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Evaluation of probiotic and antimicrobial potential of canine derived- *Lactobacilli*

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Abstract: Antibiotics are commonly used to treat infectious diseases. However, massive and inappropriate antibiotics usage cause many problems including the emergence and spread of antibiotic-resistant bacteria. To avoid this issue, in modern countries the interest of using probiotics in feed supplementation to promote health and prevent or treat intestinal infectious diseases in companion animals like dogs has been increasing. We evaluate the probiotic potential of *Lactobacilli* isolated from healthy dogs faeces. The isolated *Lactobacilli* were first confirmed by 16SrRNA sequencing, then *in vitro* tests were conducted to assess survival potential of *Lactobacilli* under simulated gastrointestinal conditions and adhesion ability to gut epithelia, effects on epithelial barrier function, anti-inflammatory activities, effects on defensin peptides (beta-defensin 3) and inhibitory effects on common pathogens. *Lactobacilli* showed considerable potential to survive in simulated gastrointestinal environmental conditions, low pH, high bile salt concentrations along with good adhesion properties with MODE-K cells. Pathogenic bacterial growth and their adhesion to MODE-K cells was significantly inhibited by *Lactobacilli*. Real-time PCR analyses further demonstrated that *L. acidophilus* strain AR1 and AR3 inhibit *Salmonella*-induced proinflammatory cytokines (IL-6, IL-8, 1β) production and reinforce expression of tight junction protein (occludin). None of the strain induce mRNA expression of beta-defensin 3 in MODE-K cells. Based on *in vitro* results the *L. acidophilus* strain AR1 has potential to be supplemented in canine feed. However, further *in vivo* studies investigating health-promoting effects are awaited.

Keywords: Probiotics, Canine, *Lactobacilli*, Feed supplementation, Infectious diseases.

1. Introduction

Probiotics are live microorganisms when administered to their host in adequate amount confer a good health effect. The animal gastrointestinal tract is very complex ecosystem on the Earth and is continuously affected by host associated [1, 2] and outside environmental factors [3]. The intestinal microbiota has significance attraction in recent years. Use of probiotics microorganisms in animal and human nutrition to improve health [4] as well

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as to control pathogenic infectious diseases has become area of great research activity [5]. As antibiotics application as feed additive or during infectious diseases has many side effects [6], i.e. long lasting changes on intestinal microbiota toward unhealthy pattern [7], intestinal barrier function damage [8] and many other problems like antibiotics residues in food stuff, bone marrow toxicity, hepatotoxicity, nephropathy, reproductive disorder, carcinogenicity etc. [9]. Nowadays probiotics are considered as antibiotics alternatives to prevent and treat the pathogenic infections [10]. For any good probiotics candidates, the adhesion abilities with intestinal epithelium, potential to fortify the barrier function, antimicrobial activities, high survival rate in gastrointestinal tract, low pH and during bile salts exposure are considered to be crucial for probiotics functionality [11-13].

Unfortunately, concerning companion animals like dogs, the amount of probiotics research is insufficient. The dog was first domesticated animal and belongs to the most popular companion animal along with cat. The reason lies in their valuable properties like better developed sense organs, social behavior and intelligence [4]. In many cultures of world, the dogs are considered part of family and because of their close contact, which has lots of implication not only to animals but also to the owner, the trend of probiotics therapeutics as well as prophylaxis applications in canine has been increasing in modern cultures [14]. Some studies have investigate the probiotics potential of Lactobacillus species in dogs [14]. *L. acidophilus* DSM13241 has potential to survive in GIT of dog and change the composition of colon microbiota toward beneficial pattern by reducing number of clostridia and increasing LAB percentage. Further it also increases the concentrations of monocytes, neutrophils, RBCs, hemoglobin, serum IgG and decreases erythrocytes fragility [15]. The application of same strain improves the fecal dry matter, fecal consistency and defecation frequency in dogs [16]. Another strain LAB20 of *L. acidophilus* isolated from dogs has ability to adhere with canine intestinal epithelial cells, HT-29 and Caco-2 cell lines. This strain improves intestinal barrier function by increasing transepithelial electrical resistance (TEER) and also shows anti-inflammatory character by attenuating LPS induced IL-8 in HT-29 cells [11]. *L. fermentum* CCM 7421 with alginite improves the dogs health by increasing hemoglobin, and serum magnesium levels, and also changes microbiota composition towards healthy pattern by decreasing and increasing the number of pathogenic (coliform, clostridia) and beneficial microbes (lactic acid bacteria) respectively [17]. *L. murinus* strain LbP2 improve the dog mental status, appetite, fecal consistency, clinical score and with other therapeutic measure, the probiotics application appear to be promising to manage canine distemper associated diarrhea [18].

