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Azithromycin pretreatment exacerbates atopic dermatitis in trimellitic anhydride-induced model mice accompanied by correlated changes in the gut microbiota and serum cytokines

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ABSTRACT

Atopic dermatitis (AD) is a common inflammatory skin disease. This study aims to investigate the effect of azithromycin (AZI) pretreatment, a common macrolide-type antibiotic, on the trimellitic anhydride (TMA) induced AD-like symptoms in mice. AZI (25 mg/kg, once daily, 5 days) was administered intragastrically before the 10-day TMA challenge. AD-like symptoms were assessed by ear thickening, scratching behavior, and pathological or immunofluorescence staining; Cytokines in the skin tissue and serum were measured by cytometric bead array; and the compositions of gut microbiota were assessed by 16S rRNA gene sequencing. AZI pretreatment accelerated the development of ear thickening and enhanced the severity of developed AD-like symptoms. AZI pretreatment promoted the infiltrations of neutrophil-like cells, T cells, and mast cells in ear skin. AZI pretreatment elevated the levels of IL-4, IL-6, and IL-17A in the ear skin of AD model mice, but it increased serum TNF- α and IL-6. AZI-pretreatment increased four gut bacterial genera (*Bacteroides*, *Candidatus_Saccharibacteria_unclassified*, *Acetatifactor*, *Firmicutes_unclassified*) but depleted three short-chain fatty acids producing gut bacterial genera (*Alistipes*, *Clostridiales_unclassified*, *Butyricicoccus*). AD-associated symptoms were positively associated with skin IL-4 and IL-17A, serum TNF- α , and IL-6, and Acetatifactor, but they negatively correlated to the three decreased gut bacterial genera (*Alistipes*, *Clostridiales_unclassified*, *Butyricicoccus*). Thus, our results demonstrate that AZI exposure deteriorates TMA-induced AD-like symptoms in mice, which is related to the imbalances of gut microbiota and skin/serum cytokines.

1. Introduction

Atopic dermatitis (AD) is a common, often persistent inflammatory skin disorder, which affects up to about 20% of children [1]. AD also affects about 3% of adults worldwide [2] and up to 10% of adults in high-income countries [3]. AD is characterized by fluctuating intensely pruritic skin lesions, which are associated with low quality of life and

high health care costs [4]. Moreover, AD also increases the risk for the development of allergic rhinitis, food allergies, and asthma, which is well-known as an “atopic march” [5].

The gut microbiota is involved in the regulation of immunity and may play a role in the development of AD. In addition to the therapeutic effects of synbiotics or probiotics in AD [6], increasing researchers have been of great interest in the effect of antibiotic exposure in AD and

Abbreviations: AZI, Azithromycin; AD, Atopic dermatitis; ANOVA, Analysis of variance; CBA, Cytometric bead array; HE, Hematoxylin-Eosin; IFN- γ , Interferon gamma; IL-17A, Interleukin 17A; IL-2, Interleukin 2; IL-6, Interleukin 6; IL-10, Interleukin 10; OTUs, Operational taxonomical units; PCoA, Principal coordinate analyses; Pre-AZI, Azithromycin pretreatment; Th1, T helper type 1; Th2, T helper type 2; Th17, T helper type 17; TMA, Trimellitic anhydride; TNF- α , Tumor necrosis factor alpha; SCFAs, Short-chain fatty acids; VEH, Vehicle.

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related atopic diseases. Azithromycin (AZI) is a broad-spectrum antibiotic that belongs to the macrolide antibacterial class, which may induce a rapid reduction of gut microbial richness and diversity [7]. Some case reports show AZI exposure may be related to allergic contact dermatitis [8–10]. Besides, it has been reported that AZI exposure is associated with an increased risk of asthma in patients with AD [11]. On the other hand, a recent study from Park et al. indicates altered gut microbiota by AZI attenuates airway inflammation in allergic asthma [12]. Thus, the risk of AZI exposure in AD remains unclear. Here, we investigated the effect of AZI pretreatment on the development of AD in the well-known trimellitic anhydride (TMA)-induced AD mouse model [13].

2. Methods

2.1. Animals and grouping

Male BALB/c mice (6 weeks old, 18–22 g body weight) were purchased from the Laboratory Animal Research Center of Zhejiang Chinese Medical University. Mice were housed in groups of 3–5 per cage at $23 \pm 2^\circ\text{C}$ and 40–60% humidity with a 12-h light–dark cycle (lights on from 8:00 to 20:00). Water and food were given *ad libitum*. This study was approved by the Institutional Animal Care and Use Committee of Zhejiang Chinese Medical University (#ZSLL-2016–83). The use and care of the mice were following the guidelines of the Animal Advisory Committee of Zhejiang Chinese Medical University and the US National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

To test the effect of AZI pretreatment on the development of AD-like symptoms, BALB/c mice were divided into two groups: the AZI pretreatment group (Pre-AZI group, AZI at 25 mg/kg were given by intragastric administration for 5 days before TMA challenge, $n = 7$), and vehicle group (VEH group, pretreatment with double distilled water instead of AZI, $n = 7$).

2.2. Chemicals and drugs

All chemicals including TMA (98 %, LOT: R28A7Y13845), isopropyl myristate (LOT: Y12M7S14582) were purchased from Shanghai Yuanye Biotechnology Co., Ltd., (Shanghai, China). Acetone was purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). TMA was dissolved in a mixed solvent of acetone and isopropyl myristate (4:1, v/v) immediately before use. Azithromycin for oral suspension (Pfizer Inc. LOT: R97426) was purchased from Zhejiang University School of Medicine Children's Hospital and suspended in double distilled water immediately before use.

2.3. Induction of AD-like symptoms by TMA

The method for induction of AD-Like symptoms by TMA is similar to the previous study [14]. Briefly, mice were sensitized with 50 μL of 5% TMA by topical application on the shaved abdominal skin. Five days later, AD-like symptoms were induced by repeatedly applying 20 μL of 2% TMA to the dorsal surface of both ears once a day for 10 days. Ear thickness was daily measured before the TMA challenge. All mice were sacrificed after the 10-day TMA challenge.

2.4. Observation of scratching behavior

Scratching behavior was observed after the last TMA challenge as previous study [15]. Mice were individually placed in a big glass beaker (3 L) to acclimate for 60 min. Immediately after the TMA challenge, each mouse was recorded by a digital video camera for 30 min in an unwatched condition. The number of scratching episodes for both ears was counted manually according to the video recordings in a blinded fashion.

