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Ellagic acid against intestinal stress via mobilizing intestinal lymphatic frequency

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Complete List of Authors:	Jin, Huimin; Hunan Agricultural University College of Animal Science and Technology, Hunan Agricultural University Che, Siyan; Hunan Agricultural University College of Animal Science and Technology, Hunan Agricultural University Wu, Kunfu; Hunan Agricultural University College of Animal Science and Technology, Hunan Agricultural University Wu, Miaomiao; Hunan Agricultural University College of Animal Science and Technology, Hunan Agricultural University

Ellagic acid prevents gut damage via ameliorating microbe-associated intestinal lymphocyte imbalance

Huimin Jin[1] †, Siyan Che[1] †, Kunfu Wu[1], Miaomiao Wu[1]*

[1]Animal Nutritional Genome and Germplasm Innovation Research Center, College of Animal Science and Technology, Hunan Agricultural University, Changsha, Hunan 410128, China.

*** Correspondence:**
Miaomiao Wu
wmm0095@163.com

† These authors have contributed equally to this work and share first authorship.

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Abstract

Inflammatory bowel disease (IBD) pathogenesis involves a sustained microbial-mediated immune response following intestinal stress. Although administration of antibiotics can be an effective therapy, the misuse of antibiotics may risk unknown drug-resistant bacteria. In this study, piglets pretreated with Ellagic acid (EA) and Ampicillin (AMP) for 14 days were injected intraperitoneally with paraquat (PQ). We found piglets lost most of their gut microbes in the AMP group, protected from subsequent intestinal damage caused by gut oxidative stress. Hence, we identified some gut microbes that may play a critical role in mediating cellular responses following cytokine stimulation in PQ -induced stress. EA preprocessing exhibited the same performance as AMP. Pretreatment of EA reduced *Streptococcus* abundance in the gut. Particularly, EA modulated intestinal lymphocyte distribution, reduced the frequency of $CD79a^+$ cells, and alleviated the upward migration of $CD3^+$ cells to the apex of the intestinal villi in the intestinal epithelium. Additionally, the intestinal immune response had been known associated closely with the abundance of *Streptococcus* in the gut. Thus, we concluded that EA has the potential to replace antibiotics to prevent microbial-mediated immune responses in the gut, and EA can be applied as a supplement candidate to alleviate the development of inflammation caused by intestinal stress.

1 Introduction

IBD has been a global healthcare problem with increasing incidence. It was indicated that genetic predisposition, the external environment, intestinal microbial flora, and immunological responses are all implicated and functionally integrated with its development[1]. Stress has long been reported anecdotally to increase disease activity in IBD both mentally and physically, in which continuing immune response to microbial dysbiosis provides colonization opportunities for pathogenic or opportunistic pathogens, and the invasion or proliferation of them is an important reason that induces IBD[2-4]. Our previous studies established that PQ caused redox imbalance in the piglet gut, increased the presence of opportunistic pathogens, and induced intestinal inflammation[5, 6]. Although the PQ is confirmed that critical for promoting intestinal damage, the role of gut microbes and intestinal epithelial immunity are not well understood in PQ-induced enteritis.

IBD is proved to result from inappropriate activation of the mucosal immune system driven by the microbiota. For instance, the presence of *Klebsiella pneumonia* and *Proteus mirabilis* is linked to an exacerbation of colitis in mouse models of IBD, and changes in the gut microbiota induced by chemically induced colitis can promote the severity of intestinal inflammation[7, 8]. Hence, manipulating the gut microbiota can be achieved by antibiotics to decrease concentrations of bacteria in the gut lumen and alter the composition of intestinal microbiota to favor beneficial bacteria, which is thought as an effective treatment for IBD[9]. However, antibiotic treatment can be associated with negative outcomes as well, especially with recurrent or prolonged

courses. Side effects of antibiotics in IBD include intolerance to treatment, *Clostridium difficile* infection, and increasing antibiotic resistance[10], and large cohort studies suggested that people exposed to antibiotics, particularly in childhood, carry a higher risk of development of IBD[11]. Additionally, antibiotics may also have a rebound effect on gut bacteria after therapy cessation of therapy, as shown in a study of patients with IBD that discovered a sharp rise in mucosal bacterial concentrations as early as one week after therapy and remaining at a level that is at least one power higher over a period of 5 months as compared to patients who had not received antibiotic treatment[12]. With awareness of the health consequences of the IBD epidemic of public, safer and more effective preventive measures are urgently waiting to be developed.

Due to their positive actions on the animal body while generating a relatively small number of side effects, especially the lower probability of microbial resistance, ingredients of natural products seem to be an excellent alternative to an antibiotic in the prevention of infectious diseases[13, 14]. More and more antibacterial ingredients in natural plant extracts are included in developing alternatives to antibiotics, which aim to be acceptable, regenerative, and eco-friendly[15-18]. EA, a phenolic lactone compound with known antioxidative and anti-inflammatory properties, is present in certain fruits and nuts[19]. We demonstrated that EA could protect against gastric ulceration and as a protective agent of the liver[5, 6]. Interestingly, EA-rich plant extracts exhibited a potent bacteriostatic effect against Gram-positive facultative anaerobic bacteria and inhibited the bactericidal activity of *C. sakazakii*, as a source of natural food preservatives for

the control of *C. sakazakii*[20, 21]. All the above indicated EA may have the potential to replace antibiotics to play anti-infective and protective effects, and the potential mechanism of EA as a gut-protecting supplement is deserved further definition.

Thus, we established a model of intestinal injury using piglets since they have physiological similarities to the human intestine and share mechanisms of human disease[22]. We found that PQ disturbed microbial homeostasis, increased pathogenic bacteria and intestinal inflammation markers. The intervention of EA had the same mitigating effects as AMP, inhibited *Streptococcus* and *Actinobacillus* while EA preserved intestinal microbial diversity, and EA shaped intestinal immune-related cells, and modulated immune responses under stress. Our results observed partly explain the mechanism of EA in relieving enteritis. It may pave the way for EA to function as a candidate supplement to enhance intestinal barrier function.

