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In Vitro and In Vivo Evaluation of *Lacticaseibacillus rhamnosus* GG and *Bifidobacterium lactis* Bb12 Against Avian Pathogenic *Escherichia coli* and Identification of Novel Probiotic-Derived Bioactive Peptides

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Abstract

Avian pathogenic *E. coli* (APEC), an extra-intestinal pathogenic *E. coli* (ExPEC), causes colibacillosis in poultry and is also a potential foodborne zoonotic pathogen. Currently, APEC infections in poultry are controlled by antibiotic medication; however, the emergence of multi-drug-resistant APEC strains and increased restrictions on the use of antibiotics in food-producing animals necessitate the development of new antibiotic alternative therapies. Here, we tested the anti-APEC activity of multiple commensal and probiotic bacteria in an agar-well diffusion assay and identified *Lacticaseibacillus rhamnosus* GG and *Bifidobacterium lactis* Bb12 producing strong zone of inhibition against APEC. In co-culture assay, *L. rhamnosus* GG and *B. lactis* Bb12 completely inhibited the APEC growth by 24 h. Further investigation revealed that antibacterial product(s) in the culture supernatants of *L. rhamnosus* GG and *B. lactis* Bb12 were responsible for the anti-APEC activity. The analysis of culture supernatants using LC–MS/MS identified multiple novel bioactive peptides (VQAAQAGDTKPIEV, AFDNTDTSLDSTFKSA, VTDTSGKAGTTKISNV, and AESSDTNLVNAKAA) in addition to the production of lactic acid. The oral administration (10^8 CFU/chicken) of *L. rhamnosus* GG significantly ($P < 0.001$) reduced the colonization (~ 1.6 logs) of APEC in the cecum of chickens. Cecal microbiota analysis revealed that *L. rhamnosus* GG moderated the APEC-induced alterations of the microbial community in the cecum of chickens. Further, *L. rhamnosus* GG decreased ($P < 0.05$) the abundance of phylum Proteobacteria, particularly those belonging to Enterobacteriaceae (*Escherichia-Shigella*) family. These studies indicate that *L. rhamnosus* GG is a promising probiotic to control APEC infections in chickens. Further studies are needed to optimize the delivery of *L. rhamnosus* GG in feed or water and in conditions simulating the field to facilitate its development for commercial applications.

Keywords APEC · *L. rhamnosus* GG · Probiotics · Peptides · Chickens · Antibiotic alternatives

Introduction

Avian pathogenic *E. coli* (APEC), an extra-intestinal pathogenic *E. coli* (ExPEC), is one of the most common bacterial pathogens of poultry [1, 2]. APEC continues to pose a formidable challenge to the poultry industry worldwide despite improvements in the poultry production systems over the years [1, 2]. APEC infects all species of poultry, including broilers, layers, breeders, and turkeys of all ages (9.52 to 36.73% prevalence), and in all types of production systems [1, 2]. In the United States (US), it is estimated that at least 30% of commercial flocks are affected by APEC at any point of time [2, 3]. APEC causes a wide range of localized and systemic infections in poultry, including yolk sac infection, omphalitis, respiratory tract infection, swollen head syndrome, septicemia, polyserositis, coligranuloma, enteritis, cellulitis, and salphingitis, collectively referred as colibacillosis [1, 2, 4]. Colibacillosis results in high morbidity and mortality (up to 20%) and decreased meat (2% decline in body weight) and egg production (loss up to 15%) [1, 2, 4]. More severely, in young chickens, APEC is associated with up to 53.5% mortality [1, 2, 4]. Further, APEC is also responsible for 36–43% of carcass condemnations at slaughter [1, 2, 4]. Altogether, APEC infections result in multi-million dollars annual losses to all facets of the poultry industry and remain as a serious impediment to the sustainable poultry production worldwide.

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APEC has been also reported as a potential foodborne zoonotic pathogen, which can be transmitted to humans through consumption of contaminated poultry products [2, 5]. In particular, APEC has genetic similarities with human ExPECs (uropathogenic *E. coli* (UPEC) and neonatal meningitis *E. coli* (NMEC)), possesses virulence genes characteristics of UPEC/NMEC, and causes urinary tract infection and meningitis in rodent models as similar to UPEC and NMEC [2]. Colicin-V (ColV) plasmids specific to APEC have been also detected in human clinical *E. coli* isolates suggesting evidence of potential foodborne transmission of APEC from poultry to humans even though concrete evidence is still lacking [2, 5]. In addition, APEC is also considered a source of antibiotic resistance genes (ARGs) to human pathogens, which can make the human infections difficult to treat [6]. Thus, APEC is a threat to both poultry and human health.

Antibiotics are commonly used to control APEC infections in poultry [2, 7, 8]. However, APEC resistance to multiple antibiotics, including tetracyclines, sulfonamides, aminoglycosides, quinolones, and β-lactams, has been reported worldwide [2, 9]. Up to 92% of APEC isolated in the US, Europe, and Australia were resistant to three or more antibiotics, particularly against tetracyclines, aminoglycosides, and sulfonamides [2, 10]. Further, many countries (particularly US and European Union) have recommended the limited use of antibiotics in food-producing animals, including poultry, with a goal of reducing the selection pressure and subsequent emergence and transmission of antibiotic-resistant bacteria to humans [2, 11]. However, limiting on-farm use of antibiotics could significantly increase morbidity and mortality, thereby compromising production efficiency, food safety, and security [12]. Therefore, there is a critical need for developing new effective alternatives to antibiotics which can enhance the poultry health and production, mitigate antibiotic resistance problem, promote antibiotic stewardship, and safeguard the human health.

Probiotics are defined as live microorganisms which when administered in adequate amounts confer health benefits to the host [13]. Probiotics exhibit antibacterial activities, promote the growth, maintain the healthy gut, and strengthen the immune system; therefore, these can serve as alternatives to antibiotics to control the bacterial infections as well as to enhance the production [14]. Probiotics exert their antibacterial effects through different mechanisms of action, such as (i) enhancement of epithelial barrier functions, (ii) competitive exclusion of pathogenic microorganisms, (iii) production of antimicrobial substances, and (iv) modulation of the host immune system [14–16]. *Lacticaseibacillus rhamnosus*, *Lactobacillus acidophilus*, *Lactobacillus johnsonii*, *Limosilactobacillus reuteri*, *Lactiplantibacillus plantarum* subsp. *plantarum*, *Limosilactobacillus fermentum*, *Lactobacillus helveticus*, *Lactobacillus gasseri*, *Bifidobacterium bifidum*, *B. lactis*, *B. infantis*, and *B. breve* are the most commonly used probiotics in humans and food animals [17–19]. These probiotics have shown antimicrobial activities against various bacterial and viral pathogens, including *Helicobacter*, *Salmonella*, *Listeria*, *Shigella*, *E. coli*, *Vibrio*, *Campylobacter,* and *Rotavirus* [16, 18]. Other probiotics have also shown proven benefits to the poultry health, particularly against *Salmonella* and *Campylobacter* [20–28]. *L. plantarum* subsp. *plantarum*, *L. reuteri*, *Ligilactobacillus salivarius*, *Lacticaseibacillus casei*, and *E. coli* Nissle 1917 reduced the *Salmonella* colonization [20, 24, 26, 28, 29]. *L. salivarius*, *L. johnsonii*, and *L. casei* reduced the colonization of *Campylobacter* [22, 27, 29]. *L. plantarum* subsp. *plantarum* reduced the APEC colonization [23–25]. However, there is overall lack of studies specifically demonstrating the activity of probiotic species against APEC. Further, information is also lacking on the bioactive substances secreted/released by these probiotics as well as their interactions with commensal microbes and/or pathogens in the gut which limits the understanding of the probiotic's mechanism(s) of action and reproducible use in industrial settings.

The objective of this study is to identify the probiotic species effective against APEC infection in poultry. Here, we identified probiotic bacteria, *L. rhamnosus* GG, effective in reducing APEC colonization in chickens. We also identified novel peptides derived from *L. rhamnosus* GG which are inhibitory to APEC growth. Further, we investigated the interactions of *L. rhamnosus* GG with commensal microbes and APEC in the gut microbiome of chickens. Our results show that *L. rhamnosus* GG can be developed as a preventative measure against APEC infections in chickens.

Materials and Methods

Bacterial Strains and Culture Conditions

The commensal and probiotic bacteria used in this study along with their culture conditions and media requirements for growth are listed in Table 1. BD GasPak™ EZ container system (Becton, Dickinson and Company, NJ, USA) or MiniMacs anaerobic workstation (Microbiology International, MD, USA) was used to grow commensal and probiotic bacteria requiring the anaerobic conditions. APEC serotype O78, primarily used in this study, was kindly provided by Dr. Johnson (University of Minnesota, Saint Paul, MN, USA) and was isolated from the lung of a turkey clinically diagnosed with colibacillosis [30]. Other APEC serotypes O1, O2, O8, O15, O18, O35, O109, and O115 were kindly provided by Drs. Nolan and Logue (University of Georgia, Athens, GA, USA). Luria–Bertani (LB) broth (BD Difco™) was used for the routine propagation of APEC serotypes.

