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Lactobacillus rhamnosus modulates murine neonatal gut microbiota and inflammation caused by pathogenic *Escherichia coli*

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Abstract

Background Pathogenic *Escherichia coli* strains produce neonatal septicemia after colonizing the neonatal gut. While the probiotic *Lactobacillus rhamnosus* GG (LGG) effectively reduces neonatal sepsis, LGG's effects on the neonatal intestinal microbiota alterations and inflammation triggered by *E. coli* are incompletely understood. We hypothesized that LGG significantly modulates the specific neonatal gut microbial populations changes and the inflammatory response elicited by the enteral introduction of septicemia-producing *E. coli*. To test this hypothesis, newborn rats were pretreated orally with LGG or placebo prior to infection with the neonatal *E. coli* septicemia clinical isolate SCB34. Amplicon 16S rRNA gene sequencing was performed on intestinal samples. Intestinal injury and expression of inflammatory mediators and apoptosis were determined.

Results Alpha diversity of gut microbiota was greater in SCB34-infected pups in comparison to sham-infected pups, these changes were not modified by LGG pretreatment. Beta diversity analyses also showed differences between SCB34-infected vs. uninfected pups. LGG pretreatment before SCB34 infection did not result in significant beta diversity changes compared to placebo. Moreover, individual genera and species abundance analyses by linear discriminant analysis effect size (LEfSe) showed significant changes in Gram-negative, Gram-positive, and anaerobic populations resulting from LGG pretreatment and SCB34 infection. LGG significantly suppressed the expression of inflammatory cytokines but did not attenuate SCB34-induced apoptosis or histologic injury.

Conclusions LGG modulates clinically significant microbiota features and inflammation triggered by pathogenic *E. coli* intestinal infection shortly after birth. This new knowledge can potentially be harnessed to design novel interventions against gut-derived neonatal sepsis.

Keywords Gastrointestinal microbiome, *Lactobacillus rhamnosus*, *Escherichia coli*, neonatal sepsis, Inflammation mediators, Animals, newborn, Probiotics

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Background

Escherichia coli is the most common Gram-negative bacterium causing neonatal sepsis [1]. Among premature newborns, *E. coli* is the predominant cause of sepsis in the first week of life, with associated mortality up to 40% [2, 3]. After ingestion, pathogenic *E. coli* strains are able to invade the neonatal intestinal epithelium and enter the bloodstream producing septicemia, as demonstrated in animal models [4].

E. coli colonizes the neonatal gut early in life, becoming a component of the newborn's intestinal microbiota shortly after birth [5]. The intestinal *E. coli* population is heterogeneous, composed of commensal strains but can also include pathogenic (Extra-Intestinal Pathogenic *E. coli*, ExPEC) strains able to invade and produce neonatal septicemia and neonatal necrotizing enterocolitis (NEC), a neonatal disease characterized by severe intestinal inflammation triggered by gut dysbiosis [6]. The neonatal gut microbiota alterations following colonization with ExPEC strains associated with neonatal septicemia have not been described in detail.

Probiotics are live microorganisms with beneficial effects to the host [7]. Probiotics modify the intestinal microbiota and modulate inflammatory responses to pathogenic bacteria [8]. Clinical trials in newborns have demonstrated a protective effect of probiotic mixtures that include *Lactobacillus* species against neonatal septicemia and NEC [9–12]. The beneficial effect of probiotic combinations against NEC is significantly greater than the benefit of administering a single probiotic strain [13, 14]. Such clinical studies have tested various probiotic strains, including species of *Lactobacillus*, *Bifidobacterium*, *Saccharomyces*, and others. Combination probiotic formulations that are effective against neonatal sepsis are thought to have synergistic effects [15]. However, the certainty of the protective effect of probiotics against sepsis, particularly in very preterm infants, is low to moderate [16]. Moreover, concerns exist about possible complications of probiotics administration, recently highlighted by a fatal episode of *Bifidobacterium longum* bacteremia in a neonate who received this probiotic strain prophylactically [17]. Therefore, a better understanding of the mechanisms by which individual probiotic strains interact in the neonatal gut is necessary to determine safety and efficacy of probiotics in newborns.

Lactobacillus rhamnosus (also known as *Lacticaseibacillus rhamnosus*) GG (LGG) is the most common *Lactobacillus* strain studied in clinical trials in newborns, which have evaluated its effects as a single probiotic or in combination with others [15]. LGG is effective in colonizing the gut of preterm infants and its adhesive capacity has been demonstrated to be superior compared to other *Lactobacillus* strains [18, 19]. LGG's effect on promoting intestinal cell survival is greater compared to other probiotic lactobacilli strains [20]. However, the mechanisms by which LGG protects against neonatal sepsis have not been studied in detail. Improved knowledge of these mechanisms is important to better define the future applications of LGG as a single or combination probiotic strain in newborns. Specifically, the role that LGG plays in regulating intestinal microbiota alterations and local inflammatory responses triggered by infection and colonization with pathogenic neonatal *E. coli* strains is incompletely understood.

Our study aimed to determine the intestinal microbiota modifications following enteral acquisition of neonatal bacteremia-producing *E. coli* in newborn rats, and the effect of LGG pretreatment on ameliorating the intestinal microbiota changes and intestinal inflammation produced by ExPEC infection in the neonatal period. We hypothesized that LGG significantly modulates the specific gut microbial populations changes and the inflammatory response elicited by the enteral introduction of septicemia-producing *E. coli*.

Methods

Bacterial strains

L. rhamnosus GG (LGG, American Type Culture Collection ATCC® 53103™, Manassas, VA) was grown anaerobically in Man, Rogosa, and Sharpe (MRS) broth. LGG mid-logarithmic cultures were prepared by adjusting concentrations in phosphate buffered saline (PBS)/0.1% gelatin using optical density (OD) measured at 600 nm, as we have previously described [21]. Titers were verified by plate quantification using the track dilution method [22]. The *E. coli* strain SCB34, a well-characterized neonatal septicemia clinical isolate that produces bacteremia after oral inoculation in newborn rats, was used for these experiments [4, 23, 24]. Mid-logarithmic SCB34 cultures grown in lysogeny broth (LB) were centrifuged at 1,900 x *g*, and resuspended in PBS/0.1% gelatin to adjust bacterial titers using OD measurements (OD of 0.7) which were confirmed by plate colony counts as we have previously described [4, 25].

In vitro *E. coli* intestinal invasion and transcytosis after LGG pretreatment

To first confirm the in vitro effects of LGG pretreatment before ExPEC infection, we performed invasion assays using T84 intestinal epithelial cells (ATCC®CCL-248™). T84 cells were grown in 24-well culture plates as previously described [23], and were pretreated with 10^8 colony forming units (CFU) of LGG per well or sterile tissue culture media (TCM) for 1 h prior to infection with 10^6 CFU *E. coli* SCB34. A modified gentamicin protection assay (substituting amikacin, given the resistance of SCB34 to gentamicin) was used to determine SCB34 percent invasion in relation to the initial inoculum, as we previously

described. Briefly, SCB34 was allowed to invade T84 monolayers for 1 h. This was followed by washing, and subsequent addition of amikacin (200 μg/mL) for two additional hours to kill extracellular bacteria. After antibiotic treatment, the T84 cells were washed, lysed with 0.1% Triton X-100, and the recovered intracellular bacteria were quantified by the track dilution method [4].

Transcytosis assays were also performed using T84 cells seeded onto 0.3 cm^2 semipermeable inserts with 3.0 μm pores. Monolayers were grown until reaching a transepithelial electrical resistance (TEER)≥1000 Ohm•cm2 measured by a voltmeter (World Precision Instruments; Sarasota, FL), as previously described [4]. Each insert was pretreated with 10^8 CFU of LGG for 1 h before SCB34 infection. Inserts were washed and infected apically with 10^5 CFU of SCB34. Inserts were incubated at 37°C, 5% CO2 and at 30-minute intervals they were transferred to collecting wells containing sterile TCM. The collecting well TCM from the previous time point was pooled for CFU quantification every 2 h [25]. After the last time point at 6 h post-infection (p.i.), TEER was recorded.