Compositions of dog intestinal microbiota changes with respect to age [19], as dogs become older the prevalence and the number of Lactobacillus sp. tend to decrease. Therefore, it is necessary to develop feed supplemented with some strains isolated from healthier dogs with good probiotics potential to improve life quality of both dogs and their owners. Our aim of this study was to asses probiotic potential of dog isolated Lactobacilli (*L. fermentum*, *L. acidophilus*,) to be used them in dogs' feedings. We conduct some basic

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necessary tests, which are considered essential for functionality of any probiotic candidate.

2. Materials and Methods

2.1. Bacterial strains and culture Conditions

Several *Lactobacilli* were successfully isolated from healthy dog faeces, purified on selective LAB agar and then identified by 16S rRNA sequencing. *L. acidophilus* (strain AR1), *L. acidophilus* (strain AR2) and *L. fermentum* (strain AR3) were chosen for further assessment. These 3 strains were anaerobically grown at 37°C in in MRS medium. The pathogenic strain, *Salmonella enterica* serovar Typhimurium strain ATCC14028 (ATCC14028), *Salmonella enterica* serovar Typhimurium strain SL1344 (SL1344), Enterotoxigenic *Escherichia coli* k88 (ETEC) (provided through courtesy of Prof. Jiufeng Wang from China Agricultural University) and *Staphylococcus aureus* (*S. aureus*) (provided by Prof. Aizhen Guo from Huazhong Agricultural University) were grown in LB medium at 37°C.

2.2. Lysozyme resistance assay

A method described by Zago et al. (2011) [20] was used to perform lysozyme resistance characteristics of tested strains. Briefly, overnight culture of each strain (AR1, AR2, AR3) was centrifuged and inoculated in 9 mL sterile electrolyte solutions (2.2 g/l^{-1} KCl, 0.22 g/l^{-1} CaCl2, 1.2 g/l^{-1} NaHCO3, 6.2 g/l^{-1} NaCl, 0.1g $/l^{-1}$ lysozyme) to simulate in vivo dilution by saliva. The bacterial suspensions without lysozyme in sterile electrolyte solution were used as a control. The survival rate was determined after 90 and 180 min and compared to control. The experiments were repeated three time in triplicate.

2.3. Bile and pH resistance assays

Overnight culture of each strain (AR1, AR2, AR3) was inoculated in MRS broth with different pH (2,3,4,5) and bile concentrations (0.05%, 0.1%, 0.2%). The inoculated MRS broth was kept at 37°C for 3 h. After 3 h the bacterial growth was measured and compared with control. The results were presented as growth percentage compared to control. The experiments were carried out in triplicate and repeated three time independently.

2.4. *In vitro* resistant to gastrointestinal conditions

A method adopted in the study of Falah et al., (2019) [21] was used to prepare simulated gastric and intestinal juices. Briefly to prepare simulated gastric and intestinal juices, pepsin (final concentration 3 g/l^{-1}, pH 2) and pancreatin (1 g/l^{-1} containing 0.2% bile salts with pH 8) was dissolved in 0.5%, w/v sterile solution of NaCl and PBS respectively. Bothe juices were sterile using 0.22 µm pore size filters. After different time of incubation period (0,30, 60, 90, and 120) with these simulated juices, the viable count of *Lactobacilli* (AR1, AR2, AR3) was calculated by agar plate count method. The assays were performed in triplicate and repeated three time.

2.5. Exopolysaccharide production test

Exopolysaccharides (EPS) production test was performed according to already described method. The tested strains were grown at 28°C in atmosphere containing 10% CO_2 for 48h, in MRS broth supplemented with 20% (v/v) tomato juice and 5g/L fructose. By visually observing the culture viscosity, the EPS producing ability of each strains was assessed. Upon agitation, a ropy liquid culture showed production of EPS [22].

