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IN VITRO CHARACTERIZATION OF PROBIOTIC PROPERTIES AND ANTI-CAMPYLOBACTER ACTIVITY OF *LACTOBACILLUS SPP*. ISOLATED FROM POULTRY, FERMENTED FOODS AND HUMAN FAECES

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ABSTRACT

The aim of the present study was to isolate and characterize indigenous lactobacilli targeting inhibition of *Campylobacter jejuni*. Lactobacilli (n=150) were isolated from poultry droppings (62), fermented foods (yogurt and pickles) (78) and human faeces (10). Selected isolates were identified to specie level by amplifying and sequencing their 16SrDNA universal primers. All isolates were screened for their anti-*Campylobacter* activity by well diffusion assay. Out of 150, 07 isolates (PL22, PL53, PL120, PL135, PL141, PL145 and PL149) had stable anti-*Campylobacter* activity at pH 7. Selected isolates (n=08) were further characterized for their tolerance to low pH and bile salts, antibiotic susceptibility, auto-aggregation and co-aggregation properties. All selected isolates had significant tolerance to low pH (>50% survival at pH 3), bile salts (0.3%), auto-aggregation and co-aggregation properties. Selected isolates were had no acquired resistance to penicillin, tetracycline, ampicillin and erythromycin. PL53, PL120 and PL149 showed significantly higher ($p<0.05$) reduction (> 3 log10) of *Campylobacter jejuni* ATCC 33291 in broth culture. It is concluded that *L. gallinarum* PL53, *L. paracasei* PL120 and *L. gallinarum* PL149 have probiotic properties and anti-campylobacter activity.

Keywords: Probiotics, *Lactobacillus*, *Campylobacter*, pH tolerance, antimicrobial activity.

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INTRODUCTION

Campylobacter is one of the most common causes of food borne infections worldwide. It is frequently associated with handling or consuming contaminated undercooked poultry products (Chaveerach *et al.*, 2004; Messaoudi *et al.*, 2011). *Campylobacter* is a commensal organism of avian gut and hence it can easily enter the food chain (Keener *et al.*, 2004; Dasti *et al.*, 2010; Awad *et al.*, 2018). Most common strategies to control *Campylobacter* are use of antibiotics (Shaughnessy *et al.*, 2011), vaccines (Kobierecka *et al.*, 2016; Meunier *et al.*, 2017), probiotics (Wine *et al.*, 2009; Ekmekciu *et al.*, 2017), bacteriocins (Svetoch *et al.*, 2011), plant extracts and phage therapy (Connerton *et al.*, 2011). Use of antibiotics result in emergence of resistance therefore probiotics may provide a better alternative to control bacterial pathogens including *Campylobacter* (Alagawany *et al.*, 2018). Probiotics are defined as "live microorganisms which when administered in sufficient quantities provide health benefits to host" (FAO/WHO, 2002). Lactobacilli and bifidobacteria are the most common probiotics. Lactobacilli are Gram positive, catalase negative, rod shaped lactic acid bacteria (Heravi *et al.*, 2011). Lactobacilli are common inhabitant of gastrointestinal tracts of humans, animals, poultry and fermented food (Wang *et al.*, 2014). Selection criteria for probiotics include the ability of organism to survive and grow in the physico-chemical barriers of gastrointestinal tracts along with antagonistic providing effects against pathogens and safety (Markowiak and Śliżewska, 2018). Probiotics control enteric pathogens of human or poultry by decreasing the pH of the gut and the secretion of antimicrobial substances such as bacteriocins and strengthening of normal flora (Abbas *et al.*, 2010). Probiotics competitively inhibit colonization of pathogens in poultry gut. Therefore, probiotics can reduce the shed of *C. jejuni* in environment which may lead to better quality poultry products (Saint-Cyr *et al.*, 2017). Although, anti-campylobacter probiotics have been isolated and characterized worldwide (Chaveerach *et al.*, 2004; Santini *et al.*, 2010; Messaoudi *et al.*, 2011; Wang *et al.*, 2014), data on anti-campylobacter probiotics are limited in Pakistan. Keeping in view the overall importance of poultry industry and public health in Pakistan, current study was designed to characterize indigenous probiotics against *C. jejuni*.

MATERIALS AND METHODS

Isolation and identification of lactobacilli: Lactobacilli were isolated from intestinal content or droppings of back yard poultry, human faeces and fermented foods (yogurt and pickles) on de Man Ragosa Sharpe (MRS) agar plates incubated for 48 hours at 37°C in anaerobic conditions using AnaeroGen anaerobic sachets (Oxoid) in anaerobic jars. Colonies showing characteristics of lactobacilli were selected, purified and identified by Gram's staining and catalase test. Isolates were identified to genera level by polymerase chain reaction (PCR) using XB5-F (5'-GCC TTG TAC ACA CCG CCC GT-3') and LbLMA1-R (5'-CTC AAA ACT AAA CAA AGT TTC-3') primers as described previously (Nawaz *et al.*, 2011). Isolates were identified to species level by amplifying and sequencing their 16SrDNA by 8FLP-F (5'-AGT TTG ATC CTG GCT CAG-3') and XB4-R (5'-GTG TGT ACA AGG CCC GGG AAC-3') universal primers as described previously (Asghar *et al.*, 2016).

Antimicrobial activity against *Campylobacter jejuni:* Antimicrobial activity of cell free supernatants (CFSs) without adjusting pH, CFS with pH 6.5 and heat treated CFS (80 °C for 10 min, pH 6.5) of lactobacilli was determined against *C. jejuni* (n=3) isolated from poultry procured from Department of Microbiology, University of Veterinary and Animal Sciences, Lahore and *C. jejuni* ATCC 33291 by well diffusion assay (Alfredson and Korolik, 2007; Wang *et al.*, 2014). *C. jejuni* (~1 McFarland) was swabbed on Mueller Hinton agar plates using sterile swabs. Wells were formed, sealed with molten agar and CFSs (100µl) of lactobacilli were added into wells. After 48 hours incubation in anaerobic condition at 42°C zones of inhibitions (mm) were measured.