2 Materials and methods

2.1 Animal and experimental design

The Animal Ethics Committee approved all experimental procedures of Hunan Agricultural University (Number: 2021042). Twenty-four Yorkshire Duroc weaned piglets at 21 days of age with 7.42 ± 0.1 kg bodyweight were acclimatized for 7 days before the experiment and housed under controlled environmental conditions (penned with hard plastic slatted flooring, and ad libitum access to experimental diet and water), and then randomly divided into three groups (8 piglets/group). During the 21-day experiment, group PQ (for negative control group, CON) was fed a basal diet formulated according to the recommended nutrient requirements of the National

Research Council (2012), while the antibiotic group (AMP, for positive control) was fed the basal diet supplemented with 24.3 mg/kg (BW) AMP (Sigma-Aldrich, Inc., St. Louis, MO), and EA group was fed the basal diet supplemented with 0.01% EA (m/m) (EA was purchased from Sai'en, Inc. Xi'an, China). On days 14 and 18 of the experiment, PQ injections were prepared by dissolving paraquat powder (Sigma-Aldrich, Inc., St. Louis, MO) in 0.9% sterile saline solution to a final dose of 4 mg/kg BW. Pigs were euthanized on 21days for blood, colon, cecal digesta, and ileum sample collection. Intestinal tissues for histomorphometric analysis were washed with sodium chloride solution and then kept in 4% paraformaldehyde; the remaining were quickly placed in liquid nitrogen and stored at -80°C for further detection.

2.2 Growth performance

During the experiment, the feed intake of piglets was recorded daily. All piglets were weighed on an empty stomach on the first day and the 21st day of the investigation. Average daily gain (ADG), average daily feed intake (ADFI), and feed to gain ratio (F /G) were calculated at the end of the experiment.

2.3 Histomorphometry

Tissues were embedded in paraffin and sectioned (4 μm), and Hematoxylin-eosin (H.E.) staining was performed and observed under a light microscope. CaseViewer.2.4 image analysis and processing system (3DHISTECH, Hungary) was

used to measure the samples' length and depth of the villi and crypts and calculate the average values.

2.4 Serum inflammatory cytokines

The collected whole blood samples were placed at room temperature for 2 hours and then centrifuged at 4000 rpm for 10 minutes. The supernatant could be taken for the detection or stored at -20°C. The contents of *TNF-α* and *IL-10* in serum were detected using Meimian ELISA Kit (Jiangsu, China), while the concentrations of *MPO,* and *IFN-γ* were determined using the Huamei ELISA Kit (Wuhan, China).

2.5 Quantitative real-time PCR (qRT-PCR)

Total *RNA* was isolated from intestinal tissues using MiniBEST Universal *RNA* Extraction Kit (TaKaRa Biotechnology, [Dalian] Co. Ltd, China). The purity of *RNA* was measured using NanoDrop 1000 (Thermo Fisher Scientific, USA). Reversing transcribed used the PrimeScriptTM RT reagent Kit with *gDNA* Eraser (TaKaRa Biotechnology, [Dalian] Co. Ltd, China). According to the manufacturer's instructions, qRT-PCR was performed using the TB Green II PCR Master Mix kit (TaKaRa Biotechnology, [Dalian] Co. Ltd, China). The corresponding primers were designed by the principle of PCR primer design and *β-actin* was used as the internal control gene. All PCR primers were purchased from Sangon Biotech (Shanghai, China). Details of the PCR primers are listed in Table S1. Quantitative real-time PCR was performed using the LightCycler®480 real-time PCR system (Roche, Switzerland)

and the *mRNA* expression of the relative samples of target genes was calculated by the $2^{-\Delta\Delta Ct}$ method. The data of each target gene was normalized to the CON group.

2.6 16S rRNA gene amplification and high-throughput sequencing

Total genomic *DNA* was extracted from ileal contents.16S *rRNA* library was constructed and specific primers amplified sequences in different regions. PCR products were purified using Qiagen Gel Extraction Kit (Qiagen, Germany). According to the manufacturer's recommendations, the true *DNA* PCR - free sample preparation kit (Illumina, USA) was used to generate the sequencing library and add the index code. Qubit @ 2.0 fluorometer (Thermo Scientific, USA) and Agilent biological analyzer 2100 system evaluated the library quality. Then, the prepared *DNA* library is sequenced by the Illumina novaseq platform to generate 250 bp paired-end reads. The sequences are clustered into operational taxonomic units (OTUs) with 97% identity. Species annotation and abundance analysis were then performed. QIIME (Version 1.7.0) calculated Alpha and Beta diversity analyses. Principal coordinate analysis (PCoA) was obtained by WGCNA, STAT, and GGploT2 package analysis in R software (2.15.3). Illumina MiSeq sequencing process, data processing, and bioinformatics analysis were performed by Beijing Nuoxin Bioinformation Technology Co., LTD. (China). 16S analysis data set is stored in NCBI, and the project name is PRJNA860285.

2.7 RNA extraction, genome-wide RNA sequencing, and transcriptomic analysis

Total *RNA* from intestinal tissue was extracted with Trizol (Thermo Scientific, USA), and the purity and degradation of *RNA* were detected. Using Agilent 2100 Bioanalyzer the *RNA* nano 6000 detection kit (Agilent Technologies, CA, USA) accurately detects the integrity and total amount of *RNA*. The *mRNA* obtained by enriching total *RNA* with Oligo (dT) magnetic beads was randomly interrupted. The fragmented *mRNA* was used as the template, reverse transcribed into *cDNA*, and the random oligonucleotide was used as the primer. PCR finally constructed the library, and then the library quality control analysis was carried out. After passing the library inspection, different libraries were pooled according to the requirements of effective concentration and target offline data volume, sequenced on Illumina novaseq 6000 platforms, and generated 150 BP paired-end readings. To ensure the quality and reliability of data analysis, the original data needs to be filtered to create clean date data. Download reference genome and gene model annotation files directly from the genome website. The reference genome index was constructed using HISAT2 (v2.0.5), and the paired-end clean reads were compared with the reference genome using hisat2. The differential expression between the two groups was analyzed by featureCounts v1.5.0-p3. Differential expression analysis was conducted using DESeq2, and adjusted p-value < 0.05 and fold change > 1.5 was considered as the threshold for significantly differential expression. All differentially expressed genes were analyzed by clusterProfiler R package to test the statistical enrichment of differential expression genes in Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways

by the cluster Profiler R package. *RNA*-Seq analysis data set is stored at NCBI under the project name PRJNA860759.

