Article

Cytoprotective Effects of *Lactobacilli* on Mouse Epithelial Cells during *Salmonella* Infection

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Abstract: Treatment of common pathogens, such as *Salmonella* species, *Escherichia coli*, *Staphylococcus aureus*, etc., is a big challenge for a practitioner. Antibiotics' side effects during their application for the treatment of infectious diseases should not be underestimated as they have many issues, such as the transfer of antibiotics-resistant genes, dysbiosis, and antibiotic-resistant strains, which is the main hurdle in the eradication of diseases. To avoid these antibiotics complications, in modern countries, the interest of using probiotics in feed supplementation to promote health and prevent or treat intestinal infectious diseases has been increasing. The purpose of the present study was to evaluate the probiotic potential of three *Lactobacilli* strains isolated from clinically healthy dogs for their further utilization as a dietary supplement for dogs to avoid pathogenic and antibiotic complication. After 16SrRNA sequencing, in vitro tests were conducted to assess the survival potential of *Lactobacilli* under simulated gastrointestinal conditions and adhesion ability to the MODE-K cell line, effects on epithelial barrier function, anti-inflammatory activities, effects on host defensin peptides (beta-defensin 3), and inhibitory effects on common pathogens. *Lactobacilli* showed considerable potential to survive in simulated gastrointestinal environmental conditions, low pH, and high bile salt concentrations along with good adhesion properties with MODE-K cells. Pathogenic bacterial growth and their adhesion to MODE-K cells were significantly inhibited by *Lactobacilli*. Real-time PCR analyses further demonstrated that the *L. acidophilus* strain AR1 and AR3 inhibit *Salmonella*-induced proinflammatory cytokine (IL-6, IL-8, IL-1β) production and reinforce the expression of tight junction protein (occludin). None of the strains induce mRNA expression of beta-defensin 3 in MODE-K cells. Based on the in vitro results, the *L. acidophilus* strain AR1 has the 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 that when administered to their host in an adequate amount confer a good health effect. The animal gastrointestinal tract is a very complex

ecosystem on the Earth and is continuously affected by host-associated [1,2] and outside environmental factors [3]. Intestinal microbiota has received significant attention in recent years. The use of probiotic microorganisms in animal and human nutrition to improve health [4] as well as to control pathogenic infectious diseases has become an area of great research activity [5]. Antibiotics' application as a feed additive or during infectious diseases has many side effects [6], i.e., long-lasting changes of the intestinal microbiota toward unhealthy patterns [7], intestinal barrier function damage [8], and many other problems, such as antibiotics residues in foodstuff, bone marrow toxicity, hepatotoxicity, nephropathy, reproductive disorder, carcinogenicity, etc. [9]. Nowadays, probiotics are considered as antibiotic alternatives to prevent and treat pathogenic infections [10]. For any good probiotics candidates, the adhesion abilities to the intestinal epithelium, potential to fortify the barrier function, antimicrobial activities, high survival rate in the gastrointestinal tract, low pH, and activity during bile salt exposure are considered to be crucial for probiotics functionality [11–13].

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

The composition of dog intestinal microbiota changes with respect to age [19]: as dogs become older, the prevalence and 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 the life quality of both dogs and their owners. This study aimed to assess the probiotic potential of dog-isolated *Lactobacilli* (*L. fermentum*, *L. acidophilus*) to be used in dog food. We conducted some basic necessary tests, which are considered essential for the functionality of any probiotic candidates.

2. Materials and Methods

2.1. Bacterial Strains and Culture Conditions

Several *Lactobacilli* were successfully isolated from healthy dog feces, purified on selective lactic acid bacterial agar, and then identified by 16S rRNA sequencing. *L. acidophilus* (strain AR1; GenBank MZ854114), *L. acidophilus* (strain AR2; GenBank MZ854118), and *L. fermentum* (strain AR3; GenBank MZ735397) were chosen for further assessment. These 3 strains were anaerobically grown at 37 °C in the De Man, Rogosa and Sharpe agar medium

(MRS). The pathogenic strain, *Salmonella enterica* serovar Typhimurium strain ATCC14028, *Salmonella enterica* serovar Typhimurium strain SL1344, Enterotoxigenic *Escherichia coli* k88 (ETEC) (provided through the 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 Lysogeny broth (LB) medium at 37 °C.

2.2. Lysozyme Resistance Assay

A method described by Zago et al. (2011) [20] was used to assess the lysozyme resistance characteristics of tested strains. Briefly, overnight culture of each strain (AR1, AR2, AR3) was centrifuged and inoculated in 9 mL of sterile electrolyte solutions (2.2 g/L KCl, 0.22 g/L CaCl$_2$, 1.2 g/L NaHCO$_3$, 6.2 g/L NaCl, 0.1 g/L 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 of each strain was determined after 90 and 180 min by the agar plate count method and compared to the control. The experiments were repeated three times 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 viable bacterial growth was measured by the agar plate count method and compared with the control. The results were presented as growth percentage compared to the control. The experiments were carried out in triplicate and repeated three times independently.

2.4. In Vitro Resistance 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, the overnight cultures of the tested strains were centrifuged and resuspended to the original volume and then each strain was inoculated to simulate gastric and intestinal juices. To prepare simulated gastric and intestinal juices, pepsin (final concentration 3 g/L, pH 2) and pancreatin (1 g/L containing 0.2% bile salts with pH 8) were dissolved in 0.5% *v/v* sterile solution of NaCl and PBS, respectively. Both juices were sterile, using 0.22 μm pore size filters. After different incubation periods (0, 30, 60, 90, and 120 min) with these simulated juices, the viable count of *Lactobacilli* (AR1, AR2, AR3) was calculated by the agar plate count method. The assays were performed in triplicate and repeated three times.

2.5. Exopolysaccharide Production Test

An exopolysaccharides (EPS) production test was performed according to an already described method of Garai-Ibabe et al., (2010). The tested strains were grown at 28 °C in an atmosphere containing 10% CO$_2$ for 48 h, in MRS broth supplemented with 20% (*v/v*) tomato juice and 5 g/L fructose. By visually observing the culture viscosity, the EPS-producing ability of each strain was assessed. Upon agitation, a ropy liquid culture showed production of EPS [22].

2.6. Co-Culture of Lactobacilli Strains (AR1, AR2, AR3) and Pathogens

Overnight cultures of the tested strains and pathogenic strains were adjusted to equal concentrations (10^7 CFU per mL) by adjusting their OD$_{600}$ values. In total, 1 mL (10^7 CFU per mL) of strain AR1 and ATCC14028 was inoculated together in fresh MRS broth to a final volume of 50 mL and named AR1-ST28. Co-cultures of AR1 with SL1344, ETEC, and *S. aureus* were named AR1-SL44, AR1-ETEC, and AR1-*S. aureus*, respectively. Co-cultures of AR2 with ATCC14028, SL1344, ETEC, and *S. aureus* were named AR2-ST28, AR2-SL44, AR2-ETEC, and AR2-*S. aureus*, respectively. Similarly, co-cultures of strain AR3 with ATCC14028, SL1344, ETEC, and *S. aureus* were named AR3-ST28, AR3-SL44, AR3-ETEC, and AR3-*S. aureus*, respectively. The viable bacterial count and pH of co-cultures and

pure cultures of each bacteria were measured by the agar plate count method at different intervals (6, 12, 24 h). The experiment was repeated three times [23].

2.7. Assessment of Pathogenic Growth Inhibitory Effects of Lactobacilli Strain (AR1, AR2, AR3) Metabolites

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

2.8. 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 (2.5×10^5/well) in 6-well plates, with each well containing 3 mL of DMEM media supplemented with 10% FBS without antibiotics, and were grown at 37 °C, 5% CO_2, and 95% air atmosphere [24].

2.9. Antibacterial Activity of Lactobacilli (AR1, AR2, AR3)-Treated MODE-K Cell Culture Supernatant

The MODE-K cells (provided by 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 their antimicrobial activity. The supernatant of MODE-K cells without *Lactobacilli* strain (AR1, AR2, AR3) exposure was used as a negative control whereas supernatant collected from wells containing only *Lactobacilli* strain (AR1, AR2, AR3, no MODE-K cells) was used as a positive control. The antimicrobial activity of the supernatants after filtering (0.25-μm pore size filters) was checked against the pathogenic strain (ATCC14028, SL1344, ETEC, *S. aureus*). A 10 μL suspension of an overnight culture of pathogens in LB broth was inoculated in 500 μL of supernatants. The viability of the pathogen was assessed by the agar plat count method after 2 h of growth incubation at 37 °C and 200 rpm shaking. The experiment was carried out in triplicate and performed three times [25].

2.10. Adhesion and Adhesion Inhibition Assays

The adhesion of probiotics with eukaryotic cells (MODE-K) was 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 times with PBS to remove unbound bacteria and were lysed using 0.2% 100 μL of Triton^TM X-100 to assess the viable bacterial count by the agar plate count method. The wells with only bacteria (no MODE-K cells) were kept as a control. The adhesion assay was expressed as a percentage of CFU per mL compared to CFU per mL with control.

Competition, inhibition, and displacement assays were performed to check the inhibitory effects of *Lactobacilli* strains (AR1, AR2, AR3) on pathogen adhesion with MODE-K

cells. *Lactobacilli* strains (AR1, AR2, AR3) were added before, at the same time, and 1 h after the addition of pathogenic strains to MODE-K cells for the inhibition, competition, and displacement assay, respectively. After 2 h of incubation for each assay, the cells were washed with PBS to remove unbound bacteria and then lysed using 0.2% 100 μL of Triton^TM X-100. The viable count of the pathogen was calculated by serial dilution and plating on LB agar plates. The adhesion was expressed as a percentage of the adhering pathogens normalized to the control [21–23].

