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RESEARCH ARTICLE

Isolation and *in vitro* Characterization of Anti-*Salmonella* Enteritidis Probiotic Potential of Indigenous Lactobacilli from Poultry

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A B S T R A C T

Aim of the present study was to isolate, identify and characterize new indigenous *Lactobacillus* strains with probiotic potential against *Salmonella* Enteritidis. From 84 isolated lactobacilli of indigenous poultry origin, 15 isolates were pre-selected for *in vitro* characterization on the basis of their activity (6.33±0.57-20.33±1.15 mm) against *S.* Enteritidis by well diffusion assay. All pre-selected isolates had variable tolerance to acidic pH (2, 3 and 4). All isolates also showed growth in MRS broth supplemented with 0.3, 1 and 1.8% bile salts. Isolates had varying degree of auto-aggregation (27.05±0.72 - 65.87±3.12%) and co-aggregation with *S.* Enteritidis (6.33±0.11 - 55.70±1.32%) within 2 hours. Safety profile of lactobacilli indicated that IKP23, IKP 111 and IKP 333 had no acquired antibiotic resistance. IKP 23, IKP 111 and IKP 333 were selected as potential probiotics on the basis of probiotic prerequisites and identified as *L. fermentum*, *L. fermentum* and *L. salivarius*, respectively by sequencing their partial 16S rRNA gene or 16S-23S intergenic spacer region. IKP 23, IKP 111 and IKP 333 inhibited *S.* Enteritidis (81, 99.3 and 93%, respectively) in co-culture experiments. This study insinuate that IKP 23, IKP 111 and IKP 333 have favourable probiotic potential and may be used for *in vivo* studies for the development of probiotics against *S.* Enteritidis.

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INTRODUCTION

Poultry is an important sector of Pakistan agricultural economy with 5.76, 10.4 and 1.3% contributions in agriculture, livestock and total GDP, respectively. In Pakistan, commercial poultry production was established in 1960s which is now providing a significant protein portion in diet on daily basis and employment to 1.5 million people (Hussain *et al.*, 2015). Problems influencing human health still arise from poultry. One major problem is the contamination of poultry products with enteric pathogens (Kamollerd *et al.*, 2016).

Salmonella Enteritidis is one of the most significant enteric pathogens colonizing poultry gut without clinical symptoms. Prolonged salmonellosis in chickens may lead to bacteremia followed by infection of ovaries, liver and spleen. Due to shedding in feces and vertical transmission, *Salmonella* disseminate through the entire flock and also cause post slaughter contaminations in poultry products (Deblais *et al.*, 2018). *Salmonella* result economic loss of billions every year (Wales and Davies, 2011). Thus, *Salmonella* not only obstacle poultry production but it can also enter into human food chain resulting in food poisoning and gastro-intestinal infections.

Salmonella control from farm to fork is a great challenge due to the emergence of antibiotic resistant strains (Deblais *et al.*, 2018). Traditional poultry production usually supplements feed with sub-therapeutic dose of antibiotics for the prevention and treatment of diseases, and increased growth performance. This may lead to the risk of antibiotic resistance in humans due to the consumption of poultry products, containing antibiotic residues (Kamollerd *et al.*, 2016). Thus, the use of antibiotics and chemicals should be prohibited in Pakistan and novel alternatives should be searched out for controlling infectious diseases (Abbas *et al*., 2017a, b, c; Abbas *et al*., 2018; Idris *et al*., 2017). Probiotics can also serve as an alternative approach to control *Salmonella* issue in poultry (Amara and Shibal, 2015).

Probiotics, which means "for life" have been described as the living supplement in feed which beneficially affect

the host's health, when administered in sufficient quantity (Sornplang and Piyadeatsoontorn, 2016). Many scientists and researchers suggested Lactic acid bacteria (LAB) as probiotics or bio-therapeutic agents. Probiotics are considered as an alternative to antibiotics especially in poultry industry. Many probiotics have been added in birds' diet (Popova, 2017). Moreover, probiotics have numerous other benefits in poultry industry including increased nutrient absorbance, better feed conversion ratio (FCR), accelerated production performance, strengthened gut microbiota, improved meat quality, better immune response, increased weight gain and competitive exclusions of pathogens (Gupta and Das, 2013).