Table 1 List of commensal and probiotic bacteria used in this study

Bacterial spp.	Media	Culture conditions	Reference/source
Enterococcus faecalis	MRS broth	37 °C, anaerobic, 16–18 h	David Francis, SDSU
Streptococcus bovis	MRS broth	37 °C, anaerobic, 16–18 h	David Francis, SDSU
Levilactobacillus brevis	MRS broth	37 °C, anaerobic, 1–2 days	David Francis, SDSU
Lactobacillus acidophilus	MRS broth	37 °C, anaerobic, 1–2 days	David Francis, SDSU
Lacticaseibacillus rhamnosus GG	MRS broth	37 °C, anaerobic, 1–2 days	ATCC, Manassas, VA, USA
Bifidobacterium longum	MRS broth + 0.05% cysteine	37 °C, anaerobic, 24 h	David Francis, SDSU
Bifidobacterium adolescentis	MRS broth + 0.05% cysteine	37 °C, anaerobic, 24 h	David Francis, SDSU
Bifidobacterium lactis Bb12	MRS broth + 0.05% cysteine	37 °C, anaerobic, 24 h	Christian Hansen Ltd., Hørsholm, Denmark
Escherichia coli Nissle 1917	LB broth	37 °C, aerobic, 10–12 h, 200 rpm	Dr. Ulrich Sonnenborn, Ardeypharm GmbH, Herdecke, Germany
Escherichia coli G58-1	LB broth	37 °C, aerobic, 10–12 h, 200 rpm	David Francis, SDSU
Bacteroides thetaiotaomicron	MRS broth	37 °C, anaerobic, 4–5 days	David Francis, SDSU

APEC serotypes stored at − 80 °C in glycerol were grown overnight in LB broth at 37 °C with shaking at 200 rpm.

Agar-well Diffusion Assay

To determine the inhibitory activity of commensal and probiotic bacteria against APEC, agar-well diffusion assay was conducted as described previously [31]. Briefly, LB agar plate was spread with 100 µL of APEC O78 (10^7 CFU/mL), and 100 µL of fully grown stationary phase whole cultures (adjusted to OD_{600}: 1) of commensal and probiotic bacteria was aliquoted into the wells bored in the agar plate. The plate was incubated at 37 °C, and zone of inhibition was measured at 12 h and 24 h post-incubation. The inhibitory activity of *L. rhamnosus* GG and *B. lactis* Bb12 was also tested with different culture volumes (200 µL, 150 µL, and 50 µL) and against other APEC serotypes as described above. Assay was also conducted with cell-free supernatants (CFSs) of *L. rhamnosus* GG and *B. lactis* Bb12 and supernatant-free *L. rhamnosus* GG and *B. lactis* Bb12 itself. CFSs were prepared by centrifugation of whole cultures at 10,000 × g for 10 min at 4 °C followed by filtration through 0.22 µm filter. The supernatant-free cultures were washed once and resuspended in PBS to check the activity of *L. rhamnosus* GG and *B. lactis* Bb12 itself. Two independent experiments were conducted.

Co-culture Assay

To determine the anti-APEC activity of *L. rhamnosus* GG and *B. lactis* Bb12 in liquid media, co-culture assay was conducted as previously described [32]. Briefly, 10^7 CFU/mL of *L. rhamnosus* GG or *B. lactis* Bb12 and APEC O78 were incubated together in 5 mL of co-culture media (contains 100% MRS and 100% LB; pH 6.75 at 0 h) at 37 °C under anaerobic conditions with shaking at 50 rpm followed by the quantification of viable APEC O78 every 12 h until 24 h. *Lactobacillus acidophilus* and *Levilactobacillus brevis* were used for the comparison of anti-APEC activity as these two *Lactobacillus* species are commonly used probiotics in animal and human studies, and several commercial probiotics currently being used in poultry industry contain these *Lactobacillus* species in their formulations. Two independent experiments were conducted.

Trans-well Migration Assay

To determine if the anti-APEC activity of *L. rhamnosus* GG and *B. lactis* Bb12 is due to bacterial cells itself or due to bacteria secreted/released products, trans-well migration assay was conducted. Assay was conducted using 0.22 µm Ultrafree-MC microcentrifuge tubes with removable filters (Millipore Sigma, MA, USA). Briefly, 16–18 h grown *L. rhamnosus* GG and *B. lactis* Bb12 cultures were aliquoted into the tube containing filter, whereas APEC O78 culture (10^7 CFU/mL) was aliquoted into the microcentrifuge tube below the filter. The filter tube was removed before aliquoting 700 µL of APEC culture into the microcentrifuge tube, then filter tube was inserted back, and 700 µL of *L. rhamnosus* GG/*B. lactis* Bb12 culture was added above the filter in the filter tube. Sufficient volume (700 µL) was added to the microcentrifuge tube to allow contact with the tube containing the filter. The tubes were incubated at 37 °C under anaerobic conditions with shaking at 50 rpm. The viability of APEC O78 was quantified at 12 h and 24 h post-incubation. Two independent experiments were conducted.

Effect of pH on Anti-APEC Activity

It is reported in studies that *Lactobacillus* and *Bifidobacterium* strains exhibit antimicrobial activity by lowering the pH of the media [32, 33]. To observe the pH change, pH of the *L. rhamnosus* GG and *B. lactis* Bb12 cultures grown in co-culture media (MRS + LB) were measured every 12 h until 48 h in a separate experiment. To determine the effect of pH on *L. rhamnosus* GG and *B. lactis* Bb12 inhibitory activity against APEC O78, co-culture media (MRS + LB) was adjusted to different pH (4.0, 4.5, 5.0, 5.5, and 6.0) using 3 M HCL and tested for anti-APEC activity as described above. Additionally, pH tolerance of APEC O78 was determined by growing APEC in LB media adjusted to different pH (4.0, 4.5, 5.0, 5.5, and 6.0) for 24 h [34]. Two independent experiments were conducted.

Characterization of Nature of Antibacterial Product(s)

To understand the nature of secreted/released product(s), CFSs of 24 h grown *L. rhamnosus* GG and *B. lactis* Bb12 cultures were subjected to heat and proteolytic enzyme treatments as described previously [35, 36]. The CFSs were subjected for heat (121 °C; autoclave) or proteinase K (1 mg/mL, 37 °C for 3 h) treatment and tested for inhibitory activity against APEC O78 in an agar-well gel diffusion assay as described above. Further, *L. rhamnosus* GG and *B. lactis* Bb12 CFSs were fractionated using a Amicon® Ultra centrifugal filter (Millipore Sigma) with mol. wt. cut-off (MWCO) of 3 kDa. The filtrates containing products less than 3 kDa were tested for inhibitory activity and compared with the inhibitory activity of unfractionated CFSs of *L. rhamnosus* GG and *B. lactis* Bb12 cultures. Two independent experiments were conducted.

Profiling of Organic Acid Production

To quantify the organic acids in the CFSs of *L. rhamnosus* GG and *B. lactis* Bb12 cultures, LC–MS/MS coupled with isotope-labeled chemical derivatization method was used as described previously [37]. For the preparation of CFSs, *L. rhamnosus* GG and *B. lactis* Bb12 were grown overnight, adjusted to OD_{600} 1.0 (~ 10^9 CFU/mL), and sub-cultured (500 µL) in fresh media (14.5 mL) for 24 h at 37 °C under anaerobic conditions. The quantity of organic acids in the CFSs of *L. rhamnosus* GG/*B. lactis* Bb12 and APEC 078 co-cultures was also determined as above. *Lactobacillus acidophilus* and *Levilactobacillus brevis* were used to compare the organic acid profiles. The LC–MS/MS Poroshell 120 SB C18 column containing solvent A, H_2O + 0.1% formic acid, and solvent B, acetonitrile (MeCN) + 0.1% formic acid, was used for the LC–MS/MS analysis. Standard solutions of acetic, propionic, butyric, and lactic acids (Sigma-Aldrich, MO, USA) were used to generate the calibration curves and quantify the concentration of organic acids in the CFSs. Sodium ^{13}C-lactic acid was used as an internal standard.

Identification of Bioactive Peptides

To identify the bioactive peptides present in *L. rhamnosus* GG and *B. lactis* Bb12 CFSs, LC–MS/MS was used as described previously [38]. To prepare the CFSs, *L. rhamnosus* GG and *B. lactis* Bb12 were grown anaerobically for 24 h, centrifuged (1000 rpm, 10 min, 25 °C), and washed with sterile water. The *L. rhamnosus* GG and *B. lactis* Bb12 pellets were resuspended in sterile water containing 2% glucose and incubated for 24 h under anaerobic conditions [38]. The *L. rhamnosus* GG and *B. lactis* Bb12 cultures were then centrifuged (1000 rpm, 10 min, 4 °C), and CFSs were separated by filtering through 0.22 µm filter. *Lactobacillus acidophilus* and *Levilactobacillus brevis* were used to compare the peptide profiles. To prepare the samples for LC–MS/MS run, CFSs (1.8 mL) were passed three times through HyperSep™ Hypercarb™ SPE cartridge (50 mg; ThermoFisher Scientific, MA, USA). The cartridge was washed twice with water (150 µL) to remove salts, and peptides were eluted (20 µL) using 50% MeCN and 0.1% trifluoroacetic acid (TFA). The elutes (0.5 µL) were injected into LC–MS/MS EasySpray C18-Fusion column set at HCD (higher energy collision dissociation) and CID (ion-trap-based collision-induced dissociation) collision energy settings. The solvent A, H_2O + 0.1% formic acid, and solvent B, MeCN + 0.1% formic acid, were used. The data generated were analyzed using Proteome Discoverer 2.2 software (ThermoFisher Scientific) using UniProt *Lactobacillus* or *Bifidobacterium* database with settings of no modifications and non-specific cleavage.

The five common highly abundant peptides in CFSs of both *L. rhamnosus* GG and *B. lactis* Bb12 (FSAVALSAVALSKPGHVNA, AESSDTNLVNAKAA, VQAAQAGDTKPIEV, AFDNTDTSLDSTFKSA, and VTDTSGKAGTTKISNV) were synthesized (GenScript, NJ, USA) and tested for anti-APEC activity by conducting kinetic time-inhibition assay as described previously [7]. Briefly, peptides dissolved in dimethyl sulfoxide (DMSO) at 200 mM concentrations were added (12 mM; final concentration of 6% DMSO) to APEC suspension (10^5 CFU/mL; LB media) in a 96-well plate. The plate was then incubated in TECAN Sunrise™ absorbance microplate reader (NC, USA) at 37 °C with OD_{600} measurement set at every 30 min for 12 h [7]. Untreated APEC (0% DMSO) and APEC treated with 6% DMSO were included as controls. DMSO at 6% in our earlier study showed no significant effect on APEC growth, only when used at 8% concentration significant effect on APEC growth was observed [7]. Three independent experiments were conducted.