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

Resistance to low pH: Tolerance of lactobacilli to low pH was determined by enumeration of lactobacilli isolates exposed to different pH (2,3,4,7) in normal saline (NS) for 90 minutes as described previously (Nawaz *et al.*, 2011). After low pH treatment, enumeration of lactobacilli was undertaken by plating 10 fold serial dilutions on MRS agar plates incubated at 37°C for 48 hours in anaerobic conditions using AnaeroGen anaerobic sachets (Oxoid) in anaerobic jars. Counts were expressed as Mean± S.D $\log_{10}$ CFU/mL.

Resistance to bile salt: Tolerance to different concentrations of bile salt was determined by inoculating 1% inoculum of fresh culture of lactobacilli isolates into MRS broth (pH 8.0) with different bile concentration (0.3, 1 and 1.8%). MRS tubes were incubated at 37°C for 48 hours followed by measuring optical density (OD) at 630 nm (Asghar *et al.*, 2016).

Aggregation abilities of lactobacilli: Ability of bacteria to auto-aggregate and co-aggregate with *C. jejuni* was assessed according to the method described by Collado *et al.*(2008). Briefly, overnight cultures of selected isolates grown in MRS broth (pH 6.5) were centrifuged at 6000 rpm for 30 minutes and the pellet was washed thrice with sterile PBS. Pellets were re-suspended in PBS (pH7.0) to prepare inoculum equivalent to 1 McFarland standard. To determine the percentage autoaggregation, bacterial suspensions (1mL) were incubated at 37°C and OD values were monitored at 0 min and 24 hours. The formula used for the calculation of auto-aggregation: $1-(A_t/A_o) \times 100$, where A_t represents absorbance at specific time and A_o absorbance at 0 minute. Co-aggregation was determined by mixing equal volumes (1mL each) of selected isolates re-suspended in PBS (~1McFarland), and *C. jejuni* followed by measuring OD at 0 min and 24 hours. The formula used for the calculation of co-aggregation: $(((A_{prob}+ A_{path})/2-A_{mix})/ A_{prob}+ A_{path}) \times 100$, where A_{prob} represents absorbance of probiotic control alone, A_{path} absorbance of pathogen alone while A_{mix} represents absorbance of mixture of pathogen and probiotic strain.

Antibiotic susceptibility testing: Antibiotic resistance against penicillin, ampicillin, ceftazidime, imipenem, meropenem, aztreonam, vancomycin, bacitracin, polymyxin B, erythromycin, gentamicin, kanamycin, chloramphenicol, tetracycline, ciprofloxacin and fusidic acid was determined by disc diffusion method (Campana *et al.*, 2017; Sharma *et al.*, 2017). Exponentially growing selected isolates, re-suspended in 0.85% NaCl (~1 McFarland) were inoculated on lactic acid bacteria susceptibility test medium using sterile swabs and antibiotic discs were placed on agar plates using disc dispenser. Plates were incubated at 37°C for 48hours followed by measuring the zone of inhibition (mm) and isolates were designated as sensitive, resistant and intermediate following the guidelines of European Food Safety Authority ($EFSA_b$, 2012)

Inhibition of *C. jejuni* in co-culture experiment: Inhibition of *C. jejuni* was determined by co-culturing with lactobacilli isolates by following the method by Wang *et al.*(2014). Equal volume (1 mL each, ~10^6CFU/mL) of fresh culture of selected isolates of lactobacilli and *C. jejuni* were inoculated in Brain heart infusion (BHI) broth and incubated under anaerobic condition at 42°C. *C. jejuni* was enumerated on charcoal cefoperazone deoxycholate agar plates while lactobacilli was enumerated on MRS agar plates at 0 min, 2 hours, 6 hours, 24 hours and 48 hours of incubation.

Statistical Analysis: Enumeration data was presented as Mean± S.D $\log_{10}$ CFU/mL and compared by one way ANOVA followed by Tukey's multiple comparison test at $p< 0.05$ by GraphPad prism 5.

RESULTS

Isolation and Identification of lactobacilli: A total of 150 lactobacilli were isolated from poultry (n=62), fermented foods (n=78) and human faeces (n=10). All the isolates were catalase negative, Gram positive rod-shaped bacteria. All isolates showed *Lactobacillus* genus specific amplification (~250bp). For specie identification 16S rDNA was amplified and PCR amplicons were sequenced by using both the forward and the reverse primers (8FLP and XB4). Sequences were submitted in NCBI GenBank. PL 53, PL 120 and PL 149 were identified as *L. gallinarum*, *L. paracasei* and *L. gallinarum*, respectively. GenBank accession numbers are these isolates are MK182967- MK182969, respectively.

Antimicrobial activity of lactobacilli: Out of 150 isolates, sixteen had activity against *C. jejuni* (indigenous isolates) and *C. jejuni (*ATCC 33291). Out of sixteen, eight isolates (PL 22, PL53, PL88, PL120, PL135, PL141, PL145 and PL149) had activity against *C. jejuni* after adjusting pH of its CFS (17.67, 14.67, 10.33, 10.67, 20.33, 15.33, 10.67, 16.00mm, respectively) and seven isolates (PL 22, PL53, PL120, PL135, PL141, PL145 and PL149) had activity after boiling its CFS at 80°C for 10 minutes (15.67, 14.67, 10.67, 15.67, 14.66, 9.67, 16.33mm, respectively) (table 2).