Probiotics are commonly used in supplementation of human and animal foods for their health benefits (Shokryazdan *et al.*, 2017). Probiotics can prevent the growth of pathogenic bacteria, such as *Salmonella* spp. and *Escherichia coli*, in gastrointestinal tracts (Casey *et al.*, 2004). Mechanism of action of probiotics, not fully elucidated, is dependent upon properties of probiotic strains. Probiotics generally control enteric pathogens by reducing gut pH in microenvironment and secretion of antimicrobial substances (bacteriocins) and strengthening of normal flora (Wang and Gu, 2010). Probiotics also modulate mucosal and systemic immune responses and act as immune boosters (Tsai *et al.*, 2005).

Keeping in mind the importance of *S.* Enteritidis in food safety and insufficiency of local probiotics, present study was designed as a first step in a multistep project to develop probiotics targeting mitigation of *S.* Enteritidis from chicken.

MATERIALS AND METHODS

Isolation of lactobacilli: Samples including caeca (n=50), ileum (n=50) and droppings (n=50) were obtained from indigenous poultry of different areas of the Punjab, Pakistan. Lactobacilli were isolated by plating serially diluted samples (10 fold) on De Man, Rogosa and Sharpe (MRS Agar) supplemented with nystatin (100 µg/100 ml) followed by 48 hours incubation at 37°C. Distinguished colonies were selected, purified and stored in MRS broth supplemented with 15% glycerol.

Identification of lactobacilli: Preliminary identification of isolates was achieved by Gram's staining and catalase test. DNAs of isolates were extracted using GeneAll DNA extraction Kit (GeneAll Biotechnology, South Korea) following the manufacturer's instructions. Genus specific polymerase chain reaction (PCR) using primers XB5-F (5'-GCCTTGTACACACCGCCCGT-3') and LbLMA1-R (5'-CTCAAAACTAAACAAAGT-3') was used for confirmation of lactobacilli. For species identification, 16S rRNA genes of selected isolates were also amplified using universal primers 8FLP-F(5'-AGTTTGATCCTGG CTCAG-3') and XB4-R (5'-GTGTGTACAAGGCCCGG GAAC-3') as described previously (Nawaz *et al.*, 2011). Amplicons of 16S rRNA amplification (~1400 bp) or 16S-23S intergenic spacer region (~250 bp) were sequenced and submitted to NCBI for obtaining GenBank accession numbers.

Screening of lactobacilli for anti-*Salmonella* Enteritidis activity: Lactobacilli were screened for their activity against *Salmonella* Enteritidis, using well diffusion assay. Briefly, a lawn of *S.* Enteritidis (0.5 McFarland) was prepared on Mueller Hinton agar plates, wells were made and sealed with molten agar. Cell free supernatant (80-100µl) of lactobacilli was added. Plates were incubated at 37°C for 24 hours in aerobic condition followed by measuring diameter of zones of inhibition (Bao *et al.*, 2010).

***In vitro* characterization of lactobacilli for their probiotic properties**

Resistance to low pH: Tolerance of lactobacilli to low pH was determined as described previously (Asghar *et al.*, 2016). Briefly, exponentially growing isolates were re-suspended (1.5×10^8CFU/ml) in phosphate buffer saline (PBS) at different pH (2, 3, 4, 7) for 90 min. Tolerance to pH was determined by re-culturing 100 µl of pH treated bacterial suspension in 10 mL MRS broth for 24 hours at 37°C, followed by the measurement of optical density (O.D) at 600 nm.

Resistance to bile salts: Tolerance of isolates to bile salts was also determined as described previously (Asghar *et al.*, 2016). Briefly, exponentially growing cultures (1%) were inoculated in MRS broth supplemented with different concentrations of bile salts (0.3%, 1.0%, and 1.8%) for 24 hours at 37°C followed by measuring the O.D. at 600 nm.

Antibiotic susceptibility profile: Antibiotic susceptibility pattern of lactobacilli was determined by disc diffusion method on MRS agar. Lactobacilli (1 McFarland) were swabbed on MRS plates and antibiotic discs were placed. Plates were incubated at 37°C for 24 hours, in anaerobic conditions followed by measuring the diameter (mm) of zones of inhibition. Results were interpreted according to breakpoints adopted from Clinical and Laboratory Standards Institute (CLSI) guidelines or European Food Safety Authority (EFSA, 2012).

Auto-aggregation and co-aggregation: Auto-aggregation and co-aggregation of lactobacilli with *S.* Enteritidis were determined as described previously (Asghar *et al.*, 2016). For auto-aggregation freshly grown *Lactobacillus* culture was re-suspended in PBS after centrifugation at 6000 rpm, followed by incubation at 37°C. In order to observe co-aggregation, equal volumes of *Lactobacillus* and *S.* Enteritidis suspensions in PBS were mixed and incubated at 37°C. OD values were recorded at 600nm after different time intervals (1 hour and 2 hours).