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2.6. Antimicrobial activity assay

The inhibitory potential of *Lactobacilli* strains (AR1, AR2, AR3) on pathogenic growth was assessed by their culture solutions, bacterial cells and supernatants by the oxford cup method. The pathogenic strains (ATCC14028, SL1344, ETEC, *S. aureus*) were poured (10^7 CFU per mL) on LB agar plates. Then oxford cups were placed and fixed. The 100 ul of culture solutions, supernatants and bacterial cells (10^7 CFU per mL) of each *Lactobacilli* strains (AR1, AR2, AR3) were poured into oxford cups and then plates were kept at 4°C for 3 h for liquid absorption. After 12 h of incubation at 37°C, the diameter of inhibition zones was measured. The experiments were repeated three time and mean inhibition diameter was calculated. Inhibition zones of > 15mm were considered as high inhibitory activity of tested strains [23].

2.7. Co-culture of *Lactobacilli* strains (AR1, AR2, AR3) and pathogens

Overnight culture of tested strains and pathogenic strains were adjusted to equal concentration (10^7 CFU per mL) by adjusting their OD_{600} values. One mL (10^7 CFU per mL) of strain AR1 and ATCC14028 were inoculated together in fresh MRS broth to final volume of 50 mL and given the name to this co culture group as AR1-ST28. Co-culture of AR1 with SL1344, ETEC and *S. aureus* were given the name as AR1-SL44, AR1-ETEC and AR1-*S. aureus* respectively. Co-culture of AR2 with ATCC14028, SL1344, ETEC and *S. aureus* were given the name as AR2-ST28, AR2-SL44, AR2-ETEC and AR2- *S. aureus* respectively. Similarly, co-culture of strain AR3 with ATCC14028, SL1344, ETEC and *S. aureus* were assigned as AR3-ST28, AR3-SL44, AR3-ETEC and AR3-*S. aureus* respectively. Viable bacterial count and pH of co-cultures and pure cultures of each bacteria were measured at different intervals (6 h, 12 h, 24 h). The experiment was repeated three time [24].

2.8. Assessment of pathogenic growth inhibitory effects of *Lactobacilli* strains (AR1, AR2, AR3) metabolites

To see whether the growth inhibitory effects on pathogens by *Lactobacilli* is mainly due to their low pH or their metabolites are also involved, the pathogens were grown in MRS broth with pH equal to 24 h culture pH of *Lactobacilli* strains (AR1, AR2, AR3). For this, the AR1 strain was grown for 24 h in MRS broth and pH of this end culture was measured. Then ATCC14028 was grown in MRS broth with pH equal to this 24 h culture pH of AR1 and given the name to this group as AR1pH-ST28. The growth of SL1344, ETEC and *S. aureus* in MRS broth with pH equal to 24 h culture pH of AR1 was given the name as AR1pH-SL44, AR1pH-ETEC and AR1pH- *S. aureus* respectively. The growth of ATCC14028, SL1344, ETEC and *S. aureus* in MRS broth with pH equal to 24 h culture pH of AR2 was given the name as AR2pH-ST28, AR2pH-SL44, AR2pH-ETEC and AR2pH-*S. aureus* respectively. Similarly, the growth of ATCC14028, SL1344, ETEC and *S. aureus* in MRS broth with pH equal to 24 h culture pH of AR3 was given the name as AR3pH-ST28, AR3pH-SL44, AR3pH-ETEC and AR3pH-*S. aureus* respectively. The viable count of these pathogens (grown with Lactobacilli and in pH equal to 24 h culture pH of different Lactobacilli) was counted by plating on LB agar plates after 12 h incubation at 37°C and compared with each other.

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2.9. Cell line and culture conditions

MODE-K (mouse epithelial cell line) cells were grown in Dulbecco's Modified Eagle's Medium supplemented with 1% antibiotic (penicillin-streptomycin) and 5% fetal bovine serum (FBS). Before infection, the cells were seeded (2.5 x 10^5/ well) in 6 well plates, each well containing 3mL DMEM media supplemented with 10% FBS without antibiotics, and were grown at 37°C, 5% CO_2 and 95% air atmosphere [25].

2.10. Antibacterial activity of *Lactobacilli* (AR1, AR2, AR3) treated MODE-K cell culture supernatant

The MODE-K cells (provided through courtesy of Prof. Deshi Shi from Huazhong Agricultural University) were grown in 6 well plates and exposed to each *Lactobacilli* strain (AR1, AR2, AR3) (100 bacteria/cell) for 6 h and then supernatants were collected to check its antimicrobial activity. The supernatant of MODE-K cells without *Lactobacilli* strain (AR1, AR2, AR3) exposure was used as negative control whereas supernatant collected from wells containing only *Lactobacilli* strain (AR1, AR2, AR3, no MODE-K cells) were used as positive control. The antimicrobial activity of the supernatants after filtering (0.25-µm pore size filters) was checked against pathogenic strain (ATCC14028, SL1344, ETEC, *S. aureus*). 10 µL suspension of overnight culture of pathogens in LB broth was inoculated in 500 µL of supernatants. The viable pathogenic counted by agar plat count method was assessed after 2 h growth incubation at 37°C and 200 rpm shaking. The experiment was carried out in triplicate and performed for three time [26].