2.11. Real-Time PCR for mRNA Expression of Tight Junction Proteins, Cytokines, and Defensin Peptides

PCR was performed to check the effects of *Lactobacilli* strains (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 the mRNA expression of Occludin, IL-8, IL-6, and IL-1β. To assess the 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 to each *Lactobacilli* strain for 1 h before addition of *S. Typhimurium* ATCC14028 and *S. Typhimurium* SL1344 at the same MOI. After 2 h of incubation, the total RNA was extracted from all treated and control groups by Trizol reagent as per the manufacturer's recommendations. To investigate the effects of *Lactobacilli* on the 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 a 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 of primers (0.5 μL of forward and 0.5 μL of reverse primer), 7 μL of nuclease-free water, 2 μL of cDNA, and 10 μL of SYBR Green Master mix (Vazyme Biotech Co., Ltd., Nanjing, China) was used. The experimental program consisted of pre-denaturation for 30 s 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 $2^{-\Delta\Delta CT}$ method of Livak and Schmittgen [26] was used to calculate the relative expression level of genes using glyceraldehyde-3-phosphate dehydrogenase as a housekeeping gene for normalization of the 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

3. Statistical Analysis

Two-way ANOVA followed by turkeys' multiple comparisons 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 was observed to be more resistant against lysozyme followed by *L. acidophilus* AR2 and *L. fermentum* AR3. *L. acidophilus* AR1 showed a considerable survival percentage of 7.22 ± 1.2% (7.33 ± 6.54 log CFU/mL) after 90 min of incubation in lysozyme. However, the survival percentage decreased to 0.63 ± 0.25% (6.67 ± 6.43 log CFU/mL). The

survival percentage of *L. acidophilus* AR2 after a 90- and 180 in incubation with lysozyme was found to be $3.88 \pm 2.20\%$ (7.01 ± 6.48 log CFU/mL) and $0.10 \pm 0.07\%$ ($6.06 \pm 5.48\%$ log CFU/mL), respectively. Whereas the survival percentage of *L. acidophilus* AR3 after a 90 and 180 min incubation with lysozyme was found to be $1.87 \pm 1.25\%$ (6.70 ± 6.29 log CFU/mL) and $0.3 \pm 0.51\%$ (4.77 ± 4.48 log CFU/mL), respectively (Figure 1).
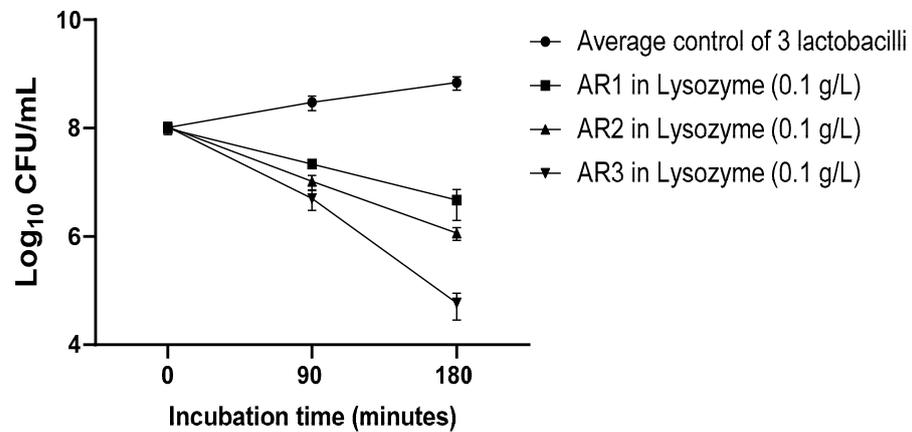

Figure 1. Lysozyme resistance assay of *L. acidophilus* AR1, *L. acidophilus* AR2, and *L. fermentum* AR3.

4.2. pH and Bile Salt Resistance

L. acidophilus AR1 showed greater resistance against the 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. The survival percentage of *L. acidophilus* AR1 at pH 5, 4, 3, and 2 was found to be $97 \pm 1\%$ (7.99 ± 6.01 log CFU/mL), $76 \pm 0.72\%$ (7.88 ± 5.85 log CFU/mL), $40 \pm 0.51\%$ (7.60 ± 5.71 log CFU/mL), and $10 \pm 0.50\%$ (7.02 ± 5.69 log CFU/mL), respectively. The survival percentage of *L. acidophilus* AR2 at pH 5, 4, 3, and 2, was calculated to be $95.63 \pm 0.86\%$ (7.98 ± 5.93 log CFU/mL), $67 \pm 1.85\%$ (7.82 ± 6.26 log CFU/mL), $32.4 \pm 1.36\%$ (7.51 ± 6.13 log CFU/mL), and $8.4 \pm 0.50\%$ (6.92 ± 5.70 log CFU/mL), respectively. Similarly, the survival percentage of *L. acidophilus* AR3 at pH 5, 4, 3, and 2 was found to be $95.2 \pm 1.36\%$ (7.97 ± 6.13 log CFU/mL), $56.5 \pm 1.85\%$ (7.75 ± 6.18 log CFU/mL), $15.7 \pm 1.07\%$ (7.19 ± 6.03), and $2.7 \pm 0.64\%$ (6.43 ± 5.80 log CFU/mL), respectively. At higher bile salt concentrations, the survival rate of each strain decreased. The survival percentage of *L. acidophilus* AR1 was observed to be $9 \pm 0.50\%$ (6.95 ± 5.69 log CFU/mL), $4.9 \pm 0.65\%$ (6.69 ± 5.81 log CFU/mL), and $1.2 \pm 0.45\%$ (6.07 ± 5.66 log CFU/mL) at the 0.05%, 0.1%, and 0.2% concentration of bile, respectively. The survival percentage of *L. acidophilus* AR2 at the 0.05%, 0.1%, and 0.2% concentration of bile was found to be $8 \pm 0.88\%$ (6.90 ± 5.94 log CFU/mL), $3.2 \pm 0.52\%$ (6.50 ± 5.72 log CFU/mL), and $0.76 \pm 0.50\%$ (5.88 ± 5.70 log CFU/mL), respectively. A similar trend of a decreasing percentage at the higher bile salt concentration was also found with *L. acidophilus* AR3 as indicated by the $4.8 \pm 0.56\%$ (6.68 ± 5.75 log CFU/mL), $0.51 \pm 0.48\%$ (5.71 ± 5.68 log CFU/mL), and $0.04 \pm 0.03\%$ (4.64 ± 4.59 log CFU/mL) survival rate at the 0.05%, 0.1%, and 0.2% concentration of bile, respectively (Figure 2).
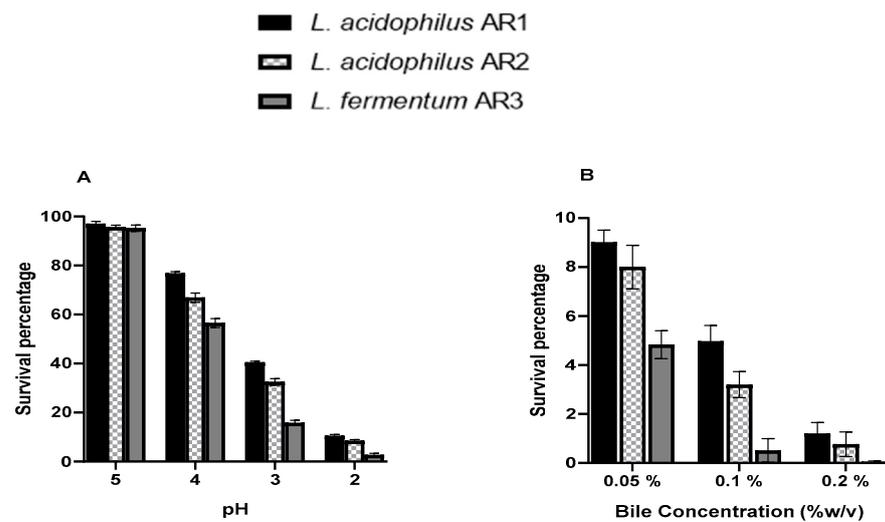
Figure 2. pH (**A**) and bile (**B**) resistance assays of *L. acidophilus* AR1, *L. acidophilus* AR2, and *L. fermentum* AR3.

4.3. Tolerance of the Simulated Condition of GIT

Figure 3 shows that all strains retained their viability during exposure to simulated gastric and intestinal juices. The *L. acidophilus* strain AR1 was found to be more resistant to simulated gastrointestinal conditions followed by the *L. acidophilus* strain AR2 and *L. fermentum* strain AR3. The survival percentage of each strain was greater in simulated intestinal juice as compared to simulated gastric juice. In simulated intestinal juice, the survival percentage of the *L. acidophilus* strain AR1 was found to be $61.6 \pm 1.65\%$ (6.93 ± 5.93 log CFU/mL), $33.7 \pm 6.5\%$ (6.78 ± 5.76 log CFU/mL), $15.3 \pm 2.22\%$ (6.55 ± 5.54 log CFU/mL), and $6.5 \pm 1.39\%$ (6.26 ± 5.51 log CFU/mL) after 30, 60, 90, and 180 min of incubation, respectively. Whereas in simulated gastric juice, the survival percentage of *L. acidophilus* strain AR1 was found to be $44.9 \pm 3.8\%$ (6.83 ± 5.84 log CFU/mL), $19.6 \pm 4.1\%$, (6.55 ± 5.69 log CFU/mL) $7.4 \pm 0.87\%$ (6.24 ± 5.39 log CFU/mL), and $2.5 \pm 0.23\%$ (5.86 ± 4.87 log CFU/mL) after 30, 60, 90, and 180 min of incubation, respectively.

In simulated intestinal juice, the survival percentage of the *L. acidophilus* strain AR2 was found to be $54.3 \pm 6.4\%$ (6.85 ± 5.64 log CFU/mL), $26.8 \pm 2.47\%$ (6.67 ± 5.70 log CFU/mL), $12.1 \pm 1.56\%$ (6.43 ± 5.61 log CFU/mL), and $5.6 \pm 0.78\%$ (6.19 ± 5.25 log CFU/mL) after 30, 60, 90, and 180 min of incubation, respectively. Whereas in simulated gastric juice, the survival percentage of the *L. acidophilus* strain AR2 was found to be $44.3 \pm 8.07\%$ (6.76 ± 5.82 log CFU/mL), $15.6 \pm 1.5\%$ (6.44 ± 5.28 log CFU/mL), $6.1 \pm 1.48\%$ (6.24 ± 5.26 log CFU/mL), and $2.1 \pm 0.28\%$ (5.37 ± 4.79 log CFU/mL) after 30, 60, 90, and 180 min of incubation, respectively.