2.5. Hematoxylin-Eosin (HE) staining

After fixed and embedded in paraffin, mouse skin was cut into 5- μm sections and mounted on positively treated slides. The slides were stained with hematoxylin and eosin for evaluation of edema and infiltration of inflammatory cells into the dermis. The severity of dermatitis was assessed on the epidermis (hypertrophy, hyperkeratosis, and infiltration by inflammatory cells) and the corium (infiltration by inflammatory cells) in a blinded fashion. Each index was scored from 0 (no symptoms), 1 (mild), 2 (moderate), to 3 (severe). The total histopathological score was expressed as the sum of the individual scores. Neutrophil-like cells were manually identified from HE staining images by their separated nuclear lobes. The number of Neutrophil-like cells was counted from five fields of view with the areas of 0.01 mm^2 for each mouse.

2.6. Toluidine blue staining

The 5- μm sections sampled from the mouse skin mounted on slides as described above. the slides were soaked in methanol for 5 min, PBS 5 min, 0.01% toluidine blue (PH = 2.5, Haokebio, Hanzhou, China) for 30 min, and ethanol for 30 s, dipped in xylene, and then coverslipped. Toluidine blue stains mast cells violet and the background blue. The number of mast cells was counted from ten fields of view with the areas of 0.03 mm^2 for each mouse.

2.7. Immunofluorescence staining

The 5- μm sections processed for immunofluorescence with CD3 epsilon (1:200, Abcam; #ab215212) and AlexaFluor488 secondary secondary fluorescent antibodies (1:200, Abcam; #ab150077). The cell nuclei were counterstained with DAPI (Meilunbio; #MA0222-L). Images of the slides were taken using an Olympus VS120-S6-W microscope. The number of CD3⁺ cells was counted from five fields of view with the areas of 0.01 mm^2 for each mouse.

2.8. Cytokine analysis

The protein levels of multiple cytokines (related to T helper type 1 (Th1), T helper type 2 (Th2), and T helper type 17 (Th17) cells) were examined by the cytometric bead array (CBA) assay (mouse Th1/Th2/Th17 cytokine kit, BD Pharmingen) according to the manufacturer's protocols. Briefly, a series of capture beads for interleukin 17A (IL-17A), interleukin 2 (IL-2), Interferon gamma (IFN- γ), interleukin 6 (IL-6), tumor necrosis factor alpha (TNF- α), and interleukin 10 (IL-10) were firstly mixed. Then co-culture the capture beads mixture, detection reagent, and 50 μL serum or tissue lysate for 2 h at room temperature, protected from light. Add 1 mL of wash buffer to each assay tube and centrifuge at 200 g for 5 min. Carefully aspirate and discard the supernatant from each assay tube. Add 300 μL of wash buffer to each assay tube to resuspend the bead pellet for analysis using a FACSCanto II system (BD Biosciences). Data were analyzed with FCAP Array software.

2.9. Microbiome analysis

Gut microbiota was collected and analyzed similarly to previous studies [16–18]. Briefly, mice were placed in an empty clean big glass beaker (3 L) for 5–10 min, and freshly dropped fecal pellets were collected aseptically. Total DNA was extracted from approximately 200 mg of feces using the QIAmp DNA microbiome kit (Qiagen, German) according to the manufacturer's protocol. Gut microbiota abundance and diversity were analyzed by sequencing of 16S rRNA gene (V3-V4 region) with Illumina MiSeq platform (MiSeq Reagents Kit v3, 600 cycles). Using QIIME 2 (<https://qiime2.org/>) [19], the clean reads were extracted from the raw-paired end reads, aligned with the SILVA database [20] and NCBI databases [21], and clustered into operational

taxonomical units (OTUs) based on a 97% similarity cutoff. Alpha diversity included the Sobs and Chao1 diversity index, and beta diversity included unweighted UniFrac distance metrics. Beta diversity metrics were then visualized using Principal coordinate analyses (PCoA) in R version 3.4.3. The sequencing reaction was conducted by Hangzhou Woosen Biotechnology Co., Ltd.

2.10. Statistics

GraphPad Prism software 8.0 was used for statistical analyses. Repeated two-way analysis of variance (ANOVA) (with Least Significant Difference Test for post hoc comparison) was used for the analysis of ear thickness data. One-way ANOVA (with Tukey test for post hoc comparison) was used for the analysis of three-group data from pathological or immunofluorescence staining. Kruskal-Wallis analysis of variance (ANOVA) (with Dunn's multiple comparisons test for post hoc comparison) was used for the analysis of the relative abundance of gut bacterial genera between groups. Spearman's rank correlation was used for correlation analyses. Student's *t*-test or Mann Whitney test was used for two-group data. All data were expressed as mean $\pm$ SEM; significance was defined as $P < 0.05$. All behavioral tests and cell countings were conducted in a blinded manner. The detailed statistical information was shown in **Supplementary Table 1**.

3. Results

3.1. AZI pretreatment aggravate AD-like symptoms in TMA-induced AD mice

Repeated application of TMA to mouse ear skins induced AD-like skin lesions with an increase in ear thickness and scratching behavior. Here we found mouse-ear thickness increased about 3-fold by TMA treatment after the 10-day challenge, and AZI pretreatment significantly promoted the increase in ear thickness to about 4-fold ($P < 0.05$, **Fig. 1A and B**). The frequency of scratching behavior in the pretreatment of the AZI group was approximately 65 times per 30 min, which was approximately twofold higher than that in the vehicle group ($P < 0.05$, **Fig. 1C**). Representative pictures of the ear were shown in **Fig. 1D**.

HE staining is one of the principal tissue stains, which provides a comprehensive picture of the microanatomy of tissues. HE staining of ear tissues further showed that AZI pretreatment increased the ear thickness ($P < 0.01$, **Fig. 2A-C**) and slightly increased the histopathological score ($P = 0.055$, **Fig. 2D**). As neutrophilic inflammation is a feature of lesional AD pathology [22], which can be identified from HE staining images by their separated nuclear lobes. A representative neutrophil-like cell was shown in the inset of **Fig. 2E**. The number of neutrophil-like cells in both Pre-AZI and Veh groups was higher than that in the normal healthy group ($P < 0.01$ for Veh group, $P < 0.001$ for Pre-AZI group, **Fig. 2E**). Moreover, the number of neutrophil-like cells in