Inhibition of *Salmonella* in broth culture: Selected lactobacilli (IKP23, IKP111 and IKP333) and *S.* Enteritidis were co-cultured in nutrient broth (10 ml) for 24 hours at 37°C and enumerated at different time intervals (6 hours and 24 hours) on MRS and Salmonella shigella agar, respectively to determine the effect of lactobacilli on *Salmonella* growth kinetics.

All data were expressed as Mean ± Standard deviation and compared by One way ANOVA followed by Tukey's multiple comparison test ($P\leq0.05$).

RESULTS

A total of 84 lactobacilli were isolated from indigenous poultry droppings, ileum and caeca and subjected to screening for anti-*Salmonella* Enteritidis potential. All isolates were Gram positive rods and catalase negative. Genus specific PCR amplification of ~250 bp amplicons confirmed all isolates as lactobacilli. Only 15 isolates, showing anti-microbial activity against *S.* Enteritidis, were selected for further analysis. As indicated in Table 1, IKP 111, IKP192 and IKP402 showed strongest activity (20.33±0.57 mm) against *S.* Enteritidis. All fifteen isolates showed varying degree tolerance to pH 4, 3 and 2 as presented in Table 2. IKP07 and IKP76 showed poor growth after exposure to acidity (pH 2, 3 and 4). All isolates were more tolerant to pH 4 as compared to pH 3 and pH 2. Similarly, all isolates were more resistant to 0.3% bile salts as compared to 1% and 1.8% bile salts (Table 3). Antibiotic susceptibility profiles of selected isolates against different antibiotics are presented in Table 4. Isolates containing acquired antibiotic resistance to penicillin (IKP183, IKP229 and IKP271), ampicillin (IKP94 and IKP402), erythromycin (IKP76, IKP94 and IKP402) and tetracycline (IKP138, IKP 271, IKP162 and IKP387) were considered safety risk and excluded from further analysis. IKP23, IKP111 and IKP333 had no acquired antibiotic resistance. Auto-aggregation and co-aggregation of lactobacilli with *S.* Enteritidis is given in Table 5. Isolates had varying degree of auto-aggregation (27.05±0.72 - 65.87±3.12%) and co-aggregation with *Salmonella* (6.33±0.11 to 55.70±1.32%) in two hours. IKP138 showed highest auto-aggregation (65.87±3.12%) while IKP111 has highest co-aggregation (55.70±1.32%) against *S.* Enteritidis. Co-culture experimentation of selected isolates (IKP 23, IKP 111 and IKP 333) with *S.* Enteritidis revealed highest inhibition (99.3%) by IKP 111, as indicated in Table 6. IKP 23, IKP 111 and IKP 333 were selected as potential probiotics on the basis of all probiotic prerequisites and identified as *L. fermentum*, *L. fermentum* and *L. salivarius*, respectively by sequencing. NCBI GenBank accession numbers of *L. fermentum* IKP23, *L. fermentum* IKP111 and *L. salivarius* IKP333 are MK350329, MK350330 and MK346270, respectively.

DISCUSSION

Salmonella is major pathogen of human and poultry. It can transmit horizontally as well as vertically in poultry and eventually to humans (Gole *et al.*, 2014). The prophylactic use of antibiotics to control *Salmonella* infections in poultry may cause alterations in gastrointestinal flora and promote emergence of antibiotic resistant strains (Saleem *et. al.*, 2018). It is dire need of time to search for alternative approaches like probiotics. As defined by Food and Agriculture organization (FAO), Probiotics are the live microbes which confer health benefits to host when ingested in adequate amounts. Lactic acid bacteria are more appropriate as probiotics because they have GRAS (Generally recognized as Save) status. World Health Organization (WHO) and FAO have already recommended the use of lactic acid bacteria strains as probiotics in animals and humans (Amara and Shibl, 2015).