In simulated intestinal juice, the survival percentage of the *L. acidophilus* strain AR3 was found to be $51.6 \pm 3.12\%$ (6.81 ± 5.51 log CFU/mL), $25.5 \pm 3.37\%$ (6.58 ± 5.56 log CFU/mL), $11.4 \pm 1.66\%$ (6.31 ± 5.41 log CFU/mL), and $4.9 \pm 1.10\%$ (6.01 ± 5.13 log CFU/mL) after 30, 60, 90, and 180 min of incubation, respectively. Whereas in simulated gastric juice, the survival percentage of the *L. acidophilus* strain AR3 was found to be $38.9 \pm 1.47\%$ (6.69 ± 5.63 log CFU/mL), $13.7 \pm 1.10\%$, (6.31 ± 5.41 log CFU/mL) $5.3 \pm 0.52\%$ (5.99 ± 5.15 log CFU/mL), and $2 \pm 0.28\%$ (5.37 ± 4.73 log CFU/mL) after 30, 60, 90, and 180 min of incubation, respectively.
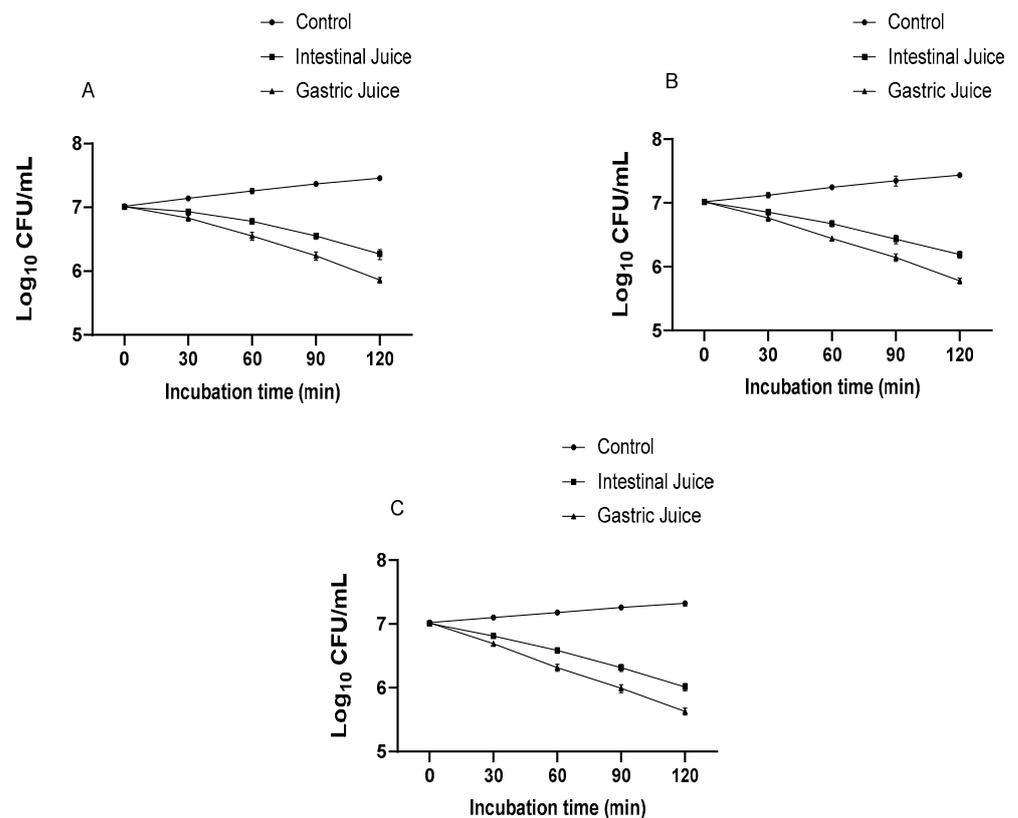
Figure 3. The survival rate of *L. acidophilus* AR1 (**A**), *L. acidophilus* AR2 (**B**), and *L. fermentum* AR3 (**C**) under simulated GIT conditions.

4.4. Exopolysaccharide Production

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

4.5. Co-Culture of Lactobacilli and Pathogens

The *Lactobacilli* strain inhibited the growth of pathogenic bacteria when grown together. Figure 4 shows the growth pattern of *L. acidophilus* AR1 pure culture and coculture with different pathogens. The growth pattern in the pure and co-culture groups was almost similar. However, in the pure culture, the growth was slightly less than the co-culture with pathogens. Similar growth patterns were observed for *L. acidophilus* AR2 (Figure 5) and *L. fermentum* AR3 (Figure 6) during co-culture assays and pure culture. However, the 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 pathogen in pure culture was more than in co-culture with *Lactobacilli* (Figure 7). The acid production patterns by each probiotic strain, as indicated by a decrease in the pH of the medium (Figures 8–10), were almost similar under each of the culture conditions (pure and co-culture assays). The pH decline was slower in the case of pure cultures of pathogenic bacteria than that of co-cultures and pure cultures of *Lactobacilli*.
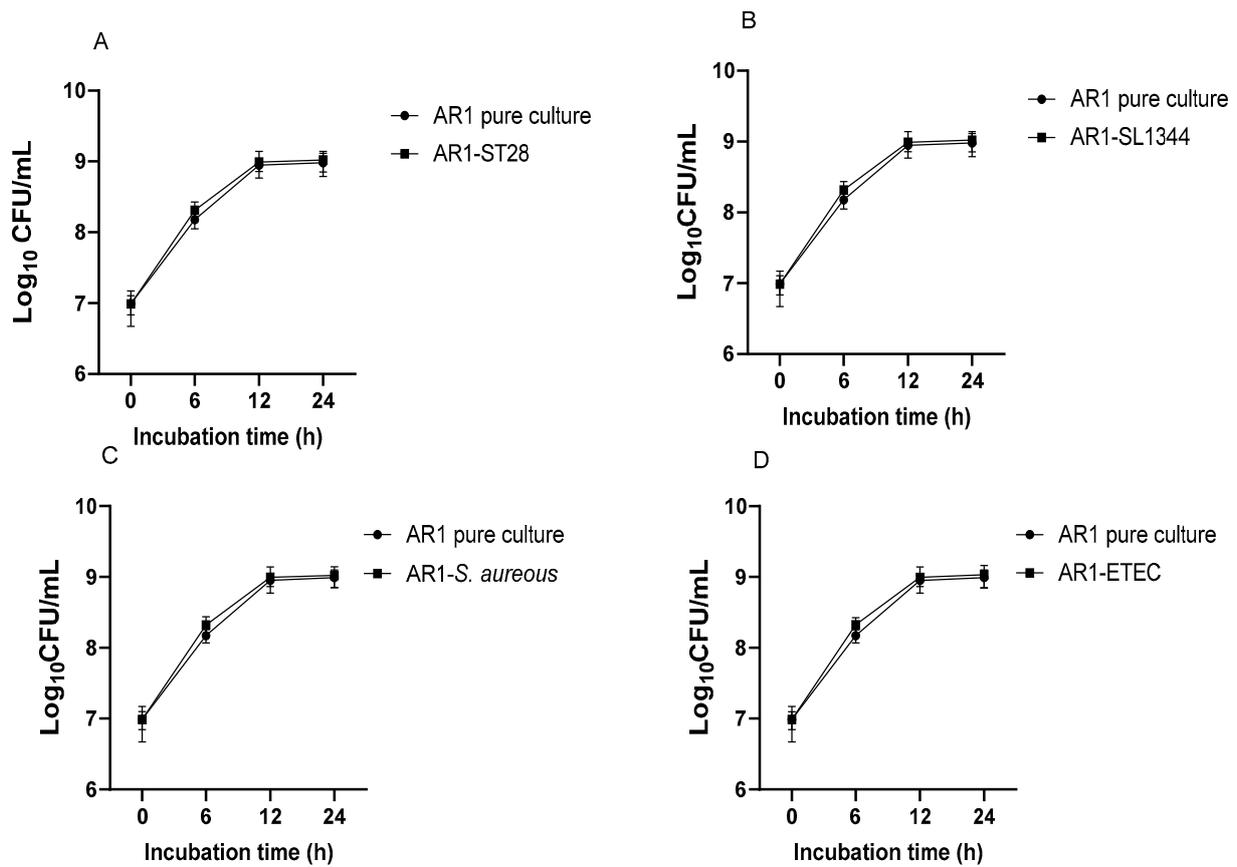
Figure 4. Growth pattern of *L. acidophilus* AR1 in pure culture and co-culture with *S. Typhimurium* 14028. (**A**), *S. Typhimurium* SL1344 (**B**), *S. aureus* (**C**), and ETEC (**D**).
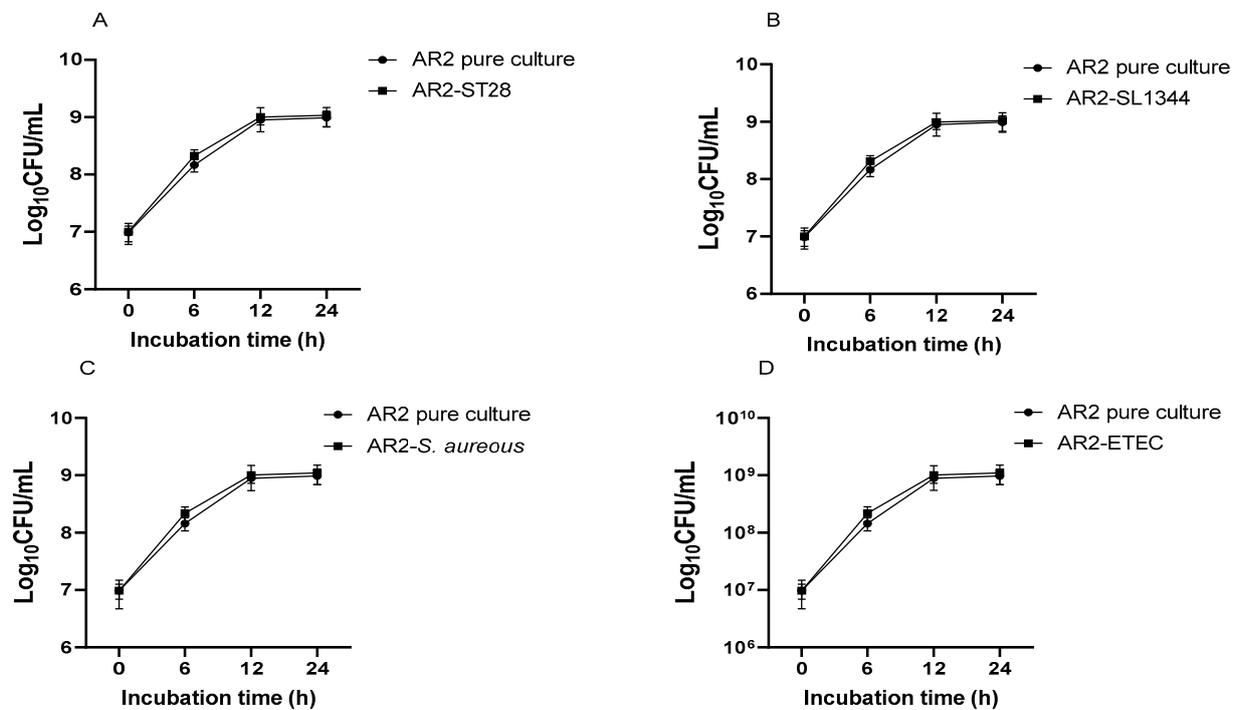