Animals and experimental design

Timed-pregnant Sprague-Dawley rats (Charles River Laboratories International Inc.) were allowed to acclimate and gave birth 7–9 days after arrival. Four individual litters were used in these studies, each for a separate experimental group. Newborn pups of both sexes within each randomly designated litter were studied. The four experimental groups were comprised as follows: Group (A) Pretreatment with LGG followed by SCB34 infection ($n=6$); group (B) Pretreatment with PBS followed by SCB34 infection ($n=5$); group (C) Pretreatment with LGG followed by PBS ($n=5$); group (D) Pretreatment with PBS followed by PBS ($n=5$). Pups within each litter were kept with their dam in separate boxes throughout the experiment. LGG was administered in a 10 μL aliquot containing 10^7 CFU per dose. Each aliquot was delivered in a non-traumatic manner, allowing the pup to suck from a pipette tip as previously described [4]. Pups received two doses of LGG four hours apart on the first day of life (DOL). Two identical doses were given on DOL 2. Immediately following the fourth LGG pretreatment dose on DOL 2, pups were infected orally with 10^6 CFU of SCB34. This inoculum size produces intestinal colonization in 100% of neonatal animals enterally inoculated with pathogenic neonatal *E. coli* strains [26], but is not high enough to produce bacteremia or mortality in pups infected with SCB34 at this age, thus allowing assessment of responses after infection. Pups in each experimental group remained with their dam in individual cages for the experiment duration, i.e., infected pups were kept in separate boxes apart from the control groups. Seven days p.i., all animals were humanely euthanized by gradual induction of deep anesthesia with inhaled isoflurane, followed by opening of the chest cavity as a secondary method, in compliance with the guidelines by the American Veterinary Medical Association [27]. Blood, distal ileum, and distal colon including stool contents were collected (the cecum was not collected). The ileum sample for one pup in Group A could not be used due to processing error. Blood from all pups was plated on LB and MRS square plates (30 μL of blood on each plate) to investigate bacteremia due to SCB34 or LGG, respectively, using the track dilution method [22]. As expected, we did not find bacteremia in any pup. Distal ileum was divided into formalin for staining, and snap-frozen for qPCR. Distal colon was snap-frozen for 16S rRNA sequencing. Animal studies were approved by the Institutional Animal Care and Usage Committee at the University of Missouri Kansas City, protocol # 1808.

Intestinal tissue qPCR

Total RNA was extracted from ileum using the RNeasy mini kit (Qiagen, Valencia, CA), and cDNA was synthesized from 1 μg of RNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Transcripts were amplified, and gene expression data were collected on a Bio-Rad 1Q5 with SYBR Green Mastermix. Pre-validated primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), intercellular adhesion molecule 1 (ICAM1), growth-regulated oncogene product 1 (GRO1, rat homolog to interleukin-8, IL-8), Toll-like receptor (TLR) 4 and single immunoglobulin interleukin-1-related receptor (SIGIRR) were purchased from Sigma. GAPDH was used as the housekeeping gene. Relative gene expression of ICAM1, GRO1, TLR4 and SIGIRR was calculated with the Pfaffl method [28]. Briefly, the difference between the Ct values (ΔCt) of each gene and the GAPDH gene is calculated for each experimental sample. Then, ΔΔCt is calculated as the difference in the ΔCt values between the experimental groups and average ΔCt values of PBS-PBS samples. The fold-change in expression is equal to 2^-(ΔΔCt).

TUNEL assay for apoptosis

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed on terminal ileum slides per manufacturer instructions (Promega, Madison, WI) as previously described [21]. Images were captured at 20X using a Zeiss LSM 510 confocal microscope with attached camera. Quantitative TUNEL evaluation was done by determining the total number of DAPI stained cells (blue) and total number of TUNEL positive cells (green) per high power field (HPF) using the ImageJ software (NIH), with at least 3 HPF per sample. Apoptotic index was calculated as the proportion of DAPI+ (blue) cells that stained for TUNEL+ (green).

Histologic grading of intestinal injury

Two separate investigators blinded to the experimental conditions (WY and JLW) graded histologic intestinal tissue using a 4-point scale to determine intestinal injury scores as previously described [29]. The severity of intestinal inflammation was classified from 0 to 4 according to this previously validated scoring system as follows: 0, intact villi; 1, Mild submucosal and/or lamina propria separation; 2, Moderate submucosal and/or lamina propria separation and/or edema in submucosa and/or muscular layers; 3, Severe submucosal and/or lamina propria separation and/or edema in submucosal and muscular layers and regional villous sloughing; 4, transmural necrosis, loss of villi. Ileum samples from 5 pups in each experimental group were formalin-fixed, paraffin-embedded and cut to prepare slides for hematoxylin and eosin staining. Slides were scanned at 40X into digital images using a Leica Biosystems Slide Scanner and analysis was performed using the system's imaging software (Aperio ImageScope, Leica Biosystems, Buffalo Grove, IL).

DNA extraction and 16S rRNA gene sequencing

For microbiome studies, the colon and stool contents (cecum not included) were flash-frozen immediately after collection. Total genomic DNA was extracted using the QIAmp DNA stool kit (Qiagen, Germantown, MD) following manufacturer's instructions. Using bacterial gDNA, the V3-V4 region of the 16S ribosomal RNA (rRNA) gene was amplified with barcoded universal bacterial primers followed by paired-end sequencing (2×300) on Illumina MiSeq platform (Illumina, San Diego, CA). The datasets generated and analyzed for this study were uploaded to the National Center for Biotechnology Information (NCBI) BioSample database under BioProject ID PRJNA1066694 https://www.ncbi.nlm.nih.gov/bioproject/1066694.

Analysis of 16S rRNA gene sequencing data

Sequencing data were processed with DADA2 v1.16, resulting in an Amplicon Sequence Variant (ASV) Table [30]. Workflow involved quality trimming, filtering, dereplication, error rate estimation, denoising, contig merging, mismatch screening, chimeric sequence removal, and ASV taxonomic classification using the SILVA reference database (SILVA 138.1, released March 10, 2021) [31]. MicrobiomeAnalyst, with the MicrobiomeAnalyst R package, was used for data analysis [32]. Preprocessing included filtering low-abundance features (20% prevalence filter, minimum count threshold of 4), removing low-variance features (interquartile range, 10% cutoff), rarefying samples, and normalizing features with rarefaction and total sum scaling (TSS). Alpha diversity indices (Observed, Simpson, ACE, Shannon) were computed, and statistical analysis employed t-tests or ANOVA. Beta diversity utilized Principal Coordinates Analysis (PCoA) with Bray-Curtis and Jaccard indices at the genus level, assessed with PERMANOVA. Differentially abundant taxa were identified using Kruskal-Wallis rank sum tests, retaining genera with p-values <0.05. Taxa with p-values <0.05 and linear discriminant (LDA) values ≥ 2 were considered significant via Linear Discriminant Analysis Effect Size (LEfSe) at each taxonomic level. LDA was employed to find a linear combination of microbial features that best separates different classes, aiding in classification and dimensionality reduction. To identify key microbial taxa with significant differences between groups, we utilized LEfSe, which ranks features based on their statistical significance and effect size [33].

Additional statistical analyses

Bacterial invasion comparisons were analyzed using Student's t-test. Bacterial transcytosis in vitro experiments were analyzed with Mann-Whitney Rank Sum Test. For analysis of expression of intestinal inflammatory markers, we determined fold changes in mRNA among experimental groups relative to expression in control pups. Values were compared using one-way ANOVA. Histopathologic scores and apoptosis indices were compared with Student's t-test. Statistical analyses were done using GraphPad Prism version 7 (GraphPad Software, San Diego, CA) and SigmaPlot version 14.0 (Systat Software, Palo Alto, CA). Statistical significance was set at $P<0.05$.

Results

LGG pretreatment decreases invasion and transcytosis of *E. coli* SCB34 across intestinal epithelial cells in vitro

We first explored whether LGG impairs the ability of neonatal *E. coli* clinical isolate SCB34 to invade and transcytose intestinal epithelium. We observed that LGG pretreatment for 1 h before SCB34 infection significantly decreased SCB34 invasion into intestinal epithelial cells (Fig. 1A). Moreover, LGG pretreatment of T84 intestinal monolayers completely prevented SCB34 transcytosis (Fig. 1B).

Baseline mean TEER values of polarized intestinal cells treated with TCM or LGG before SCB34 infection were comparable at 1051.5 (SD±25.3) Ohm•cm2 and 1062.0 (SD±32.3) Ohm•cm2, respectively. At 6 h p.i., TEER increased in both groups to mean values of 1677.0 [SD±173.7] Ohm•cm^2 and 1909.5 [SD±198.9] Ohm•cm^2, respectively ($P<0.001$, Fig. 1C). These results demonstrate that LGG pretreatment ameliorates intestinal epithelial invasion of neonatal *E. coli*, and effectively interferes with SCB34's transcytosis without altering transepithelial electrical resistance.