2.8 Immunofluorescence

The ileum tissue was routinely embedded in paraffin and sliced, dewaxed with a hierarchical alcohol concentration of xylene, washed with distilled water for 5min, then heated for antigen repair, and then sealed with 5% BSA for 60min. Primary antibody CD79a (1:50, Thermo Fisher Scientific, USA), CD11b (1:50, Abcam, USA), CD3 (1:50, Proteintech Group, USA) incubated 4°C. The next day, remove and rewarm for 30 min, washed three times with PBS, add CoraLite594-conjugated goat anti-rabbit IgG H&L (Abcam, USA) fluorescent antibody, and incubate for 1 h at 37°C and avoid light, wash three times with PBS and add DIPI working solution to stain nuclei at 37°C for 10-20 min, finally add buffered glycerol to seal the film. Fluorescence microscopy (Motic, China) detected staining after sealing.

2.9 Statistical analysis

All results were statistically analyzed by GraphPad Prism 8 software and SPSS 22.0 statistical software and expressed as mean ± SEM. Analysis of variance (ANOVA) was used to determine the significant differences between the groups, and a t-test was used to evaluate the differences between the two groups. Statistical differences were accepted when $P < 0.05$.

3 Results

3.1 EA prevented intestinal damage induced by Paraquat.

We first sought to determine the role of microbes in the subsequent injury of PQ-induced stress and whether EA can prevent it. Piglets were pretreated with EA and AMP for 14 days, followed by an intraperitoneal injection of PQ (Figure. 1A). Notably, as demonstrated in Figure 1, when AMP clarified gut microbes, damage to the intestine by PQ production was effectively prevented, suggesting that gut microbes are involved in subsequent responses to stress. EA supplementation effectively ameliorated growth performance limited by PQ, including daily gain and feed intake of piglets (Figure. 1B-D, $P < 0.05$). Markers include serum cytokines and their related *mRNA* in tissues that reflect intestinal injury were detected. Similar to the AMP processing group, serum *TNF-a*, *IL-10,* and *MPO* levels in the EA group were significantly different from those in the non-treated group. Meanwhile, *mRNA* expression levels of *IFN-γ, IL-10, IL-22*, and *IL-6* in ileum and colon tissues moderated significantly after EA treatment (Figure .1E-G).

In addition, crypt depth and villus height were quantified. EA was found to significantly affect intestinal stress, displayed by the ratio of villus height to crypt depth induced by PQ in ileum (Figure. 1H-J). H&E staining showed that the pathological condition of the ileum was improved by EA treatment, villus height was higher than that of the CON group, and epithelial cells were arranged more closely. EA significantly affected intestinal inflammation, and reduced inflammatory cell infiltration (Figure. 1K). These data suggested that gut microbes may play a key

role in subsequent injury from PQ stress, and treatment of EA improved intestinal stress resistance of piglets.