Figure 5. Growth pattern of *L. acidophilus* AR2 in pure culture and co-culture with *S. Typhimurium* ATCC14028 (**A**), *S. Typhimurium* SL1344 (**B**), *S. aureus* (**C**), and ETEC (**D**).
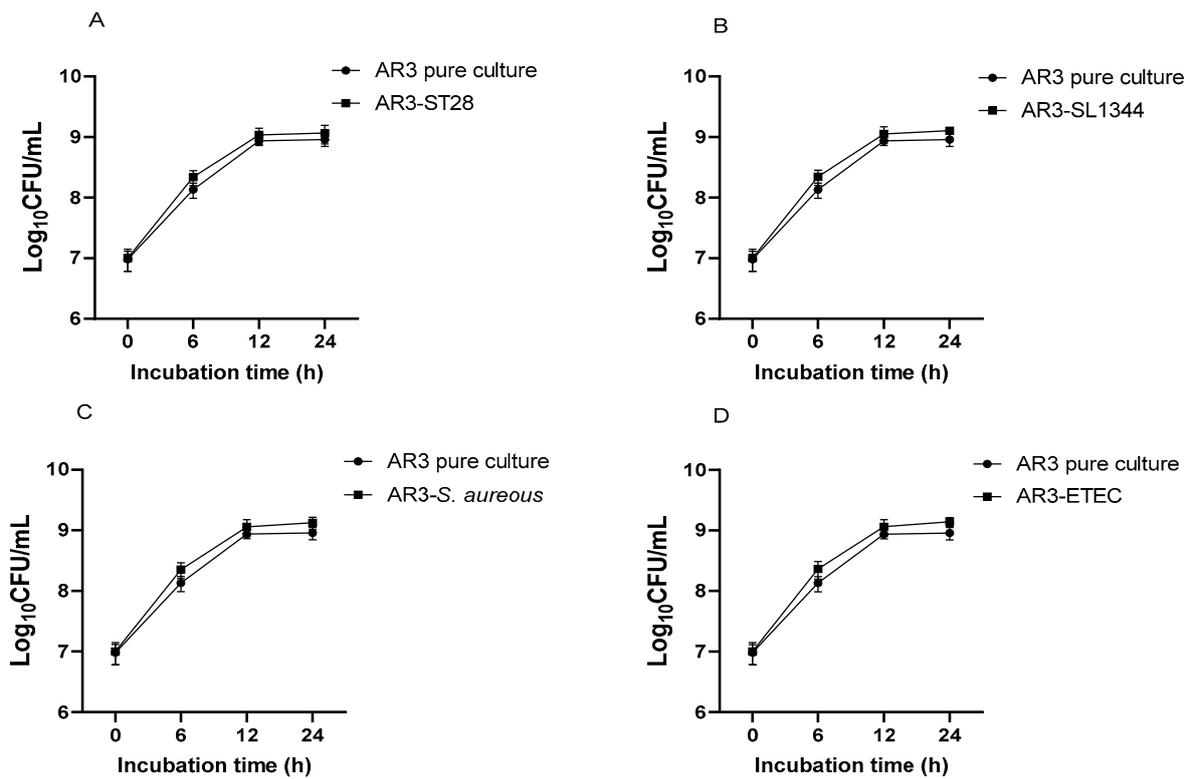
Figure 6. Growth pattern of *L. acidophilus* AR3 in pure