Cell Culture Studies

The anti-APEC activity of *L. rhamnosus* GG and *B. lactis* Bb12 CFSs was studied in cell culture model using polarized HT-29 (Human Colorectal Adenocarcinoma Cell Line; ATCC HTB-38) cells, maintained in complete Dulbecco's modified Eagle's medium (DMEM, Gibco, MA, USA) supplemented with 10% fetal bovine serum (FBS, Gibco), 2 mM L-glutamine, 5 mM galactose, 1% penicillin–streptomycin (PS) solution, and 0.1 mM non-essential amino acids (NEAA) [39]. To evaluate the effect of CFSs of *L. rhamnosus* GG and *B. lactis* Bb12 on APEC O78 adhesion to HT-29 cells, 10% CFSs from the 24 h grown *L. rhamnosus* GG and *B. lactis* Bb12 cultures were added to the wells containing HT-29 monolayers, which was incubated for 3 h as described previously [40]. The CFSs diluted to 10% were used as they were non-toxic to HT-29 cells as well as non-inhibitory to APEC growth at this concentration (data not shown). Prior to treatment with CFSs, the polarized HT-29 cells were washed and incubated for 2 h in DMEM containing no antibiotics and FBS. After treatment, the HT-29 cells were washed with DPBS, infected with APEC O78 (MOI 100) and incubated for 3 h. For infection, the logarithmic phase grown APEC O78 was pelleted, washed, and resuspended in DMEM at OD_{600} 0.05 (5×10^7 CFU/mL). The infected HT-29 cells were washed three times, and the adherent APEC O78 was enumerated after lysis with 0.5% Triton X-100 followed by serial dilution (ten-fold) and plating on LB agar plate.

To determine the effect of CFSs on APEC O78 invasion, the HT-29 cells were pre-treated with CFSs and infected with APEC O78 as described above. Following 3-h incubation with APEC O78, the HT-29 cells were washed three times and treated with DMEM containing 150 µg/mL gentamicin for 1 h. The HT-29 cells were washed twice with DPBS, lysed and invaded APEC O78 was quantified as described above. Two independent experiments were conducted with three replicates in each experiment. The effect of pre-treatment (3 h) of *L. rhamnosus* GG and *B. lactis* Bb12 cells itself after separation of culture supernatant by centrifugation and washing as above was also determined. The washed *L. rhamnosus* GG and *B. lactis* Bb12 pellets were resuspended in DMEM at OD_{600} 1.0 (~ 10^9 CFU/mL) prior to adding into the wells containing HT-29 monolayers, and procedure as above was followed.

Scanning Electron Microscopy (SEM)

To determine the modes of action of *L. rhamnosus* GG and *B. lactis* Bb12, APEC O78 was treated with CFSs of *L. rhamnosus* GG and *B. lactis* Bb12 and imaged using Hitachi S-4700 scanning electron microscope as described previously [7]. Briefly, APEC O78 culture adjusted at OD_{600} 1.0 (1×10^9 CFU/mL) was treated with CFSs prepared from 24 h grown *L. rhamnosus* GG and *B. lactis* Bb12 for 2 h at 37 °C with shaking at 200 rpm. Following treatment, culture was processed for SEM as described previously [7].

Efficacy of *L. rhamnosus* GG and *B. lactis* Bb12 in Chickens

Animal study was approved by The Ohio State University Institutional Animal Care and Use Committee (IACUC, protocol # 2010A00000149). Chickens were euthanized using CO_2 following American Veterinary Medical Association (AVMA) guidelines. Standard animal husbandry practices were followed throughout the experiment. Feed and water were provided ad libitum.

The efficacy of *L. rhamnosus* GG and *B. lactis* Bb12 and their combination (1:1) was tested in 1-day-old specific pathogen free (SPF) Leghorn chickens ($n = 10$/group). From day 1, *L. rhamnosus* GG and *B. lactis* Bb12 were administered orally (200 µL in PBS; 10^8 CFU/chicken), once a day, until day 14. On day 7, chickens were infected orally with rifampicin-resistant (Rif^r) APEC O78 (7.5×10^7 CFU/chicken) as described previously [23, 24, 41]. Before the infection, random cloacal swabs were collected from each group ($n = 2$/group) to confirm the absence of APEC. Chickens infected with APEC but not treated with probiotic (positive control, PC) and not infected with APEC and not treated with probiotic (negative control, NC) were included as controls. On day 15, chickens were euthanized, and tissues (cecum, liver, and heart) were aseptically collected for APEC quantification. The tissues were homogenized in PBS, and the suspensions were ten-fold serially diluted and plated on MacConkey agar plates containing 50 µg/mL rifampicin. Body weight of chickens was measured at days 1 and 15.

The *L. rhamnosus* GG-specific quantitative polymerase chain reaction (qPCR) was performed to assess the presence of *L. rhamnosus* GG in the cecum of *L. rhamnosus* GG-treated chickens as previously described [42]. The primers (Table S1) were obtained from Integrated DNA Technologies (IDT). The qPCR (two-step) was performed using Maxima SYBR Green/ROX qPCR master mix (ThermoFisher Scientific) following the manufacturer's instructions in a RealPlex^2 Mastercycler® (Eppendorf, CT, USA) with single cycle of 95 °C for 10 min and 40 cycles of amplification with 95 °C for 15 s denaturing and 60 °C for 1 min annealing temperatures. PureLink™ Microbiome DNA Purification Kit (ThermoFisher Scientific) was used to extract the microbial DNA from the cecal contents (approximately 0.2 g) of the chickens. RNase A treatment (2–3 µL of 100 mg/mL solution per sample; Qiagen, MD, USA) was performed to remove the RNA. DNA quantity and quality were measured using NanoDrop 2000c Spectrophotometer

(ThermoFisher Scientific). The standard *L. rhamnosus* GG qPCR curve was used to enumerate the *L. rhamnosus* GG which was generated by making ten-fold serial dilutions of *L. rhamnosus* GG DNA extracted (MasterPure™ DNA Purification Kit; Epicentre, WI, USA) from OD_{600} 1.0 *L. rhamnosus* GG (~ 10^9 CFU/mL) culture. The qPCR was also performed for microbial DNA extracted from cecal contents of NC chickens to confirm the specificity of *L. rhamnosus* GG primers.

Cecal Microbiome Analysis

To investigate the impact of *L. rhamnosus* GG treatment on the cecal microbiome of chickens, 16S rRNA-based microbiome study was conducted as previously described [43, 44]. DNA was extracted from 0.2 g of cecal contents using PureLink™ Microbiome DNA Purification Kit (ThermoFisher Scientific) and treated with RNase A (2–3 µL of 100 mg/mL solution per sample; Qiagen). DNA quantity and quality were measured using NanoDrop 2000c Spectrophotometer (ThermoFisher Scientific). The extracted DNA samples were subjected to 16S rRNA V4-V5 sequencing at the molecular and cellular imaging center (MCIC) (https://mcic.osu.edu/genomics/illumina-sequencing). Amplicon libraries were prepared using IFU KAPA HiFi HotStart ReadyMixPCR Kit (Roche, NJ, USA), and PCR clean-up was performed using Agencourt AMPure XP beads (BECKMAN COULTER Life Sciences, CA, USA). Nextera XT DNA Library Preparation Kit (Illumina, CA, USA) was used to generate Illumina library, and sequencing was performed using Illumina MiSeq platform generating paired end 300-bp reads.

For the microbiota analysis, QIIME (Quantitative Insights Into Microbial Ecology) 2 bioinformatics platform [45] (https://qiime2.org/) was used. Quality control of the raw reads was performed using FastQC 0.11.8 (Babraham Bioinformatics). Trimmomatic-0.33 was used to trim the adaptor and other Illumina-specific sequences (http://www.usadellab.org/cms/?page=trimmomatic). The trimmed sequences (fastq.gz) were imported into the QIIME 2 as a manifest file format (PairedEndManifestPhred33V2). The feature table construction and additional filtering of the sequences was performed using DADA2 [46]. The taxonomic analysis was performed using Naive Bayes classifiers trained on Silva 132 99% OTUs (silva-132–99-nb-classifier.qza) database. The phylogenetic diversity was analyzed using align-to-tree-mafft-fasttree pipeline, and alpha (Shannon's diversity index) and beta diversity (Bray–Curtis distance) were analyzed using core-metrics-phylogenetic pipeline (https://docs.qiime2.org/2019.7/tutorials/moving-pictures/). The statistical difference ($P < 0.05$) in the taxonomic composition between the *L. rhamnosus* GG treated, PC (APEC infected but not treated with *L. rhamnosus* GG), and NC (non-APEC infected and non-*L. rhamnosus* GG treated) groups was determined using the Man-Whitney *U* test. The alpha and beta diversity were analyzed using the Kruskal–Wallis and PERMANOVA tests ($P < 0.05$), respectively.

Statistical Analysis

The statistical significance ($P < 0.05$) of bacterial viability reduction and inhibition of adhesion and invasion was calculated using two-way ANOVA followed by the Bonferroni post-test. The statistical significance ($P < 0.05$) of treatment on reduction of APEC load and increment in body weight was calculated using the Man-Whitney *U* test.