2.11. Adhesion and adhesion inhibition assays

The adhesion of probiotic with eukaryotic cells (MODE-K) were assessed by agar plate count methods. The MODE-K cells were grown in 24 well plates. At appropriate confluency, the cells were exposed to *Lactobacilli* strain (AR1, AR2, AR3) at different concentrations (10^6 CFU per mL, 10^7 CFU per mL, 10^8 CFU per mL) and incubated at 37°C, 5% CO_2 and 95% air atmosphere for 2 h. After 2 h, the cells were washed 4-5 time with PBS to remove unbound bacteria and were lysed using 0.2% 100 µL Triton ™ X-100 to count viable bacterial count by agar plat count method. The wells with only bacteria (no MODE-K cells) were kept as a control. The adhesion assay was expressed as percentage of CFU per mL compared to CFU per mL with control.

Competition, inhibition and displacement assays were performed to check inhibitory effects of *Lactobacilli* strain (AR1, AR2, AR3) on pathogens adhesion with MODE-K cells. *Lactobacilli* strain (AR1, AR2, AR3) were added before, at same time and 1 h after addition of pathogenic strains to MODE-K cells for inhibition, competition and displacement assay respectively. After 2 h incubation for each assay, the cells were washed with PBS to remove unbound bacteria and then lysed using 0.2% 100 µL Triton ™ X-100. The viable count of pathogen was calculated by serial dilution and plating on LB agar plates. The adhesion was expressed as percentage of adhering pathogens normalized to control [21, 24].

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2.12 Real time PCR for mRNA expression of tight junction proteins, cytokines and defensin peptides

Real time PCR was performed to check effects of *Lactobacilli* strain (AR1, AR2, AR3) and pathogenic strain (*S. Typhimurium* ATCC14028 and *S. Typhimurium* SL1344) on different gene expression (Occludin, IL-8, IL-6, IL-1β) using MODE-K cells. First the MODE-K cells were exposed for 2 h before total RNA extraction, to all bacteria separately (100 bacteria/cells) to check mRNA expression of Occludin, IL-8, IL-6, IL-1β. To assess anti-inflammatory activity of *Lactobacilli* strain (AR1, AR2, AR3) and reinforcement of tight junction protein (occludin) during *S. Typhimurium* ATCC14028 and *S. Typhimurium* SL1344, the MODE-K cells were exposed with each *Lactobacilli* strain 1 h before addition of *S. Typhimurium* ATCC14028 and *S. Typhimurium* SL1344 at same MOI. After 2 h incubation the total RNA was extracted from all treated and control groups by Trizol reagent as per manufacturer recommendations. To investigate the effects of *Lactobacilli* on gene expression of beta-defensin 3, the confluent MODE-K cells were exposed to each tested strain (100 bacteria/ cell) for 6 h before total RNA extraction. Using FastKing RT Kit (Tiangen Biotech Co., Ltd, Beijing, China) the cDNA was made from this extracted RNA after checking its quality and quantity. For qPCR the 20 µL reaction mixture containing 1 µL primers (0.5 µL forward and 0.5 µL reverse primer), 7 µL nuclease free water, 2 µL cDNA and 10 µL SYBR Green Master mix (Vazyme Biotech Co., Ltd, Nanjing, China) was used. The experimental program consisted of pre-denaturation for 30 sec at 95 °C, followed by denaturation cycles (40) for 10 s at 95 °C, annealing for 30 s at 56 °C and extension at 72 °C for 30 s. The ΔΔCt method of Livak and Schmittgen [27] was used to calculate relative expression level of genes using glyceraldehyde-3-phosphate dehydrogenase as a house keeping gene for normalization of expression level of genes. The used primer sequences are listed in table 1.