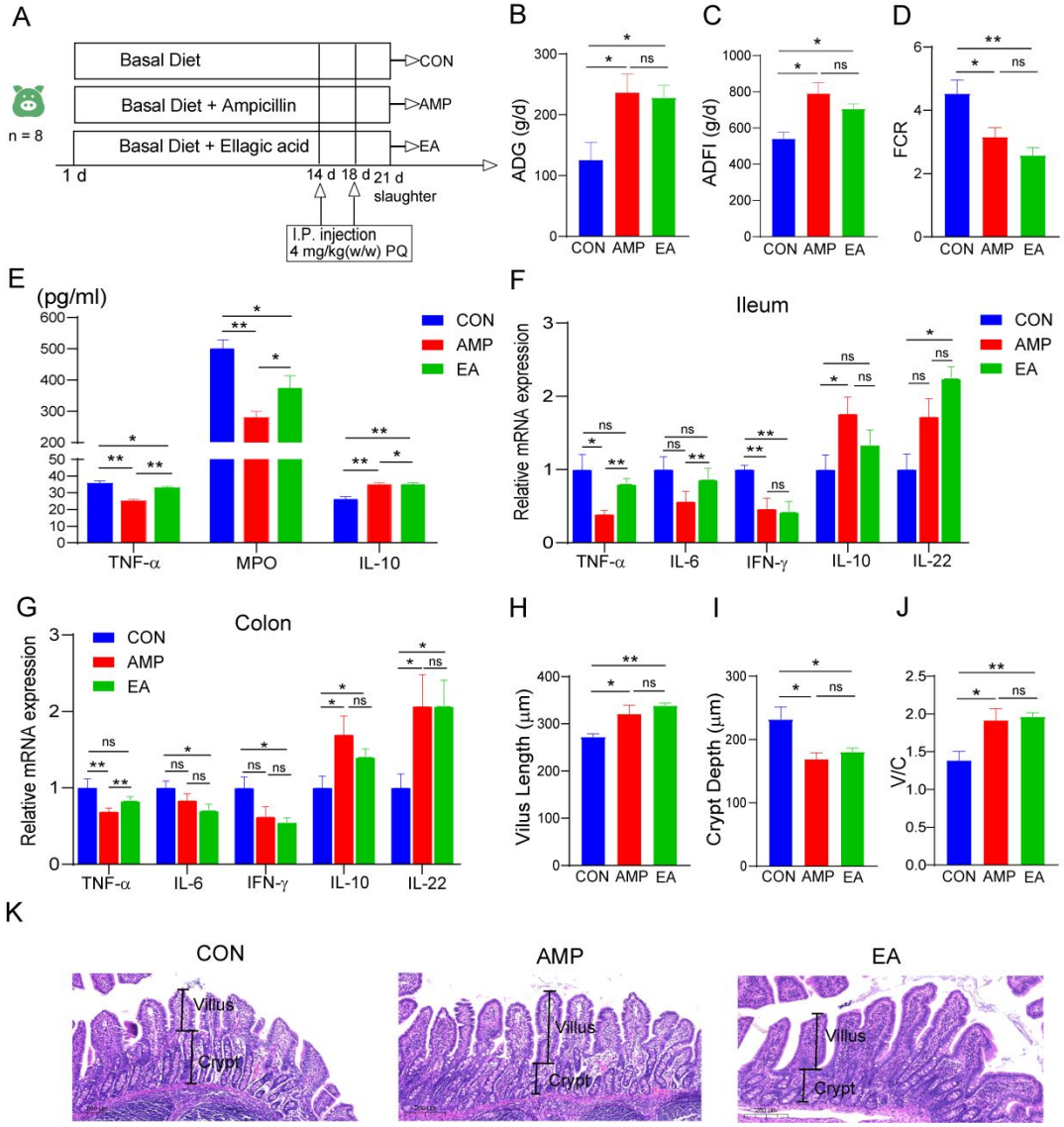

Figure 1. EA prevented intestinal damage induced by Paraquat. (**A**) A schematic of the animal treatment strategy. The piglets were sacrificed after subjection to the different treatments on the 21st day, and n represents the number of animals in each group. The daily weight gain (**B**), average daily feed intake (**C**), and feed conversion rate (**D**) of piglets during the trial period. (**E**) ELISA determined serum levels of *TNF-a, IL-10*, and *MPO* (pg/ml). (**F**) Relative *mRNA* expression levels of *TNF-α,*

IL-6, IFN-γ, IL-10, and *IL-22* in ileum. (**G**) Relative *mRNA* expression levels of *TNF-α, IL-6, IFN-γ, IL-10,* and *IL-22* in colon. (**H-J**) Statistical map of villi and crypt depth in ileum of piglets. (**K**) Histological examination of ileum with H&E staining in piglets. Scale bars, 100 mm. All data are expressed as the mean ± SEM (n = 8). Comparisons were performed by t-test or analysis of variance (ANOVA). **P* < 0.05, ***P* < 0.01.

3.2 EA selectively inhibited gut microbes.

We next asked how EA pretreatment affected gut microbes. As expected, EA treatment preserved gut microbial diversity, that different from AMP. Beta diversity analysis used the Bary Curtis distance measure to detect structural changes in intestinal flora in samples, which showed the EA group had a wider variety ($P < 0.05$), while only slight differences in Alpha diversity indexes richness index (ACE and Chao1) (Figure. 2A&B). The disparity between the three groups of intestinal flora, principal coordinate analysis (PCoA) based on the Unweighted_Unifrac differences showed that the microbial community of the AMP group showed a trend of separation from the other groups ($P = 0.003$) (Figure. 2C). Stre*ptococcus* and *Actinobacillus* blooms in only PQ-treated gut, while the intervention of EA and AMP reduced their abundance surprisingly. *Streptococcus* and *Actinobacillus* in the EA group were down-regulated to only 0.255-fold and 0.73-fold as in the CON group, respectively. In addition, EA upgraded the abundance of *Lactobacillus* and *Clostridiales* (Figure. 2D&E). These results indicated that EA has antibiotic-like effects (reduces opportunistic pathogens), but does not indiscriminately attack other populations.