Results

L. rhamnosus GG and *B. lactis* Bb12 Induced Strong Zone of Inhibition Against APEC Serotypes

Of the several whole cultures of commensal and probiotic bacteria tested (Table 1), *L. rhamnosus* GG and *B. lactis* Bb12 induced large zone of inhibition against APEC 078 at 12 h (14.5 ± 0.5 and 13.5 ± 0.5) and 24 h (12.5 ± 0.5 and 11.5 ± 0.5) post-incubation in agar-well diffusion assay (Table 2). *Enterococcus faecalis*, *Levilactobacillus brevis*, *Bifidobacterium adolescentis*, and *Bacteroides thetaiotaomicron* also induced zone of inhibition at 12 h (9.5 ± 0.5, 9.5 ± 0.5, 12.5 ± 0.5, and 9.5 ± 0.5, respectively); however, no zone of inhibition was observed at 24 h. The decrease in inhibition at 24 h might be due to lack of continuous production of inhibitory substances in solid media by commensal and probiotic bacteria as stationary phase grown cultures were used in the assay. Slight but not measurable zone of inhibition was also observed with *Lactobacillus acidophilus*,

Table 2 Zone of inhibition induced by commensal and probiotic bacteria against APEC O78

Bacterial spp.	Zone of inhibition (mm ± SD)	
	12 h	24 h
Enterococcus faecalis	9.5 ± 0.5	0.0 ± 0.0
Streptococcus bovis	0.0 ± 0.0	0.0 ± 0.0
Levilactobacillus brevis	9.5 ± 0.5	0.0 ± 0.0
Lactobacillus acidophilus	0.0 ± 0.0	0.0 ± 0.0
Lacticaseibacillus rhamnosus GG	14.5 ± 0.5	12.5 ± 0.5
Bifidobacterium longum	0.0 ± 0.0	0.0 ± 0.0
Bifidobacterium adolescentis	12.5 ± 0.5	0.0 ± 0.0
Bifidobacterium lactis Bb12	13.5 ± 0.5	11.5 ± 0.5
Escherichia coli Nissle 1917	0.0 ± 0.0	0.0 ± 0.0
Escherichia coli G58-1	0.0 ± 0.0	0.0 ± 0.0
Bacteroides thetaiotaomicron	9.5 ± 0.5	0.0 ± 0.0

Streptococcus bovis, and *Bifidobacterium longum* (data not shown). No zone of inhibition was observed with *E. coli* Nissle 1917 and *E. coli* G58-1.

The zone of inhibition induced by *L. rhamnosus* GG and *B. lactis* Bb12 against APEC 078 was volume dependent. Large zone of inhibition was observed when incubated with 200 µL (18.5 ± 0.5 and 17.5 ± 0.5) of culture volume followed by 150 (16.5 ± 0.5 and 15.5 ± 0.5), 100 µL (14.5 ± 0.5 and 13.5 ± 0.5), and 50 µL (10.5 ± 0.5 and 10.0 ± 1.0). Further, *L. rhamnosus* GG and *B. lactis* Bb12 also induced similar zone of inhibition against other multiple pre-dominant APEC (O1, O2, O8, O15, O18, O35, O109, and O115) serotypes (Table S2).

No Viable APEC Was Detected When APEC Was Incubated with *L. rhamnosus* GG and *B. lactis* Bb12

The growth of APEC was not compromised in co-culture (100% MRS + 100% LB) media as compared to LB media (Fig. S1A). The significant reduction ($P < 0.001$) in viable APEC was observed at 12 h when incubated with *L. rhamnosus* GG and *B. lactis* Bb12 in co-culture media, whereas no reduction was observed when incubated with *L. acidophilus* and *L. brevis*. At 24 h, no viable APEC was recovered when incubated with *L. rhamnosus* GG and *B. lactis* Bb12, whereas slight reduction (< 2 logs) was also observed when incubated with *L. acidophilus* and *L. brevis* (Fig. 1A).

L. rhamnosus GG and *B. lactis* Bb12 Secreted/Released Products Are Responsible for Anti-APEC Activity

As observed in co-culture assay, both *L. rhamnosus* GG and *B. lactis* Bb12 CFSs significantly ($P < 0.001$) reduced the viable APEC population at 12 h, and no viable APEC was detected at 24 h (Fig. 1B) in trans-well migration assay. Further, *L. rhamnosus* GG and *B. lactis* Bb12 CFSs also induced the zone of inhibition similar to *L. rhamnosus* GG and *B. lactis* Bb12 whole culture in agar-well diffusion assay (Table 3). However, no zone of inhibition was induced by *L. rhamnosus* GG and *B. lactis* Bb12 cells itself after CFSs were separated and cells resuspended in PBS. Further, the heat- and proteolysis-treated CFSs of *L. rhamnosus* GG and *B. lactis* Bb12 retained the anti-APEC activity similar to untreated CFSs (Table 3). Similarly, fractionated (< 3 kDa) CFSs of *L. rhamnosus* GG and *B. lactis* Bb12 also exhibited the anti-APEC activity similar to unfractionated CFSs (Table 3), suggesting that secreted/released products are heat stable, proteolysis resistant, and of low mol. wt. in size.

The shortened bacterial cells measuring ~ 0.5–1 µM with bulbous swelling were observed after treatment with

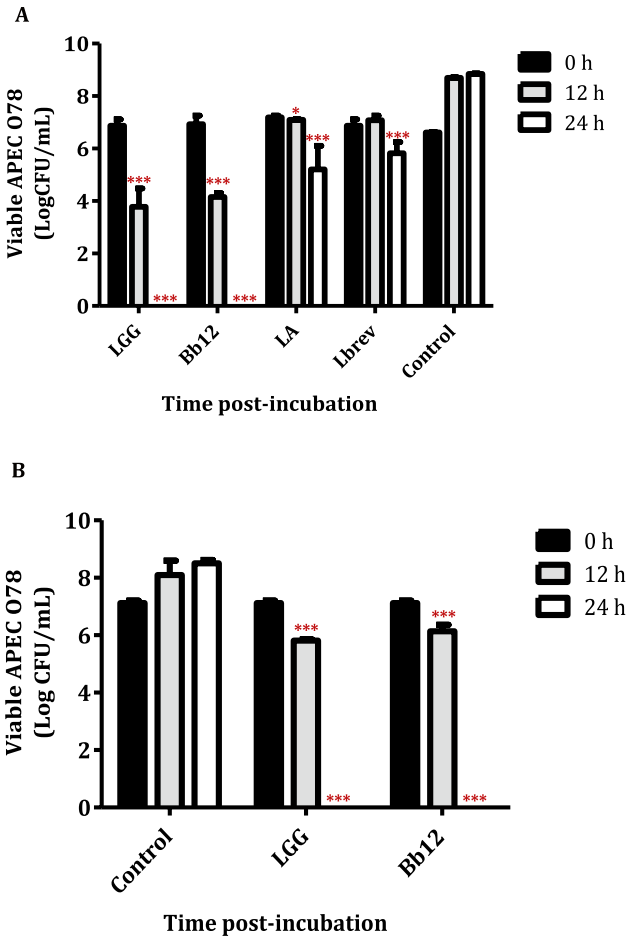

Fig. 1 **A** Viability of APEC in co-culture assay when incubated together with different probiotics. APEC O78 culture grown alone in co-culture media was used as a control. **B** Viability of APEC in trans-well migration assay. *L. rhamnosus* GG and *B. lactis* Bb12 cultures were aliquoted into the tube containing filter, whereas APEC O78 culture was aliquoted in to the microcentrifuge tube below the filter. APEC O78 culture grown with APEC O78 culture above the filter was used as a control. LGG, *Lacticaseibacillus rhamnosus* GG; Bb12, *Bifidobacterium lactis* Bb12; LA, *Lactobacillus acidophilus*; Lbrev, *Levilactobacillus brevis*; $^{*}P < 0.05$, $^{***}P < 0.001$, two-way ANOVA Bonferroni post-test

L. rhamnosus GG and *B. lactis* Bb12 CFSs as compared to untreated bacterial cells which measured ~ 1.5–2 µM in length (Fig. 2).

Table 3 Anti-APEC activity of *L. rhamnosus* GG and *B. lactis* Bb12 cell-free supernatants (CFSs)

	Zone of inhibition (mm ± SD)			
	CFS*	121 °C	Proteinase K	< 3 kDa filtrate
L. rhamnosus GG	13.0 ± 0.5	12.5 ± 0.5	12.0 ± 0.0	13.0 ± 0.0
B. lactis Bb12	13.0 ± 1.0	13.5 ± 0.5	11.5 ± 0.5	13.5 ± 0.5