culture and co-culture with *S. Typhimurium* ATCC14028 (**A**), *S. Typhimurium* SL1344 (**B**), *S. aureus* (**C**), and ETEC (**D**).
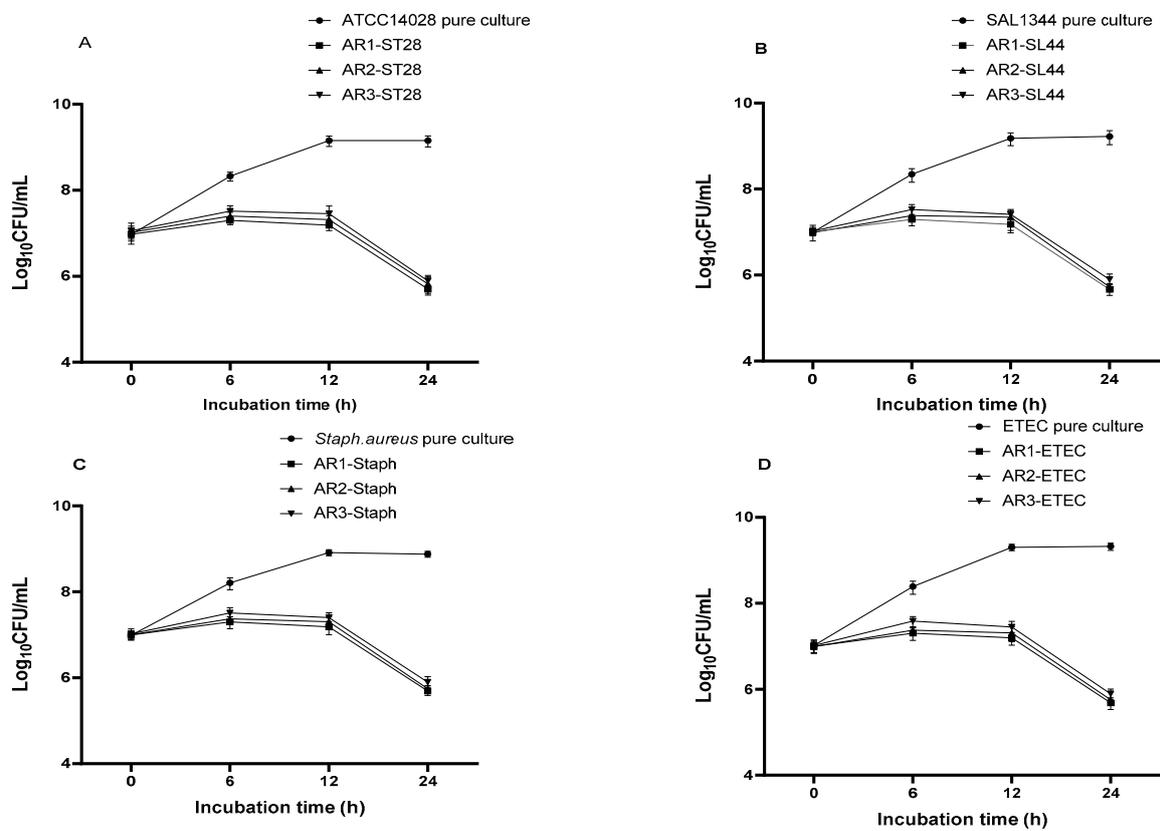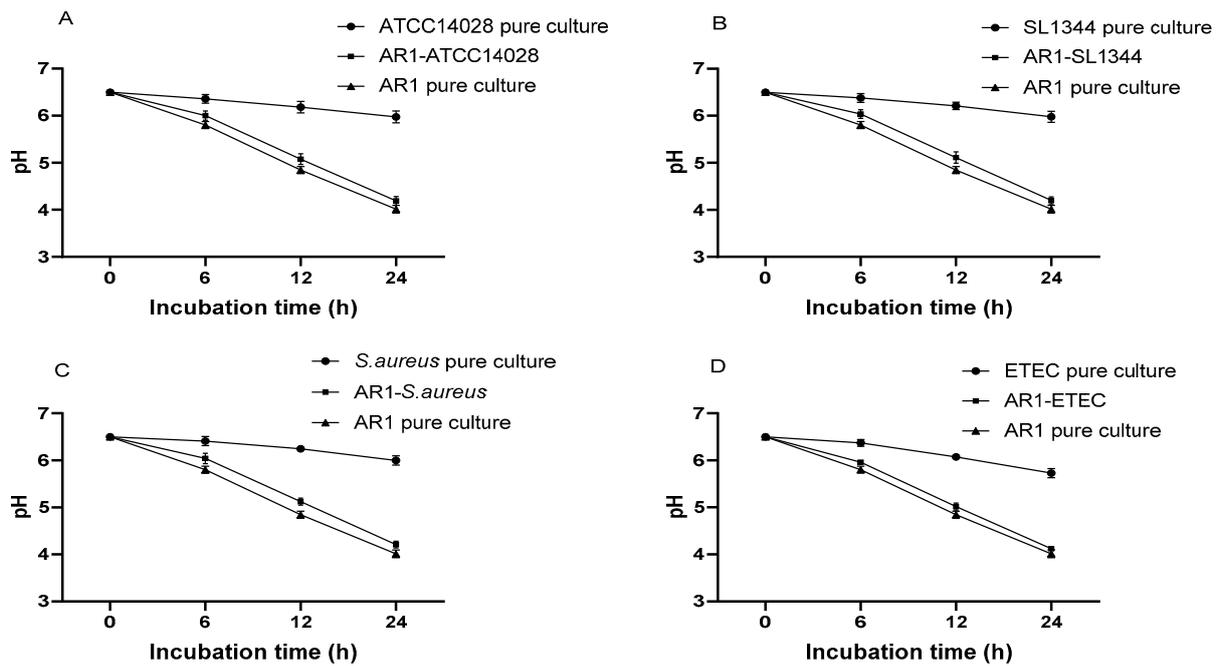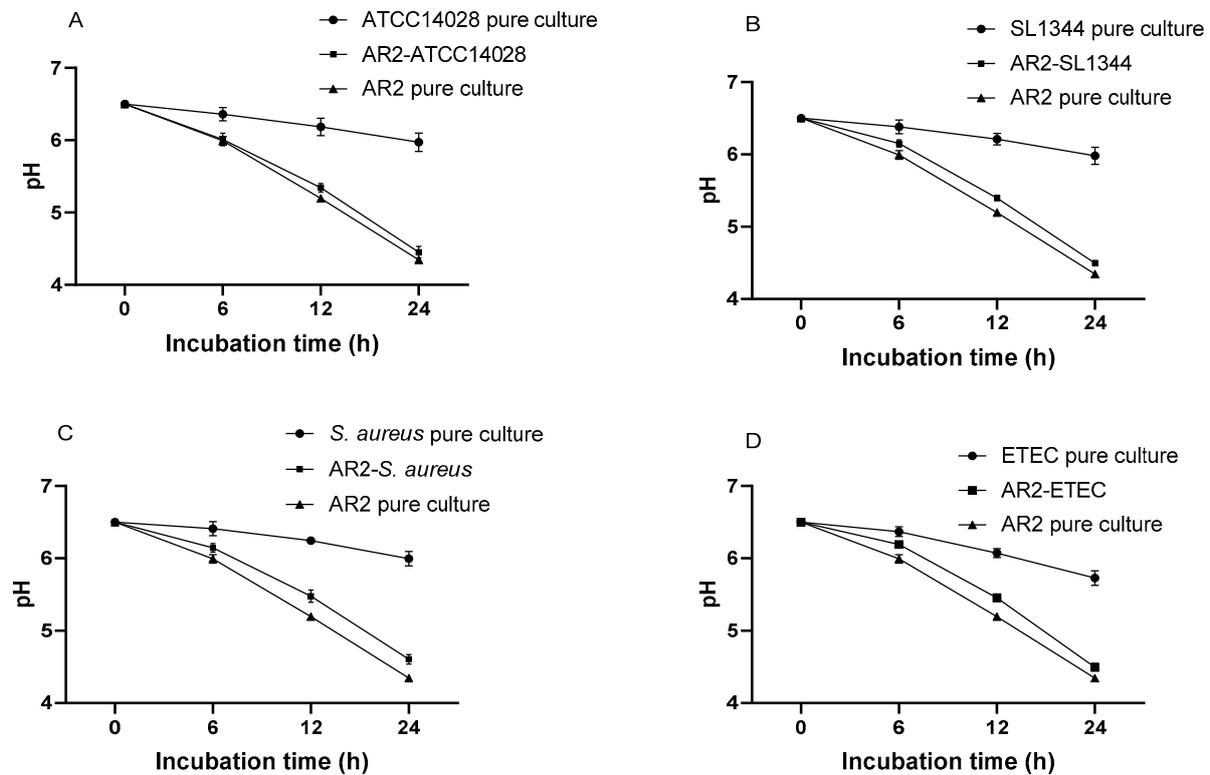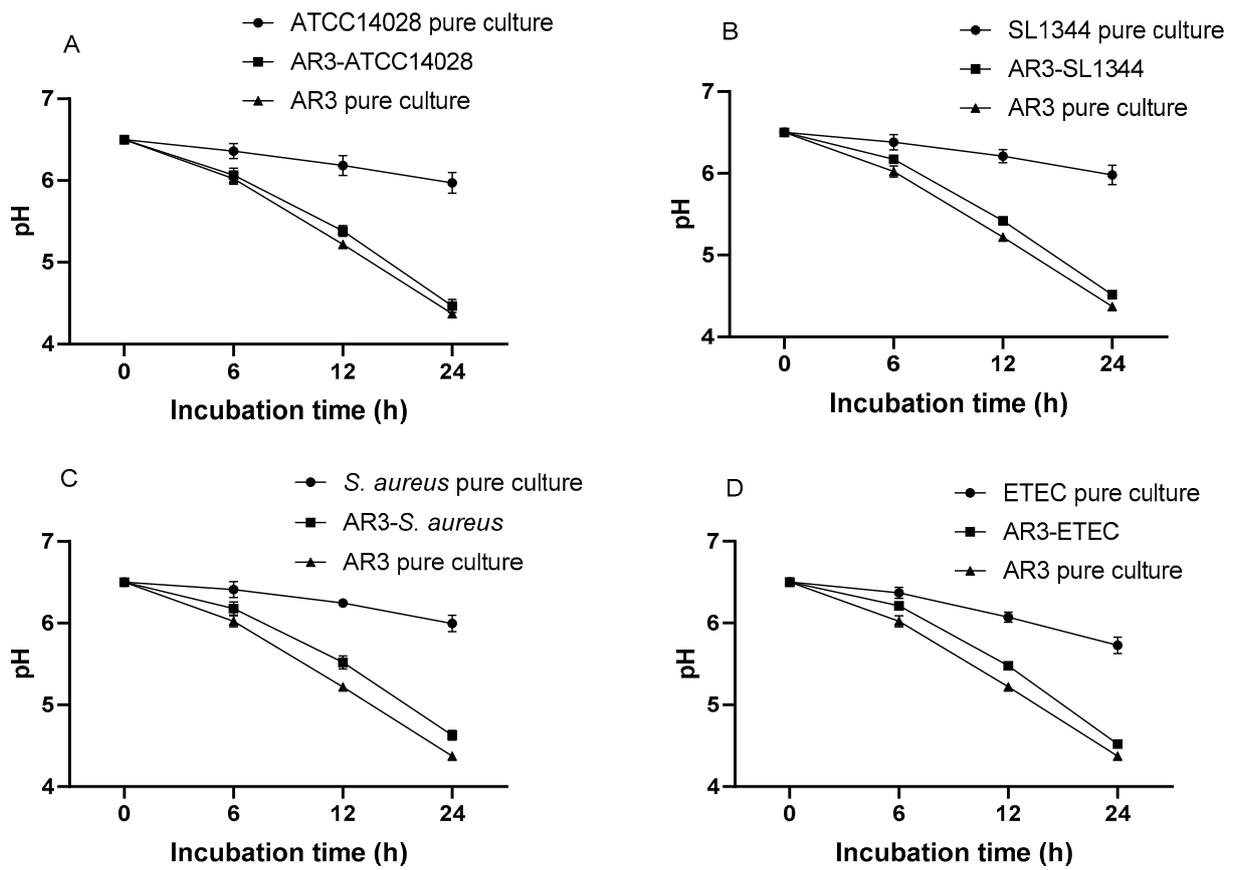