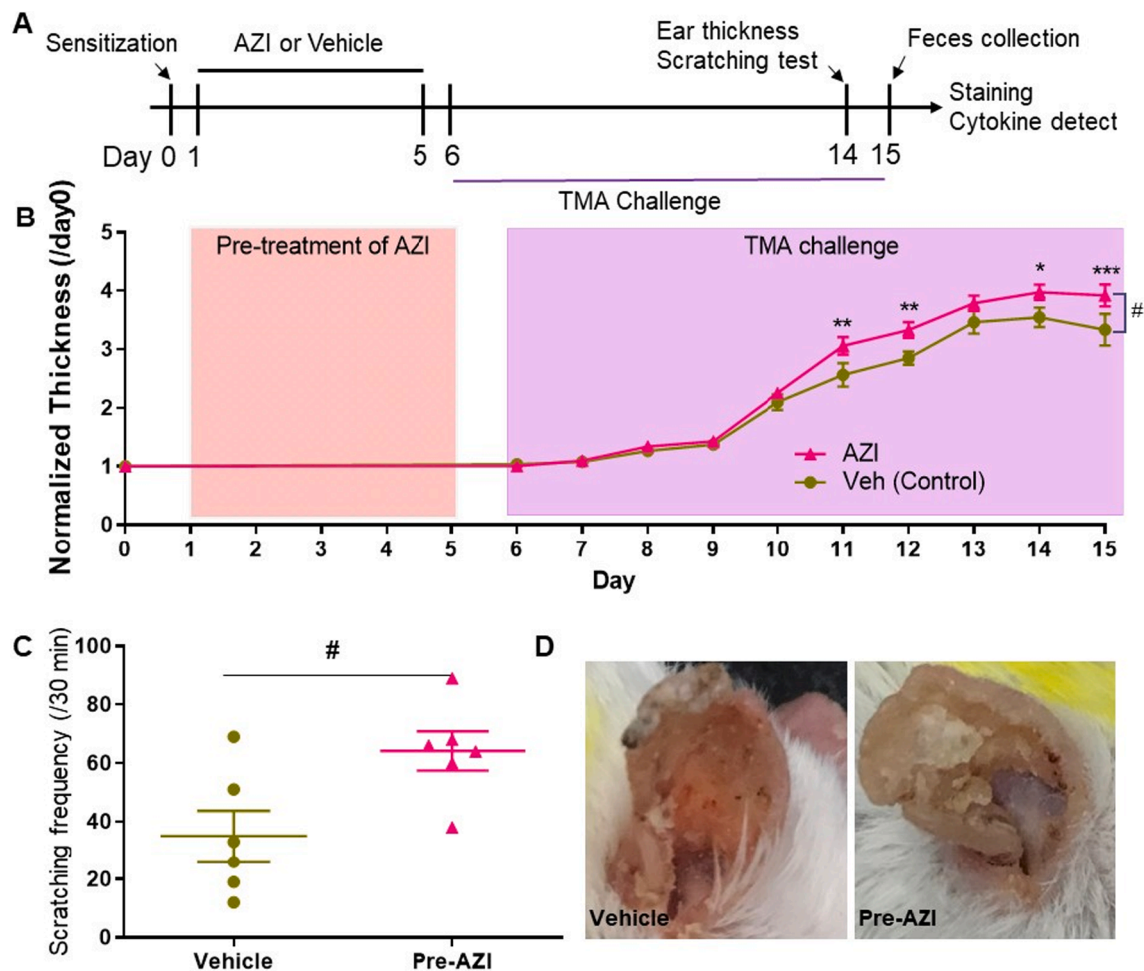


Fig. 1. AZI pretreatment aggravated AD-like symptoms in TMA-induced AD mice. (A) Schematic diagram of experimental design. (B) Normalized ear thickness (N = 7). (C) Scratching frequency (N = 6). (D) Representative pictures of the ear. * $P < 0.05$, ** $P < 0.01$ compared with vehicle for each time points. # $P < 0.05$ compared with vehicle. Veh: pre-treated with water; Pre-AZI: pre-treated with Azithromycin. Repeated two-way analysis of variance (ANOVA) (with Least Significant Difference Test for post hoc comparison) was used for B; Student's *t*-test was used for C.

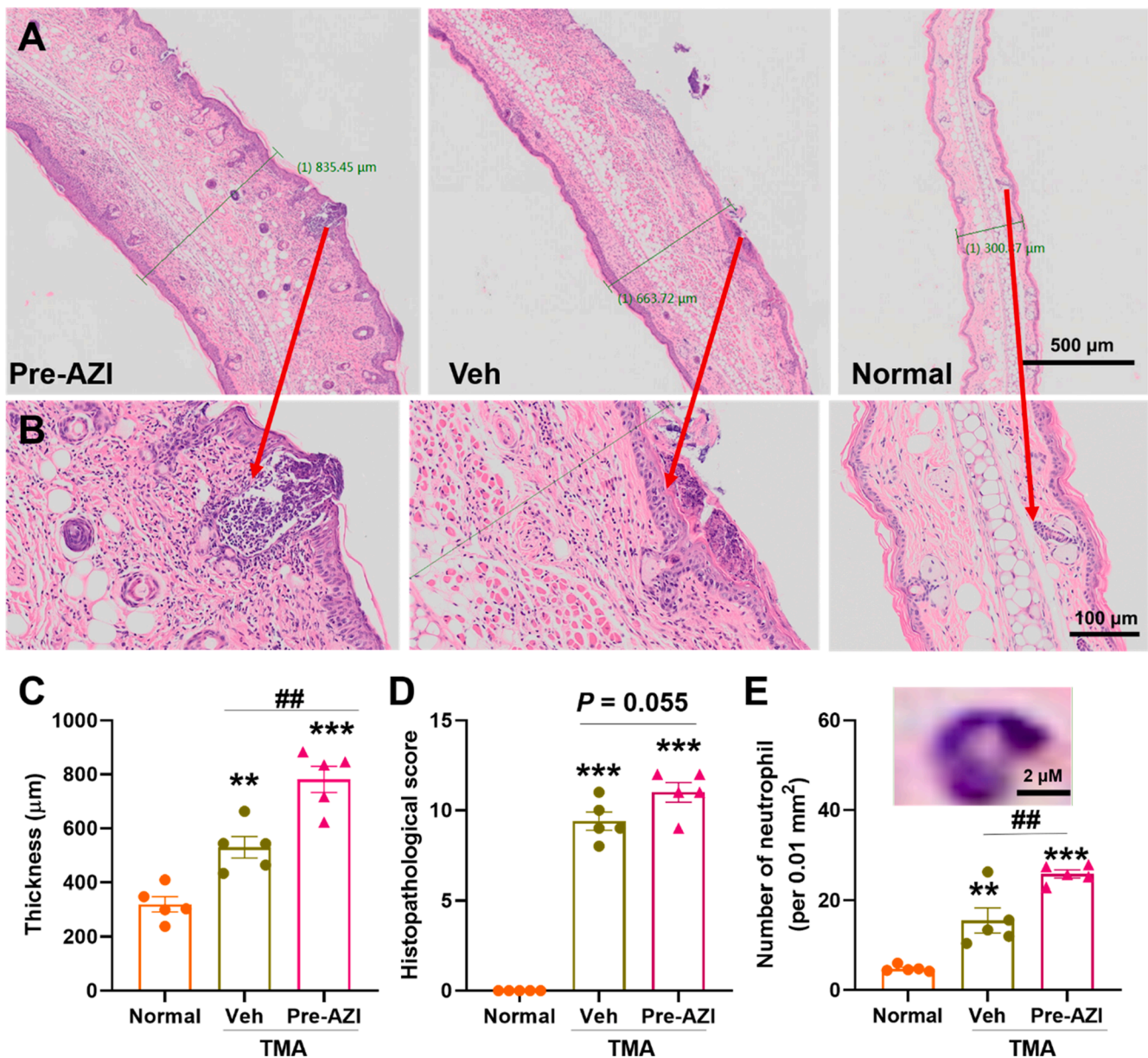


Fig. 2. HE staining of ear sections. (A) HE staining images; (B) enlarged pictures of A. (C) the thickness of the ears. (D) the histopathological score. (E) the number of neutrophil-like cells. *** $P < 0.001$ compared with healthy normal mice; # $P < 0.05$ compared with vehicle-treated AD model mice. Veh: pretreated with water; Pre-AZI: pretreated with Azithromycin; Normal: BALB/c mice without any treatments, including the TMA. $N = 5$ for each group. One-way analysis of variance (ANOVA) (with Tukey test for post hoc comparison) was used for C-E.