*Not subjected to heat and proteolysis treatments and fractionation

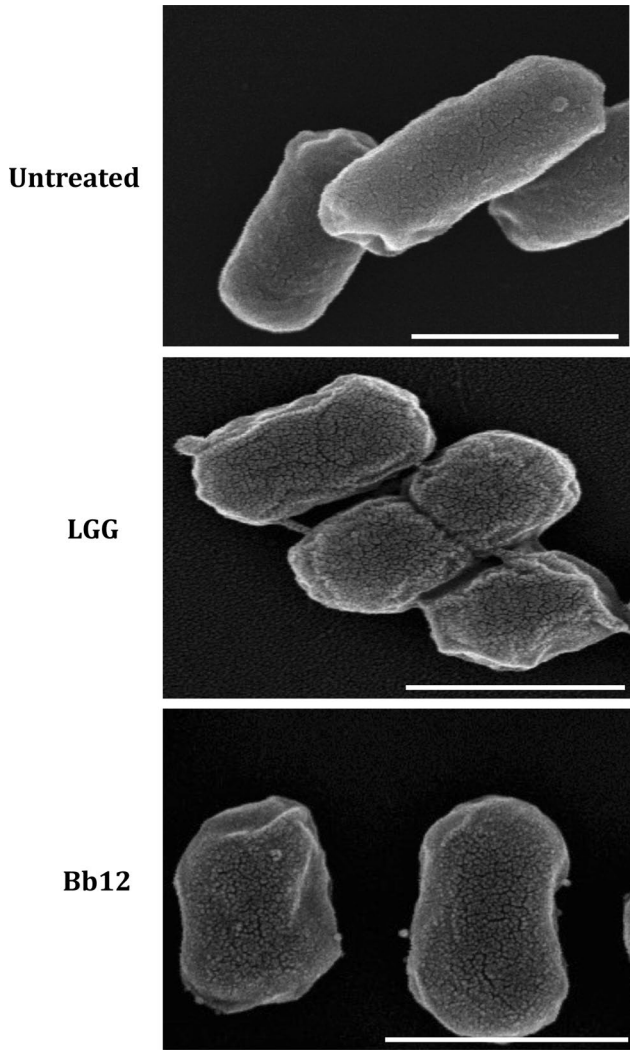

Fig. 2 Scanning electron microscopy (SEM) images showing morphology of untreated APEC or APEC treated with CFSs (cell-free supernatants) of *L. rhamnosus* GG and *B. lactis* Bb12. APEC was treated with CFSs prepared from 24 h grown culture of *L. rhamnosus* GG and *B. lactis* Bb12 for 2 h at 37 °C with shaking at 200 rpm. Bars: 1 µM

Activity of *L. rhamnosus* GG and *B. lactis* Bb12 Is pH-Independent

It has been previously shown that probiotic lactic acid bacteria exert inhibitory effect against pathogenic bacteria by lowering the pH [32, 33]. Therefore, we monitored the changes in pH when APEC was co-cultured with *L. rhamnosus* GG, *B. lactis* Bb12, *L. acidophilus,* and *L. brevis*. At 24 h, the lowest pH was observed when APEC was co-cultured with *B. lactis* Bb12 (4.12), followed by *L. rhamnosus* GG (4.37), *L. acidophilus* (4.66), and *L. brevis* (4.96) (Table 4). We further quantified the viability of APEC in co-culture media adjusted to different pH (ranging 4.0 to 6.0) in order to determine the effect of pH on *L. rhamnosus* GG and *B. lactis* Bb12 anti-APEC activity. No significant effect on the viability of APEC was observed, except at pH 4.0 (Fig. S1B); however, significant number (~5.4 logs) of APEC was still viable even at pH 4 after 24 h compared to no viable APEC recovered when incubated with *L. rhamnosus* GG and *B. lactis* Bb12 (Fig S1B). APEC was also pH-tolerant up to pH 4.0 when incubated for 24 h in LB media alone adjusted to different pH (ranging 4.0 to 6.0; data not shown). These studies suggest that pH alone is not responsible for anti-APEC activity of *L. rhamnosus* GG and *B. lactis* Bb12.

Table 4 pH of co-culture media in the presence of *L. rhamnosus* GG, *B. lactis* Bb12, *L. acidophilus,* and *L. brevis*

	pH (mean ± SD)			
	12 h	24 h	36 h	48 h
L. rhamnosus GG	4.47 ± 0.06	4.37 ± 0.06	4.39 ± 0.01	4.35 ± 0.03
B. lactis Bb12	4.82 ± 0.06	4.12 ± 0.01	4.09 ± 0.01	4.11 ± 0.01
L. acidophilus	5.42 ± 0.03	4.66 ± 0.02	4.54 ± 0.01	4.43 ± 0.06
L. brevis	5.12 ± 0.07	4.96 ± 0.04	4.83 ± 0.03	4.74 ± 0.04

pH of the co-culture media at 0 h: 6.75

L. rhamnosus GG and *B. lactis* Bb12 Contain Lactic Acid and Multiple Small Peptides in Their Cell-Free Supernatants

LC–MS/MS coupled with isotope-labeled chemical derivatization method was used to quantify the organic acids produced by *L. rhamnosus* GG, *B. lactis* Bb12, *L. acidophilus,* and *L. brevis* [37]. The standard curves of lactic ($y = 0.0006x$-0.2761, $R^2 = 0.9865$), acetic ($y = 0.0006x$-0.3419, $R^2 = 0.9807$), propionic ($y = 0.00012x$-0.577, $R^2 = 0.9839$), and butyric acids ($y = 0.0017x$-0.6008, $R^2 = 0.9855$) were generated to quantitate the concentration of organic acids in CFSs (Fig. S2A–D). Lactic acid was predominantly present in all CFSs (Fig. 3A). The highest concentration of lactic acid was observed in CFS of *B. lactis* Bb12 (0.090 M) followed by *L. rhamnosus* GG (0.067 M), *L. brevis* (0.059 M), and *L. acidophilus* (0.044 M). Interestingly, higher concentrations of lactic acid were produced by *L. rhamnosus* GG (0.26 M), *B. lactis* Bb12 (0.24 M), and *L. acidophilus* (0.19 M) when co-cultured with APEC compared to monoculture (Fig. 3B).

LC–MS/MS analysis of CFSs eluted through HyperSep™ Hypercarb™ SPE cartridge was also performed to identify the bioactive molecules secreted/released by *L. rhamnosus* GG, *B. lactis* Bb12, *L. acidophilus,* and *L. brevis* [38]. At HCD (higher energy collision dissociation) setting, 57 peptides (Dataset 1) were identified, whereas 152 peptides were identified at CID (ion-trap-based collision-induced dissociation) setting (Dataset 2). A total of 33 peptides (Table 5) of

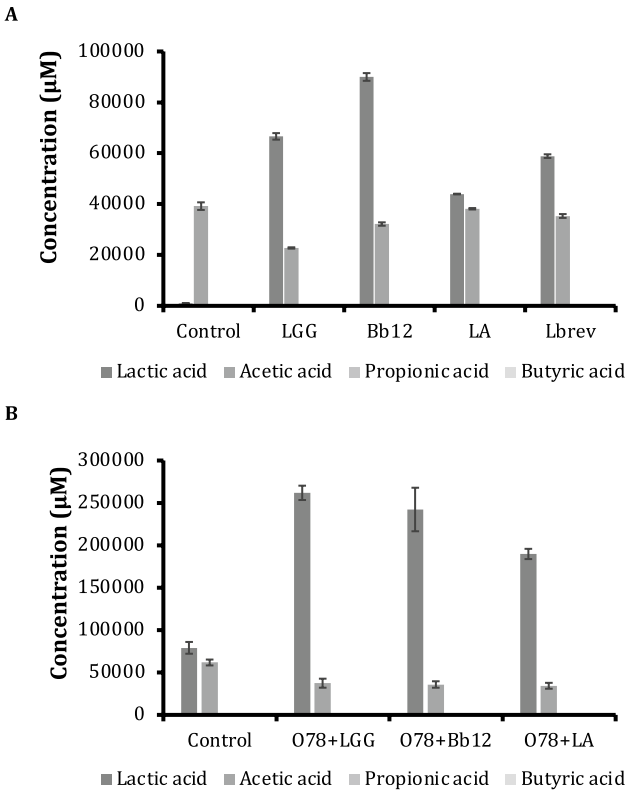

Fig. 3 Concentration of organic acids in cell-free supernatants (CFSs) of different probiotics when cultured alone (**A**) or co-cultured with APEC (**B**). MRS media were used as a control in monoculture study, and APEC O78 culture grown alone in co-culture media was used as a control in co-culture study. LGG, *Lacticaseibacillus rhamnosus* GG; Bb12, *Bifidobacterium lactis* Bb12; LA, *Lactobacillus acidophilus*; Lbrev, *Levilactobacillus brevis*

mol. wt. less than 3 kDa were identified in common in both HCD and CID settings. Consistent with strong anti-APEC activity of *L. rhamnosus* GG and *B. lactis* Bb12 compared to *L. acidophilus* and *L. brevis*, these peptides were mostly present in CFSs of *L. rhamnosus* GG and *B. lactis* Bb12.

L. rhamnosus GG and *B. lactis* Bb12 Cell-Free Supernatants Reduced the Adhesion and Invasion of APEC in HT-29 Cells

The HT-29 cells were pre-treated with 10% CFSs (concentration non-toxic to HT-29 cells and non-inhibitory to APEC growth) for 3 h to determine the effect of CFSs on adhesion and invasion of APEC. Both the CFSs significantly reduced ($P < 0.05$) the percent of original inocula of APEC adhered and invaded in HT-29 cells (Fig. 4). However, no effect on the adhesion and invasion was observed when HT-29 cells were pre-treated with *L. rhamnosus* GG and *B. lactis* Bb12 cells itself after CFSs were separated and cells resuspended in DMEM (data not shown).