Table 1: primers sequences used for real time PCR

Genes	Forward sequence	Reverse sequence
Occludin	CACACTTGCTTGGGACAGAG	TAGCCATAGCCTCCATAGCC
Defb3	GCTAGGGAGCACTTGTTTGC	TTGTTTGAGGAAAGGAGGCA
IL-8	CGGCAATGAAGCTTCTGTAT	CCTTGAAACTCTTTGCCTCA
IL-6	CAAAGCCAGAGTCCTTCAGAG	GCCACTCCTTCTGTGACTCC
IL-1β	GGGCCTCAAAGGAAAGAATC	TACCAGTTGGGGAACTCTGC
GAPDH	AGCTTGTCATCAACGGG AAG	TTTGATGTTAGTGGGGTCT CG

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3 Statistical analysis

Two-way ANOVA followed by turkey multiple comparison and student t-test was performed to analyze statistically significant ($P<0.05$) data. Version 8.0.1 of GraphPad Prism was used to visualize the data.

4. Results

4.1 Tolerance to lysozyme

L. acidophilus AR1 has more resistant against lysozyme followed by *L. acidophilus* AR2 and *L. fermentum* AR3. The *L. acidophilus* AR1 showed considerable survival percentage (22%) after 90 min incubation in lysozyme (Fig. 1).

4.2 pH and bile salt resistance

L. acidophillus AR1 has more resistant against low pH and high bile salt concentration followed by *L. acidophilus* AR2 and *L. fermentum* AR3. The survival rate of each strain increased as pH increased and bile salt concentration decreased (Fig. 2).

4.3 Tolerance to simulated condition of GIT

Fig.3 shows that all strains retained their viability during exposure to simulated gastric and intestinal juices. *L. acidophilus* strain AR1 was found to be more resistant to simulated gastrointestinal conditions followed by *L. acidophilus* strain AR2 and *L. fermentum* strain AR3.

4.4 Exopolysaccharide production

The *L. acidophilus* strain AR1 shown more ropy character in MRS broth, supplemented with 20% (v/v) tomato juice and 5g/L fructose, followed by *L. acidophilus* strain AR2 and *L. fermentum* strain AR3.

4.5 Antimicrobial activity

Fig. 4. shows the antimicrobial activity of *Lactobacilli* cultures solutions, bacterial cells and their supernatants against pathogens. The antimicrobial activity of culture solutions of all *Lactobacilli* strain has high inhibitory activity than supernatants and bacterial cells. The *Lactobacilli* alone has no antimicrobial activity. The culture of *L. acidophilus* strain AR1 has highest inhibitory activity as indicated by 19.03 ± 0.2 mm inhibition zone against *S. Typhimurium* strain ATCC14028 followed by *S. Typhimurium* strain SL1344 (17.47 ± 0.55 mm inhibition zone), *S. aureus* (15.43 ± 0.70 mm inhibition zone) and ETEC (14.90 ± 0.46 mm inhibition zone) (Fig.4A). The culture of *L. acidophilus* strain AR2 has highest inhibitory activity (18.80 ± 0.85 mm inhibition zone) against *S. Typhimurium* SL1344 followed by *S. Typhimurium* ATCC14028 (17.67 ± 0.61 mm inhibition zone), *S. aureus* (14.57 ± 0.9 mm inhibition zone) and ETEC (13.90 ± 1.03 mm of inhibition zone) (Fig.4B). The culture and supernatant of *L. fermentum* strain AR3 has less antimicrobial activity as compared to strains of *L. acidophilus* (Fig.4C).

4.6 Coculture of *Lactobacilli* and pathogens

The *Lactobacilli* strain inhibited the growth of pathogenic bacteria when grown together. Fig. 5 shows growth pattern of *L. acidophilus* AR1 pure culture and coculture with different pathogens. The growth pattern in pure and co-culture groups was almost similar, however in pure culture the growth was slightly less than co-culture with pathogens. Similar growth

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patterns were observed for *L. acidophilus* AR2 (Fig. 6) and *L. fermentum* AR3 (Fig 7) during co-culture assays and pure culture. However, growth inhibitory effects of each *Lactobacillus* on different pathogens were found to be different. The pathogenic bacterial growth was inhibited in co-culture with *Lactobacilli*. After 24 h the viable count of each pathogens in pure culture was more than in co-culture with *Lactobacilli* (Fig. 8). Acid production patterns by each probiotic strain, as indicated by decrease in pH of medium (Fig. 9, 10, 11), were almost similar under each of culture conditions (pure and co-culture assays). The pH decline was slower in case of pure cultures of pathogenic bacteria than that of co-cultures and pure cultures of *Lactobacilli***.**

4.7 Inhibitory effects of *Lactobacilli* metabolites on pathogenic growths

The viable count of pathogens was found to be more when grown in pH equal to 24 h culture pH of *Lactobacilli* than that of co-culture with *Lactobacilli*. Pathogens growth was inhibited more when grown together with *Lactobacilli* than in low pH equal to 24 h *Lactobacilli* culture pH suggesting the antimicrobial activity of *Lactobacilli* metabolites as shown in Fig. 12.