Table 1: Antibacterial activity of selected lactobacilli against *Salmonella* Enteritidis

Isolate	Isolate source	Antimicrobial activity (mm) expressed as Mean ± S.D
IKP07	Poultry dropping	6.33±0.57[a]
IKP23	Poultry dropping	17.33±0.57[b]
IKP41	Poultry dropping	16.33±1.52[b]
IKP76	Poultry dropping	18±01[b]
IKP94	Poultry dropping	11.66±0.57[c]
IKP111	Poultry dropping	20.33±0.57[d]
IKP138	Poultry dropping	14.66±1.15[e]
IKP162	Poultry dropping	9.33±0.57[f]
IKP183	Poultry cecum	13.33±0.57[e]
IKP192	Poultry cecum	20.33±0.57[d]
IKP229	Poultry cecum	8.66±0.57[f]
IKP271	Poultry cecum	12±1[c]
IKP333	Poultry ileum	19.66±1.15[d]
IKP387	Poultry ileum	17±1[b]
IKP402	Poultry ileum	20.33±1.15[d]

[a,b,c,d,e,f] Different superscripts in different rows of same columns show statistically significant difference at P≤0.05.

Table 2: pH tolerance in lactobacilli

Lactobacilli	Optical density (Mean± Standard Deviation)			
	pH 7	pH4	pH3	pH2
IKP07	0.474±0.01[a]	0.15±0.01[b]	0.12±0.05[b]	0.135±0.03[b]
IKP23	0.584±0.04[a]	0.56±0.05[a]	0.494±0.07[a]	0.336±0.02[b]
IKP41	0.334±0.03[a]	0.321±0.03[a]	0.261±0.04[b]	0.232±0.05b[b]
IKP76	0.574±0.04 [a]	0.18±0.05[b]	0.170±0.07[b]	0.116±0.02[b]
IKP94	0.592±0.01[a]	0.54±0.09 [a]	0.486±0.07[b]	0.386±0.02[c]
IKP111	0.777±0.02 [a]	0.683±0.0[b]	0.543±0.03[c]	0.418±0.07[d]
IKP138	0.297±0.06[a]	0.290±0.05[a]	0.178±0.01[b]	0.188±0.01[b]
IKP162	0.333±0.04[a]	0.315±0.03[a]	0.214±0.07[b]	0.166±0.05[b]
IKP183	0.567±0.03[a]	0.522±0.04[a]	0.517±0.01[a]	0.445±0.02[b]
IKP192	0.498±0.04[a]	0.476±0.05[a]	0.414±0.04[a]	0.368±0.06[b]
IKP229	0.444±0.01[a]	0.456±0.07[a]	0.398±0.04[b]	0.354±0.01[b]
IKP271	0.614±0.04[a]	0.598±0.05[a]	0.534±0.07[b]	0.522±0.02[b]
IKP333	0.587±0.01[a]	0.566±0.03[a]	0.497±0.06[b]	0.479±0.03[b]
IKP387	0.602±0.05[a]	0.555±0.01[a]	0.423±0.01[b]	0.405±0.03[b]
IKP402	0.543±0.05[a]	0.476±0.04[b]	0.434±0.02[b]	0.398±0.01[c]

[a,b,c,d] Different superscripts in different columns of same row show statistically significant difference at P≤0.05.

Table 3: Growth of selected lactobacilli in MRS broth supplemented with different concentrations of bile salts within 24 hours

Lactobacilli	Optical density (Mean± Standard Deviation)			
	MRS broth	0.30%	1.0%	1.8%
IKP07	1.23±0.02[a]	0.365±0.01[b]	0.134±0.03[c]	0.121±0.09[c]
IKP23	1.40±0.08[a]	1.210±0.05[a]	0.714±0.09[b]	0.432±0.05[c]
IKP41	1.11±0.04[a]	0.411±0.01[b]	0.165±0.10[c]	0.174±0.07[c]
IKP76	1.276±0.02[a]	1.052±0.08[b]	0.654±0.06[c]	0.444±0.01[d]
IKP94	0.987±0.03[a]	0.744±0.05[b]	0.543±0.09[c]	0.298±0.05[d]
IKP111	1.31±0.02[a]	0.823±0.10 [b]	0.462±0.04[c]	0.171±0.08[c]
IKP138	1.163±0.07[a]	0.567±0.05[b]	0.397±0.09[c]	0.243±0.05[d]
IKP162	1.534±0.02[a]	1.341±0.04[b]	0.598±0.06[c]	0.519±0.01[d]
IKP183	1.007±0.06[a]	0.698±0.01b[b]	0.619±0.02[b]	0.567±0.03[c]
IKP192	1.345±0.01[a]	0.897±0.01[b]	0.555±0.03[c]	0.256±0.08[d]
IKP229	0.925±0.10[a]	0.845±0.09[a]	0.390±0.03[b]	0.314±0.02[b]
IKP271	1.211±0.02[a]	0.921±0.08[b]	0.777±0.06[c]	0.476±0.06[d]
IKP333	1.083±0.11[a]	0.611±0.04[b]	0.582±0.07[b]	0.531±0.05[b]
IKP387	1.29±0.04[a]	0.883±0.05[b]	0.546±0.02[c]	0.478±0.02[c]
IKP402	1.170±0.07[a]	0.609±0.10[b]	0.455±0.06[c]	0.286±0.07[d]

[a,b,c,d] Different superscripts in different columns of same row show statistically significant difference at P≤0.05.