Table 5 List of peptides identified in *L. rhamnosus* GG and *B. lactis* Bb12 CFSs using LC–MS/MS

Sequence	Theo. MH + [Da]	Accession number
EVKALAEKVLKK	1355.86	A0A0R2DJY6
SAVALSAVALSKPGHVNA	1691.94	C2JZA7
AVALSAVALSKPGHVNA	1604.91	C2JZA7
VALSAVALSKPGHVNA	1533.87	C2JZA7
FSAVALSAVALSKPGH-VNA*	1839.01	C2JZA7
VAGVTLASASTLDKDIKD	1803.97	C2JYJ6
LKDVLSSYLSTSSSSTSK	1977.00	A0A180C684
ALSAVALSKPGHVNA	1434.81	C2JZA7
AQNGNTNKIEVDNIVYK	1919.98	A0A179YFC2
VAGVTLASASTLDKDVKE	1803.97	A0A0R2DLD3
VIVVVAAIGGGLNNK-GKSSS	1870.08	A0A179YAS6
DEVKALAEKVLKK	1470.89	A0A0R2DJY6
GNDTPADSAVKARIV	1513.80	K8QAJ2
HDVIQNALNAK	1222.65	A0A249DEL5
LSSYLSTSSSSTSK	1521.73	A0A180C684
FSQATNAYFIKGA	1417.71	A0A2A5L4H0
AADKSQVKVGVLQL	1455.85	C2K1D8
AESSDTNLVNAKAA*	1390.68	A0A179YN16
ATLAGVGVSGFAATTVHA	1629.86	A0A179XCY0
ALDVDGIIAQLKDA	1441.79	A0A0H0YQJ8
VQAAQAGDTKPIEV*#	1426.75	A0A179YFC2
VNAAQNGNTNKIEVD-NIVYK	2204.13	A0A179YFC2
VNAAQNGNTNKIEVDNI	1813.90	A0A179YFC2
SINRDDYNKAVSDGQDKL	2037.98	A0A2A5L4H0
QSQFAQEQSEAAKATQA	1822.86	A0A179YJG3
AFDNTDTSLDSTFKSA*#	1719.77	A0A180C684
AIAAITDTMKKEGLAE	1661.88	K0N9I2
DANKIKEQLEEVGAT-VTLK	2086.14	A0A0H0YQJ8
DTSGKAGTTKISNV	1378.72	A0A1Z2F669
EVASKTNDIAGDGTTTA	1650.78	A0A0R1WMV7
GLALITAVPQVVRA	1407.87	A0A179Y5L8
VTDTSGKAGTTKISNV*#	1578.83	A0A1Z2F669
NKVGPKEYIPELNKSL	1829.02	A0A179YFC2

*Selected peptides for synthesis; #Selected peptides inhibitory to APEC

L. rhamnosus GG Reduced the Colonization of APEC in Cecum of Chickens

The efficacy of *L. rhamnosus* GG and *B. lactis* Bb12 and their combination (1:1) was tested in chickens by administering orally (10^8 CFU/chicken) for 14 days. The *L. rhamnosus* GG treatment significantly reduced ($P < 0.001$; 1.6 logs) the APEC load in cecum 7 days post-infection as compared to APEC infected but not probiotic treated (PC; positive control)

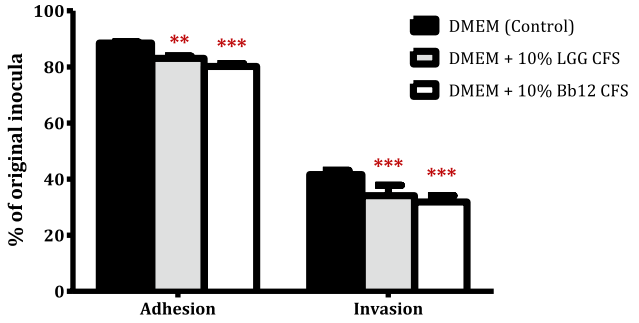

Fig. 4 Percent of original inocula of APEC O78 adhered and invaded in HT-29 cells when pretreated with cell-free supernatants (CFSs) of *L. rhamnosus* GG and *B. lactis* Bb12. LGG, *Lacticaseibacillus rhamnosus* GG; Bb12, *Bifidobacterium lactis* Bb12; DMEM, Dulbecco's modified Eagle medium; $^{**}P < 0.01$, $^{***}P < 0.001$, two-way ANOVA Bonferroni post-test

chickens (Fig. 5A). On the other hand, only 0.6 log APEC reduction was observed in *B. lactis* Bb12-treated chickens. No *L. rhamnosus* GG-treated chickens were positive for APEC in internal organs (liver and heart), whereas 10% and 20% of chickens were APEC positive in *B. lactis* Bb12-treated and untreated groups, respectively (data not shown). Surprisingly, the combination treatment with *L. rhamnosus* GG and *B. lactis* Bb12 only resulted in 0.4 log reduction in APEC load.

L. rhamnosus GG treatment also significantly ($P < 0.05$; 12 g in 2 weeks) increased the body weight gain of chickens as compared to not APEC-infected and not probiotic-treated (NC; negative control) chickens (Fig. 5B), whereas no significant increase in body weight was observed in chickens treated with *B. lactis* Bb12 (6.6 g) or *L. rhamnosus* GG and *B. lactis* Bb12 combination (2.6 g).

We performed *L. rhamnosus* GG-specific qPCR to quantitate *L. rhamnosus* GG in the cecum of *L. rhamnosus* GG-treated chickens [42]. The standard *L. rhamnosus* GG qPCR curve was generated (Fig. S3A) and used to quantitate *L. rhamnosus* GG in cecum. At day 15, ~ 6.3 logs of *L. rhamnosus* GG (on average) were enumerated in cecum (Fig. S3B). No amplification of *L. rhamnosus* GG was observed in cecal contents of NC chickens. These results suggest that *L. rhamnosus* GG can resist the low gastric pH and high intestinal bile salt concentrations of the chicken's gut.

L. rhamnosus GG Reduced the Enterobacteriaceae (Escherichia-Shigella) Abundance in Cecum of Chickens

The analysis of alpha diversity (or Shannon index) revealed no significant difference in the microbial richness between the treatment groups (Fig. 6A). However, the microbial community of APEC infected but not treated (PC) chickens was dissimilar to *L. rhamnosus* GG treated and non-infected and non-treated (NC) chickens when beta diversity was analyzed

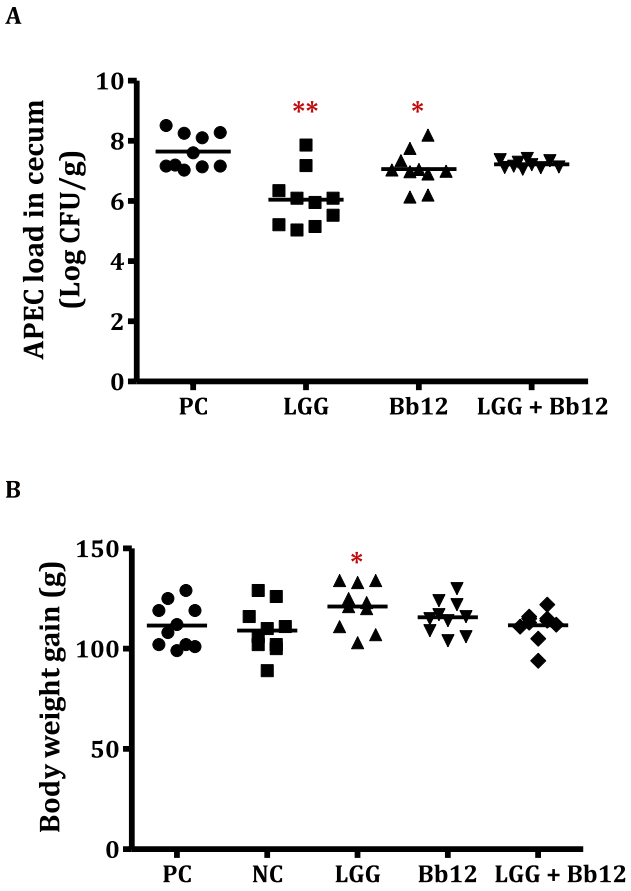

Fig. 5 **A** APEC load in cecum of chickens treated with *L. rhamnosus* GG, *B. lactis* Bb12 or *L. rhamnosus* GG and *B. lactis* Bb12 combination compared to PC (APEC infected but not probiotic treated; positive control) group. **B** Body weight gain of chickens treated with *L. rhamnosus* GG, *B. lactis* Bb12 or *L. rhamnosus* GG and *B. lactis* Bb12 combination compared to PC and NC (non- APEC infected and non-probiotic treated; negative control) groups. LGG, *Lacticaseibacillus rhamnosus* GG; Bb12, *Bifidobacterium lactis* Bb12; $^{*}P < 0.05$, $^{**}P < 0.01$, Man-Whitney *U* test

using Bray–Curtis dissimilarity index (Fig. 6B). The microbial communities of *L. rhamnosus* GG-treated and NC chickens were similar, suggesting that *L. rhamnosus* GG moderated the APEC-induced alterations of microbial community in the cecum of chickens.

The *L. rhamnosus* GG treatment significantly ($P < 0.05$) increased (80.22 to 92.98%) the Firmicutes abundance, whereas decreased (19.72 to 6.11%) the Proteobacteria abundance as compared to PC chickens (Fig. 6C). Specifically, in Firmicutes, the abundance of bacteria belonging to Erysipelotrichia (3.64 to 14.23%) class or Erysipelotrichales (3.64 to 12.99%) order was increased. On the other hand, in Proteobacteria, the abundance of bacteria belonging to Gammaproteobacteria (19.72 to 6.72%) class or Enterobacteriales (19.57 to 6.11%) order was decreased. At the family level, the abundance of Enterobacteriaceae (19.57 to 6.11%) and