In-vitro evaluation for probiotic potential

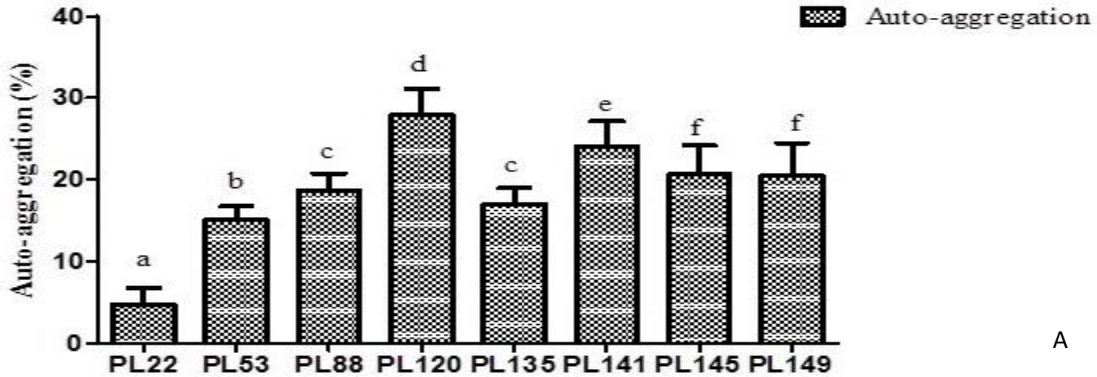
pH tolerance: Selected isolates showed different level of tolerance to pH 3, 4 and 7 as presented in Table 3. PL 120 and Pl 145 exhibited maximum $\log_{10}$ CFU/ml values after 90 minutes pH stress in saline solution with pH 3 and 4. PL120, PL145 and PL149 showed significantly higher ($p<0.05$) tolerance to pH 2 (5.75±0.52, 5.25±0.55 and 5.05±0.55 $\log_{10}$ CFU/mL values, respectively) as compared to PL22, PL53, PL88, PL135 and PL141 (4.80±0.50, 3.33±0.54, 4.46±0.22, 4.90±0.32 and 3.10±0.20 $\log_{10}$ CFU/mL values, respectively).

Tolerance to bile salt: Tolerance of selected isolates to different concentration of bile salts (0.3, 1 and 1.8%) is presented in Table 4. All isolates were resistant and able to grow at 0.3% bile salt. PL22, PL120 and PL135 showed significantly higher ($p<0.05$) growth (1.31±0.01, 1.28±0.09 and 1.05±0.05 O.D, respectively) as compared to PL53, PL88, PL141, PL145 and PL149 (0.95±0.01, 0.77±0.07, 0.41±0.10, 0.41±0.15 and 0.81±0.16 O.D, respectively). PL120 also showed growth (O.D 0.58±0.10) in MRS broth supplemented with 1% bile salt. None of the isolates had significant growth (O.D > 0.3) at 1.8% bile salt.

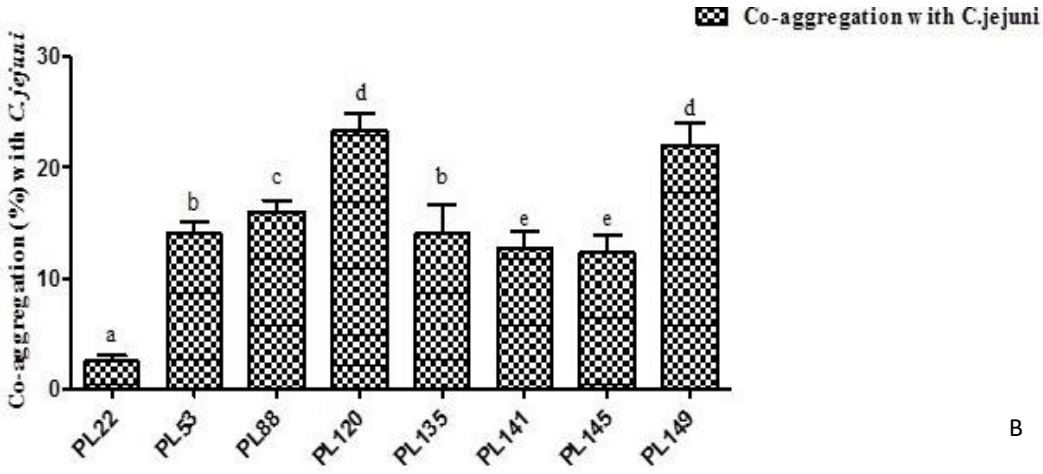
Auto-aggregation and co-aggregation test: Auto-aggregation and co-aggregation of lactobacilli with *C. jejuni* is represented in Fig.1. PL120 showed the highest ($p<0.05$) auto-aggregation (27.83%) as compared to PL22, PL53, PL88, PL135, PL141, PL145 and PL149 (4.78, 15.12, 18.67, 17.00, 24.00, 20.67 and 20.53%, respectively). PL120 and PL149 showed the highest ($p<0.05$) co-aggregation potential with *C. jejuni* (23. 33 and 2.00%, respectively) while PL22 showed the lowest (2.5%) as compared to PL53, PL88, PL135, PL141 and PL145 (14.02, 16.00, 14.00, 12.73 and 12.33%, respectively).

Antibiotic susceptibility testing: Antibiotic sensitivity profile of the selected isolates (n=8) is given in Table 5. PL53, PL120 and PL149 were sensitive to penicillin, ampicillin, erythromycin, gentamicin, chloramphenicol and tetracycline indicating absence of acquired resistance against these antibiotics. PL22 was resistant to penicillin, ampicillin, erythromycin and tetracycline while PL88 and PL135 were resistant to tetracycline. Resistance to bacitracin, tetracycline, ciprofloxacin and fusidic acid was variable.

Inhibition of *Campylobacter jejuni* growth co-culture with lactobacilli isolates: Inhibition of *Campylobacter jejuni* growth co-culture with lactobacilli isolates is presented in Figure 2. The lactobacilli isolate PL 53, PL 120 and PL 149 showed >3 log reduction in *C. jejuni* count in co-culture experiment while lactobacilli count increased considerably after 48 hours. PL53 showed the highest ($p<0.05$) log reduction against *C. jejuni* (3.37) as compared to PL120 (3.19).