Figure 7. Growth pattern of *S. Typhimurium* ATCC14028 (**A**), *S. Typhimurium* SL1344 (**B**), *S. aureus* (**C**), and ETEC (**D**) in pure and co-culture with different *Lactobacilli*.

Figure 8. pH decline patterns of pure cultures of *L. acidophilus* AR1 and different pathogens, and co- cultures of *L. acidophilus* AR1 with *S. Typhimurium* ATCC14028 (**A**), *S. Typhimurium* SL1344 (**B**), *S. aureus* (**C**), and ETEC (**D**).

Figure 9. pH decline patterns of pure cultures of *L. acidophilus* AR2 and different pathogens, and co-cultures of *L. acidophilus* AR2 with *S. Typhimurium* ATCC14028 (**A**), *S. Typhimurium* SL1344 (**B**), *S. aureus* (**C**), and ETEC (**D**).

Figure 10. pH decline patterns of pure cultures of *L. acidophilus* AR3 and different pathogens, and co-cultures of *L. acidophilus* AR3 with *S. Typhimurium* ATCC14028 (**A**), *S. Typhimurium* SL1344 (**B**), *S. aureus* (**C**), and ETEC (**D**).

*4.6. Inhibitory Effects of Lactobacilli Metabolites on Pathogenic Growth*

The viable count of pathogens was found to be more when grown in pH equal to the 24 h culture pH of *Lactobacilli* than that of co-culture with *Lactobacilli*. Pathogen growth was inhibited more when grown together with *Lactobacilli* than in low pH equal to the 24 h *Lactobacilli* culture pH, suggesting the antimicrobial activity of *Lactobacilli* metabolites. The viable count of *S. Typhimurium* ATCC14028 was significantly less (7.1 ± 6.19 log CFU/mL) during co-culture with *L. acidophilus* AR1 as compared to the viable count (7.3 ± 6.49 log CFU/mL) when grown in medium with pH equal to the 24 h *L. acidophilus* AR1 culture pH. Similar observations were noticed when *S. Typhimurium* ATCC14028, *S. Typhimurium* SL1344, and *S. aureus* were grown with the *L. acidophilus* strain AR1 and AR2, and in medium with pH equal to the 24 h *L. acidophilus* strain AR2 and AR2 culture pH. No significant difference was observed in the viable count of pathogens in the case of the *L. fermentum* strain AR3 (Figure 11).
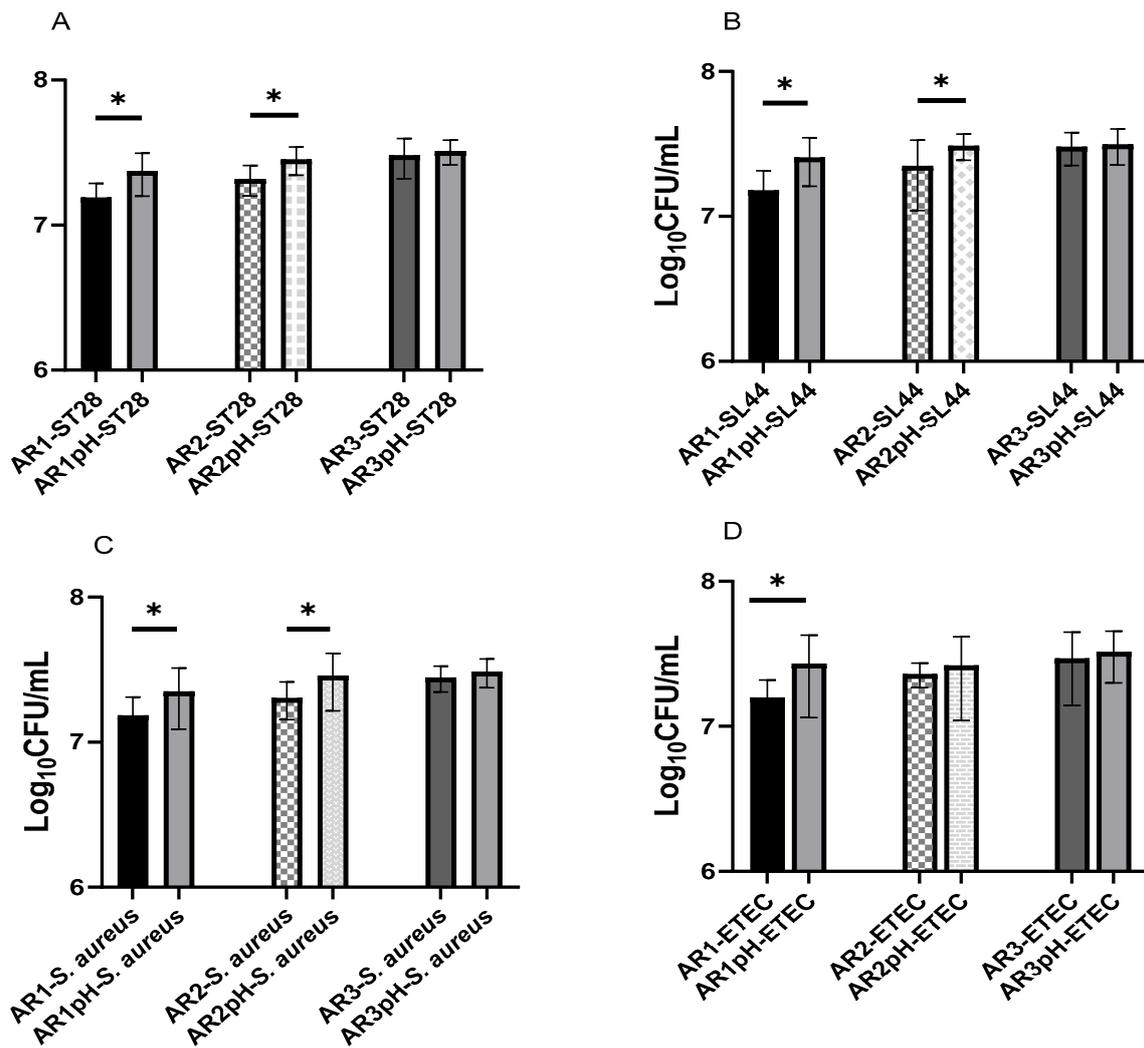
Figure 11. Viable count of *S. Typhimurium* ATCC14028 (**A**), *S. Typhimurium* SL1344 (**B**), *S. aureus* (**C**), and ETEC (**D**) after 12 h of growth in co-culture with *Lactobacilli* and low pH equal to the 24 h culture pH of *Lactobacilli*. Star (*) represents significant difference between the groups.

4.7. Host Defensin Peptide Assessment

No antimicrobial activity of *Lactobacilli*-treated MODE-K cell culture supernatant was observed. A significant difference was not observed in the viable count of pathogenic bacteria when grown in *Lactobacilli* culture supernatant and MODE-K cell culture supernatant collected after *Lactobacilli* stimulation (Figure 12).
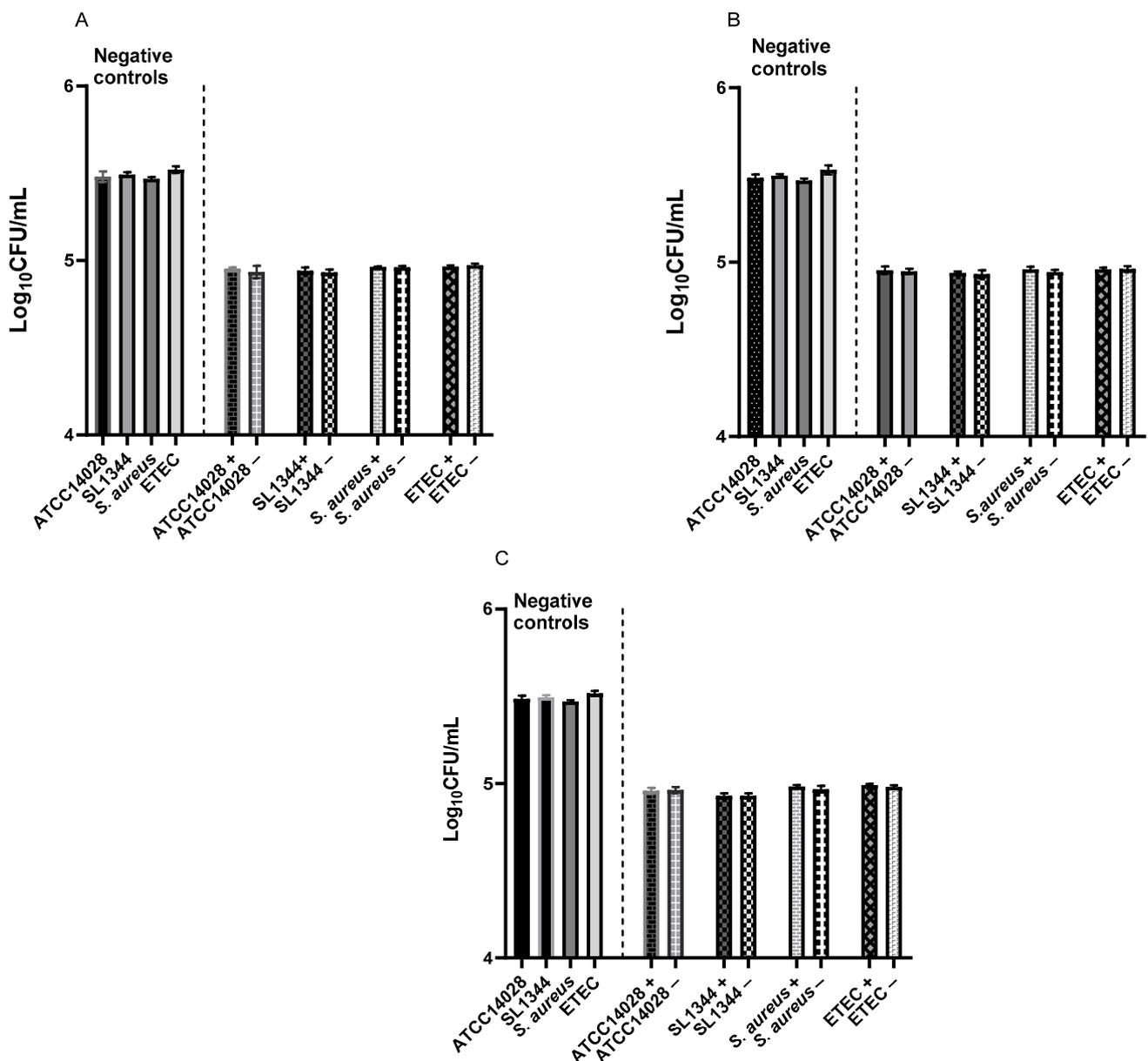
Figure 12. Antimicrobial activity of *Lactobacilli* culture supernatant and MODE-K cell culture supernatant collected after stimulation with *L. acidophilus* AR1 (**A**), *L. acidophilus* AR2 (**B**), and *L. fermentum* AR3 (**C**). Positive sign (+) represents *Lactobacillus* culture supernatant and negative sign (−) represents *Lactobacillus*-treated MODE-K cell culture supernatant.

