracasei*	ZA32Cl	+	-	-
6	*L. paracasei*	ZA78Cl	++	+	-
7	*L. salivarius*	ZA67C	+	-	-
8	*L. salivarius*	ZA68Cl	+++	++	-
9	*L. salivarius*	ZA74Cl	++	++	-
10	*L. jhonsonii*	ZA61C	++	++	-
11	*L. jhonsonii*	ZA64Cl	++	++	-
12	*L. jhonsonii*	ZA79Cl	+	-	-
13	*L. agilis*	ZA62Cl	++	++	-
14	*L. fermentum*	ZA66Cl	+++	++	-
15	*L. sakei*	ZA80C	++	+	-
16	*L. curvatus*	ZA81Cl	+	+	-

+ or – sign indicates growth or no growth, respectively.

Table 5. Antimicrobial activity of *Lactobacillus* strains against enteric pathogens.

S.No.	Strain	Zone of inhibition (mm), Mean ± SE				p - value
		Strain ID	*S. aureus*	*S.* Typhimurium	*E. coli*	
1	*L. paracasei*	ZA15SI	14.5 ± 0.76	11.2 ± 0.60	11.8 ± 0.73	0.033
2	*L. paracasei*	ZA16C	11.5 ± 0.50	12.0 ± 0.58	11.7 ± 0.67	0.832
3	*L. paracasei*	ZA27SI	11.7 ± 0.88	11.5 ± 0.87	11.5 ± 0.50	0.985
4	*L. paracasei*	ZA30SI	21.7 ± 0.67	12.2 ± 0.73	11.3 ± 0.67	0.000
5	*L. paracasei*	ZA32CL	18.2 ± 0.60	10.3 ± 1.20	12.3 ± 0.60	0.002
6	*L. paracasei*	ZA78Cl	11.7 ± 0.88	11.7 ± 0.44	12.7 ± 0.88	0.593
7	*L. salivarius*	ZA67C	20.8 ± 0.60	12.8 ± 0.44	14.3 ± 0.73	0.000
8	*L. salivarius*	ZA68Cl	21.2 ± 0.73	10.2 ± 0.73	12.5 ± 0.76	0.000
9	*L. salivarius*	ZA74Cl	18.3 ± 0.88	10.5 ± 1.26	12.2 ± 0.73	0.003
10	*L. jhonsonii*	ZA61C	21.8 ± 0.44	12.2 ± 0.73	11.8 ± 0.73	0.000
11	*L. jhonsonii*	ZA64Cl	12.2 ± 1.01	14.7 ± 0.88	13.2 ± 0.60	0.193
12	*L. jhonsonii*	ZA79Cl	12.2 ± 0.60	10.5 ± 1.32	10.5 ± 1.32	0.525
13	*L. agilis*	ZA62Cl	15.5 ± 0.76	12.8 ± 1.01	12.7 ± 0.88	0.115
14	*L. fermentum*	ZA66Cl	21.8 ± 0.83	11.7 ± 0.33	11.2 ± 0.93	0.000
15	*L. sakei*	ZA80C	15.7 ± 0.88	10.3 ± 1.76	12.2 ± 0.73	0.053
16	*L. curvatus*	ZA81Cl	22.8 ± 1.01	12.0 ± 1.15	14.2 ± 0.73	0.001

Data represented as Mean ± SD, each in triplicate. All parameters were calculated using one-way ANOVA; *P* value < 0.05 taken as significant.

Discussion

The emerging bacterial resistance is an outcome of extensive antimicrobial drugs use in poultry. To counter the present situation, an alternate approach is needed to minimize the use of antimicrobial agents with chicken health-promoting effects. Probiotics are the group of healthy microorganism, which has both antibacterial and growth promoting activities. Therefore, the present study was designed to explore and characterize indigenous *Lactobacillus* species among broilers.

A total of 220 samples were collected from various regions of the chicken gastrointestinal tract. These samples were streaked on *Lactobacillus* specific MRS agar, a selective media used for growth and isolation. The isolated colonies were morphologically identified as Gram-positive, small rods as reported earlier [19-20]. Many factors were considered in order to use *Lactobacillus* species as probiotics such as survivability under the dynamic changes to tolerate acid, bile salt concentration, the adherence to the epithelial surface and produce its role against other disease causing agents [21]. The main site of HCl production in chickens is proventriculus later it passes to gizzard with a pH range of 2.5 to 4.74 and feed takes 1 to 3 hours to pass through these organs depends on particle size of feed [22] while in caeca and colon the pH ranges between 5.60 to 5.83 and 6.08 to 6.58 respectively, so the probiotic must sustain in these stress conditions of gastrointestinal tract [23].

In this study *Lactobacillus* species were assessed for survival under varying environmental conditions. To classify a Lactobacillus as strong probiotic strain the isolate must be tolerant to high-acid, bile and salt concentrations as reported earlier [14, 16, 25]. All 16 strains showed survival rate of > 90% and > 80% at 0.2% and 0.3% bile salt concentration respectively (Table 4). Moreover, except for *L. paracasei* (ZA32Cl), *L. salivarius* (ZA67C) and *L. johnsonii* (ZA79Cl), all other strains tolerated NaCl concentration [2.0% (0.34 mol/L) and 4.0% (0.68 mol/L)]. However, Only 8 strains [(*L. paracasei* (n = 4), *L. salivarius* (n = 2), *L. fermentum* (n = 1) and *L. curvatus* (n=1)] showed 90% survival rate at pH 3 (Table 2). Our findings are relatively better as reported earlier where *L. reuteri, L. salivarius* and *L. animalis* survived only for 4 h at pH 3 [24, 25]. Other studies also reported higher survival rate of *Lactobacillus* species at 0.3% bile salt concentration [14, 19, 30]. The observed osmotolerance (up to 1mol/L) was comparable with the previous studies [16, 33, 34].

Antimicrobial activity was performed using agar well diffusion method. In the present study isolated and characterized *Lactobacilli* strains from the chicken were tested for antimicrobial properties against poultry pathogens. All strains showed strong to moderate antimicrobial activity against *S. aureus*, *Salmonella* Typhimurium and *E. coli*. *L. acidophilus, L. plantarum* and *L. rhamnosus* showed moderate antimicrobial activity against *E. coli* while other researchers reported a relatively higher antimicrobial activity of *Lactobacillus* species against *E. coli* [41]. Our results are in comparison with the finding, which showed moderate activity against *Salmonella* species [35]. While our results are contrary to other studies who reported higher antimicrobial activity of *Lactobacillus* species against *Salmonella* than *E. coli* [36]. Among

different *Lactobacillus* species, *L. salivarius* isolated from chicken showed better antagonism *in-vitro* against various poultry pathogens including *Salmonella spp. and E. coli* [37-39]. The antagonism observed in this study may be an outcome of immunomodulatory response through antimicrobial metabolites produced by isolated strains [40].

Conclusion

Our results suggest that conventional culture-dependent techniques are important for preliminary screening of probiotics strains however, molecular-based assessment is essential for species-level identification. Six isolated and well-characterized probiotic strain may have commercial potential to overcome the development of antibiotic resistance in poultry industry.

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