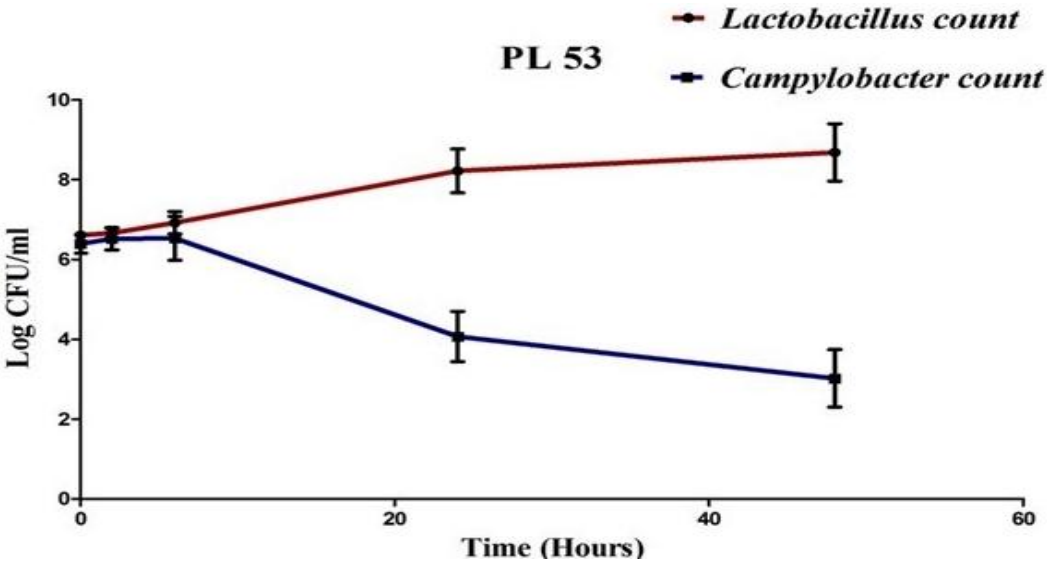


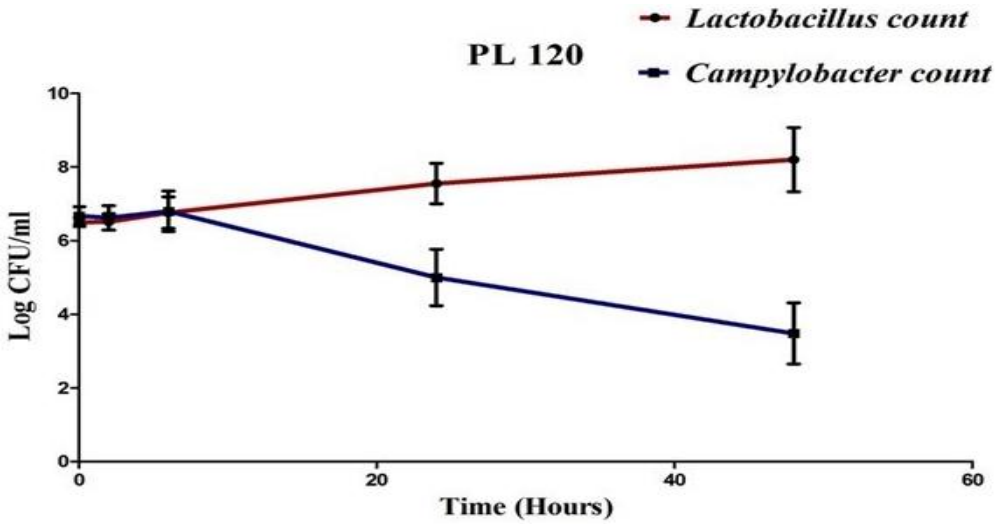
A



B

Figure 1. A) Auto-aggregation (%) of selected lactobacilli strains B) Co-aggregation (%)of selected lactobacilli strains with *C. jejuni* [a,b,c,d,e,f]: Isolates having different superscript differ significantly (p<0.05)





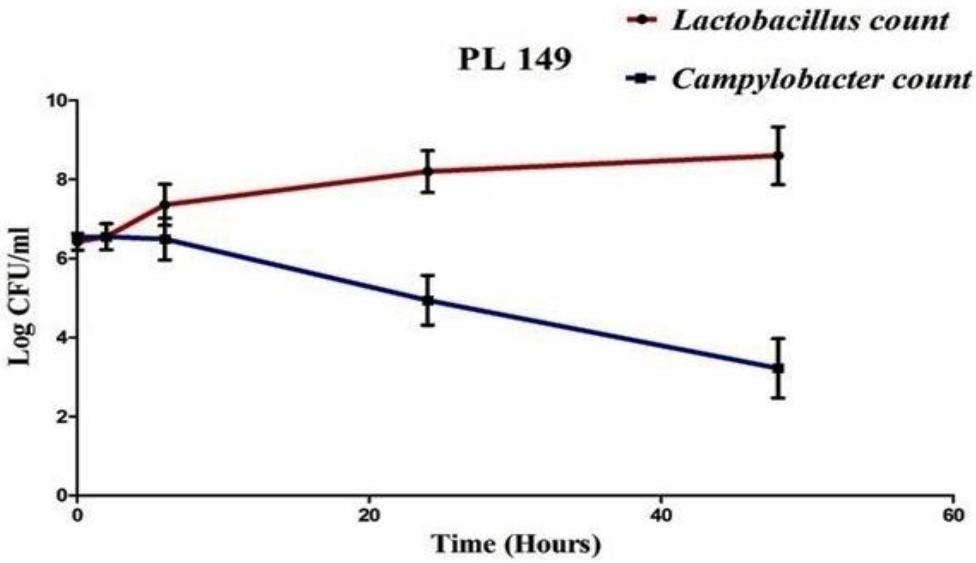


Figure 2. Log CFU/ml count of lactobacilli and *Campylobacter jejuni*in co-culture experiment (PL 53, PL 120 and PL 149, respectively).

Table 1.Antimicrobial activity of lactobacilli isolates against *Campylobacter jejuni* (n=4) expressed in mm.