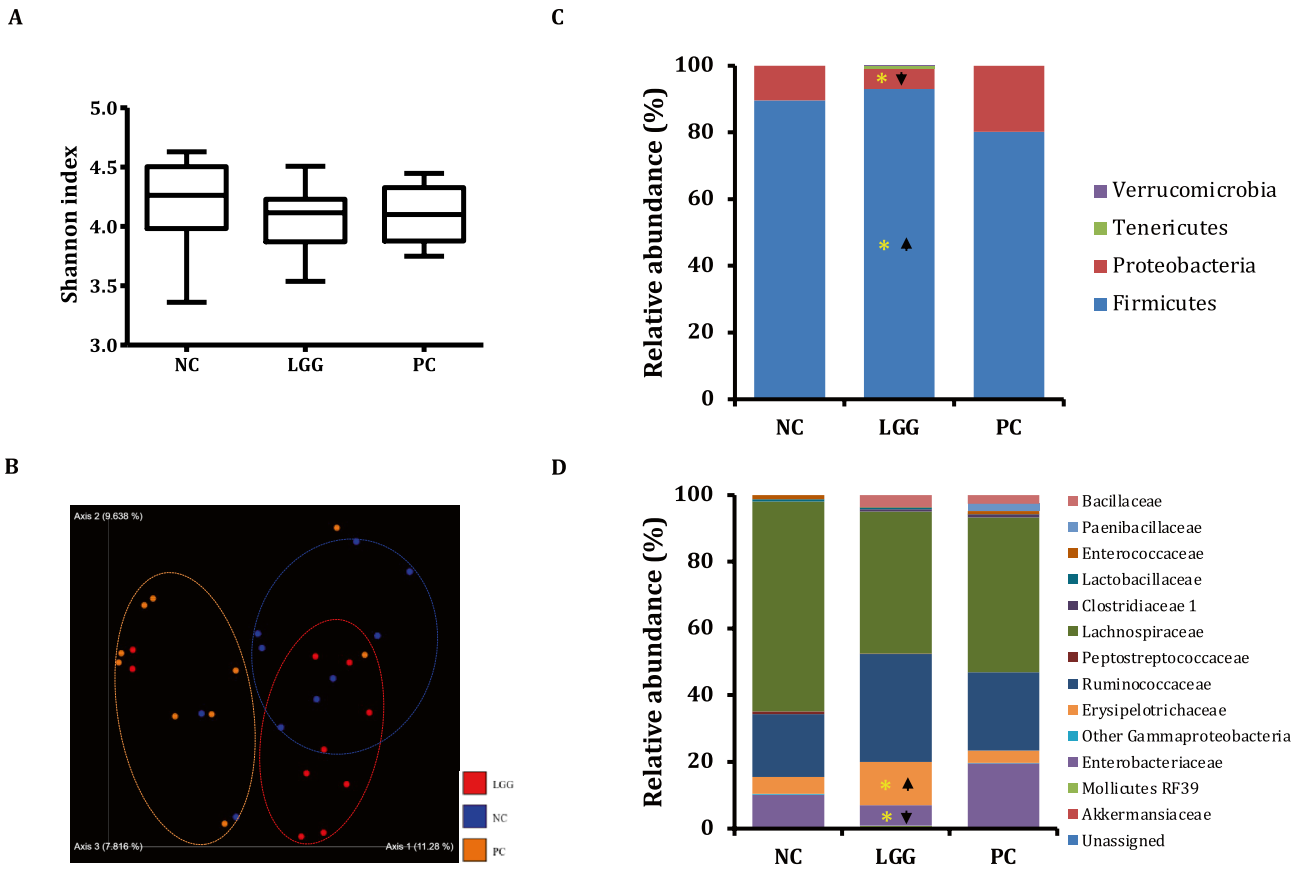

Fig. 6 Alpha diversity (Shannon index) (**A**) and beta diversity (Bray–Curtis dissimilarity index) (**B**) of cecal microbial community of chickens treated with *L. rhamnosus* GG compared to PC (APEC infected but not probiotic treated; positive control) and NC (non-APEC infected and non-probiotic treated; negative control) groups. Relative abundance of cecal microbiota at the phylum (**C**) and family (**D**) level in chickens treated with *L. rhamnosus* GG compared to PC and NC groups. LGG, *Lacticaseibacillus rhamnosus* GG; *$P < 0.05$, Man-Whitney *U* test

Enterococcaceae (1.03 to 0.09%) was significantly decreased ($P < 0.05$), whereas the abundance of Erysipelotrichaceae was significantly ($P < 0.05$) increased (3.64 to 12.99%) (Fig. 6D). At the genus level, the abundance of *Escherichia-Shigella* (16.45 to 4.20%), *Enterococcus* (1.03 to 0.09%), *Flavonifractor* (6.73 to 2.24%), and Lachnospiraceae (uncultured) (4.51 to 0%) was significantly decreased ($P < 0.05$), whereas abundance of *Erysipelatoclostridium* (3.60 to 12.93%), *Negativibacillus* (0 to 1.54%), DTU089 (0 to 1.02%), *Butyricicoccus* (1.08 to 2.45%), *Blautia* (0 to 2.10%), and *Lactobacillus* (0.03 to 0.56%) was increased (Table 6).

Compared to NC chickens, the abundance of Bacillales (0 to 3.61%) order was significantly increased in *L. rhamnosus* GG-treated chickens. At the family level, the abundance of Bacillaceae (0 to 3.61%), Clostridiaceae 1 (0 to 0.64%), and Ruminococcaceae (18.73 to 32.35%) was increased, whereas abundance of Lachnospiraceae (62.85 to 42.58%) was significantly decreased (Fig. 6D). At the genus level, the abundance of *Bacillus* (0 to 3.61%), DTU089 (0 to 1.02%), and *Negativibacillus* (0 to 1.54%) was significantly ($P < 0.05$) increased.

The abundance of bacteria belonging to Clostridia (82.51 to 70.77%) class or Clostridiales (82.51 to 70.77%) order was significantly ($P < 0.05$) decreased in PC chickens as compared to NC chickens. At the family level, the abundance of Lachnospiraceae (62.85 to 46.38%) was decreased (Fig. 6D). At the genus level, the abundance of *Candidatus Soleaferrea* (1.31 to 0.34%) and *Caproiciproducens* (0.77 to 0.05%) was significantly decreased, whereas abundance of *Flavonifractor* (2.68 to 6.73%) and Lachnospiraceae (uncultured) (0 to 4.51%) was significantly increased (Table 6).

Peptides Identified in the Cell-Free Supernatants of *L. rhamnosus* GG and *B. lactis* Bb12 Are Inhibitory to APEC

Out of 33 peptides identified by LC–MS/MS both at HCD and CID settings, five highly abundant common peptides present in both *L. rhamnosus* GG and *B. lactis* Bb12 were tested for anti-APEC activity. Three peptides (VQAAQAGDTKPIEV, AFDNTDTSLDSTFKSA, and VTDTSGKAGTTKISNV)

Table 6 Relative abundance (%) of bacteria at the genus level in different treatment groups

Genus	Relative abundance (%)		
	NC	***L. rhamnosus* GG**	**PC**
Akkermansia	0.00	0.09	0.00
Escherichia-Shigella	10.16	4.20*	16.45
[Clostridium] innocuum group	0.00	0.06	0.04
Erysipelatoclostridium	5.13	12.94*	3.60
[Eubacterium] coprostanoligenes group	0.99	1.36	1.34
Subdoligranulum	0.00	0.52	0.00
Ruminococcus 1	0.00	0.22	0.00
Ruminococcaceae UCG-014	0.00	0.04	0.00
Ruminiclostridium 9	3.46	5.02	7.73
Ruminiclostridium 5	0.35	1.10	0.48
Oscillibacter	0.49	0.17	0.48
Negativibacillus	0.00	1.54*,***	0.00
Flavonifractor	2.68	2.24*	6.73**
DTU089	0.00	1.02*,***	0.00
Caproiciproducens	0.77	0.28	0.05**
Candidatus Soleaferrea	1.31	0.76	0.34**
Butyricicoccus	1.17	2.45*	1.08
Anaerotruncus	2.03	4.93	2.33
Clostridioides	0.93	0.14	0.00
Lachnospiraceae (uncultured)	0.00	0.00*	4.5**
[Ruminococcus] torques group	24.38	18.19	23.13
[Ruminococcus] gauvreauii group	0.00	1.57	0.09
Sellimonas	0.77	0.27	4.04
Lachnoclostridium	0.00	1.11	0.00
Blautia	5.97	2.10*	0.00
Clostridium sensu stricto *1*	0.00	0.64	0.90
Lactobacillus	0.75	0.56*	0.03
Enterococcus	1.21	0.09*	1.03
Paenibacillus	0.00	0.00	2.19
Bacillus	0.00	3.61***	2.56

*Bacteria significantly ($P < 0.05$) altered in *L. rhamnosus* GG-treated group as compared to APEC infected but not treated (PC) group; **Bacteria significantly ($P < 0.05$) altered in PC group as compared to non-infected and non-treated (NC) group; ***Bacteria significantly ($P < 0.05$) altered in *L. rhamnosus* GG group as compared to NC group

were completely inhibitory to APEC (Fig. 7) at 12 mM concentration. The mass spectrometry (MS) peaks for these peptides are shown in Figs. S4 and S5. Peptide AESSDTNLVNAKAA was slightly inhibitory to APEC, whereas FSAVALSAVALSKPGHVNA did not affect the APEC growth.

Discussion

The efficacy of *L. rhamnosus* GG has been demonstrated to reduce infections caused by different bacterial pathogens in different animal hosts [40, 47–55]. *L. rhamnosus* GG administration reduced the *S. infantis* colonization in jejunum and its translocation to internal organs of piglets [47, 53], *S.* Typhimurium colonization in jejunum of piglets [48], and *S.* Typhimurium-induced deaths in mouse model [49]. Similarly, *L. rhamnosus* GG reduced the jejunal and ileal lesions caused by *S. enterica* serovar 4,[5],12:i: in piglets [50]. Further, the culture supernatant of *L. rhamnosus* GG increased the resistance to systemic *E. coli* K1 infection in neonatal rats by reducing intestinal bacterial colonization, translocation, and dissemination to extra-intestinal sites [40, 55]. The mortality of mice was reduced when *L. rhamnosus* GG was administered in experimental model of septic peritonitis by preventing systemic bacteremia [51, 54]. *L. rhamnosus* GG supplementation also reduced the mortality in fish (red tilapia) challenged with *Aeromonas veronii* [52]. Our results demonstrate that *L. rhamnosus* GG is also a promising preventative against APEC infection in chickens.