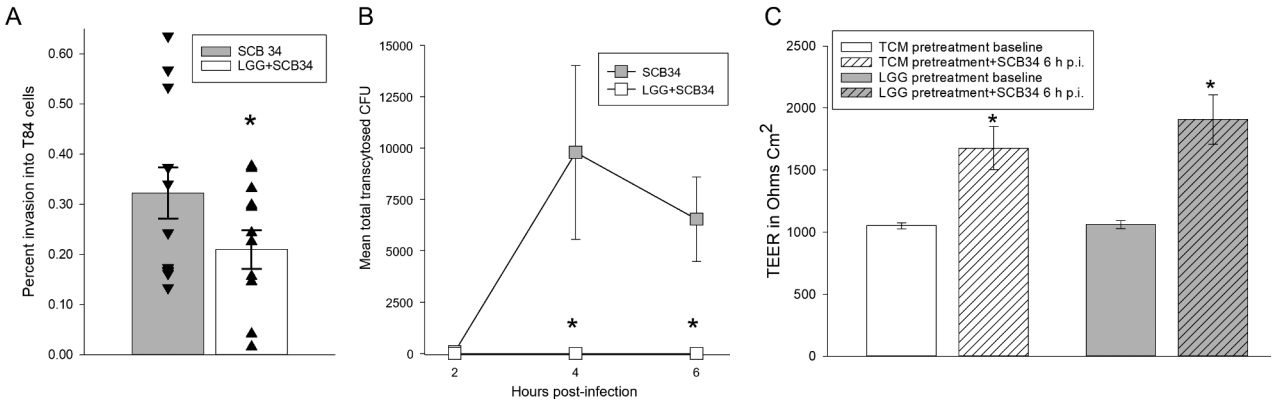

Fig. 1 LGG decreases invasion and transcytosis of intestinal cells by neonatal *E. coli* bacteremia isolate SCB34. (**A**) Percent invasion into T84 intestinal epithelial cells by SCB34 is significantly decreased by pretreatment with LGG. Each condition was tested in triplicate and experiments were repeated three times (T-test, $*p < 0.05$, $n = 12$ replicate wells per group). (**B**) Transcytosis of SCB34 across T84 cells is completely abolished by pretreatment with LGG before infection. Each condition was tested in duplicate, and experiments were repeated twice (Mann-Whitney Rank Sum Test, $*P < 0.03$, $n = 4$ replicates per group). (**C**) Baseline transepithelial electrical resistance (TEER) is similar between T84 monolayers pretreated with sterile tissue culture media (TCM) or LGG, and significantly increases 6 h after SCB34 infection in both groups (T-test, $*P < 0.001$comparing each SCB34-infected group with their respective control, $n = 4$ replicates per group)

Pretreatment with LGG modifies key intestinal microbiota features of neonatal rats orally infected with *E. coli* SCB34

To investigate intestinal microbiota alterations resulting from enteral acquisition of SCB34 in the neonatal period, we compared 16S rRNA sequencing data of colonic microbiota from SCB34-infected pups versus controls. We also determined the effect of LGG pretreatment on the intestinal microbiota changes produced by oral infection with *E. coli* SCB34.

SCB34-infected groups showed significantly greater microbiota alpha diversity compared to noninfected controls, regardless of LGG pretreatment (Fig. 2A-D). This is shown in more detail in pairwise comparisons between SCB34-infected vs. uninfected pups shown in Fig. 2E-H, which demonstrate an effect of infection on microbiota community structure between these two groups that included both LGG and placebo pretreated pups. In the absence of *E. coli* infection, LGG pretreatment overall did not significantly modify microbiota alpha diversity compared to control (PBS-PBS) pups, except for a minor difference in evenness suggested by Shannon index, as shown in pairwise comparisons in Fig. 2I-L.

Beta diversity measured by Bray-Curtis index showed that microbiota abundance in pups pretreated with LGG before SCB34 infection was not significantly different compared to the microbiota abundance of placebo-pretreated pups before SCB34 infection (Fig. 3A). The PCoA plot indicated some overlap between these groups that received LGG and PBS prior to infection, suggesting that the overall microbial communities in these groups were not markedly separated. Microbial abundance of both SCB34-infected groups differed from the two groups not infected with SCB34 (Fig. 3A). The two SCB34-infected groups were also clearly separated from both control groups per Jaccard index; LGG pretreatment did not modify this effect (Fig. 3B). These beta diversity differences between the infected vs. noninfected groups are shown in more detail in Fig. 3C-D. In addition, beta diversity analysis did not show differences between the groups that only received PBS (PBS-PBS) or LGG (LGG-PBS), but were not infected with SCB34 (Fig. 3E-F). Finally, marked differences were observed in the composition of gut microbes at genus level among the four groups (Fig. 4).

To investigate specific differences in abundance of individual genera, we performed linear discriminant analysis effect size (LEfSe) comparisons. LEfSe analyses demonstrated that SCB34-infected pups had lower abundance of *Staphylococcus.* LGG administration also reduced *Staphylococcus* abundance, with a greater decrease in *E. coli*-infected pups (Fig. 5A). These results indicate that the reduction in *Staphylococcus* is likely a synergistic effect of both LGG and SCB34 infection. We also found that SCB34-infected groups had greater abundance of the genus *Lactobacillus*, regardless of LGG pretreatment (Fig. 5B).

Interestingly, we found lower abundance of *Escherichia-Shigella* genera in both SCB34-infected groups compared to controls, and these changes were not modified by LGG (Fig. 5C). Importantly, we did find a striking effect of LGG pretreatment, regardless of SCB34 infection, on decreasing specific anaerobe populations including *Clostridium* and *Romboutsia* (Fig. 5D-E). Moreover, LGG pretreatment completely reversed the increase in abundance of the anaerobic genus *Veillonella* caused by SCB34 infection (Fig. 5F). Further analysis of all four groups at the species level showed that *Lactobacillus intestinalis* and *Enterococcus avium* had the greatest

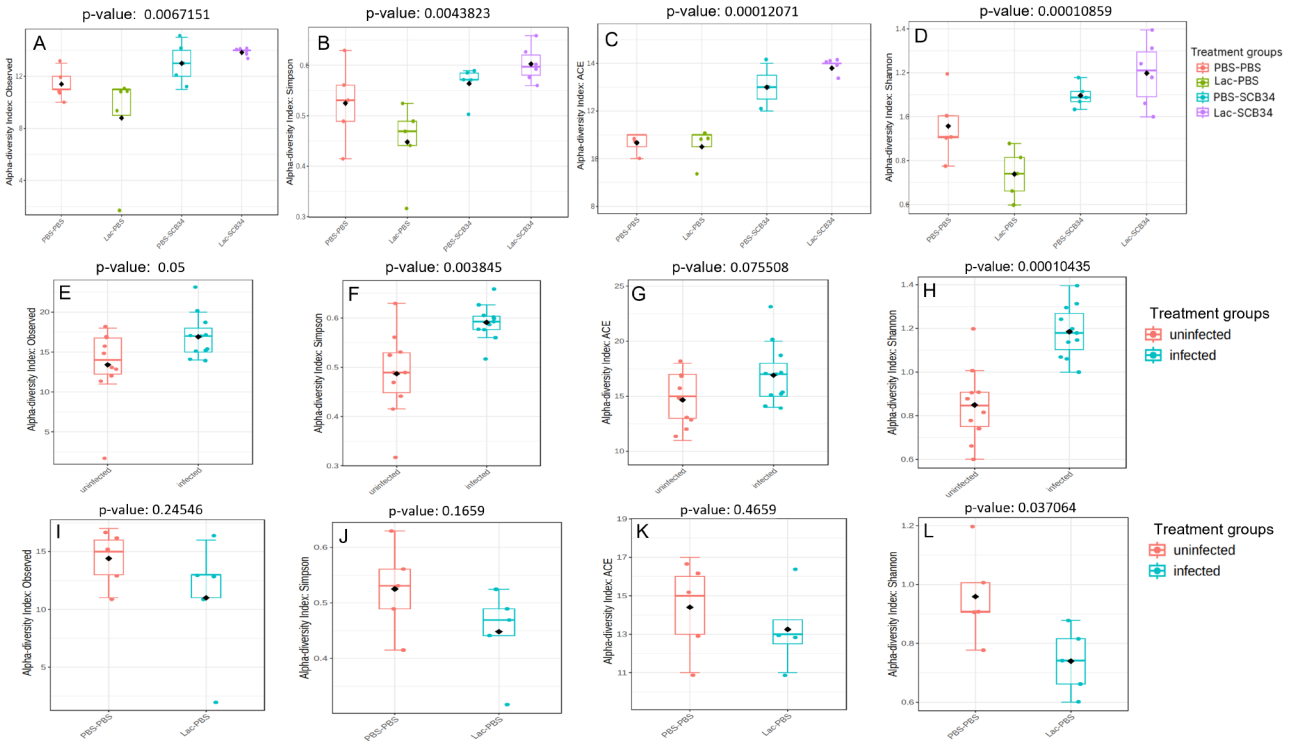

Fig. 2 Intestinal microbiota alpha diversity modifications caused by neonatal *E. coli* infection and LGG pretreatment. (**A-D**) Alpha diversity of neonatal intestinal microbiota is greater in SCB34-infected pups. Observed, Simpson, ACE, and Shannon indices show significant differences in alpha diversity among the four study groups ($p < 0.01$). Pretreatment with LGG did not modify the increased alpha diversity associated to SCB34 infection. Experimental groups: PBS-PBS, $n = 5$; Lac-PBS, $n = 5$; PBS-SCB34, $n = 5$; Lac-SCB34, $n = 6$. The increase in alpha diversity found in all SCB34-infected pups is shown in more detail in pairwise comparisons of infected pups vs. uninfected pups ($n = 11$ and $n = 10$, respectively) (**E-H**). Each comparison group included both LGG and placebo pretreated pups. Differences are particularly demonstrated by Shannon index ($p < 0.001$). On the other hand, pairwise analysis of pups pretreated with placebo ($n = 5$) or LGG ($n = 5$) prior to sham infection showed no significant differences when analyzed with various alpha diversity indices (**I-L**)

abundance in the LGG-SCB34 infected group, whereas *Romboutsia ilealis* and *Haemophilus parainfluenzae* had a lower abundance in this treatment group compared to the PBS-SCB34 group (Fig. 6A-D). We then focused our analysis on pairwise comparisons between the LGG-SCB34 and the PBS-SCB34 groups only, and demonstrated a significantly lower abundance in the genus *Staphylococcus* in the LGG-pretreated group (Fig. 7A), and no difference between these two groups in regard to the genera *Lactobacillus* or *Escherichia-Shigella* (Fig. 7B-C). The decrease in anaerobic populations as a result of LGG pretreatment. was confirmed in these pairwise comparisons (Fig. 7D-F). At the species level, we confirmed the increased abundance of *Lactobacillus intestinalis* in the LGG-SCB34 group (Fig. 8A), and a marked decrease in *Romboutsia*, *Veilonella parvulam* and *Morganella morganii* by pretreatment with LGG prior to SCB34 infection (Fig. 8B-C).