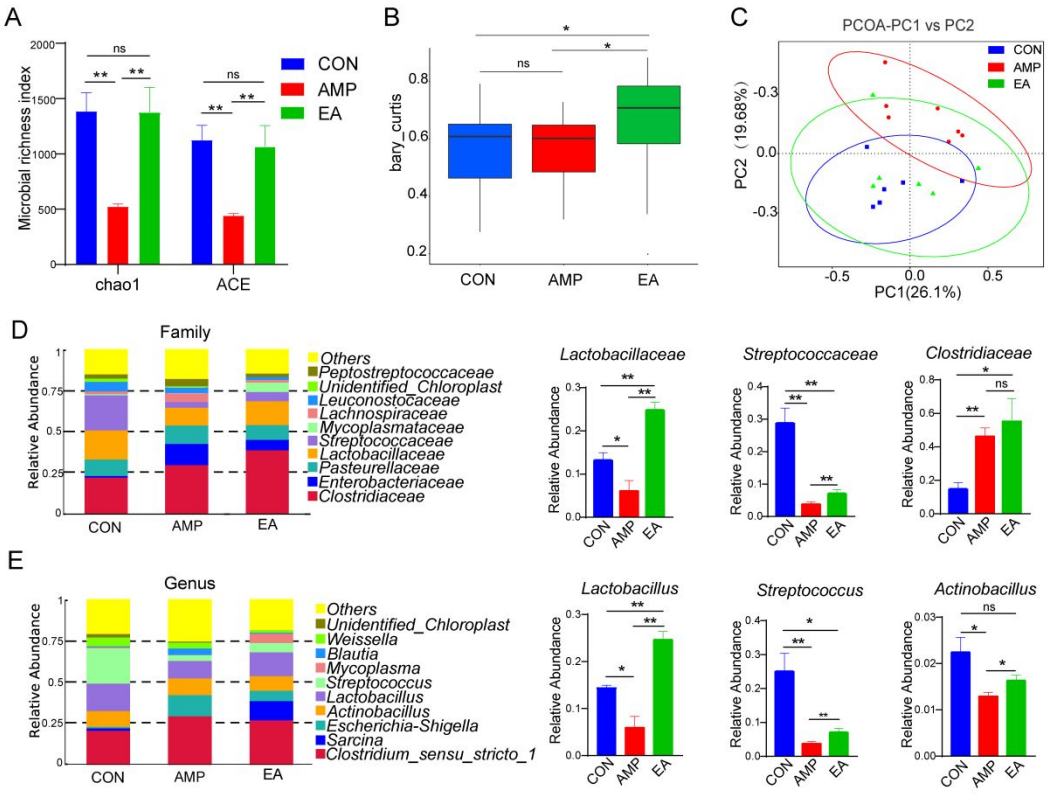

Figure 2. EA selectively inhibited gut microbes. (**A**) Microbial alpha diversity is based on the entire OTU table (as analyzed by chao1 and ACE index); the distribution and density of samples are displayed in a bar graph. *P*-values are from the Wilcoxon rank-sum test; (**B**) Beta diversity index of the ileum microbiota of weaned pig. Box plots show means and quartiles; (**C**) Principal Coordinate Analysis (PCoA) plot of Beta diversity. Relative abundance of top 10 (**D**) families, and (**E**) genera in the CON, AMP, and EA groups. **P* < 0.05, ***P* < 0.01.

3.3 EA interrupted lymphocyte-related gene expression.

After establishing the positive effects of EA on gut microbes, we tried to gain mechanistic insights into the role of EA in response to intestinal damage. Here, we studied differentially expressed *mRNAs* in the colon tissues of each group. Volcanic maps of transcriptome sequencing data show the distribution of differentially

expressed genes (DEGs) between the two groups. There were significant differences in gene expression patterns between EA treatment and no-treatment, among which 2243 genes, including 1267 up-regulated genes and 976 down-regulated genes (Figure. 3A). To further understand the potential biological functions of differential gene expression and the possible pathways involved, we annotated the functions of DEGs by Gene Ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment. Functional annotations based on GO and KEGG indicated that most differentially expressed genes were involved in immune responses. The first 20 DEGs in the KEGG pathway were down-regulated (Figure. 3B&S1B, number of DEGs > 2). EA treatment down-regulated T cell receptor signaling pathway, B cell receptor signaling pathway, Toll-like receptor signaling pathway, and NOD-like receptor signaling pathway (Figure. 3B). In addition, the IgA - producing intestinal immune network is the same pathway after EA and AMP treatment (Figure. 3B&S1B). Figures 3C and S1C showed the down-regulated DEGs (number of DEGs > 2) enrichment of 10 top GO. The results showed that EA treatment was immune-related biological processes associated with DEGs down-regulation (Figure. 3C), suggesting that the protective effects of EA on intestinal stress are associated with immune-related gene expression reprogramming and may achieve intestinal stress resistance by perturbing intestinal immune cells.

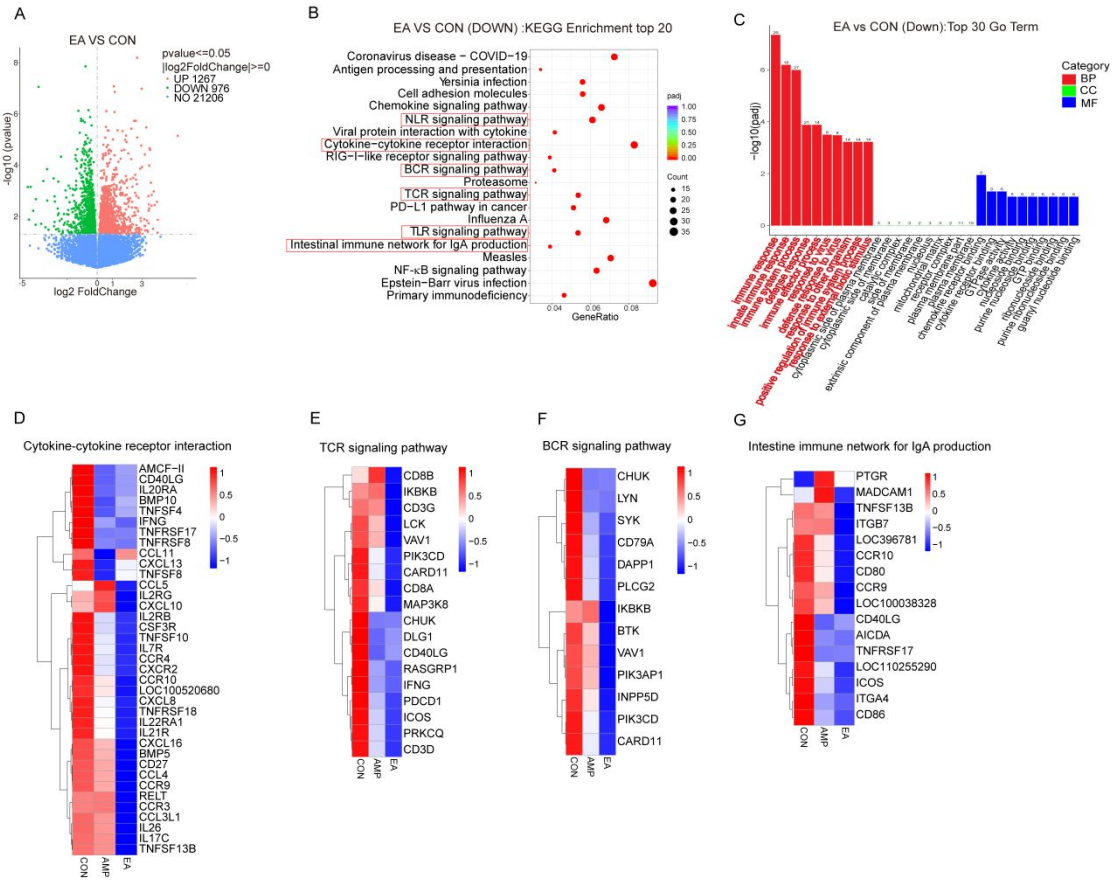

Figure 3. EA interrupted lymphocyte-related gene expression. (**A**) The differentially expressed genes in different treatment groups were represented by a volcano plot using a cut-off value of $P \leq 0.05$ and fold change ≥ 0. Red dots represent up-regulated genes, green dots represent down-regulated ones, and blue dots represent not significantly regulated genes. (**B**) EA Group has downgraded the deg KEGG notes for the top 20. The X-axis represents the KEGG enrichment fraction; Y-axis represents pathway items. The circle's color indicates the *P*-value and the size indicates the number of DEGs. The process is redder and more prominent, meaning that the enrichment degree of this pathway is higher. (**C**) EA group downgrades the GO annotation of the first 30 DEGs. The categories represent biological processes, cellular components, and molecular functions. Heatmap of DEGs enriched in cytokine-cytokine receptor interaction signaling pathway (**D**), T cell receptor