Isolate	Source	Antimicrobial activity (mm)				
		C. jejuni (18 C)	*C. jejuni* (19 C)	*C. jejuni (23 C)*	*C. jejuni* (ATCC 33291)	Mean ZOI activity ±S.D
PL 05	Poultry	9.33±0.58	8.67±0.58	8.50±0.50	10.23±0.25	9.18±0.83
PL 13	Poultry	8.43±0.51	9.33±0.58	8.77±0.40	15.17±0.76	10.43±2.92
PL 22	Poultry	21.67±1.53	10.43±0.51	24.00±1.00	19.00±1.00	18.78±5.44
PL 33	Poultry	11.33±1.19	16.57±1.25	14.00±1.00	12.33±0.58	13.56±2.25
PL 53	Poultry	15.00±1.00	13.68±0.58	23.33±1.53	15.67±1.15	16.92±4.06
PL 77	Poultry	8.33±0.58	12.68±1.15	9.00±1.00	8.33±0.58	9.58±2.02
PL 88	Poultry	23.33±1.53	14.00±1.00	23.67±1.53	12.67±1.15	18.42±5.45
PL 93	Poultry	26.67±1.53	23.67±1.53	14.33±1.53	23.67±1.53	22.08±5.02
PL 102	Yogurt	8.33±0.58	10.00±1.00	16.67±1.53	9.67±1.53	11.17±3.54
PL 105	Yogurt	9.67±1.15	9.67±1.53	8.67±1.15	26.33±1.53	13.58±7.79
PL 111	Yogurt	11.67±1.53	10.67±1.15	9.67±0.58	15.33±1.53	11.83±2.48
PL 120	Human	30.33±1.53	14.67±1.53	10.67±2.08	15.67±0.58	17.83±7.90
PL 135	Human	31.67±1.53	24.00±2.65	20.33±2.08	21.00±2.65	24.25±5.08
PL 141	Human	19.67±1.53	16.33±1.53	14.67±1.53	15.00±1.00	16.42±2.39
PL 145	Human	20.33±1.53	16.67±2.08	23.33±3.79	11.33±2.52	18.50±3.70
PL 149	Poultry	27.33±0.58	16.67±1.53	13.00±2.00	16.67±0.58	18.042±5.71

S.D= standard deviation
ZOI= Zone of inhibition

Table 2. Activity of cell free supernatants (without adjusting pH, pH 6.5 and after heat treatment, pH 6.5) of selected isolates against *Campylobacter jejuni* (ATCC 33291) as determined by well diffusion assay expressed as Mean ± SD.

Isolate	Antimicrobial activity (mm) (Mean ±SD)		
	Without adjusting pH	With pH 6.5	After heat treatment (pH 6.5)
PL 22	18.33±0.58	17.67±0.58	15.67±0.58
PL 53	15.33±0.58	14.67±0.58	14.67±0.58
PL 88	12±1.00	10.33±0.58	NZ
PL 120	15.33±0.58	10.67±0.58	10.67±0.58

PL 135	21±1.00	20.33±0.58	15.67±0.58
PL 141	15±1.00	15.33±0.58	14.66±0.58
PL 145	10.67±0.58	10.67±0.58	9.67±0.58
PL 149	16.67±0.58	16±1.00	16.33±0.58

S.D= standard deviation

Table 3. Survival of selected isolates after 90 minutes pH stress in 0.85% saline solution represented as log_{10} CFU/ml.

Isolate	Log_{10} CFU/mL (Mean±S.D)			
	pH 7	**pH 4**	**pH 3**	**pH 2**
PL 22	7.52±0.52[a]	7.06±0.54[a]	5.77±0.43[a]	4.80±0.50[a]
PL 53	6.16±0.55[b]	6.11±0.68[b]	5.75±0.63[a]	3.33±0.54[b]
PL 88	6.30±0.52[b]	5.96±0.53[c]	5.91±0.52[a]	4.46±0.22[c]
PL 120	7.43±0.62[a]	7.26±0.53[a]	7.14±0.34[b]	5.75±0.52[d]
PL 135	7.40±0.42[a]	6.22±0.51[b]	6.03±0.54[c]	4.90±0.32[a]
PL 141	7.17±0.66[a]	6.74±0.54[e]	6.05±0.55[c]	3.10±0.20[e]
PL 145	7.26±0.35[a]	7.12±0.38[a]	7.16±0.45[b]	5.25±0.55[f]
PL 149	7.50±0.50[a]	6.04±0.55[b]	5.50±0.30[d]	5.05±0.55[g]

[a,b,c,d,e,f,g]: Isolates having different superscript differ significantly ($p<0.05$)

S.D= standard deviation

Table 4. Mean± standard deviation growth of selected isolates in MRS broth with different bile salt concentrations after 24 hours represented at OD at 630nm.

Isolate	Tolerance to bile salts (O.D)			
	MRS	**0.3%**	**1%**	**1.8%**
PL 22	1.91±0.05[a]	1.31±0.01[a]	0.33±0.01[a]	0.29±0.01[a]
PL 53	1.45±0.04[b]	0.95±0.01[b]	0.46±0.01[b]	0.33±0.01[a]
PL 88	1.02±0.01[c]	0.77±0.07[c]	0.30±0.05[a]	0.28±0.06[a]
PL 120	1.64±0.01[d]	1.28±0.09[d]	0.58±0.10[c]	0.36±0.04[a]
PL 135	1.46±0.13[b]	1.05±0.05[b]	0.39±0.25[a]	0.27±0.09[a]
PL 141	1.48±0.06[b]	0.41±0.10[e]	0.33±0.02[a]	0.21±0.09[a]
PL 145	1.08±0.04[c]	0.41±0.15[e]	0.38±0.06[a]	0.21±0.12[a]
PL 149	1.10±0.10[c]	0.81±0.16[c]	0.39±0.10[a]	0.28±0.05[a]

[a,b,c,d,e]: Isolates having different superscript differ significantly ($p<0.05$)

S.D= standard deviation

OD= Optical density

Table 5. Antibiotic susceptibility pattern of selected isolates (n=8) expressed as zone of inhibition (mm).