the Pre-AZI group was higher than that in the Veh group ($P < 0.01$, Fig. 2E). The Representative HE staining section of each mouse was proved in Supplementary Fig. 1.

3.2. AZI pretreatment promoted T cell and mast cell infiltration in the ear skin of TMA-induced AD mice

AD is a common form of T-cell-mediated skin disease. As shown in Fig. 3, we found the infiltration of CD3^+ T cells was rare in the ear of normal healthy mice (Fig. 3A), while it was common and severe in the diseased ears in both Veh and Pre-AZI groups ($P < 0.001$ for both compared with the normal group, Fig. 3B-D). The infiltration of CD3^+ T cells in the Pre-AZI group was much severer than that in the Veh group, which is evidenced by a higher number of CD3^+ T cells in the Pre-AZI group than that in the Veh group ($P < 0.01$, Fig. 3D). The representative immunofluorescence staining section of each mouse was proved in

Supplementary Fig. 2.

Mast cells are also believed to be involved in the pathogenesis of AD [23]. As shown in Fig. 4, we found the number of mast cells was low in the ears of normal healthy mice (Fig. 4A), while it was increased in the diseased ears of both Veh and Pre-AZI group ($P < 0.01$ for Veh group, $P < 0.001$ for Pre-AZI group, Fig. 4B-D). The infiltration of mast cells in the Pre-AZI group was much severer than that in the Veh group, which is evidenced by a higher total number of mast cells in the Pre-AZI group than these in the Veh group ($P < 0.05$, Fig. 4D) Representative immunofluorescence staining section of each mouse were proved in Supplementary Fig. 3.

3.3. AZI pretreatment exacerbated cytokine imbalances in the ear skin and serum

The cytokine balance in the skin is considered important in AD [24].

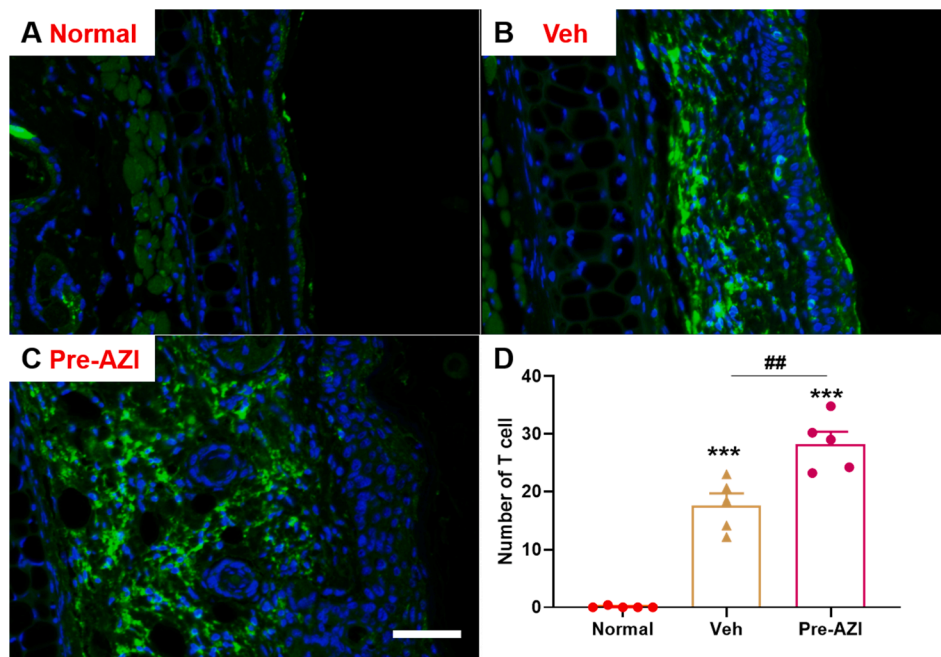


Fig. 3. CD3 immunofluorescence staining of ear sections. (A) Normal group; (B) Vehicle group. (C) Pre-AZI group. (D) the total number of mast cells in 10 fields of view. *** $P < 0.001$ compared with healthy normal mice; ## $P < 0.01$ compared with vehicle-treated AD model mice. Veh: pretreated with water; Pre-AZI: pretreated with Azithromycin; Normal: BALB/c mice without any treatments, including the TMA. $N = 5$ for each group. The scale bar means 50 μm . One-way analysis of variance (ANOVA) (with Tukey test for post hoc comparison) was used for D.