signaling pathway (**E**), B cell receptor signaling pathway (**F**), and IgA-producing intestinal immune network-related KEGG pathways (**G**). Red indicates increased expression and blue indicates decreased expression.

3.4 EA-shaped intestinal epithelial lymphocytes.

The location frequency of immune cells may reflect the stress state of the gut. We sought to investigate the role of EA on the distribution of immune cells in the core. We measured the frequency of $CD79a^+$ and $CD11b^+$ positive cells and the distribution frequency of $CD3^+$ cells and various segments of the crypt-villus axis and found that EA treatment attenuated PQ - induced $CD79a^+$ B cell infiltration, despite little effects on $CD11b^+$ leukocytes, while AMP treatment reduced $CD11b^+$ leukocytes infiltration and had a minor influence on $CD79a^+$ B cells (Figure. 4A-D). The distribution changes of $CD3^+$ cells in villi axis of ileum crypt showed that the percent of T cells in EA treatment mostly clustered at the bottom of villi, AMP treatment mostly clustered at the bottom and middle of villi, and only in CON group mostly clustered at the top of villi (Figurer. 4E&F). The above data suggest that EA and AMP can reduce the recruitment and migration of ileal immune cells in stress-exposed piglets.

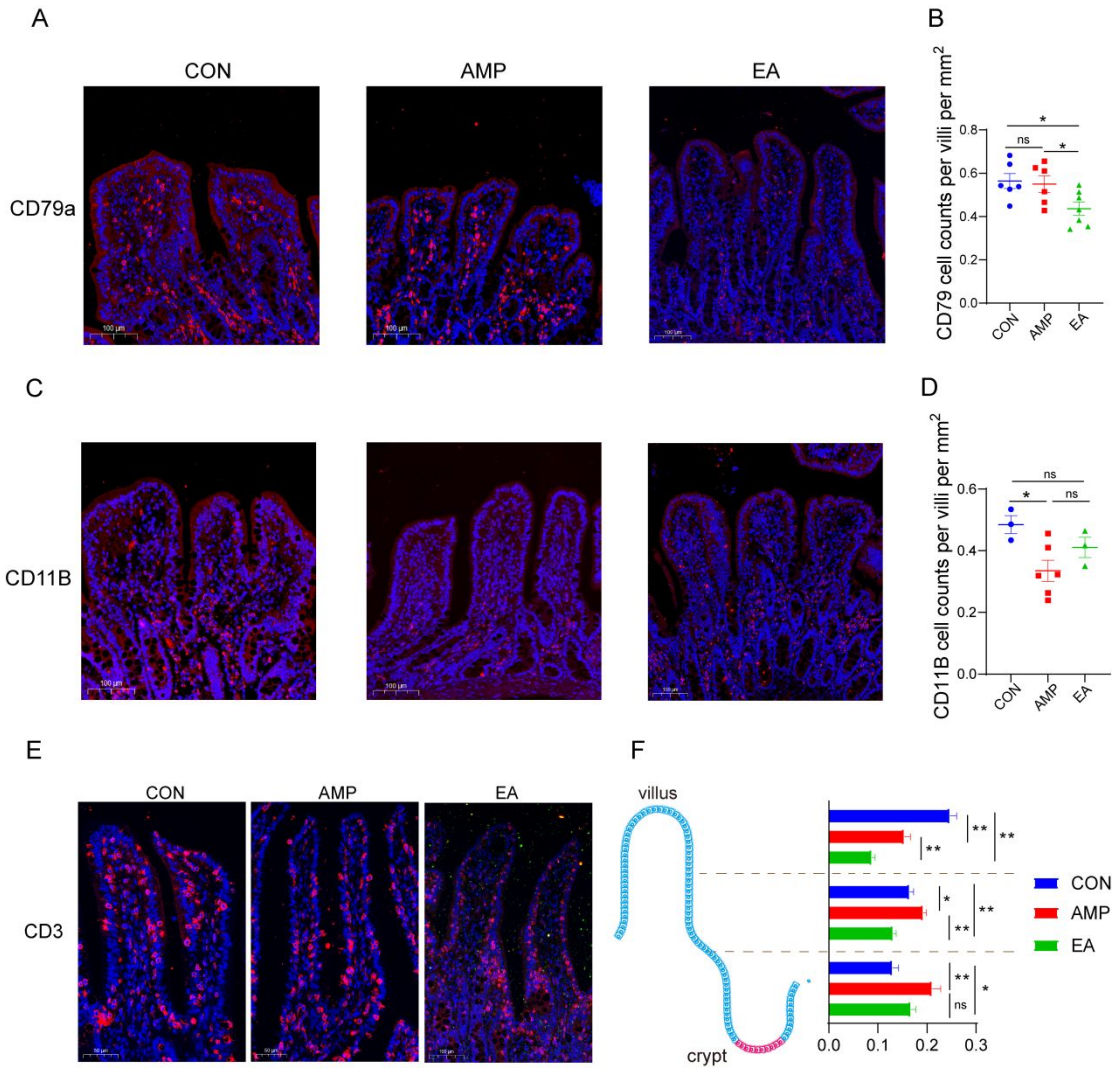

Figure 4. EA shaped gut immune cells. (**A, C**) A representative image of CD79a B cell and $CD11b^+$ macrophages in intestinal infiltration. $CD79a^+$ B cells and $CD11b^+$ macrophages are shown in blue red and intestinal epithelial cell nuclei. (**B, D**) Intestinal frequencies of $CD79a^+$ B cells and $CD11b^+$ macrophages. (**E**) A representative image of $CD3^+$ T cell migration in the intestinal tract. $CD3^+$ T cells are shown in Red and intestinal epithelial cell nuclei in blue. (**F**) The distribution frequency of $CD3^+$ T cells along each ileum crypt and villus axis segment in different treatment groups. All data are expressed as the mean ± SEM (n = 8). Comparisons were performed by t-test or analysis of variance (ANOVA). All data are expressed as