Lactobacilli can kill or reduce pathogen by reduction in gut pH due to lactic acid production, secretion of antimicrobial bacteriocins and H_2O_2, competitive exclusion of pathogen and strengthening normal flora (Wang and Gu, 2010). Present study searched out 84 indigenous probiotic lactobacilli from poultry birds. Different researches have also reported the isolation of lactobacilli from poultry and fermented food products in Pakistan (Asghar *et al.*, 2016; Arif *et al.*, 2018; Saleem *et al.*, 2018).

Table 4: Antibiotic resistance profile of lactobacilli

Selected Isolates	Resistance Profile
IKP07	BAC^R, VAN^R
IKP23	VAN^R
IKP41	-
IKP76	PEN^R, ERY^R, BAC^R, FUS^R, KAN^R, IMP^R, VAN^R
IKP94	AMP^R, ERY^R , VAN^R
IKP111	VAN^R
IKP138	AMP^R, POL^R, BAC^R, CIP^R, TET^R, VAN^R
IKP162	TET^R, Van^R
IKP183	PEN^R, BAC^R, KAN^R, Van^R
IKP192	ERY^R, VAN^R
IKP229	PEN^R, AMP^R, POL^R, IMP^R, VAN^R
IKP271	PEN^R, POL^R, BAC^R, TET^R, VAN^R
IKP333	VAN^R
IKP387	CHL^R, TET^R, VAN^R
IKP402	ERY^R, POL^R, AMP^R, CHL^R, CIP^R, IMP^R, VAN^R

R: Resistant, PEN: Penicillin, AMP: Ampicillin, IMP: Imipenem, VAN: Vancomycin, BAC: Bacitracin, POL: Polymyxin B, ERY: Erythromycin, GEN: Gentamicin, KAN: Kanamycin, CHL: Chloramphenicol, TET: Tetracycline, CIP: Ciprofloxacin, FUS: Fusidic acid.

Table 5: Auto aggregation and co-aggregation of lactobacilli at different time intervals

Lactobacilli	Percent auto-aggregation and co-aggregation (Mean±S.D)			
	1 hour		2 hours	
	Auto aggregation	Co-aggregation	Auto aggregation	Co-aggregation
IKP07	16.22 ± 0.1^{a}	17.10 ± 0.30^{a}	27.05 ± 0.72^{a}	19.11 ± 0.26^{a}
IKP23	28.09 ± 1.31^{b}	23.33 ± 0.82^{b}	51.23 ± 0.93^{b}	26.20 ± 0.75^{b}
IKP41	34.25 ± 0.65^{c}	27.44 ± 0.10^{c}	45.70 ± 0.99^{c}	25.80 ± 0.46^{b}
IKP76	14.50 ± 0.55^{a}	29.05 ± 0.77^{c}	33.60 ± 1.20^{d}	24.30 ± 0.40^{b}
IKP94	29.32 ± 0.47^{b}	21.10 ± 0.94^{b}	46.53 ± 0.32^{c}	33.40 ± 0.10^{c}
IKP111	41.10 ± 0.55^{d}	40.10 ± 0.34^{d}	60.70 ± 2.44^{e}	55.70 ± 1.32^{d}
IKP138	50.10 ± 0.35^{e}	14.50 ± 0.22^{e}	65.87 ± 3.12^{f}	20.01 ± 0.68^{a}
IKP162	24.20 ± 0.47^{f}	18.57 ± 0.18^{a}	37.90 ± 1.40^{g}	24.80 ± 0.90^{b}
IKP183	$25.30\pm0.1.44^{f}$	22.19 ± 0.96^{b}	42.13 ± 0.39^{c}	25.16 ± 0.87^{b}
IKP192	14.20 ± 0.14^{a}	08.34 ± 0.43^{f}	25.40 ± 1.28^{a}	07.14 ± 0.09^{e}
IKP229	37.15 ± 0.89^{g}	17.28 ± 0.41^{a}	46.40 ± 0.98^{c}	17.90 ± 0.08^{a}
IKP271	33.30 ± 0.59^{c}	19.25 ± 0.74^{a}	44.60 ± 0.77^{c}	23.45 ± 0.88^{b}
IKP333	27.88 ± 0.20^{b}	17.88 ± 0.19^{a}	41.40 ± 0.82^{h}	26.40 ± 0.34^{b}
IKP387	15.10 ± 0.10^{a}	10.39 ± 0.52^{f}	37.40 ± 0.40^{g}	6.33 ± 0.11^{e}
IKP402	28.09 ± 0.80^{b}	16.49 ± 0.45^{a}	51.23 ± 1.50^{b}	19.87 ± 0.21^{a}