Additional genera with greater abundance identified by LEfSe analyses included *Haemophilus*, *Rothia*, *Streptococcus*, *Proteus*, and *Enterococcus* in both SCB34-infected groups (Supplementary Figures S1A-E). In addition, we identified specific genera that were discriminative factors among the four groups (Supplementary Fig. S2). At the species level, those with greatest abundance among the four groups are shown in Supplementary Fig. S3, with *Lactobacillus intestinalis* predominating in the LGG-SCB34 group. A clustered heatmap illustrates the relative abundance of the fourteen most prevalent genera in each sample among the four experimental groups (Supplementary Fig. S4). Taken together, our findings demonstrate that SCB34 inoculation significantly modifies the intestinal microbiota of newborn pups infected enterally with this neonatal pathogen. LGG also modulated important components of the neonatal intestinal microbiota, and pretreatment with this probiotic significantly modified relevant SCB34-induced microbiota changes at the genus level.

Neonatal pups pretreated with LGG prior to oral inoculation with SCB34 show decreased intestinal inflammatory responses

We next investigated the effects of LGG pretreatment on neonatal intestinal cytokine expression following oral SCB34 inoculation. Intercellular adhesion molecule 1 expression in endothelial cells facilitates tissue

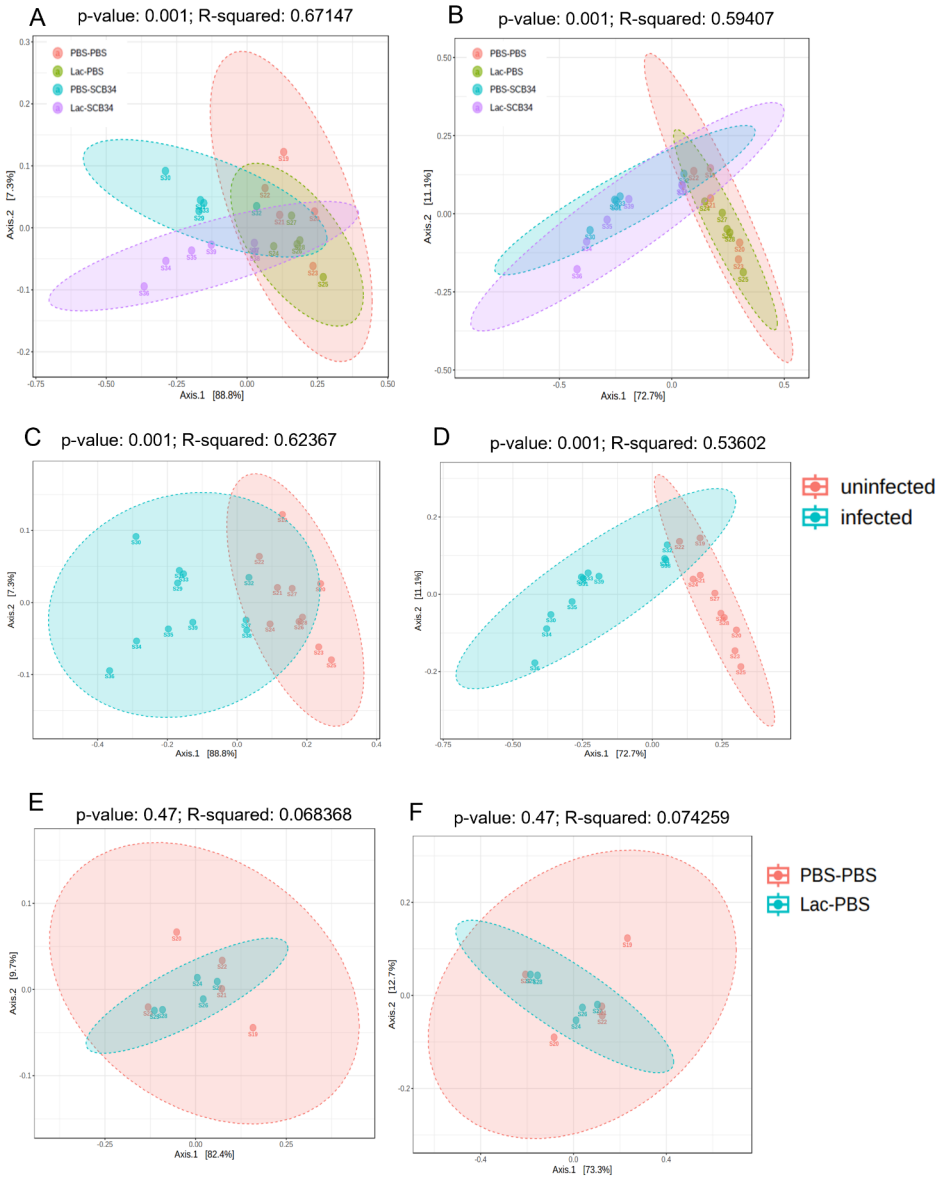

Fig. 3 Microbiota beta diversity of SCB34-infected pups shows significant differences compared to uninfected pups. Differences in microbiota abundance are present between both SCB-infected groups vs. the non-infected groups as demonstrated by PCoA (Principal Coordinates Analysis) using the Bray-Curtis index (**A**). This analysis did not demonstrate differences in microbial abundance in SCB34-infected pups that received LGG vs. placebo prior to infection. Jaccard index analysis (**B**) shows that the dissimilarities between both SCB-infected groups vs. the non-infected groups are also independent of LGG treatment. Experimental groups: PBS-PBS, $n=5$; Lac-PBS, $n=5$; PBS-SCB34, $n=5$; Lac-SCB34, $n=6$. All analysis performed with PERMANOVA. Pairwise comparisons of beta diversity between all infected pups vs. all placebo pretreated pups ($n=11$ and $n=10$, respectively) show significant differences between the two groups by both Bray-Curtis and Jaccard indices (**C-D**, respectively) ($P<0.01$). Additional pairwise comparisons between the two non-infected groups show no differences in beta diversity by Bray-Curtis and Jaccard indices (**E-F**, respectively)

penetration of neutrophils from the vascular bed, and is a reliable marker for inflammation. GRO1 is the murine equivalent of human IL8, which is induced with intestinal inflammation in neonates. Toll like receptor 4 (TLR4) recognizes lipopolysaccharide present in Gram-negative bacteria, and is strongly implicated in mediating necrotizing enterocolitis associated with intestinal dysbiosis in preterm neonates. SIGIRR is a major inhibitor of TLR4 signaling and NEC in the neonatal intestine. As such an imbalance between pro- and anti-inflammatory genes is characteristic of diseases characterized by intestinal inflammation in the preterm gut, thus was quantified here. As shown in Fig. 9A and B, intestinal expression of ICAM1 and GRO1 was significantly greater in SCB34-infected pups as compared to noninfected animals. LGG