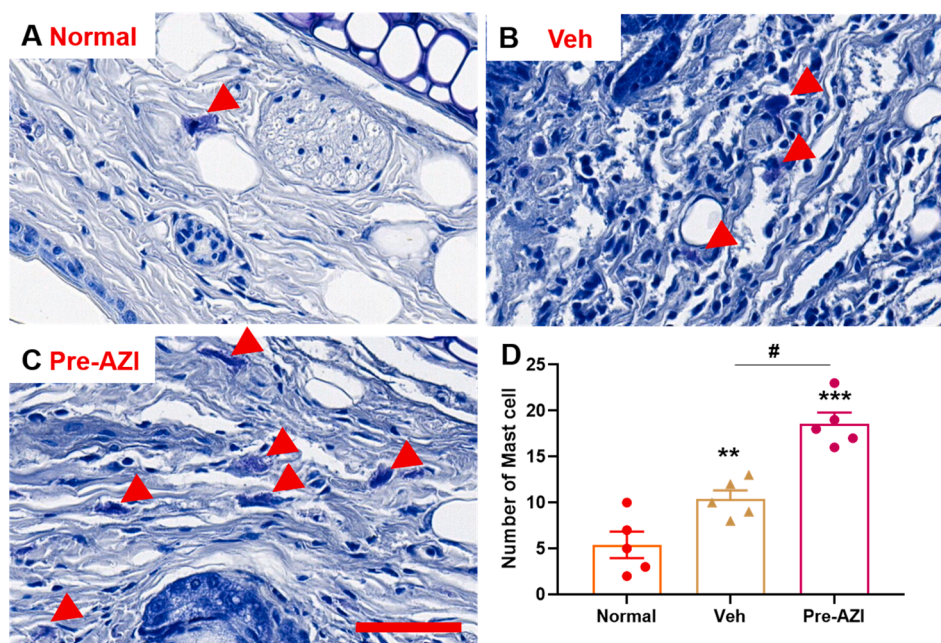


Fig. 4. Toluidine blue staining for ear sections. (A) Normal group; (B) Vehicle group. (C) Pre-AZI group. (D) the total number of mast cells in 10 fields of view. * $P < 0.05$, *** $P < 0.001$ compared with healthy normal mice; # $P < 0.01$ compared with vehicle-treated AD model mice. Veh: pretreated with water; Pre-AZI: pretreated with Azithromycin; Normal: BALB/c mice without any treatments, including the TMA. $N = 5$ for each group. The scale bar means 50 μm . One-way analysis of variance (ANOVA) (with Tukey test for post hoc comparison) was used for D.

As shown in Fig. 5, the levels of Th2-related IL-4 ($P < 0.01$, Fig. 5A), Th17-related IL-17A ($P < 0.05$, Fig. 5G), and another proinflammatory cytokine IL-6 ($P < 0.05$, Fig. 5F) were significantly elevated in Pre-AZI group when compared with the Veh group. Of note, the IL-4/IFN- γ in the Pre-AZI group was higher than that in the Veh group ($P < 0.05$, Fig. 5C). However, AZI pretreatment had no significant effect on the levels of IFN- γ ($P > 0.05$, Fig. 5B), IL-2 ($P > 0.05$, Fig. 5D), TNF- α ($P < 0.001$, Fig. 5E), and IL-10 ($P > 0.05$, Fig. 5H).

Specific serum cytokines, such as TNF- α [25], may also contribute to AD. As shown in Fig. 6, AZI pretreatment increased the levels of serum TNF- α ($P < 0.001$, Fig. 6E) and IL-6 ($P < 0.05$, Fig. 6F), but it had no significant effect on the levels of serum IL-4 ($P > 0.05$, Fig. 6A), IFN- γ ($P > 0.05$, Fig. 6B), IL-4/IFN- γ ($P > 0.05$, Fig. 6C), IL-2 ($P > 0.05$, Fig. 6D), IL-17A ($P > 0.05$, Fig. 6G) and IL-10 ($P > 0.05$, Fig. 6H).

3.4. AZI pretreatment induced a perturbation in the gut microbiota in TMA-induced AD mice

To examine whether the AD exacerbating effects of AZI were associated with gut microbiota, the bacterial 16S rRNA V3-V4 region in feces was sequenced. The alpha diversity indices for the Sobs and Chao1 indexes were significantly different between AZI pretreatment and normal groups ($P < 0.01$ for Sobs and $P < 0.01$ for Chao1, Fig. 7A and B). Beta diversity was assessed by PCoA on UniFrac distance matrices. The profiles of the gut microbiota were clearly different among the three groups at family and genus levels (Supplementary Fig. 4). AZI significantly influenced the gut microbiota composition and separated the control and AZI-pretreated model mice by PCoA (Fig. 7C). The AD model also induced separation in the gut microbiota composition of the normal

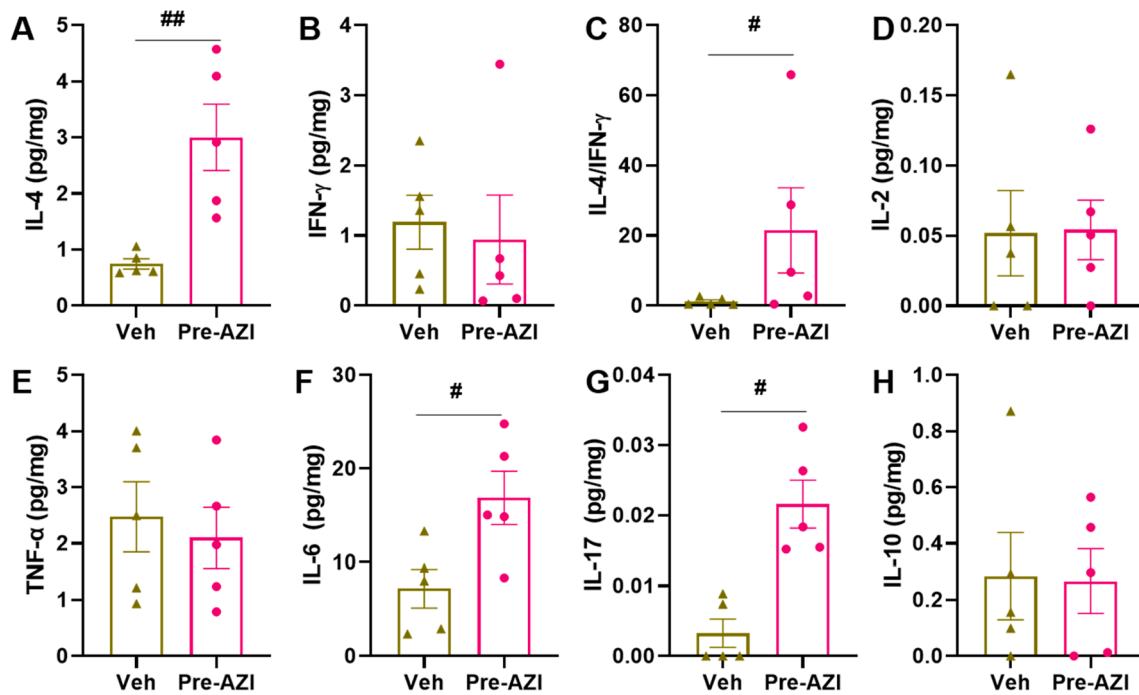


Fig. 5. The effect of AZI pretreatment on the level of ear tissue Th1/Th2/Th17-Related Cytokines in TMA-induced AD mice. (A) the level of IL-4; (B) the level of IFN- γ ; (C) the ratio of IL-4/IFN- γ ; (D) the level of IL-2; (E) the level of TNF- α ; (F) the level of IL-6; (G) the level of IL-17A; (H) the level of IL-10. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with vehicle. Veh: vehicle; Pre-AZI: AZI pretreatment. N = 5 for each group. Mann Whitney test was used for A and C; Student's *t*-test was used for the others.