the mean ± SEM (n = 8). Comparisons were performed by t-test or ANOVA. *P < 0.05, **P < 0.01.

3.5 Environmental factor correlation analysis

Based on transcriptome and 16S analysis, we had identified that *Streptococcus* in both EA and AMP groups was inhibited, in which intestinal stress was relieved. There were four main pathways by that EA alleviates intestinal stress, Cytokine-Cytokine receptor interaction signaling Pathway, IgA-produces intestinal immune network, T Cell receptor signaling Pathway, and B cell receptor signaling Pathway. Spearman correlation analysis was performed to clarify the relationship between the significant differential genes and the level of intestinal bacteria treated by EA. We discovered the interactions between essential genes in these pathways and pathogens (such as *Streptococcus*) (Figure. 5). The expression of these chemokines and cytokines was enhanced in the intestinal tract of stressed piglets, which could be prevented by pre-treatment with EA. The resulting heatmap shows that *Streptococcus* was a common bacterium positively correlated with the differential genes. In addition, multiple genes were negatively correlated with *Bifidobacterium*. Therefore, our data suggest that downregulation of PQ - induced intestinal stress by EA triggers intestinal epithelial sensory signals, mobilizes B lymphocytes, increases IgA production, and suppresses *Streptococcus* abundance, thereby reducing microbial-mediated subsequent intestinal immune responses.

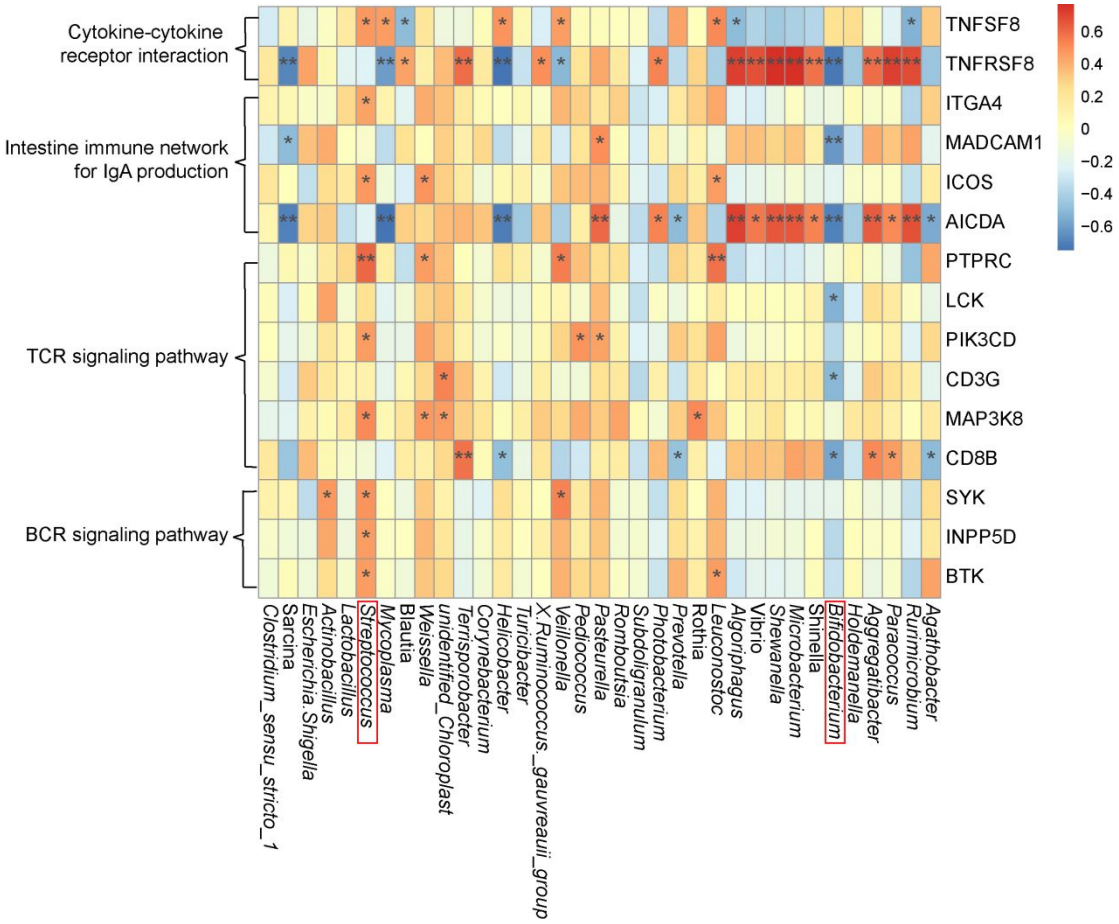

Figure 5. The intestinal inflammation induced by stress exposure in piglets is related to the intestinal flora - Spearman correlation heat map of bacterial genera and environmental factors. The color range from blue to red is a negative to a positive correlation. Significant correlations are expressed by $*P < 0.05$ and $**P < 0.01$.