^{a,b,c,d,e,f,g,h} Different superscripts in different rows of same column show statistically significant difference at P≤0.05.

Table 6: Inhibition of *Salmonella* Enteritidis by lactobacilli in broth cultures at different time intervals

Isolates	Log_{10}CFU/ml (Mean ± S.D) of *Salmonella* co-cultured with lactobacilli				
	0 min	6 hour	24 hour	Mean Log reduction	% reduction
IKP23	5.21±0.46	5.09 ± 0.10^{a}	4.47 ± 0.55^{a}	0.74^{a}	81^{a}
IKP111	6.11±0.18	5.66 ± 0.47^{b}	3.95 ± 0.11^{b}	2.16^{b}	99.3^{b}
IKP333	5.78±0.40	5.23 ± 0.15^{c}	4.62 ± 0.45^{b}	1.16^{c}	93^{c}

^{a,b,c} Different superscripts in different rows of same column show statistically significant difference at P≤0.05.

Evaluation of *in vitro* probiotic potential is based upon tolerance to acidic pH and high bile salts concentration, adherence to epithelial cells, auto-aggregation, co-aggregation, antibiotic resistance profile, antimicrobial and antagonistic effect against potentially pathogenic bacteria (Shokryazdan *et al.*, 2017). Present study screened three potential probiotics including *L. fermentum* IKP 23, *L. fermentum* IKP 111 and *L. salivarius* IKP 333, as evident by the *in vitro* tolerance to acidic conditions (pH 2, 3 and 4) and bile salts (0.3, 1 and 1.8%) for at least 90 minutes and inhibition of *S.* Enteritidis both in co-culture and well diffusion experiments. Similar results have been also declared previously employing the same strategy (Cálix-Lara *et al.*, 2014; Dec *et al.*, 2014; Asghar *et al.*, 2016). Whereas other researchers have declared anti-*Salmonella* potential of lactobacilli using different strategies like spot test (Garriga *et al.*, 1998), inhibition of *Salmonella* invasion using HT29 human intestinal cell line (Casey *et al.*, 2004) and competitive exclusion of *Salmonella* in gut (La Ragione *et al.*, 2004). *Salmonella* inhibition capability of isolates is in accordance to the previous studies (Makras *et al.*, 2006). In this way, growth kinetics of S. Enteritidis was determined when co-cultured with lactobacilli. *L. salivarius* can produce acetic acid and lactic acid as a result of sugar fermentation in poultry feed, resulting in inhibition of pathogenic bacteria. Another *in vitro* study also declared probiotic potential of *L. salivarius* isolated from ducks (Sumarsih *et al.*, 2014). *In vivo* study by Choi *et al.*, (2011), also declared immune enhancing effect of *L. salivarius*. Complete inhibition of *H. pylori* by *L. salivarius* both in mixed cultures and infected gnotobiotic murine model has been also reported previously (Aiba *et al.*, 1998). Similarly, *L. fermentum* have been also isolated previously from fermented plant material (Morita *et al.*, 2008). Hypocholesterolemic effect of *L. fermentum* as a probiotic have been also evaluated previously (Pereira *et al.*, 2003).

Acquired antibiotic resistance in lactobacilli poses a significant threat to public health. Probiotic lactobacilli should lack resistance against antibiotics so that they may not transfer it to pathogens. Present study revealed resistant lactobacilli against tetracycline, erythromycin, ampicillin, chloramphenicol, penicillin and vancomycin which were excluded from further analysis. This resistance may be adopted during the course of time by acquiring resistant genes harboring plasmids from other resistant pathogens. Acquired antibiotic resistance have also been previously characterized in lactobacilli (Saleem *et al.*, 2018).