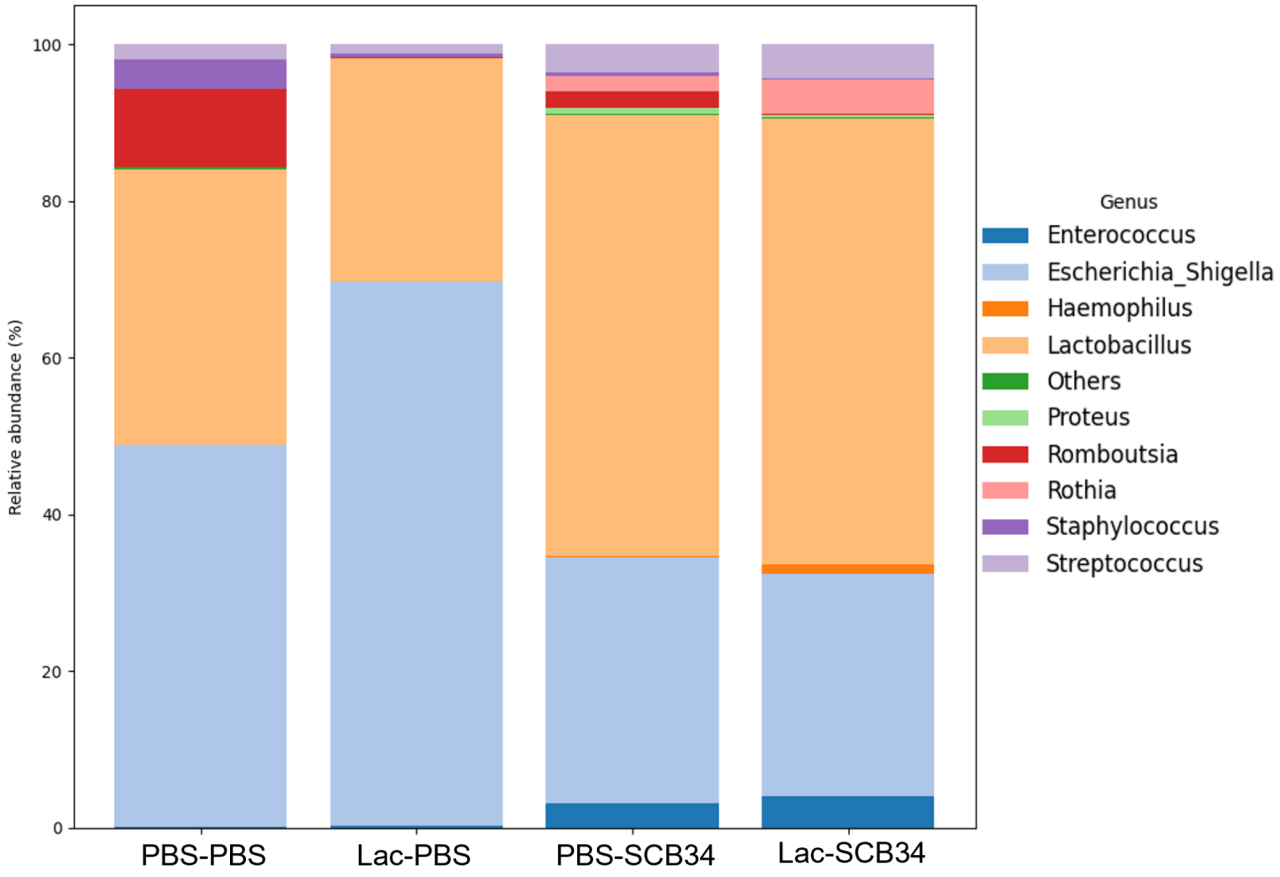

Fig. 4 Bacterial composition at the genus level in each experimental group. Overall bacterial composition characteristics show similarities among both SCB34-infected groups. These two groups had a distinct bacterial composition compared to both groups of sham-inoculated pups

pretreatment decreased this inflammatory response to SCB34 infection.

To further explore intestinal pro-inflammatory and anti-inflammatory pathways regulated by LGG in SCB34-infected newborn pups, we determined expression of TLR4 and SIGIRR in response to SCB34 infection following pretreatment with LGG or placebo. Only the SCB34-infected pups that were pretreated with placebo showed a significant increase in intestinal TLR4 expression compared to noninfected pups pretreated with placebo (Fig. 9C). The increase in TLR4 expression after SCB34 infection was not observed in pups that received LGG pretreatment. Moreover, expression of SIGIRR, a broad negative regulator of TLR signaling, increased in animals pretreated with LGG before SCB34 infection (Fig. 9D). These results provide evidence that LGG pretreatment significantly ameliorates intestinal inflammatory responses elicited by *E. coli* SCB34 by promoting expression of genes that inhibit pro-inflammatory TLR4 signaling in the neonatal gut.

SCB34 infection induces histologic intestinal injury and apoptosis regardless of LGG pretreatment

We further assessed the effects of LGG pretreatment on intestinal injury and apoptosis induced by enteral infection with neonatal *E. coli* SCB34. Intestinal apoptosis was significantly increased in SCB34-infected pups (Fig. 10). This effect was not ameliorated by LGG. Importantly, LGG administration by itself was not associated with increased intestinal apoptosis.

Histologic injury scores were also greater in both groups infected with SCB34 (Fig. 11); LGG pretreatment did not significantly modify the histopathologic injury scores induced by SCB34 infection. Overall, our results demonstrate that SCB34 infection was a significant determinant of intestinal injury and apoptosis in these neonatal animals. Pretreatment with LGG in the setting of this infection did not result in protection against tissue apoptosis and injury.

Discussion

We demonstrate that LGG decreases invasion and transcytosis of clinical neonatal *E. coli* isolate SCB34 across intestinal epithelial cells *in vitro*. LGG is known to

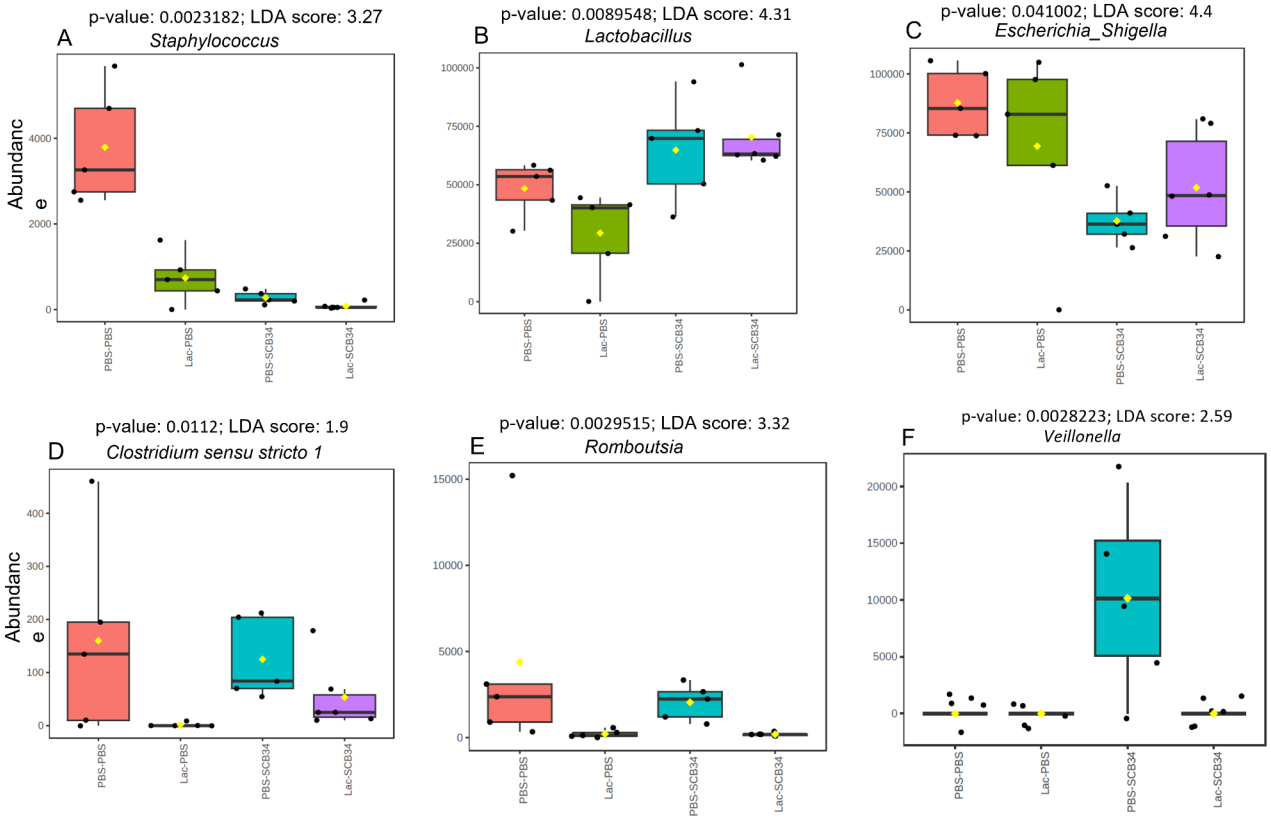

Fig. 5 LEfSe analyses demonstrated relevant genera that significantly discriminated the four study groups. Linear discriminant analysis (LDA) score cutoff was (log10) > 2.0. Genera with significant differences ($p < 0.003$) included *Staphylococcus* (**A**), *Lactobacillus* (**B**), and *Escherichia-Shigella* (**C**) (PBS-PBS, $n = 5$; Lac-PBS, $n = 5$; PBS-SCB34, $n = 5$; Lac-SCB34, $n = 6$). LEfSe analyses also demonstrate significant differences in populations of anaerobic genera among the four study groups. Significant differences ($p < 0.02$) were found for *Clostridium* (**D**), *Romboutsia* (**E**), and *Veillonella* (**F**)