4 Discussion

WHO predicted that drug-resistant diseases could cause 10 million deaths each year by 2050, and antimicrobial resistance could force up to 24 million people into extreme poverty in the next ten years if no actions are taken[23]. Antibiotic misuse also challenges the ecological balance, which proved that antibiotic resistance genes could be collected in places including sediments, groundwater, and drinking water[22]. In addition, the indiscriminate use of antibiotics in animal production plays an

expressive role in the antibiotic resistance crisis, and brings unpredictable dietary safety risks[24,25]. However, despite all the above disadvantages of antibiotics, it is inevitable to apply antibiotics in the treatment of disease since they have become the cornerstone of modern medicine, which is used to combat infections and save millions of lives every year in the world[26]. Hence, considering the importance of antibiotics and the prevalence of drug-resistant bacteria, it is of great significance to find safer and environmentally-friendly alternatives to antibiotics. We found here that EA reduced *Streptococcus* abundance, modulated intestinal lymphocyte distribution, and prevented PQ-induced stress in the gut and subsequent adverse immune reactions in the gut of piglets. Hence, EA can be used as a safer natural plant antibacterial ingredient and developed as an alternative to antibiotic. However, the Spearman Analysis revealed the gut microbiota showed a strong correlation with lymphocytes, in which *Streptococcus* and *Bifidobacterium* positively and negatively correlate with intestinal lymphocyte receptors, respectively. We thus hypothesize that EA supplementation maintains the homeostasis of intestinal lymphocytes. Regrettably, since EA was not intervened on germ-free animal, we could not tell whether EA was directly utilized by intestinal epithelial lymphocytes and subsequently responded. In addition, the crosstalk between microbiota and lymphocytes needs to be further studied.

The rise of opportunistic pathogens or colonization of pathogenic bacteria contributes to enteritis development under stress. It has been confirmed PQ could weaken gut immunity, and increase susceptibility to Gram-negative bacteria[27].

Streptococcus and *Actinobacillus* are the most invasive groups of bacteria, dominating in the intestinal tract under PQ stress and triggering inflammatory responses by changing the microbial community[28]. Link to the fact that *Streptococcus* induced macrophages to release pro-inflammatory cytokines and chemokines, such as *TNF-α* and *IL-6*, worsened enteritis, and reversed intestinal damage by treating broad-spectrum antibiotics AMP, demonstrating that microbes play a decisive role in PQ - induced enteritis. EA exhibited the same positive effects as AMP, inhibited the expansion of pathogenic bacteria ($P < 0.05$), and significantly attenuated markers associated with intestinal inflammation. Meanwhile, EA preserved microbial diversity, as the demonstrated in previous studies, EA promoted the growth of beneficial gut bacteria such as *Lactobacillus* and inhibited the growth of pathogens such as enteropathogenic *Escherichiacoli* and *Pseudomonas aeruginosa*[29, 30]. Combine with our study, EA suppressed the abundance of *Streptococcus*, reduced infiltration of inflammatory cells, and attenuated PQ - induced intestinal stress. All above proved that EA has the potential to replace antibiotics as a more environmentally friendly and healthier plant extract.

Cytokine - Cytokine pathway mediates immune cell recruitment and intracellular signaling mechanisms, indicating the development of inflammation[31]. The gene ontology enrichment function analysis results showed that the biological processes involved in immunity response in a decreasing trend when EA pretreatment. In addition, the KEGG results also verified the downregulation of T cell and B cell receptor signaling pathways by EA. We speculate that changing the number and

distribution of intestinal epithelial lymphocytes is the critical role of EA in the mechanism of intestinal epithelial cells, which are against luminal pathogens since the distribution of immune cells. It directly reflects the immune status of the gut, and the migration of lymphocytes also vividly shows the process of fighting pathogens[32]. EA alleviated the increased number of intestinal B cells and the marked translocation of T cells (migration from the tip to the tip of the villi) caused by stressor exposure. The gut microbiota participates in the production of intestinal IgA[33], which explained the present study by the same efficacy of EA as AMP after stress exposure, which ascribe to the mobilizing of IgA-producing immune response on the intestinal epithelium. Additionally, EA also alleviated intestinal inflammation by regulating lymphocyte number and migration. These results all support that EA has to promote effect as antibiotics and has the opportunity to become a safe food supplement to improve the intestinal defense.

IgA - produces intestinal immune network pointed *Streptococcus* as a pathogen, and Toll-like receptors (TLRs) and Nod-like receptors (NLRs) act as sensors of *Streptococcus* infection to activate innate immune responses when *Streptococcus* breaks through microbial and chemical barriers and reaches intestinal epithelial cells[34], TLRs signal transduction leads to the expression of inflammatory factors and the mobilization of chemokines in the intestinal epithelium and carries out antibacterial responses by producing various antimicrobial peptides and pro-inflammatory cytokines[35]. Macrophages located in the subepithelial region specialize in phagocytosis of pathogenic microorganisms[36]. These responses clear the infection

through an innate immune response that directly kills or inhibits the pathogen's replication. Intestinal dendritic cells collect bacteria in mucus and migrate to lymph nodes, recruiting T or B lymphocytes for adaptive immune defense[37]. We observed in the Environmental factor correlation analysis that *Streptococcus* was associated with the above immune processes, suggesting EA enhances intestinal resistibility and against intestinal stress, possibly via intervening in the interaction between *Streptococcus* and epithelial immune cells.

5 Conclusion

Pre-treatment of EA prevented PQ - induced intestinal stress by modulating gut microbes and epithelial immune responses. Our finding indicated that EA has important implications for preventing intestinal damage and regulating microbiota after intestinal stress. It may provide new insights into the prevention of IBD and other diseases by acting *Streptococcus*-targeted alternative anti-drugs insights.

Author contributions

Miaomiao Wu: Conceptualization, Supervision, Review, and editing of the manuscript. Huimin Jin and Siyan Che: Investigation, Data curation, Writing-Original draft preparation. Kunfu Wu: Methodology, Investigation.

Declaration of competing interest

The authors declare that they have no financial or business conflicts of interest.

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