Auto-aggregation and co-aggregation are also important pre-requisites for the selection of probiotics. Auto-aggregation is an indicator of adhesion capacity of lactobacilli to epithelial cells, resulting in mitigation of pathogen adhesion. *Lactobacillus* strains showed significant auto-aggregation. Similar results have also been reported previously (Bao *et al.*, 2010; Asghar *et al.*, 2016). Co-aggregation of probiotics indicates their ability to inhibit pathogens. IKP 76 and IKP 387 carried good capability to co-aggregate with *S.* Enteritidis. Similar co-aggregation pattern of lactobacilli against *Salmonella* have been also reported previously (Collado *et al.*, 2007; Asghar *et al.*, 2016). Thus, these isolates can be employed as potential probiotics against *S.* Enteritidis in poultry after *in vivo* evaluations. Various studies have determined *in vivo* probiotic potential of lactobacilli (Garriga *et al.*, 1998). During *in vivo* evaluations, effect of lactobacilli on parameters *i.e.*, body weight, feed conversion ratio, immune response, phosphorus availability and gut morphology may also be examined (Asghar *et al.*, 2016).

Conclusions: It is concluded that *L. fermentum* IKP 23, *L. fermentum* IKP 111 and *L. salivarius* IKP 333 may be used as potential probiotics in poultry to control and mitigate *Salmonellae* as an alternative of antibiotics, after further *in vivo* evaluations.

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Authors contribution: MN, AAA and MDA designed the project. IK collected samples and executed experiments. MN and IK analyzed data. MN and AAA prepared manuscript. All authors revised the manuscript and approved the final version

REFERENCES

Abbas A, Iqbal Z, Abbas RZ, *et al.,* 2017a. Immunomodulatory activity of *Pinus radiata* extract against coccidiosis in broiler chicken. Pak Vet J 37:145-9.

Abbas A, Iqbal Z, Abbas RZ, *et al.,* 2017b. *In vivo* anticoccidial effects of *Beta vulgaris* (sugar beet) in broiler chickens, Microb Pathog 111:139-44.

Abbas A, Iqbal Z, Abbas RZ, *et al*., 2017c. Immunomodulatory effects of *Camellia sinensis* against coccidiosis in chickens. J Anim Plant Sci 27:415-21.

Abbas A, Abbas RZ, Masood S, *et al*., 2018. Acaricidal and insecticidal effects of essential oils against ectoparasites of veterinary importance. Boletín Latinoamericano Y Del Caribe De Plantas Medicinales Y Aromáticas 17: 441 – 452.

Aiba Y, Suzuki N, Kabir AM, *et al*., 1998. Lactic acid-mediated suppression of *Helicobacter pylori* by the oral administration of *Lactobacillus salivarius* as a probiotic in a gnotobiotic murine model. Am J Gastroenterol 93:2097-101.

Amara A and Shibl A, 2015. Role of Probiotics in health improvement, infection control and disease treatment and management. Saudi Pharm J 23:107-14.

Arif A, Nawaz M, Rabbani M, *et al*., 2018. Screening, characterization and physicochemical optimization of phosphorus solubilization activity of potential probiotic *Lactobacillus spp*. Pak Vet J 38:316-20.

Asghar S, Arif M, Nawaz M, *et al*., 2016. Selection, characterisation and evaluation of potential probiotic *Lactobacillus spp.* isolated from poultry droppings. Benef Microbes 7:35-44.

Bao Y, Zhang Y, Zhang Y, *et al*., 2010. Screening of potential probiotic properties of *Lactobacillus fermentum* isolated from traditional dairy products. Food Control 21:695-701.

Cálix-Lara TF, Rajendran M, Talcott ST, *et al*., 2014. Inhibition of *Escherichia coli* O157: H7 and *Salmonella enterica* on spinach and identification of antimicrobial substances produced by a commercial Lactic Acid Bacteria food safety intervention. Food Microbiol 38:192-200.

Casey PG, Casey GD, Gardiner GE, *et al*., 2004. Isolation and characterization of anti-Salmonella lactic acid bacteria from the porcine gastrointestinal tract. Lett Appl Microbiol 39:431-8.

Choi HJ, Kim JY, Shin MS, *et al*., 2011. Immuno-enhancing Effects of *Lactobacillus salivarius* JWS 58 and *Lactobacillus plantarum* JWS 1354 isolated from duck. Korean J Vet Res 51:281-8.