Antibiotic	Disc conc	Antibiotic susceptibility (ZOI, mm)							
	(µg)	**PL22**	**PL53**	**PL88**	**PL120**	**PL135**	**PL141**	**PL145**	**PL149**
PEN	**10 iu**	9 (R)	30 (S)	36 (S)	35 (S)	30 (S)	29 (S)	31 (S)	24 (S)
AMP	**10**	9 (R)	30 (S)	20 (S)	22 (S)	26 (S)	31 (S)	30 (S)	22 (S)
CAZ	**30**	NZ (R)	NZ (R)	NZ (R)	NZ (R)	NZ (R)	NZ (R)	NZ (R)	NZ (R)
IMP	**10**	13 (R)	NZ (R)	33 (S)	NZ (R)	NZ (R)	NZ (R)	NZ (R)	28 (S)
MEM	**10**	NZ (R)	NZ (R)	NZ (R)	NZ (R)	8 (R)	NZ (R)	NZ (R)	NZ (R)
AZT	**30**	NZ (R)	NZ (R)	NZ (R)	NZ (R)	NZ (R)	NZ (R)	NZ (R)	NZ (R)
VAN	**30**	NZ (R)	NZ (R)	NZ (R)	NZ (R)	NZ (R)	NZ (R)	NZ (R)	NZ (R)
BAC	**10 U**	NZ (R)	25 (S)	13 (R)	17 (I)	20 (S)	27 (S)	29 (S)	8 (R)
POL	**300 U**	NZ (R)	NZ (R)	NZ (R)	NZ (R)	NZ (R)	NZ (R)	NZ (R)	18 (I)
ERY	**30**	NZ (R)	36 (S)	48 (S)	40 (S)	42 (S)	36 (S)	39 (S)	26 (S)
GEN	**30**	17 (I)	22 (S)	16 (I)	20 (S)	20 (S)	20 (S)	22 (S)	20 (S)
KAN	**30**	NZ (R)	8 (R)	NZ (R)	NZ (R)	NZ (R)	NZ (R)	9 (R)	NZ (R)
CHL	**30**	30 (S)	36 (S)	33 (S)	31 (S)	38 (S)	32 (S)	38 (S)	30 (S)
TET	**30**	NZ (R)	30 (S)	9 (R)	34 (S)	12 (R)	30 (S)	32 (S)	24 (S)
CIP	**5**	8 (R)	NZ (R)	NZ (R)	19 (I)	21 (S)	8 (R)	8 (R)	14 (I)

FUS	**10**	NZ (R)	20 (S)	NZ (R)	NZ (R)	NZ (R)	22 (S)	22 (S)	12 (R)

ZOI= Zone of inhibition, NZ= No zone of inhibition, R= Resistant, I= Intermediate, S= Sensitive

PEN: penicillin, AMP: ampicillin, CAZ: ceftazidime, IMP: imipenem, MEM: meropenem, AZT: aztreonam, VAN: vancomycin, BAC: bacitracin, POL: polymyxin B, ERY: erythromycin, GEN: gentamicin, KAN: kanamycin, CHL: chloramphenicol, TET: tetracycline, CIP: ciprofloxacin, FUS: fusidic acid.

DISCUSSION

C. jejuni is one of the major food-borne bacterial pathogens. European Food Safety Authority (EFSA) have reported poultry meat to be the main source accounting for up to 80% of human Campylobacteriosis (Authority, 2011). In recent years, due to the increased concern on antibiotic resistance, it is important to develop alternatives of antibiotics such as probiotics (Awad *et al.*, 2018).

Out of 150 isolates in this study, only 16 showed activity against *C. jejuni*. Activity of lactobacilli is generally because of bacteriocins and lactic acid (Campana *et al.*, 2017). Out of sixteen, eight isolates (PL05, PL13, PL33, PL77, PL93, PL102, PL105 and PL111) had activity because of acids; therefore, those isolates were excluded from further analysis. Out of eight remaining isolates, activity of seven isolates was retained after boiling at 80°C for 10 minutes. Although lactobacilli having activity against *C. jejuni* have also been reported previously, there is limited data available for anti-*Campylobacter* probiotic development from Pakistan (Stern *et al.*, 2006; Santini *et al.*, 2010; Saint-Cyr *et al.*, 2017).

For probiotic organisms, it is imperative to tolerate and grow in physicochemical barriers of host gut, colonize the intestinal epithelium and be safe. Physicochemical barriers of poultry gut include low pH in poultry stomach and bile salts in intestines which can be simulated *in-vitro.* Tolerance of lactobacilli to low pH (pH 3.0) and bile salt (0.3%) indicate their ability to be probiotic. Results of the present study revealed that selected isolates (PL22, PL53, PL88, PL120, PL135, PL141, PL145 and PL149) had variable tolerance to pH and bile salt. Lactobacilli resistant to pH and bile salt have also been reported in many previous studies in many parts of the world including Pakistan (Keener *et al.*, 2004; Modesto *et al.*, 2009; Abbas *et al.*, 2010; Dec *et al.*, 2014; Wang *et al.*, 2014).

Auto-aggregation tests depicts the ability of bacteria to clump with itself and form a film over the epithelial cells while the co-aggregation potential indicates the ability of probiotics to clump with the pathogen and preventing its binding to the epithelial cells (Wine *et al.*, 2009; Li *et al.*, 2015). Selected isolates (PL22, PL53, PL88, PL120, PL135, PL141, PL145 and PL149) showed variable auto-aggregation (4.78, 15.12, 18.67, 27.83, 17.00, 24.04, 20.67 and 20.53%, respectively) and co-aggregation (2.50, 14.02, 16.00, 23.33, 14.00, 12.73, 12.33 and 22%, respectively). Lactobacilli preventing the attachment of *C. jejuni* to epithelial cells has been reported in many previous studies (Tareb *et al.*, 2013; Campana *et al.*, 2017). In this study, co-aggregation against *C. jejuni* of PL53, PL88, PL120, PL135, PL141, PL145 and PL149 was higher than reported previously of *Lactobacillus acidophilus* W37 (4.33%), *L. paracasei* W56 (13.94%) and *L. lactis* W58 (3.42%) (Campana *et al.*, 2017). Our co-aggregation results against *C. jejuni* were lower than *L. rhamnosus* CNCM-I-3698 (61.20%) and *L. farciminis* CNCM-I-3699 (53.60%) (Tareb *et al.*, 2013).