4.8. Adhesion and Adhesion Inhibition Assays

Each probiotic strain showed an adhesion ability with MODE-K cells. The adhesion percentage of each probiotic strain increased as the initial concentrations increased (Figure 13). The maximum adhesion (7.5 ± 0.2%) was observed for the *L. acidophilus* strain AR1 followed by the *L. acidophilus* strain AR2 (7 ± 0.25%) and *L. fermentum* strain AR3 (5.8% ± 0.27) at the highest inoculated concentration (10^8 CFU per mL). The probiotic strain inhibited pathogen adhesion at all concentrations in a concentration-dependent manner. The pathogenic strain's adhesion percentage decreased considerably when the inoculated concentration of probiotic strains was increased. The maximum inhibitory effects of *Lactobacilli* on pathogen adhesion were observed during the inhibition assay followed by the competition and displacement assays. The adhesion percentage of *S. Typhimurium* ATCC14028 was decreased to 21.1 ± 0.85%, 50.1 ± 0.86%, and 63.3 ± 0.65% during the

displacement, competition, and inhibition assays, respectively, with the *L. acidophilus* strain AR1. The *L. acidophilus* strain AR2 also inhibited the adhesion of *S. Typhimurium* ATCC14028. The adhesion percentage of *S. Typhimurium* ATCC14028 was decreased to 19.8 ± 1.20%, 47.6 ± 1.30%, and 62.3 ± 0.63% during the displacement, competition, and inhibition assays, respectively, with the *L. acidophilus* strain AR2. Similarly, the adhesion percentage of *S. Typhimurium* ATCC14028 was decreased to 16.7 ± 1.25%, 45.1 ± 1.60%, and 59.3 ± 1.97% during the displacement, competition, and inhibition assays, respectively, with the *L. acidophilus* strain AR3. Similar results were noted in the case of the other pathogens during the displacement, competition, and inhibition assays with these *Lactobacilli* (Figure 13).
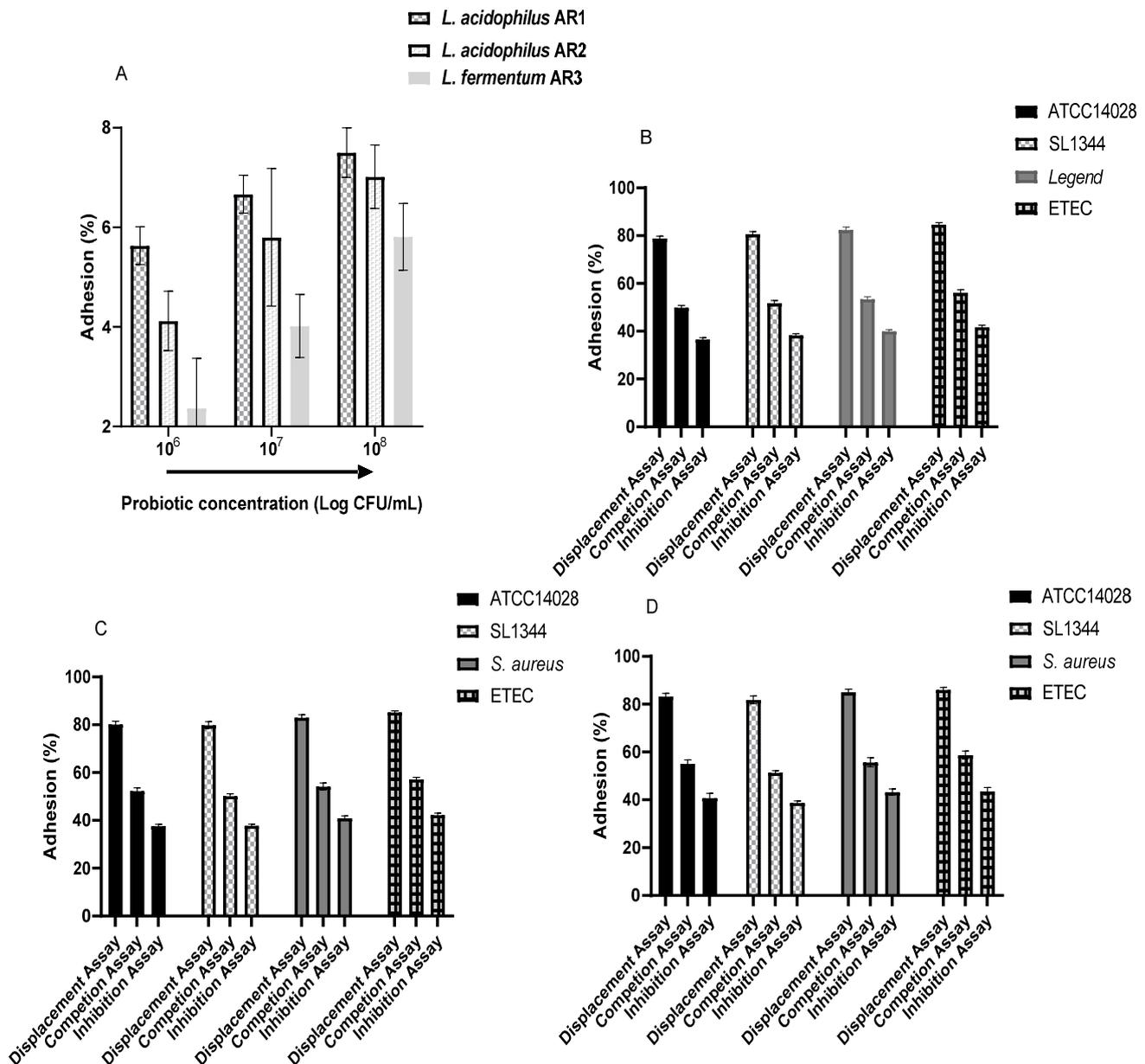

Figure 13. Adhesion of *Lactobacilli* with MODE-K cells (**A**) and inhibitory effects on the adhesion of different pathogenic with MODE-K cells by *L. acidophilus* AR1 (**B**), *L. acidophilus* AR2 (**D**), and *L. fermentum* AR3 (**C**).

4.9. 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 significantly inhibited the *Salmonella*-induced proinflammatory cytokines. The tight junction protein (occludin) was significantly down-regulated by both strains of *Salmonella* and *L. acidophilus* strain AR1 and *L. fermentum* strain AR3 pre-treatment significantly curtailed this effect (Figure 14). As far as host defensin peptide (beta-defensin 3) is concerned, none of the strains induced mRNA expression of beta-defensin 3 in MODE-K cells (Figure 15).
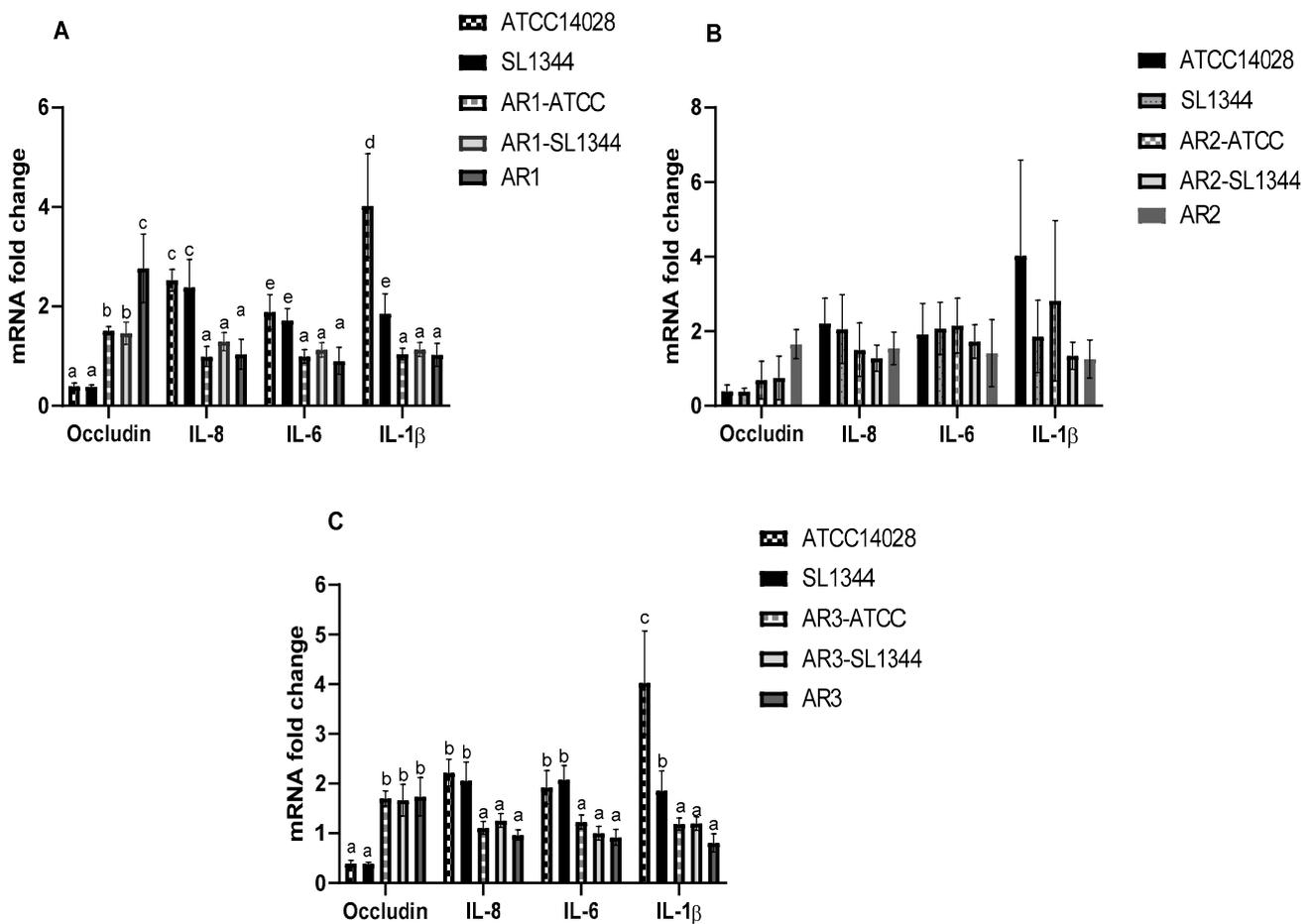
Figure 14. Modulatory effects of *L. acidophilus* AR1 (**A**), *L. acidophilus* AR2 (**B**), and *L. fermentum* AR3 (**C**) on different gene expression during pathogenic infection. Different letters show significant values.
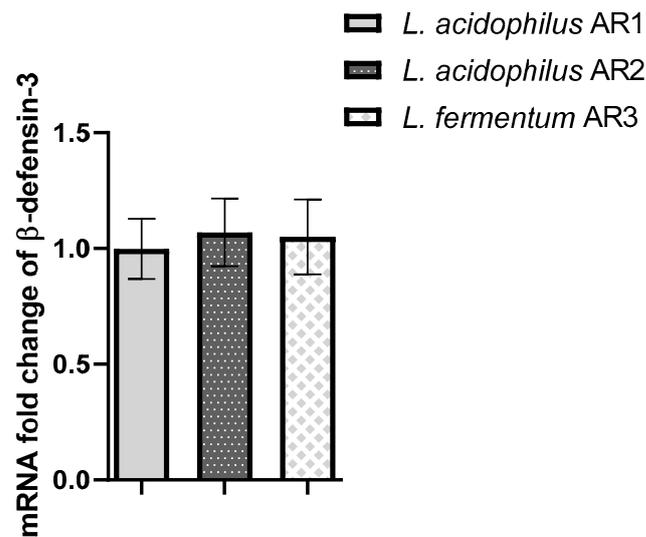
Figure 15. Represents the effect of *L. acidophilus* AR1, *L. acidophilus* AR2, and *L. fermentum* AR3 on beta-defensin 3 mRNA expression. No significant difference was observed with respect to the control (1).