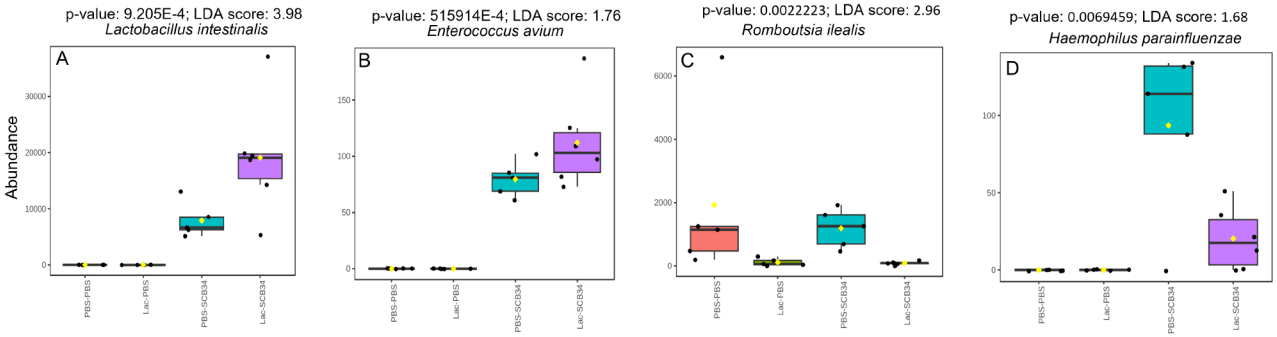

Fig. 6 LEfSe analysis at the species level shows significant differences of relevant microbial populations. Pre-treatment by LGG before SCB34 infection shows an increase in *Lactobacillus intestinalis* (**A**) and *Enterococcus avium* (**B**), and significant decreases in *Romboutsia ilealis* (**C**) and *Haemophilus parainfluenzae* (**D**)

enhance intestinal epithelial barrier function by stabilizing tight junctions altered during infection [34]. Our results support those obtained using other neonatal *E. coli* invasive strains [35, 36]; thus, strengthening the generalizability of LGG's protective effect against ExPEC dissemination in the neonatal host.

The specific effects of LGG on the microbiota and local inflammatory responses in the neonatal gut after infection with septicemia-producing neonatal *E. coli* had not been previously investigated. We found that LGG did not reverse the increase in alpha diversity caused by SCB34. We also did not observe an effect of LGG pretreatment before SCB34 infection on microbiota composition assessed by beta diversity. The increase in alpha diversity in both groups of SCB34-infected pups is intriguing, given that decreased intestinal microbiota

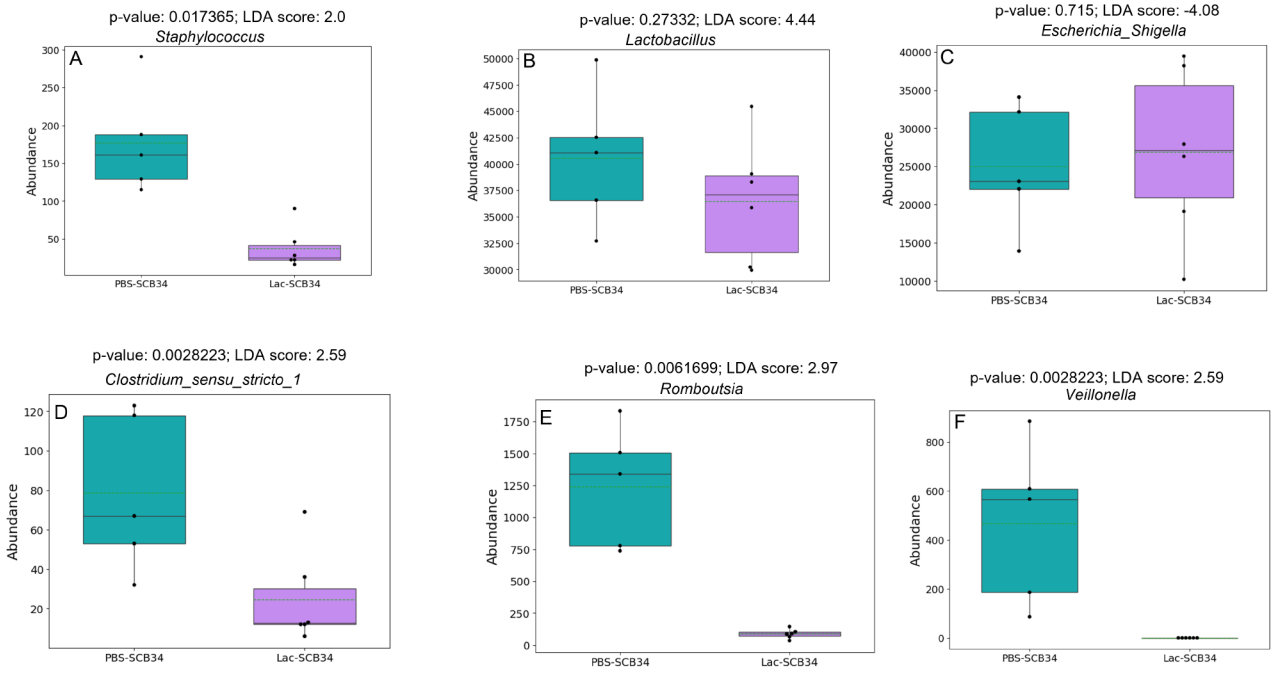

Fig. 7 LEfSe pairwise comparisons between SCB34-infected groups show significant differences at the genus level. Significant decreases of *Staphylococcus* and anaerobic populations are demonstrated, while comparisons do not show significant differences of *Lactobacillus* or *Escherichia-Shigella* populations between the groups

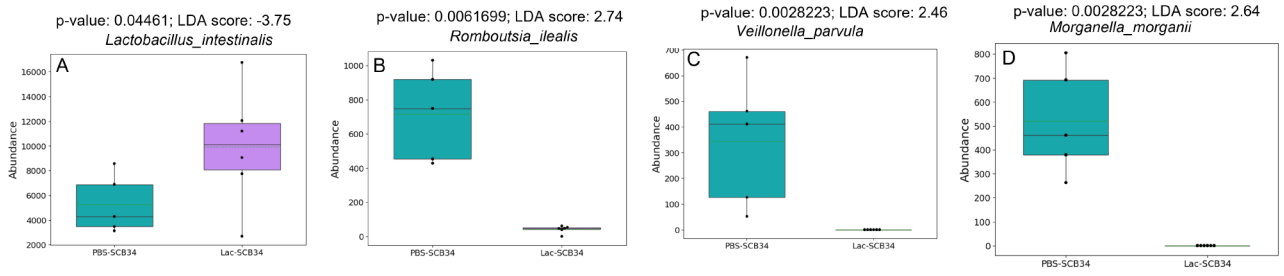

Fig. 8 Pairwise LEfSe comparisons at the species level demonstrate significant differences between the PBS-SCB34 and the LGG-SCB34 infected groups. LGG pretreatment was associated to an increase in *Lactobacillus intestinalis* (**A**), and a decrease in *Romboutsia ilealis* (**B**), *Veillonella parvula* (**C**), and *Morganella morganii* (**D**)

diversity is a hallmark of dysbiosis, which has generally been associated with greater susceptibility to septicemia arising from the gut in newborns, particularly in those born preterm [37]. Nevertheless, alterations in microbiota diversity preceding sepsis are not a uniform finding, and these changes become less pronounced closer to a septicemia episode [38, 39]. Moreover, studies on neonatal microbiota alterations preceding sepsis have been done in newborns with sepsis caused by various individual organisms [37–40]. The microbiota composition in the first month of life is highly dynamic and influenced by multiple factors, including the introduction of pathogenic strains [41]. In our controlled animal study, strain level variation may account for an increase in alpha diversity in the SCB34 group. A meta-analysis of both richness and evenness may increase the understanding of the changes in alpha diversity resulting from SCB34 infection. This is one of the limitations of our study as it is solely based on 16S rRNA analysis. While our results expand the understanding of relevant microbiota changes in response to colonization with septicemia-producing *E. coli*, and the modulation of these changes by LGG, future studies of functional and metagenomic characterization could provide additional information.