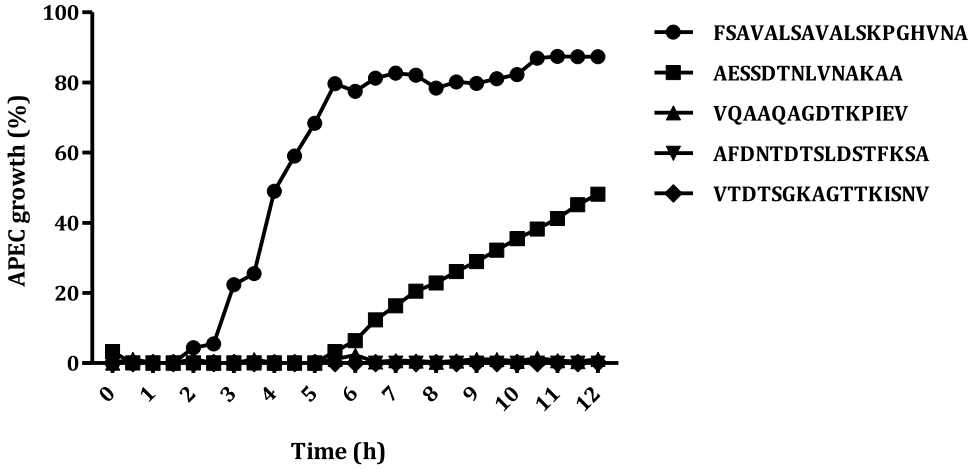

Fig. 7 Growth (%) of APEC when treated with different peptides at 12 mM concentrations. Peptides were added to the APEC suspension in a 96-well plate, and plate was incubated in TECAN Sunrise™ absorbance microplate reader at 37 °C with OD_{600} measurement set at every 30 min for 12 h

Previously, several antimicrobial peptides have been isolated and characterized from *L. rhamnosus* GG and other *Lactobacillus* sps. A 37.3 kDa postbiotic, HM0539, was identified in *L. rhamnosus* GG (ATCC 53,103) supernatant through LC–MS/MS analysis [56]. HM0539 showed beneficial effects against *E. coli* K1 infection in neonatal rats by promoting maturation of intestinal defense; however, effect on growth of *E. coli* K1 was not evaluated. Similar to our finding, multiple small peptides (NPSRQERR, PDENK, VHTAPK, MLNERVK, YTRGLPM, GKLSNK, and LSQKSVK) of < 1 kDa mol. wt. were identified in *L. rhamnosus* GG conditional media; they also showed growth inhibitory activity against Enteroaggregative *E. coli* (EAEC) O42 [57] and APEC serotypes [58]. Two major secreted proteins, p75 (major secreted protein 1; Msp1) and p40 (major secreted protein 2; Msp2), resembling cell wall hydrolases were identified in *L. rhamnosus* GG supernatant with reported functions in promoting the survival and growth of intestinal epithelial cells [59]. In another study, a 1.3 kDa peptide was isolated from supernatant of *L. gasseri* SF1109 with anti-bacterial, anti-biofilm, and immunomodulatory activities against *Pseudomonas aeruginosa* and *E. coli* [60]. A 1.1 kDa peptide (NVGVLXPPXLV; acidocin LCHV) was purified from supernatant of *L. acidophilus* n.v. Er 317/402 strain Narine that has broad spectrum of activity against Gram-positive and Gram-negative pathogens [61]. Peptides (SGADTTFLTK, LVGKKVQTE, and GTLIGQDYK) isolated from supernatant of *L. plantarum* CECT 749 have also displayed antifungal activity against *Aspergillus parasiticus* and *Penicillium expansum* [62]. These findings suggest that small peptides have potential to be developed as new therapeutics against APEC infections. From this study, we identified three novel peptides (VQAAQAGDTKPIEV, AFDNTDTSLDSTFKSA, and VTDTSGKAGTTKISNV) in the cell-free supernatant of *L. rhamnosus* GG that have anti-APEC activity (Fig. 7, Table 5). Further characterization of these three bioactive peptides is necessary to develop them as potential new anti-APEC therapeutics. In the current study, we only tested five highly abundant peptides for their bioactivity. Testing of additional peptides identified from this study can identify additional new anti-APEC therapeutic candidates.

The abundance of bacteria belonging to phylum Proteobacteria, particularly Enterobacteriaceae family (*Escherichia-Shigella*), was decreased in gut microbiota of chickens treated with *L. rhamnosus* GG (Fig. 6, Table 6). The increase in phylum Proteobacteria which includes many opportunistic bacteria is associated with low productivity and pro-inflammatory cytokine profile in chickens [63]. The Proteobacteria abundance was also decreased when *L. rhamnosus* GG was supplemented in mice having dysbiosis of colon microbiota induced by experimental sepsis [64]. Similar to our finding, the abundance of *Akkermansia*, a genus belonging to phylum Firmicutes, was increased in those mice treated with *L. rhamnosus* GG [64]. The *L. rhamnosus* GG treatment in those mice reduced the sepsis-induced mortality by modulating the microbiota dysbiosis, likely by decreasing the Enterobacteriaceae and Enterococcaceae abundance, similar to what we observed in our study [64]. Firmicutes abundance was also increased in pre-weaning piglets treated with *L. rhamnosus* GG [65]. *L. rhamnosus* GG treatment in those piglets was proven beneficial for intestinal health as it enhanced the biological, physical, and immunological barriers of intestinal mucosa [65]. On contrary to Proteobacteria, the increase in phylum Firmicutes is associated with high productivity and anti-inflammatory cytokine profile in chickens [63]. The abundance of bacteria belonging to genus *Escherichia* was also decreased in gut microbiota of children who consumed *L. rhamnosus* GG indicating the ability of *L. rhamnosus* GG to prevent bacterial infections [66]. The increased abundance of bacteria belonging to Erysipelotrichaceae family was observed in *L. rhamnosus* GG treated chickens in our study, which is reported to be associated with improved growth and feed conversion in chickens [67]. These results indicate that *L. rhamnosus* GG can modulate the gut microbiota composition in different hosts to resist bacterial infections. Interestingly, *Flavonifractor* abundance was also increased in *S.* Typhimurium-infected chickens [68], similar to

what we observed in APEC-infected chickens in our study. This suggests that *Flavonifractor* could be a potential gut microbial marker to monitor enteric infections in chickens; however, further investigations are needed to establish this cause-and-effect relationship.

The adhesion and invasion of APEC to HT-29 cells was reduced when pre-treated with sub-inhibitory concentration of *L. rhamnosus* GG supernatant (Fig. 4). It is possible that pre-treatment of *L. rhamnosus* GG supernatant enhanced the integrity of HT-29 colorectal epithelial cells [40], thus improving the epithelial barrier function and decreasing the adhesion and invasion of APEC. Similar to what we observed in our study, pre-treatment of *L. rhamnosus* GG supernatant reduced the adhesion, invasion, and translocation of *E. coli* K1 to human colorectal epithelial (Caco-2) monolayer cells [40]. The pre-treatment of *L. rhamnosus* GG supernatant also inhibited the adherence of *S. aureus* to primary human keratinocytes [69] and adhesion and invasion to human osteoblast (HOB) cells [70]. However, in contrast to our finding, pre-treatment of *L. rhamnosus* GG cells itself decreased the intracellular invasion of *S. infantis* in porcine jejunal epithelial (IPEC-J2) cells [53] and adhesion, invasion, and transcytosis of *E. coli* K1 in Caco-2 cells [55]. Interestingly, the simultaneous addition (no-pre-treatment) of *L. rhamnosus* GG also reduced the adhesion, invasion, and translocation of *C. jejuni* to chicken (B10X1) and pig (CLAB) small intestinal epithelial cell lines [71]. These findings indicate that *L. rhamnosus* GG itself or its cell-free supernatant can exhibit anti-bacterial effects to competitively exclude different pathogens at infection sites, thereby preventing the diseases.

As reported in other studies [32, 33], *L. rhamnosus* GG effect against APEC can be multi-factorial that includes production of lactic acid, secretion/release of small peptides, and others. The shortened cells with bulbous swelling were observed in SEM after APEC was treated with *L. rhamnosus* GG supernatant (Fig. 2). Similar morphology was observed when *E. coli* was treated antimicrobial peptides, gramicidin S, and α-helical peptidyl-glycylleucine-carboxyamide (PGLa) [72], indicating the likely damage of the bacterial cell envelope upon treatment with *L. rhamnosus* GG CFS.

In summary, our study evaluated different probiotic and commensal bacteria and identified *L. rhamnosus* GG as a potential preventative measure against APEC infection in chickens. We identified multiple small novel bioactive peptides that can be developed as non-antibiotic therapeutics against APEC in the future. We also uncovered *L. rhamnosus* GG interactions with APEC and commensal microbes in the gut microbiota of chickens which can facilitate the understanding of mechanism behind *L. rhamnosus* GG antibacterial effects. Our future studies will optimize the delivery of *L. rhamnosus* GG in feed or water, test the efficacy of *L. rhamnosus* GG under conditions mimicking the field, and evaluate the identified peptides in chickens.

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Author Contribution Conceptualization: GR, DK, and GC. Methodology: GR, DK, GC, YH, LD, and VS. Formal analysis and investigation: GR, DK, GC, YH, LD, and VS. Writing-original draft preparation: DK. Writing-review and editing: GR and DK. Funding acquisition: GR. Resources: GR. Supervision: GR.

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Availability of Data and Material All data generated or analyzed during this study are included in this published article (and its supplementary information files).

Declarations

Ethics Approval Animal study was approved by The Ohio State University Institutional Animal Care and Use Committee (IACUC, protocol # 2010A00000149).

Consent to Participate Not applicable.

Consent for Publication All authors read and approved the final manuscript.

Conflict of Interest The authors declare no competing interests.

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