4.8 Host defensin peptide assessment

No antimicrobial activity of *Lactobacilli* treated MODE-K cell culture supernatant was observed. The significant difference was not observed in viable count of pathogenic bacteria when grown in *Lactobacilli* culture supernatant and MODE-K cell culture supernatant collected after *Lactobacilli* stimulation (Fig. 13)

4.9 Adhesion and adhesion inhibition assays

Each probiotic strain has adhesion ability with MODE-K cells. The adhesion percentage of each probiotic strain increased as initial concentrations is increased (Fig. 14). The maximum adhesion (7.5 ± 0.2%) was observed for *L. acidophilus* strain AR1 followed by *L. acidophilus* strain AR2 (7 ± 0.25%) and *L. fermentum* strain AR3 (5.8% ± 0.27) at highest inoculated concentration (10^8 CFU per mL). Probiotic strain inhibited pathogens adhesion at all concentrations in concentration depended manner. The pathogenic strains adhesion percentage decreased considerably when inoculated concentration of probiotic strains is increased. The maximum inhibitory effects of *Lactobacilli* on pathogens adhesion were observed during inhibition assay followed by competition and displacement assays (Fig. 14).

4.10 Real time PCR for mRNA expression of tight junction proteins, cytokines and defensin peptides

The real time PCR results showed that both strains of *Salmonella* significantly increased proinflammatory cytokines (IL-8, IL-6, IL-1β) mRNA expression. The *L. acidophilus* strain AR1 and *L. fermentum* strain AR3 statistically significant inhibited the *Salmonella* induced pro-inflammatory cytokines. The tight junction protein (occludin) was significantly downregulated by both strain of *Salmonella* and *L. acidophilus* strain AR1 and *L. fermentum* strain AR3 pre-treatment significantly curtail this effect. As for as host defensin peptide (beta-defensin 3) is concerned none of strain induced mRNA expression of beta-defensin 3 in MODE-K cells (Fig. 15)

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5 Discussion

The present study was conducted to evaluate probiotic potential of *Lactobacilli* (*L. acidophilus* AR1, *L. acidophilus* AR2, *L. fermentum* AR3) isolated from dog feces. Resistance to the harsh environment of the GI tract is an important step towards selection of potential probiotic candidates. The first barrier which should be overcome is the mouth which contains high lysozyme concentration, then low pH and digestive enzymes of stomach and antimicrobial action of bile of intestine [28]. Our results showed that the isolate have potential to survive in harsh GI tract environment. The isolated *Lactobacilli* showed considerable number even after 180 min incubation with lysozyme which is considered a severe treatment [29]. Survival rate of *Lactobacilli* in presence of lysozyme has also been reported by many other studies [20, 29, 30]. To reach at the end of the GIT, the stomach acid is one of major issue for microorganisms. Therefore, pH is considered to be crucial for selection of probiotics [31]. When food is swallowed the stomach pH range from 3-6 depending on food composition, so pH range of 2-5 is usually examined to evaluate pH resistance potential of probiotics[21]. In various other studies it has been reported that *Lactobacilli* can survive well in low pH [32-34]. Results in our hand are in concordance with these facts showing that the new isolated *Lactobacilli* can survive under low pH. Several mechanisms are associated with *Lactobacilli* to survive in low pH like, protein and DNA damage repair, several metabolic pathways, proton pumps, neutralization processes, changes of cell membrane composition and cell density [32, 35-38]. Moreover, the survivability in low pH is also associated with production of polysaccharide which protect the microorganisms against lethal pH effects [39] which was found to be positive in this study. Our results are in agreement with Aziz and Falah who reported that *L. fermentum* can survive in low pH [21, 40].