Our LefSe analysis showed that LGG administration decreased abundance of *Staphylococcus*, particularly in the presence of *E. coli* infection. These modifications may indicate a protective effect, given that staphylococci are important neonatal sepsis pathogens and predominate in the preterm intestinal microbiome early in life [42]. Interestingly, LGG administration alone did not modify overall abundance of the genera *Lactobacillus* or

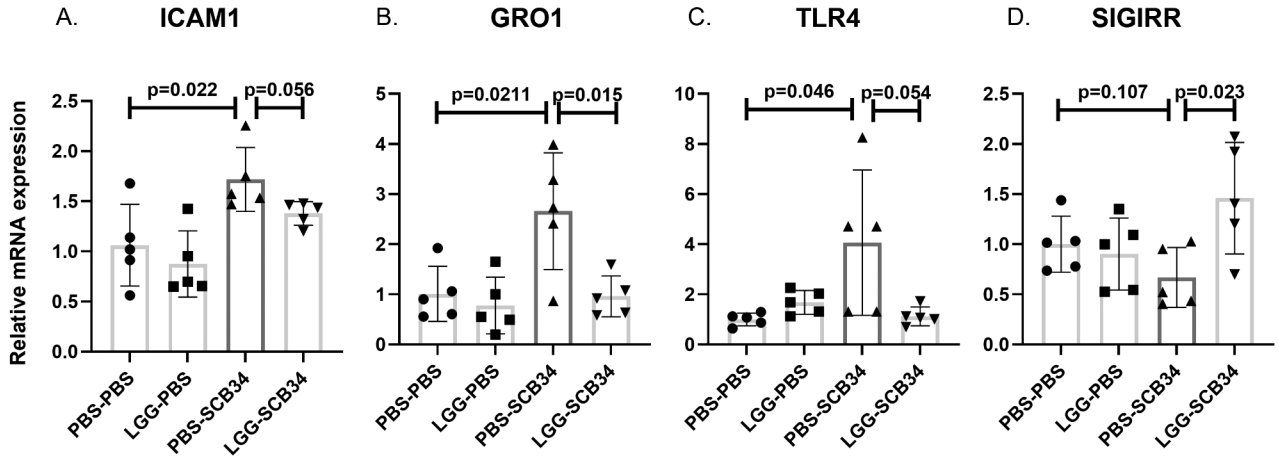

Fig. 9 LGG pretreatment ameliorates intestinal inflammatory responses of neonatal rats orally inoculated with *E. coli* SCB34. Expression of inflammatory cytokines ICAM1 (**A**) and GRO1 (**B**) was determined in intestinal tissue homogenates of animals infected with invasive *E. coli* isolate SCB34 following pretreatment with LGG or placebo. Pretreatment with LGG decreased the expression of ICAM1 and GRO1, which were significantly upregulated in response to SCB34 infection alone ($p < 0.05$, t-test, pairwise comparison). Intestinal expression of TLR4 was also upregulated in response to SCB34 infection compared to uninfected PBS-PBS control ($p < 0.05$, t-test, pairwise comparison) and this effect was also dampened in pups pretreated with LGG compared to PBS pretreatment (**C**). SIGIRR, a broad negative regulator of TLR-dependent inflammatory signaling, was significantly increased only in neonatal animals pretreated with LGG before SCB34 infection and not in placebo pretreated pups (**D**) ($p < 0.05$, t-test, pairwise comparison) ($n = 5$ pups per experimental group). The Y axis represents relative expression of each gene to the GAPDH housekeeping gene expression among the experimental groups

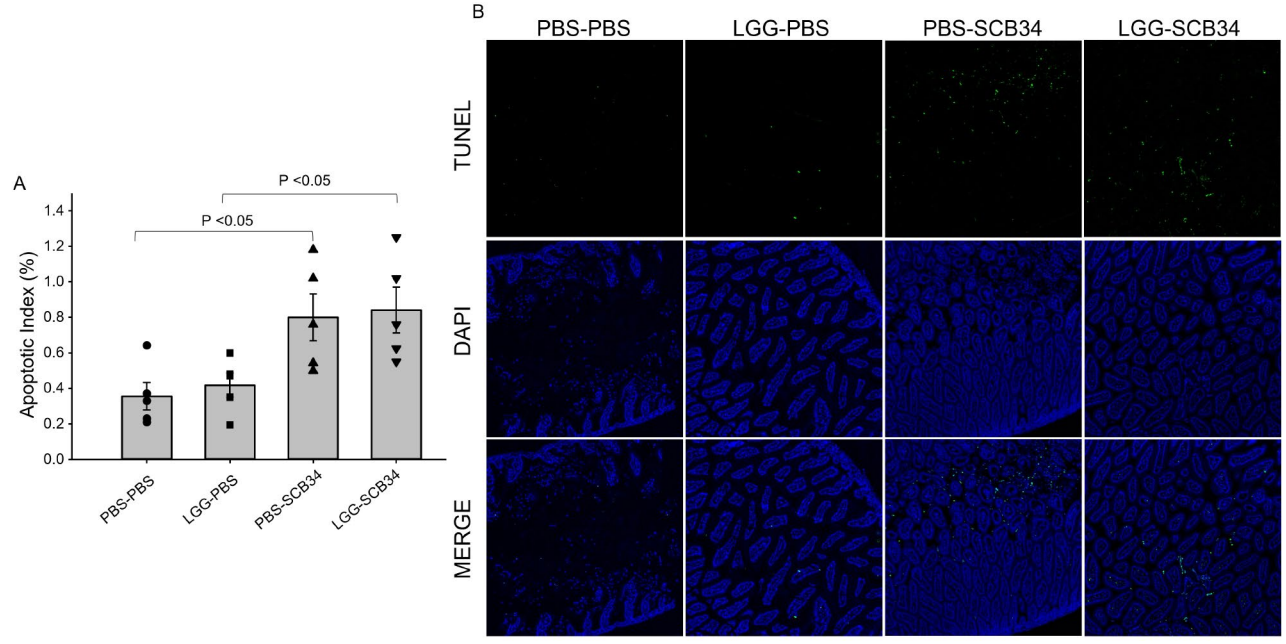

Fig. 10 Intestinal apoptosis is increased in pups infected with *E. coli* SCB34. Apoptotic index (**A**) was significantly greater in both groups of SCB34-infected newborn pups. The increased apoptosis was not affected by LGG pretreatment ($n = 5$ pups per treatment group, one way ANOVA comparisons). (**B**) Representative intestinal ileal sections demonstrate increased apoptosis evidenced by TUNEL staining (green) of samples from both groups of SCB34-infected pups, as compared to PBS-PBS or LGG-PBS groups. Images obtained with LSM 510 confocal laser scanning microscope (Carl Zeiss, Germany) at 20x magnification

Escherichia-Shigella, but SCB34 significantly changed the abundance of these genera. However, further analysis at the species level showed that the abundance of *Lactobacillus intestinalis* increased with LGG pretreatment prior to SCB34 infection. *Lactobacillus intestinalis* has shown to be protective against intestinal inflammation and against toxin-induced intestinal barrier damage [43]. It is possible that LGG modulates other species of lactobacilli, and consequently, the neonatal intestinal response to pathogenic bacterial such as *E. coli*. Other

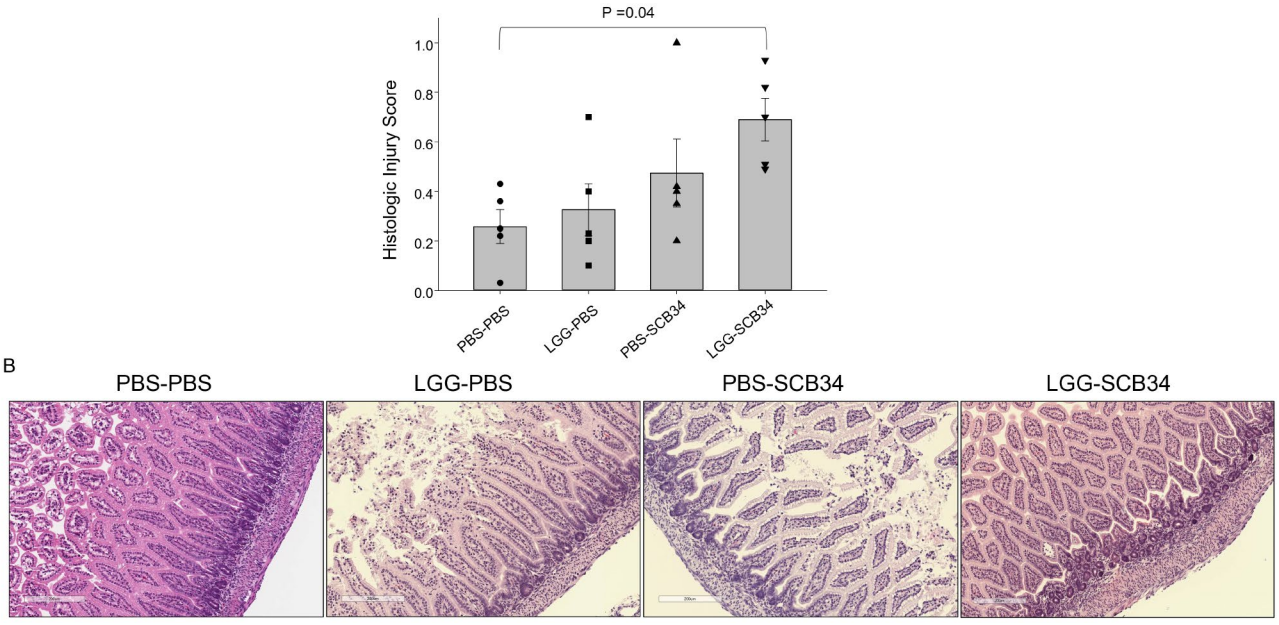

Fig. 11 Histologic injury scores are greater as result of *E. coli* SCB34 infection. Intestinal tissue injury scores (**A**) were increased in pups infected with SCB34 regardless of LGG pretreatment, with the LGG-SCB34 group showing the greatest scores, which were significantly higher compared to the PBS-PBS control group ($n = 5$ pups per treatment group, one way ANOVA comparisons, $p < 0.05$). (**B**) Hematoxylin and eosin staining of representative intestinal ileal sections demonstrate mild damage with only slight submucosal epithelium separation evidenced in both SCB34-infected pups samples, which are absent in the PBS-PBS and LGG-PBS groups (scale bar = 200 µm)