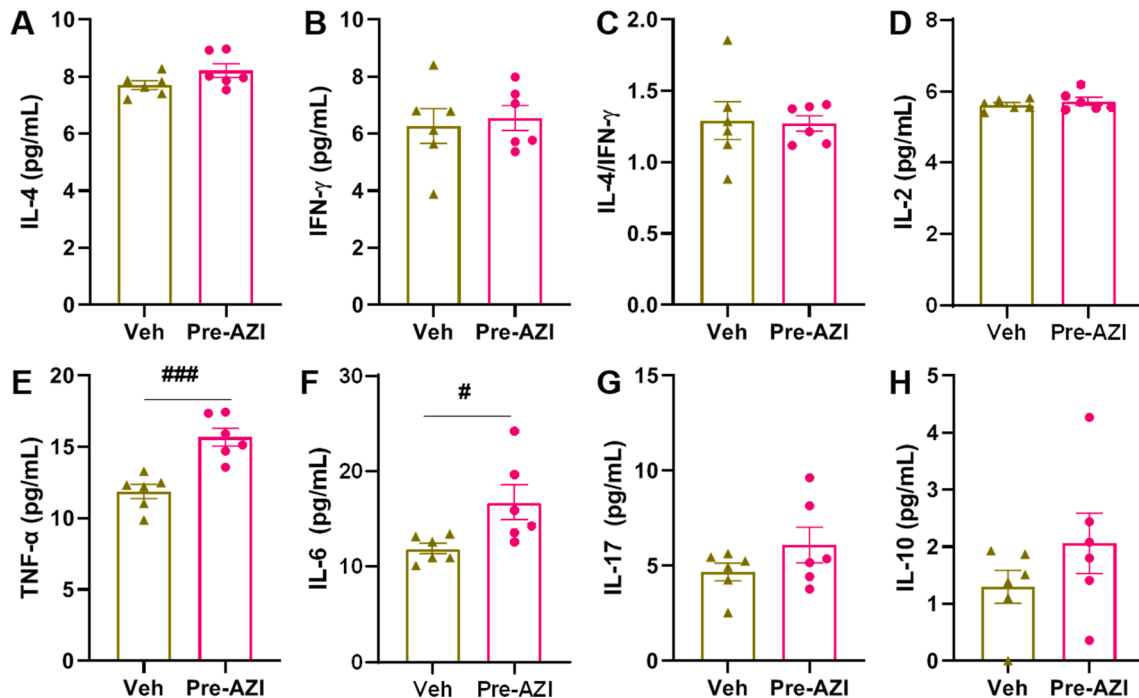


Fig. 6. The effect of AZI pretreatment on the level of serum Th1/Th2/Th17-Related Cytokines in TMA-induced AD mice. (A) the level of IL-4; (B) the level of IFN- γ ; (C) the ratio of IL-4/IFN- γ ; (D) the level of IL-2; (E) the level of TNF- α ; (F) the level of IL-6; (G) the level of IL-17A; (H) the level of IL-10. $^*P < 0.05$, $^{**}P < 0.01$ compared with vehicle. Veh: vehicle; Pre-AZI: AZI pretreatment. N = 6 for each group. Mann Whitney test was used for F; Student's *t*-test was used for the others.

group (Fig. 7C). The heatmap of 15 altered genera in five groups was shown in Fig. 7D. In comparison with the vehicle-treated model group, four genera (*Bacteroides*, *Candidatus_Saccharibacteria_unclassified*, *Acetatifactor*, *Firmicutes_unclassified*) significantly increased and three genera (*Alistipes*, *Clostridiales_unclassified*, *Butyrivibrio*) were significantly

decreased in the AZI-pretreated model group. In comparison with the healthy normal group, one genus (*Bacteroides*) significantly increased eight genera (*Bacteroidales_unclassified*, *Duncanella*, *Muribaculum*, *Parabacteroides*, *Ligilactobacillus*, *Muribaculaceae_unclassified*, *Alis-tipes*, *Clostridiales_unclassified*) decreased in the AZI-pretreated model