Transferable antibiotic resistance pose a threat to the commensal microflora from probiotic strains and therefore it is also an important criteria for the selection of probiotic strains to determine the safety of the administered beneficial microbe (Campana *et al.*, 2017). Lactobacilli (of humans, poultry and fermented foods) resistant to penicillin, ampicillin, vancomycin, erythromycin, gentamicin, tetracycline and ciprofloxacin have been reported from previous studies (Heravi *et al.*, 2011; Sharma *et al.*, 2017). All isolates were sensitive to erythromycin, ampicillin, penicillin and tetracycline except PL22 and PL88 and PL135.

Three isolates PL53, PL120 and PL149 were finally selected as potential probiotic organisms on the basis of all the characters studied. PL22, PL88 and PL135 were not potential probiotics because it acquired antibiotic resistance.

PL141 and PL145 were excluded from the study due to their low tolerance to bile salts and co-aggregation properties. PL53, PL120 and PL149 ability to inhibit *C. jejuni* in broth culture was evaluated *in-vitro*. All three isolates were able to inhibit *C. jejuni* count by more than 3 log values. Inhibition of *C. jejuni* by lactobacilli have also been reported in *Lactobacillus plantarum* N8, *L. plantarum* N9, *L. plantarum* ZL5, *L. paracasei* ZL4, *L. acidophilus* ATCC4356, *L. salivarius* in previous studies (Saarela *et al.*, 2002; Chaveerach *et al.*, 2004; Wine *et al.*, 2009; Wang *et al.*, 2014; Campana *et al.*, 2017).

On comparison of selected isolates, *L. gallinarum* PL 149 (poultry) had the maximum activity even after adjusting its pH and tolerated 0.3% concentration of bile salt. While *L. paracasei* PL 120 had good probiotic potential including tolerance to low pH (pH 2), tolerance to bile salt concentrations (1%) and aggregation potential while comparatively less log reduction of *C. jejuni*. *L. paracasei* PL 120 was isolated from human so it may be not effective in poultry. *L. gallinarum* PL 53 isolated from poultry had maximum log reduction of *C. jejuni* in co-culture experiment, the

tolerance to pH 3, bile salt concentration (up to 1%) while low aggregation potential as compared to selected three strains.

Conclusion: It is concluded that *L. gallinarum* PL53, *L. paracasei* PL120 and *L. gallinarum* PL149 may have anti-campylobacter probiotic potential and should be evaluated further *In Vivo* for their possible use as probiotics.

Authors' contribution: MK, AAA, MN and ARA conceived and designed study. MK executed the experiments. MK and MN analysed the data. MK and MN prepared the manuscript. All authors critically revised the manuscript for important intellectual contents and approved the final version.

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REFERENCES

Abbas, M.A., E. Spackman, D.E. Swayne, Z. Ahmed, L. Sarmento, N. Siddique, K. Naeem, A. Hameed, and S. Rehmani (2010). Sequence and phylogenetic analysis of H7N3 avian influenza viruses isolated from poultry in Pakistan 1995-2004. Virol. J. 7(1): 137.

Alagawany, M., M.E.A. El-Hack, M.R. Farag, S. Sachan, K. Karthik, and K. Dhama (2018). The use of probiotics as eco-friendly alternatives for antibiotics in poultry nutrition. Environ. Sci. Pollut. R. 25(11): 10611-10618.

Alfredson, D.A., and V. Korolik (2007). Antibiotic resistance and resistance mechanisms in *Campylobacter jejuni* and *Campylobacter coli*. FEMS Microbiol. Lett. 277(2): 123-132.

Asghar, S., M. Arif, M. Nawaz, K. Muhammad, M. Ali, M. Ahmad, S. Iqbal, A. Anjum, M. Khan, and J. Nazir (2016). Selection, characterisation and evaluation of potential probiotic *Lactobacillus spp*. isolated from poultry droppings. Benef. Microbes. 7(1): 35-44.

Authority, E.F.S. (2011). EFSA issues advice on reduction of *Campylobacter* in chickens. EFSA J. 3(6): 223.

Awad, W.A., C. Hess, and M. Hess (2018). Re-thinking the chicken–*Campylobacter jejuni* interaction: a review. Avian Pathol. 47(4): 352-363.

Campana, R., S. van Hemert, and W. Baffone (2017). Strain-specific probiotic properties of lactic acid bacteria and their interference with human intestinal pathogens invasion. Gut pathog. 9(1): 12.

Chaveerach, P., L. Lipman, and F. Van Knapen (2004). Antagonistic activities of several bacteria on in vitro growth of 10 strains of *Campylobacter jejuni/coli*. Int. J. Food Microbiol. 90(1): 43-50.

Collado, M.C., J. Meriluoto, and S. Salminen (2008). Adhesion and aggregation properties of probiotic and pathogen strains. Eur. Food Res. Technol. 226(5): 1065-1073.

Connerton, P., A. Timms, and I. Connerton (2011). *Campylobacter* bacteriophages and bacteriophage therapy. J. Appl. Microbiol. 111(2): 255-265.

Dasti, J.I., A.M. Tareen, R. Lugert, A.E. Zautner, and U. Groß (2010). *Campylobacter jejuni*: a brief overview on pathogenicity-associated factors and disease-mediating mechanisms. Int. J. Med. Microbiol. 300(4): 205-211.

Dec, M., A. Puchalski, R. Urban-Chmiel, and A. Wernicki (2014). Screening of *Lactobacillus* strains of domestic goose origin against bacterial poultry pathogens for use as probiotics. Poult. Sci. 93(10): 2464-2472.

Ekmekciu, I., U. Fiebiger, K. Stingl, S. Bereswill, and M.M. Heimesaat (2017). Amelioration of intestinal and systemic sequelae of murine *Campylobacter jejuni* infection by probiotic VSL# 3 treatment. Gut pathog. 9(1): 17.

FAO/WHO. (2002). Guidelines for the evaluation of probiotics in food. Food and Agriculture Organization of the United Nations and World Health Organization Working Group Report. In: Food and Agriculture Organization Rome.