5. Discussion

The present study was conducted to evaluate the 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 the selection of potential probiotic candidates. The first barrier that should be overcome is the mouth, which contains a high lysozyme concentration, then the low pH and digestive enzymes of the stomach and the antimicrobial action of bile of the intestine [27]. Our results showed that the isolates have the potential to survive in the harsh GI tract environment. The isolated *Lactobacilli* showed a considerable number even after 180 min of incubation with lysozyme. A 180 min incubation with lysozyme is considered a severe treatment during probiotics lysozyme resistance assays [28]. The survival rate of *Lactobacilli* in the presence of lysozyme has also been reported by many other studies [20,28,29]. To reach the end of the GIT, stomach acid is one of the major issues for microorganisms. Therefore, pH is considered to be crucial for the selection of probiotics [30]. When food is swallowed, the stomach pH ranges from 3–6 depending on the food composition, so a pH range of 2–5 is usually examined to evaluate the pH resistance potential of probiotics [21]. In various other studies, it has been reported that *Lactobacilli* can survive well in low pH [31–33]. The results of our study are also in concordance with these facts, showing that the new isolated *Lactobacilli* can survive under low pH. Several mechanisms are associated with the survival of *Lactobacilli* in low pH, such as protein and DNA damage repair, several metabolic pathways, proton pumps, neutralization processes, and changes of the cell membrane composition and cell density [31,34–37]. Moreover, the survivability in low pH is also associated with the production of polysaccharides, which protect the microorganisms against lethal pH effects [38], 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 [21,39].

The probiotic functions in the duodenum are compromised because of the secretion of bile salts, which act as antimicrobial molecules [21]. Therefore, to evaluate LAB's potential as a probiotic, their resistance to bile salts is essential. Resistance to bile salts has been considered a condition for bacterial metabolic activity and colonization in the intestine of the host [30]. Bile salt concentrations of 0.15% to 0.5% are usually investigated [40], and to select a strain considered to have good bile resistant ability, a limit of a 0.3% bile concentration was established by Mathara et al. (2008) [41]. In this study, the tested strains showed resistance against different concentrations of bile salts. These findings were consistent

with Hashemi et al. (2014) [38], Sagdic et al. (2014) [42], and Vasiee et al. (2018) [30], who found that LAB can resist 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 the bile salt-resistant activity of probiotics is related to bile salt hydrolases enzymes [38]. A study was carried out on wild-type and bile salt hydrolase (bsh) mutant pairs of *L. plantarum* and *L. amylovorus*, which provided a correlation between bile salt hydrolysis and bile tolerance. The findings demonstrate that mutated cells were much more vulnerable to bile and bile salts and exhibited less growth rates when bile salts were present [43]. The protective effects of the food matrix are another proposed factor that contributes to the survival of microorganisms in the presence of higher bile salt concentrations [44]. Further, we evaluated the survival potential of isolated *Lactobacilli* under simulated GIT conditions. Our results showed that the isolated *Lactobacilli* have a considerable survival rate under simulated gastric juice; however, the survival rate of all strains under simulated gastric juice of pH 2 was found to be less than the pH resistance assay at pH 2. It could be due to the antimicrobial action of pepsin. Similarly, the isolated *Lactobacilli* can also maintain their viable count under simulated intestinal juice containing pancreatin and bile salts. A similar finding in the case of *L. fermentum* strain 4–17 was also reported by Falah et al.'s (2019) [21]. Many other studies have also reported that *Lactobacilli* can maintain their viable count under simulated conditions of GIT [28,45].

Pathogenic bacterial growth inhibition is one of the striking properties of probiotics. Many studies of LAB have shown their broad spectrum of antimicrobial activities [46,47]. The present study indicated that these *Lactobacilli* can inhibit the growth of selected common pathogens based on co-culture assays. 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 0–24 h), but *Lactobacilli*'s inhibitory effect on the pathogen viable count was not as prominent during the first 12 h of the co-culture period; however, the pathogen 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*'s growth inhibitory effects on pathogens are not only by their low pH but their metabolites also have antimicrobial activity. Fayol-Messaoudi et al.'s (2005) study also reported that non-lactic acid molecules of LAB inhibited pathogen growth [48]. Similar findings were also reported by Wang et al. (2018) [23]. LAB can produce a variety of antimicrobial molecules, such as bacteriocin, hydrogen peroxide, bacteriocin-like compound, and extracellular organic acids [49–54].

Probiotics' colonization potential is one of the most important properties recommended by WHO/FAO. Most probiotics' important functions, such as immune modulation or antagonisms to harmful microbes, are linked to their intestinal colonization, which is usually investigated in vitro using simulated intestinal cells [21]. Intestinal adhesion and colonization are mediated by the interaction between bacterial cell surface molecules and gut epithelial cell receptors and are highly variable for different bacterial strains. García-Ruiz et al. (2014) reported 0.37 to 12.2% adhesion of wine-isolated LAB with Caco-2 cells whereas LAB isolated from Sardinian dairy products showed 3 to 20% adhesion with Caco-2 cells [45]. Our results are in line with these findings showing that the adhesion property of probiotics is strain-specific with maximum adhesion of *L. acidophilus* AR1 (7.5 $\pm$ 0.20%) followed by *L. acidophilus* AR2 (7 $\pm$ 0.25%) and *L. fermentum* AR3 (5.8 $\pm$ 0.27%) with MODE-K cells. The adhesion percentage of these LAB depended on the initial concentrations and significantly increased when the initial inoculated concentration was 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 the inoculated bacterial concentration increased [23]. The pathogenic infections are usually associated with their adhesion to the host epithelium, which sometimes results in destruction of the epithelium, facilitating entry of the pathogens and their toxins into the host organ [23]. LAB has the potential to inhibit pathogens' adhesion to host cells by

producing different kinds of adhesive surface molecules (e.g., enolases, glyceraldehyde-3-phosphate, pyruvate dehydrogenase) [55]. These adhesive molecules assist LAB adhesion to host cells as well as in contesting and preventing pathogenic bacterial attachment and colonization [56–58]. Our results are consistent with these reports showing inhibitory effects of dog-isolated LAB on the adhesion of different pathogens with MODE-K cells. The adhesion percentage of all tested pathogens was less during the inhibition assay followed by the competition and displacement assays. This is because during pre-addition of probiotic *Lactobacilli*, they occupy most of the host cellular receptors. The anti-infection properties of LAB against pathogens may also be due to their ability to secrete different kinds of antimicrobial compounds (such as organic acids, primarily lactic acid, hydrogen peroxide, volatile compounds, such as diacetyl and ethanol, carbon dioxide, and bacteriocins), and bio-surfactant production [59]. These anti-infective properties of LAB have been reported in various studies. *Lactobacillus* can inhibit the adhesion of different pathogens (*Staphylococcus aureus*, *Listeria monocytogenes*, *Clostridium difficile*, *S. enterica* serovar Typhimurium) with Caco-2 cells [60]. Similar results of LAB's potential to inhibit the adhesion of pathogens with epithelial cells have also been reported by many other researchers [21,23,28,45,61]. However, our finding of LAB's anti-infective potential did not match with Nowak and Motyl (2017), Wang et al. (2018), and Falah et al., (2019). Anti-infection relies on both pathogenic and probiotic strains and should be assessed on a case-by-case [28,62]. The intestinal epithelial barrier prevents systemic entry of toxins and bacteria and many other unwanted molecules by acting as a physical and biochemical barrier. Therefore, its integrity and function are very important. Many studies have reported that LAB has the potential to improve the intestinal barrier damage induced by enteric pathogens [63–68].

Our qPCR results indicated that LAB significantly inhibits the *Salmonella*-decreased gene expression of occludin. Further, we checked the anti-inflammatory effects of these LAB during *Salmonella* infection as pathogenic-induced proinflammatory cytokines have been reported to be associated with barrier damage by pathogens [69]. Our results indicated that *Salmonella* significantly induced proinflammatory cytokines and our LAB alleviated this effect. Several other reports of LAB study during *Salmonella* infection have also shown that LAB ameliorates the intestinal barrier damage and proinflammatory cytokine production induced by *Salmonella* [64,70]. Our results did not match with findings of other researchers [71], where they reported that *L. fermentum* AGR1487 has negative effects on epithelial barrier integrity. This could possibly be due to strain differences and we used the MODE-K cell line and Anderson used the Caco-2 cell line.

We also evaluated dog-isolated *Lactobacilli*'s potential to induce antimicrobial peptide (such as host defensin peptides) production by MODE-K cells, based on previous study of probiotics (LAB) induction of antimicrobial peptides in their hosts [72–75]. 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 the strains induced mRNA expression of defensin peptides (beta-defensin 3) in the MODE-K cell line. These results were in disagreement with the reports of Wang et al. (2018) and Zhang et al. (2011) [23,75]. This could be due to the difference of the probiotic species and cell lines used in these studies. Our *Lactobacilli* were *L. acidophilus* and *L. fermentum* and we used the MODE-K cell line. Wang used *L. plantarum* and the Caco-2 cell line whereas Zhang used *L. salivariu* and took samples by biopsy from neonatal pigs.

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