The probiotic functions in duodenum is compromised because of secretion of bile salts which act as antimicrobial molecules [21]. Therefore, to evaluate LAB potential as a probiotic their resistance to bile salts is essential. Resistant to bile salts has been considered as a condition for bacterial metabolic activity and colonization in intestine of host [31]. Bile salts concentration of 0.15% to 0.5% is usually investigated [41] and in order to select a strain considered to have good bile resistant ability, Mathara et al. (2008) established a limit of 0.3% concentration of bile [42]. In this study the tested strains showed resistance against different concentrations of bile salts. These findings were consistent with Vasiee et al.[31], Sagdic et al.[43] and Hashemi et al.[39] who found that LAB can resist the bile acids very well. When bacteria are exposed to bile salts their cellular homeostasis is disturbed leading to cell death. It has been proposed that bile salts resistant activity of probiotics is related to bile salts hydrolases enzymes [39]. A study carried out on wild-type and bile salt hydrolases (bsh) mutant pairs of *Lb. plantarum* and *Lb. amylovorus* has provided a correlation between bile salt hydrolysis and bile tolerance. Findings demonstrate that mutated cells were much more vulnerable to bile and bile salts and exhibited less growth rates when bile salts are present [44]. The protective effects of food matrix is another proposed factor that contribute in survival of microorganism in presence of

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higher bile salts concentrations [45]. Further we evaluated survival potential of isolated *Lactobacilli* under simulated GIT conditions. Our results showed that the isolated *Lactobacilli* have considerable surviving rat under simulated gastric juice, however survival rate of all strains under simulated gastric juice of pH 2 was found to be less than pH resistance assay at pH 2. It could be due to antimicrobial action of pepsin. Similarly, the isolated *Lactobacilli* can also maintain their viable count under simulated intestinal juice containing pancreatin and bile salts. Falah et al.,2019 [21] study also reported similar finding in case of *L. fermentum* strain 4-17. Many other studies have also been reported that the *Lactobacilli* can maintain their viable count under simulated conditions of GIT [29, 46]. Pathogenic bacterial growth inhibition is one of striking property of probiotics. Many studies of LAB have been shown their broad spectrum of antimicrobial activities [47, 48]. The present study indicated that these *Lactobacilli* have ability to inhibit growth of selected common pathogens based on co-culture assays and oxford cup method. The isolated *Lactobacilli* culture solutions and their supernatants showed inhibition zone of different diameter with prominent effects against *Salmonella* but *Lactobacilli* cells not showed any inhibition zone. These results indicated that *Lactobacilli* cells have no antimicrobial activity rather the pathogenic growth inhibitory effects are mainly mediated by their metabolites or low pH conditions. The antimicrobial activity of *Lactobacilli* culture solutions was significantly fount to be more than their supernatants indicating the fact that antimicrobial activity is not only mediated by low pH of *Lactobacilli* but continuous metabolites production of *Lactobacilli* also has significant role against growth of pathogens. These findings were further clarified when results of co-culture assays were analyzed. In our co-culture assays, the pH decline pattern of MRS broth of pure culture of *Lactobacilli* and their respective co-cultures with pathogens was almost similar (from 0 h-24 h) but *Lactobacilli* inhibitory effect on pathogens viable count was not as prominent during first 12 h of co-culture period, however pathogens viable count was less during this period than respective pure cultures of pathogens. Moreover, when pathogenic bacteria were grown in pH equal to 24 h *Lactobacilli* culture pH, their viable count was more than their co-culture assays with *Lactobacilli*. These findings indicate that *Lactobacilli* growth inhibitory effects on pathogens is not only by their low pH but their metabolites also have antimicrobial activity. Fayol-Messaoudi et al., 2005 study also reported that non lactic acid molecules of LAB inhibited pathogen growth [49]. Similar kind of findings were also reported by Wang et al., 2018 [24]. LAB can produce a variety of antimicrobial molecules like bacteriocin, hydrogen peroxide, bacteriocin-like compound, extracellular organic acids [50-55].

Probiotics colonization potential is one of most important property recommended by WHO/FAO. Most of probiotics important functions like immune modulation or antagonisms to harmful microbes are linked to their intestinal colonization which are usually investigated in vitro using simulated intestinal cells [21]. Intestinal adhesion and colonization are mediated by interaction between bacterial cells surface molecules and gut epithelial cells receptors and is highly variable for different bacterial strains.