Heravi, R.M., H. Kermanshahi, M. Sankian, M. Nassiri, A.H. Moussavi, L.R. Nasiraii, and A. Varasteh (2011). Screening of lactobacilli bacteria isolated from gastrointestinal tract of broiler chickens for their use as probiotic. Afr. J. Microbiol. Res. 5(14): 1858-1868.

Keener, K., M. Bashor, P. Curtis, B. Sheldon, and S. Kathariou (2004). Comprehensive review of *Campylobacter* and poultry processing. Compr. Rev. Food Sci. Food Saf. 3(2): 105-116.

Kobierecka, P.A., A.K. Wyszyńska, J. Gubernator, M. Kuczkowski, O. Wiśniewski, M. Maruszewska, A. Wojtania, K.E. Derlatka, I. Adamska, and R. Godlewska (2016). Chicken anti-*Campylobacter* vaccine–comparison of various carriers and routes of immunization. Front. Microbiol. 7: 740.

Li, Q., X. Liu, M. Dong, J. Zhou, and Y. Wang (2015). Aggregation and adhesion abilities of 18 lactic

acid bacteria strains isolated from traditional fermented food. Int. J. Agric. Policy Res. 3(2): 84-92.

Markowiak, P., and K. Śliżewska (2018). The role of probiotics, prebiotics and synbiotics in animal nutrition. Gut pathog. 10(1): 21.

Messaoudi, S., G. Kergourlay, A. Rossero, M. Ferchichi, H. Prévost, D. Drider, M. Manai, and X. Dousset (2011). Identification of lactobacilli residing in chicken ceca with antagonism against *Campylobacter*. Int. Microbiol. 14(2): 103-110.

Meunier, M., M. Guyard-Nicodème, E. Vigouroux, T. Poezevara, V. Beven, S. Quesne, L. Bigault, M. Amelot, D. Dory, and M. Chemaly (2017). Promising new vaccine candidates against *Campylobacter* in broilers. PLoS ONE 12(11): e0188472.

Modesto, M., M.R. D'Aimmo, I. Stefanini, P. Trevisi, S. De Filippi, L. Casini, M. Mazzoni, P. Bosi, and B. Biavati (2009). A novel strategy to select *Bifidobacterium* strains and prebiotics as natural growth promoters in newly weaned pigs. Livest. Sci. 122(2-3): 248-258.

Nawaz, M., J. Wang, A. Zhou, C. Ma, X. Wu, and J. Xu (2011). Screening and characterization of new potentially probiotic lactobacilli from breast-fed healthy babies in Pakistan. Afr. J. Microbiol. Res. 5(12): 1428-1436.

Saarela, M., L. Lähteenmäki, R. Crittenden, S. Salminen, and T. Mattila-Sandholm (2002). Gut bacteria and health foods—the European perspective. International J. Food Microbiol. 78(1-2): 99-117.

Saint-Cyr, M.J., N. Haddad, B. Taminiau, T. Poezevara, S. Quesne, M. Amelot, G. Daube, M. Chemaly, X. Dousset, and M. Guyard-Nicodème (2017). Use of the potential probiotic strain *Lactobacillus salivarius* SMXD51 to control Campylobacter jejuni in broilers. Int. J. Food Microbiol. 247: 9-17.

Santini, C., L. Baffoni, F. Gaggia, M. Granata, R. Gasbarri, D. Di Gioia, and B. Biavati (2010). Characterization of probiotic strains: an application as feed additives in poultry against *Campylobacter jejuni*. Int. J. Food Microbiol. 141: S98-S108.

Sharma, C., S. Gulati, N. Thakur, B.P. Singh, S. Gupta, S. Kaur, S.K. Mishra, A.K. Puniya, J.P.S. Gill, and H. Panwar (2017). Antibiotic sensitivity pattern of indigenous lactobacilli isolated from curd and human milk samples. Biotech. 7(1): 53.

Shaughnessy, R.G., K.G. Meade, B.A. McGivney, B. Allan, and C. O'Farrelly (2011). Global gene expression analysis of chicken caecal response to *Campylobacter jejuni*. Vet Immunol. Immunopathol. 142(1-2): 64-71.

Stern, N., E. Svetoch, B. Eruslanov, V. Perelygin, E. Mitsevich, I. Mitsevich, V. Pokhilenko, V. Levchuk, O. Svetoch, and B. Seal (2006). Isolation of a *Lactobacillus salivarius* strain and purification of its bacteriocin, which is inhibitory to *Campylobacter jejuni* in the chicken gastrointestinal system. Antimicrob. Agents Chemother. 50(9): 3111-3116.

Svetoch, E.A., B.V. Eruslanov, V.P. Levchuk, V.V. Perelygin, E.V. Mitsevich, I.P. Mitsevich, J. Stepanshin, I. Dyatlov, B.S. Seal, and N.J. Stern (2011). Isolation of *Lactobacillus salivarius* 1077 (NRRL B-50053) and characterization of its bacteriocin and spectra of antimicrobial activity. Appl. Environ. Microbiol.

Tareb, R., M. Bernardeau, M. Gueguen, and J.-P. Vernoux (2013). In vitro characterization of aggregation and adhesion properties of viable and heat-killed forms of two probiotic *Lactobacillus strains* and interaction with foodborne zoonotic bacteria, especially *Campylobacter jejuni.* J. Med. Microbiol. 62(4): 637-649.

Wang, G., Y. Zhao, F. Tian, X. Jin, H. Chen, X. Liu, Q. Zhang, J. Zhao, Y. Chen, and H. Zhang (2014). Screening of adhesive lactobacilli with antagonistic activity against *Campylobacter jejuni*. Food Control 44: 49-57.

Wine, E., M.G. Gareau, K. Johnson-Henry, and P.M. Sherman (2009). Strain-specific probiotic (*Lactobacillus helveticus*) inhibition of *Campylobacter jejuni* invasion of human intestinal epithelial cells. FEMS Microbiol. Lett. 300(1): 146-152.