Collado MC, Meriluoto J and Salminen S, 2007. Measurement of aggregation properties between probiotics and pathogens: in vitro evaluation of different methods. J Microbiol Meth 71:71-4.

Deblais L, Helmy YA, Kathayat D, *et al*., 2018. Novel imidazole and methoxybenzylamine growth inhibitors affecting *Salmonella* cell envelope integrity and its persistence in chickens. Sci Rep 8:13381.

Dec M, Puchalski A, Urban-Chmiel R, *et al*., 2014. Screening of *Lactobacillus strains* of domestic goose origin against bacterial poultry pathogens for use as probiotics. Poult Sci 93:2462-72.

EFSA, 2012. Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. EFSA J 10:2740.

Garriga M, Pascual M, Monfort J, *et al.*, 1998. Selection of lactobacilli for chicken probiotic adjuncts. J Appl Microbiol 84:125-32.

Gole VC, Roberts JR, Sexton M, *et al*., 2014. Effect of egg washing and correlation between cuticle and egg penetration by various *Salmonella* strains. Int J food Microbiol 182:18-25.

Gupta AR and Das S, 2013. The benefits of probiotics in poultry production: an overview. Int J Livest Res 3:18-22.

Hussain J, Rabbani I, Aslam S, *et al*., 2015. An overview of poultry industry in Pakistan. Worlds Poult Sci J 71:689-700.

Idris M, Abbas RZ, Masood S, *et al*., 2017. The potential of antioxidant rich essential oils against avian coccidiosis. World's Poult Sci J 73:89-104

Kamollerd C, Surachon P, Maunglai P, *et al*., 2016. Assessment of probiotic potential of *Lactobacillus reuteri* MD5-2 isolated from ceca of Muscovy ducks. Korean J Vet Res 56:1-7.

La Ragione R, Narbad A, Gasson M, *et al*., 2004. In vivo characterization of *Lactobacillus johnsonii* FI9785 for use as a defined competitive exclusion agent against bacterial pathogens in poultry. Lett Appl Microbiol 38:197-205.

Makras L, Triantafyllou V, Fayol-Messaoudi D, *et al*., 2006. Kinetic analysis of the antibacterial activity of probiotic lactobacilli towards *Salmonella enterica* serovar Typhimurium reveals a role for lactic acid and other inhibitory compounds. Res Microbiol 157:241-7.

Morita H, Toh H, Fukuda S, *et al*., 2008. Comparative genome analysis of Lactobacillus reuteri and Lactobacillus fermentum reveal a genomic island for reuterin and cobalamin production. DNA Res15:151-61.

Nawaz M, Wang J, Zhou A, *et al*., 2011. Characterization and transfer of antibiotic resistance in lactic acid bacteria from fermented food products. Curr Microbiol 62:1081-9.

Pereira DIA, McCartney AL and Gibson GR, 2003. An in vitro study of the probiotic potential of a bile-salt-hydrolyzing lactobacillus fermentum strain, and determination of its cholesterol-lowering properties. Appl Environ Microbiol 69:4743-52.

Popova T, 2017. Effect of probiotics in poultry for improving meat quality. Curr Opin Food Sci 14:72-7.

Saleem N, Nawaz M, Ghafoor A, *et al*., 2018. Phenotypic and molecular analysis of antibiotic resistance in lactobacilli of poultry origin from Lahore, Pakistan. Pak Vet J 38:409-13.

Shokryazdan P, Faseleh Jahromi M, Liang JB, *et al*., 2017. Probiotics: from isolation to application. J Am Coll Nutr 36:666-76.

Sornplang P and Piyadeatsoontorn S, 2016. Probiotic isolates from unconventional sources: a review. J Anim Sci Technol 58:26.

Sumarsih S, Sulistiyanto B, Sutrisno C, *et al*., 2014. Characteristic of *Lactobacillus* Isolated from Pengging Duck's intestines as probiotics. Int J Poult Sci 13:47-51.

Tsai CC, Hsih HY, Chiu HH, *et al*., 2005. Antagonistic activity against *Salmonella* infection in vitro and in vivo for two *Lactobacillus* strains from swine and poultry. Int J Food Microbiol 102:185-94.

Wales A and Davies R, 2011. A critical review of *Salmonella* Typhimurium infection in laying hens. Avian Pathol 40:429-36.

Wang Y and Gu Q, 2010. Effect of probiotic on growth performance and digestive enzyme activity of Arbor Acres broilers. Res Vet Sci 89:163-7.