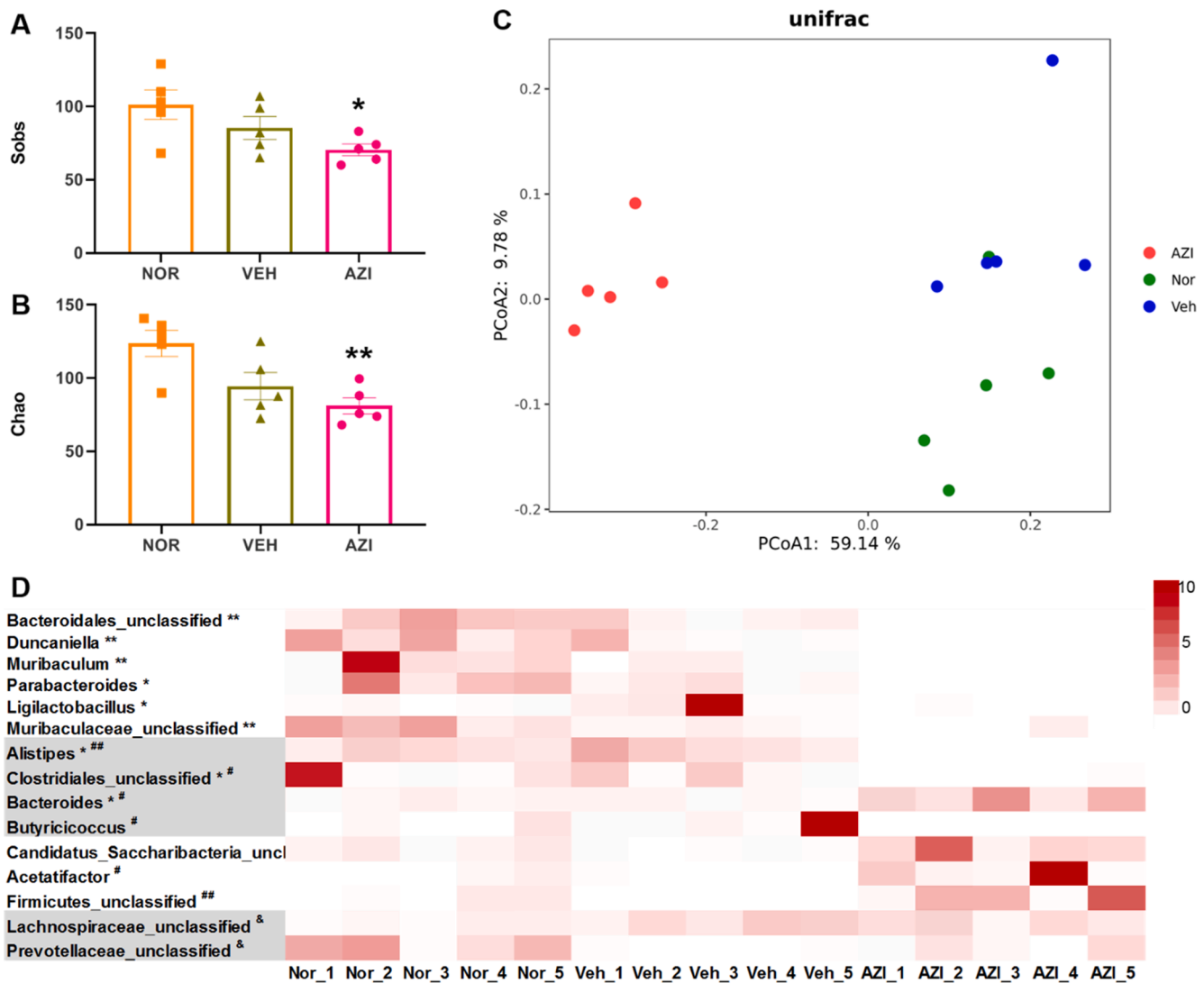


Fig. 7. Alterations in gut microbiota caused by AZI pretreatment. Effects of AZI pretreatment on Sobs (A) and Chao (B) indices. (C) PCoA score plots of gut microbiota based on UniFrac distance matrices. (D) the heat map of differential microbial communities at the genus level. * $P < 0.05$, ** $P < 0.01$ compared AZI-pretreated AD model group to healthy normal mice; # $P < 0.05$, ## $P < 0.01$ compared vehicle-pretreated AD model group to AZI-pretreated AD model group; & $P < 0.05$ compared vehicle-pretreated AD model group to healthy normal mice. Veh: pretreated with water; AZI: pretreated with Azithromycin; Normal: BALB/c mice without any treatments, including the TMA. $N = 5$ for each group. One-way analysis of variance (ANOVA) (with Tukey test for post hoc comparison) was used for A and B; Kruskal-Wallis analysis of variance (ANOVA) (with Dunn's multiple comparisons test for post hoc comparison) was used for D.

group. Besides, in comparison with the healthy normal group, one genus (*Lachnospiraceae_unclassified*) significantly increased, and one genus (*Prevotellaceae_unclassified*) significantly decreased in the vehicle-pretreated model group. Of note, six genera (*Bacteroidales_unclassified*, *Duncaniella*, *Muribaculum*, *Parabacteroides*, *Alistipes*, *Butyricoccus*) were not found in all AZI pretreated model mice, but they were found in most mice of the other two groups.

3.5. Correlation among histopathological ear thickness, level of cytokines, and relative abundance of gut bacterial genera

To interpret the relationship among severity of AD, level of serum cytokines, and relative abundance of gut microbiota at the genus level, we did Spearman's rank correlation analysis among histopathological ear thickness, scratching frequency, level of serum cytokines, and relative abundance of gut microbiota at the genus level. As shown in Fig. 8, the levels of TNF- α and IL-6 were positively correlated with the histopathological ear thickness; the levels of IL-6 were positively correlated with the scratching frequency; the relative abundance of five genera

(*Bacteroidales_unclassified*, *Duncaniella*, *Alistipes*, *Clostridiales_unclassified*, *Butyricoccus*) was negatively correlated with the histopathological ear thickness, while one genus (*Acetatifactor*) was positively correlated with the histopathological ear thickness; the relative abundance of six genera (*Bacteroidales_unclassified*, *Duncaniella*, *Parabacteroides*, *Alistipes*, *Clostridiales_unclassified*, *Butyricoccus*) was negatively correlated with the scratching frequency, while three genera (*Candidatus_Saccharibacteria_unclassified*, *Acetatifactor*, *Firmicutes_unclassified*) was positively correlated with scratching frequency. On the other hand, the level of TNF- α was negatively correlated with the relative abundance of six genera (*Bacteroidales_unclassified*, *Duncaniella*, *Parabacteroides*, *Alistipes*, *Clostridiales_unclassified*, *Butyricoccus*) but positively correlated with the relative abundance of three genera (*Bacteroides*, *Acetatifactor*, *Firmicutes_unclassified*); the level of IL-6 was negatively correlated with the relative abundance of eight genera (*Bacteroidales_unclassified*, *Duncaniella*, *Muribaculum*, *Parabacteroides*, *Ligilactobacillus*, *Alistipes*, *Clostridiales_unclassified*, *Butyricoccus*) but positively correlated with the relative abundance of four genera (*Bacteroides*, *Candidatus_Saccharibacteria_unclassified*, *Acetatifactor*, *Firmicutes_unclassified*). The