significant changes at the species level that LGG produced when administered prior to SCB34 infection were the decrease of *Romboutsia ilealis* and *Veillonella parvula*, both anaerobes of clinical relevance [44, 45], as well as the Gram- negative opportunistic pathogen, *Morganella morganii*. Changes in neonatal intestinal lactobacilli populations in the presence of pathogenic bacteria have been only partially studied. Singer et al. showed that only specific lactobacilli species counteracted dysbiosis in the neonatal gut after infection by *Klebsiella pneumoniae*, and LGG did not have such effect [46]. Our results highlight the need for additional investigation on the role of individual *Lactobacillus* species in newborns colonized with pathogenic *E. coli*. We speculate that early acquisition of pathogenic *E. coli* strains, while a risk factor for bacteremia, may also contribute to maintain lactobacilli populations normally present in the developing neonatal gut [47], and possibly to their increase as seen in rodents as their intestinal microbiota matures [48]. This possible effect of pathogenic *E. coli* could also explain the decreased abundance of the genera *Escherichia-Shigella*, given that *Proteobacteria* become less prominent as the intestinal microbiome matures [49, 50]. SCB34 acquisition also increased the abundance of the anaerobe genus *Veillonella*. An increase in anaerobe populations has also been associated with neonatal intestinal microbiota maturation [50, 51]. Remarkably, our results showed that, regardless of pathogenic *E. coli* infection, LGG decreased abundance of anaerobic populations, both at the genus and at the species level. Given that a predominance of gut anaerobes has been found to be protective against neonatal sepsis [37], our findings suggest against a beneficial LGG effect from this standpoint. The effects of lactobacilli on dysbiosis are strain-specific [46]; it is thus possible that only selected beneficial effects may be associated specifically to LGG [52]. Similarly, acquisition of pathogenic *E. coli* may modify some populations deemed beneficial, as discussed above, while also affecting other populations that have been associated with dysbiosis such as enterococci.

Our study also showed that LGG modulates SCB34-induced intestinal inflammatory responses. LGG prevented SCB34-induced TLR4 expression, and expression of downstream proinflammatory cytokine GRO1 (IL-8 murine equivalent). IL-8 inhibition by LGG has been demonstrated in intestinal epithelial cells exposed in vitro to inflammatory stimuli [53].While other strains of lactobacilli were shown in a study to increase in vitro production of IL-8 in intestinal cells, LGG did not produce this effect [54], which is in keeping with our results. Moreover, our study demonstrates for the first time LGG's effects on the neonatal intestinal inflammatory response to neonatal ExPEC in vivo. We also demonstrate that LGG decreased ICAM1 expression induced by SCB34. We have previously shown LGG's protective effect against increased expression of inflammatory markers in neonatal animals experiencing formula feeding injury [55]. This supports the evidence of LGG's

anti-inflammatory effect against other adverse stimuli in newborns. In addition to decreasing expression of intestinal inflammatory mediators, our current study also reveals that LGG increased expression of SIGIRR, a negative regulator of TLR4 and other members of the IL-1R/TLR receptor family [56, 57], in the presence of SCB34 infection. This is relevant, given that increased expression of SIGIRR is protective against NEC, a life-threatening neonatal intestinal inflammatory disease triggered by intestinal dysbiosis that frequently leads to septicemia [29].

We did not find evidence of protection by LGG against intestinal apoptosis or injury induced by SCB34 infection. Reassuringly, and in keeping with previous studies, LGG alone was not associated with these detrimental effects. LGG has been shown to protect against chemically induced apoptosis of intestinal cells in vitro and in murine intestine [58]. Our previous studies showed that LGG ameliorated NEC-induced apoptosis and injury in the neonatal gut [21]. The difference may be explained by the more severe, multifactorial injury produced in our experimental NEC model, which LGG can overcome. *E. coli* injury in our colonization model presented herein is milder, making a possible LGG benefit not significantly apparent. These results are novel, given that LGG's effects against intestinal apoptosis and injury triggered by infection with septicemia-producing *E. coli* had not been explored. While injury in the SCB34-infected groups was overall mild, it is intriguing that the greatest injury scores were found in the LGG-SCB34 group. While differences were not statistically significant, further studying the effects of LGG in the setting of greater disease severity is an important next step that we will be able to investigate using our model. The study of additional mechanisms, such as the effects of LGG on intestinal epithelial restitution and proliferation in the neonatal gut, is also important to include in future investigations, given the evidence available on the role of probiotics in intestinal mucosal healing in other intestinal pathologies [59].

Our study has some limitations. Sample size is small, but despite this, significant differences in microbiota composition were demonstrated. Other studies have also shown significant microbiota changes among similar size groups evaluating other conditions [60]. Another study limitation is the assessment of a single time point after infection. However, this time point is particularly relevant as it mirrors the time of establishment of *E. coli* colonization in newborns (not earlier than the 5th day of life) [5, 61], and the age at which the risk for *E. coli* septicemia is highest in newborns. Future studies are needed to further characterize the interactions of pathogenic *E. coli* over time in the neonatal gut, and additional associated host responses.

In conclusion, our results support our initial hypothesis on the distinct role of LGG in regulating specific neonatal intestinal microbiota and intestinal inflammatory responses during infection with pathogenic *E. coli*. These findings contribute to the understanding of the protective mechanisms specific to LGG against neonatal *E. coli* sepsis, which have not been studied in detail. Additional studies are needed to optimize the use of probiotics in neonates based on improved understanding of their unique mode of action.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12866-024-03598-6.

Supplementary Fig. S1. Linear discriminant analysis (LDA) effect size (LEfSe) identified genera with greatest differences in prevalence among the four study groups. LDA score cutoff was (log10) > 2.0. Genera with significant differences ($p < 0.002$) included *Haemophilus* (S1A), *Rothia* (S1B), *Streptococcus* (S1C), *Proteus* (S1D), and *Enterococcus* (S1E).

Supplementary Fig. S2. Linear discriminant analysis (LDA) Effect Size (LEfSe) analysis at the genus level. A histogram of LDA scores was plotted to identify statistically significant dominant bacteria at genus level among the treatment groups (LDA values ≥ 2, $p < 0.05$). Different colors indicate microbiome taxa enriched in every corresponding group.

Supplementary Fig. S3. LEfSe analysis at the species level. A histogram of LDA scores shows the statistically significant dominant bacteria at the species level among the treatment groups (LDA values ≥ 2, $p < 0.05$), with *Lactobacillus intestinalis* predominating in the LGG-SCB34 experimental group.

Supplementary Fig. S4. Clustered abundance heatmap at genus level. The heatmap displays relative abundance distribution of dominant genera among all experimental groups. Each value from 0 to 1 corresponds to the proportion of a specific genus relative to the total microbial composition within each sample, normalized to a scale from 0 to 1 to aid with interpretation.

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Author contributions

H.X. processed and analyzed microbiota data, prepared data visualization, and revised the manuscript for critical intellectual content. S.U. performed microbiota samples sequencing, analyzed microbiome data, critically reviewed the manuscript for intellectual content. C.Z. processed and analyzed microbiota data, performed data visualization. W.Y. performed protein expression assays and analysis, and prepared histological images and analyzed them. I.A. prepared samples for sequencing and microbiota analyses. J.L.W. processed animal samples, prepared bacterial cultures, performed in vitro experiments, processed tissue samples and acquired and interpreted histologic images. V.S. supervised key portions of the project and critically revised the manuscript for intellectual content. S.C.B. conceptualized and supervised the project, performed animal and bacterial in vitro experiments, analyzed data, wrote the manuscript and revised it for intellectual content.All authors read and approved the final manuscript.

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Data availability

The datasets generated and analyzed for this study were uploaded to the National Center for Biotechnology Information (NCBI) BioSample database

under BioProject ID PRJNA1066694 https://www.ncbi.nlm.nih.gov/bioproject/1066694.

Declarations

Ethics approval and consent to participate
The animal studies described in this manuscript were approved by the Institutional Animal Care and Use Committee of the University of Missouri Kansas City, protocol number 1808.

Consent for publication
Not applicable.

Clinical trial number
Not applicable.

Competing interests
The authors declare no competing interests.

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