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García-Ruiz et al. (2014) reported 0.37 to 12.2% adhesion of wine isolated LAB with Caco-2 cells [29] whereas LAB isolated from Sardinian dairy products showed 3 to 20% adhesion with Caco-2 cells [46]. Our results are in line with these findings showing that the adhesion property of probiotics is a strain specific with maximum adhesion of *L. acidophilus* AR1 (7.5 ± 0.20 %) followed by *L. acidophilus* AR2 (7 ± 0.25 %) and *L. fermentum* AR3 (5.8 ± 0.27%) with MODE-K cells. The adhesion percentage of these LAB depends on initial concentrations and significantly increased when initial inoculated concentration is increased. This concentration dependent relationship verifies the findings of Wang et al. 2018 who reported that *Lactobacillus* (*L. plantarum* ZLP001) adhesion with IPEC-J2 significantly increased as inoculated bacterial concentration is increased [24]. The pathogenic infections are usually associated with their adhesion to host epithelium which sometime results in destruction of epithelium facilitating the pathogens and their toxin entry into host organ [24]. LAB have potential to inhibit pathogens adhesion to host cells by producing different kinds of adhesive surface molecules (e.g., enolases, glyceraldehyde-3-phosphate, pyruvate dehydrogenase) [56]. These adhesive molecules assist LAB adhesion to host cells as well as in contesting and preventing pathogenic bacterial attachment and colonization [57-59]. Our results are in consistent with these reports showing inhibitory effects of dog isolated LAB on adhesion of different pathogens with MODE-K cells. The adhesion percentage of all tested pathogenic was less during inhibition assay followed by competition and displacement assays. Because during pre-addition of probiotic *Lactobacilli*, they occupy most of host cellular receptors. Anti-infection properties of LAB against pathogens may also be due to their ability to secrete different kinds of antimicrobial compound (such as organic acids, primarily lactic acid, hydrogen peroxide, volatile compounds such as diacetyl and ethanol, carbon dioxide and bacteriocins), and bio-surfactants production [60]. These anti-infective properties of LAB have been reported in various studies. Nowak & Motyl, 2017 reported that *Lactobacillus* can inhibit adhesion of different pathogens (*Staphylococcus aureus*, *Listeria monocytogenes, Clostridium difficile*) with Caco-2 cells and in his other study he reported adhesion inhibition of *S. enterica* serovar Typhimurium with Caco-2 cells by LAB [61]. Similar results of LAB potential to inhibit adhesion of pathogens with epithelial cells have also been reported by many other researchers [21, 24, 29, 46, 62]. However, our finding of LAB anti-infective potential did not match with Nowak & Motyl, 2017 [62], Wang et al., 2018 [24] and Falah et al., 2019 [21]. AS Anti-infection relies on both pathogenic and probiotic strains and should be assessed on a case-by-case basis [29, 63]. Intestinal epithelial barrier prevents systemic entry of toxins and bacteria and many other unwanted molecules by acting as physical and biochemical. Therefore, its integrity and function are very important. Many studies have reported that LAB have potential to improve the intestinal barrier damage induced by enteric pathogens [64-69].

Our qPCR results indicated that LAB significantly inhibit the *Salmonella* decreased gene expression of occludin. Further we checked anti-inflammatory effects of these LAB during *Salmonella* infection as pathogenic induced proinflammatory cytokines have been reported to be associated with

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barrier damage by pathogens [70]. Our results indicated that *Salmonella* significantly induced pro-inflammatory cytokines and our LAB alleviate this effect. Several other reports of LAB study during *Salmonella* infection have also been shown that LAB ameliorate the intestinal barrier damage and pro-inflammatory cytokines production induced by *Salmonella* [65, 71]. Our results did not match with Anderson et al., 2013 [72] who reported that *L. fermentum* AGR1487 has negative effects on epithelial barrier integrity. Possibly it could be due to strain difference and cell line, we use MODE-K cell line and Anderson used Caco-2 cell line.

We also evaluated the dog isolated *Lactobacilli* potential to induce antimicrobial peptides (like host defensin peptides) production by MODE-K cells, based on previous study of probiotics (LAB) induction of antimicrobial peptides in their hosts [73-76]. Our results indicated that dog isolated *Lactobacilli* treated MODE-K cell culture supernatant has no antimicrobial activity. These findings were verified by our qPCR results that none of strain induce mRNA expression of defensin peptides (beta-defensin 3) in MODE-K cell line. These results were in disagreement with reports of Wang et al., 2018 [24] and Zhang et al. 2011 [76]. It possibly could be due to difference of probiotic species and cell lines used in these studies. Our *Lactobacilli* were *L. acidophilus* and *L. fermentum* and we used MODE-K cell line. Wang used *L. plantarum* and Caco-2 cell line whereas Zhang used *L. salivariu* and took samples by biopsy from neonatal pigs.

Competing interest statement

Authors declare no competing interest exist

Authors contribution

GZ and RA conducted the experiments and wrote the manuscript, ZJ, YM, YX, WM helped GZ and RA, QT supervised, design the experiment, and reviewed the manuscript.

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