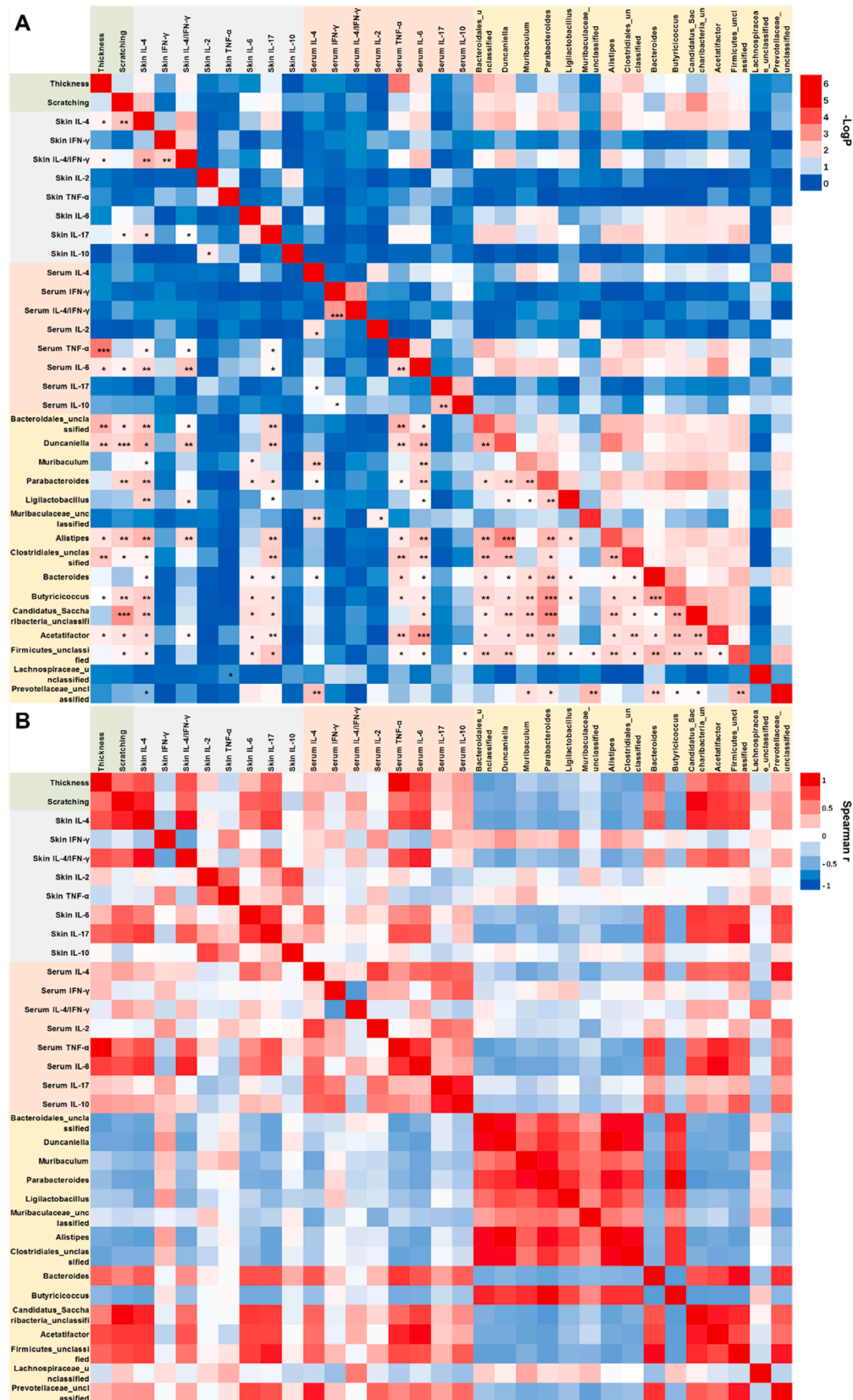


Fig. 8. Correlation among AD symptoms, level of cytokines, and relative abundance of gut bacterial genera. The correlation P-value matrix (A) and r-value matrix (B) that calculated by Spearman's rank correlation. Spearman's rank correlation was used.

detailed correlation P-value matrix and r-value matrix calculated by Spearman's rank correlation was also shown in **Supplementary** Figures 5 and 6.

4. Discussion

AD is a common inflammatory skin disease related to the imbalance of gut microbiota. AZI is a broad-spectrum antibiotic that can induce an imbalance of gut microbiota. Here we found: (1) AZI pretreatment increased the AD-associated ear thickening, enhanced the frequency of scratching behaviors, and aggravated the pathological changes of diseased skin, and exacerbated the infiltration of immune cells in the diseased skin (including neutrophil-like cells, T cells, and mast cells); (2) AZI pretreatment elevated the levels of IL-4, IL-6 and IL-17A in the diseased skin of AD model mice, but it increased the levels of TNF- α and IL-6 in the serum; (3) AZI pretreatment also increased four gut bacterial genera (*Bacteroides*, *Candidatus Saccharibacteria unclassified*, *Acetatifactor*, *Firmicutes unclassified*) but depleted three short-chain fatty acids producing gut bacterial genera (*Alistipes*, *Clostridiales unclassified*, *Butyricicoccus*); (4) AD-associated symptoms were positively associated with skin IL-4 and IL-17A, serum TNF- α and IL-6, and *Acetatifactor*, but they negatively correlated to the three decreased gut bacterial genera (*Alistipes*, *Clostridiales unclassified*, *Butyricicoccus*). Our results demonstrate that AZI pretreatment deteriorates the severity of the TMA-induced AD model in mice, which is related to the imbalances of gut microbiota and skin/serum cytokines.

Previous studies mainly focused on the relationship between antibiotic exposure during prenatal or early postnatal life and the risk of AD. For example, Dom et al. found prenatal exposure to antibiotics may increase the risk of AD [26]. Mubanga et al. found antibiotic use in both prenatal and early postnatal life is associated with the risk of AD in early childhood [27]. On the other hand, a systematic review conducted by Tsakok et al. found exposure to antibiotics in the first year of life, but not prenatally, is more common in children with eczema [28]. Thus, these results provide a link between antibiotic exposure and the risk of AD. However, there are still many confounding factors, such as the use of specific antibiotics. Here, we tested the effect of the treatment of AZI (25 mg/kg for 5 days) on the development of AD in 6-week old mice, which is similar to the use of AZI in the clinic. We found AZI pretreatment enhanced the ear thickness from day 11 (when the AD-like symptoms were developing) and increased scratching frequency, enhanced the histopathological score, and exacerbated the infiltration of several immune cells (including neutrophil-like cells, T cells, and mast cells) in the diseased ear skin on day 15 (when the AD symptoms were developed). These results demonstrate that preceding exposure to AZI not only accelerated the development of AD-like symptoms but also enhanced the severity of developed AD. Thus, our results provide the first direct evidence that AZI pretreatment deteriorates TMA-induced AD-like symptoms in mice. Further clinic studies are warranted to verify if preceding exposure to AZI may increase the risk of AD and deteriorate the severity of AD in humans.

It is well-known that AD is a common inflammatory skin disease predominately related to a Th1/Th2 imbalance with a predominantly Th2 immune response [29]. The ratio of Th2-related IL-4 and Th1-related IFN- γ is frequently used as an index of Th1/Th2 balance [30]. Here we found AZI pretreatment increased the ratio of skin IL-4/IFN- γ by the level of IL-4 without affecting that of IFN- γ in TMA-induced AD model mice. These data are consistent with our pathological data that AZI pretreatment increased T cells infiltration in the ear skins. Besides, AZI pretreatment increased the level of IL17A in ear tissue of TMA-induced AD model mice, which may also exaggerate AD [31]. Of note, AD-associated ear thickness was positively associated with the levels of skin IL-4 and the ratio of IL-4/IFN- γ , while the scratching frequency was positively correlated with skin IL-4 and IL17A. Thus, these data suggest AZI pretreatment increase of Th2 and Th17 immune responses in the diseased ear skin in TMA-induced mouse model of AD, which also

supports the AD-deteriorating effect of AZI pretreatment.

On the other hand, inconsistent with the skin cytokines, AZI pretreatment only increased the level of TNF- α and IL-6 in serum of the AD model mice, but it did not affect the levels of IL-4, and IL17 as well as the ratio of IL-4/IFN- γ in the serum of AD model mice. Of note, AD-associated ear thickness was positively associated with the serum levels of TNF- α and IL-6; and the scratching frequency was positively correlated with the serum level of IL-6. Thus, these data suggest that AZI pretreatment did not induce a systematical (serum) enhancement of Th2 and Th17 responses in TMA-induced AD model mice. Our results support previous studies that TNF- α and IL-6 may contribute to the severity of AD [24] and may up-regulate the level of IL-4 in immune cells [32,33]. Interestingly, the level of serum TNF- α and IL-6 is positively correlated with skin IL-4 level, skin IL-17A level, and the ratio of skin IL-4/IFN- γ but not skin IL-6 level in AD model mice. These data suggest the increase of skin IL-6 might be a consequence of local skin inflammation rather than an accompanying phenomenon of increased serum IL-6 (or vice versa) in TMA-induced AD model mice. Altogether, our data suggest the mechanism for AZI pretreatment deteriorating AD may be related to the increases of serum TNF- α and IL-6 levels as well as the enhancements of skin Th2 and Th17 responses, though their causal relationship remains unclear.

The gut microbiota is involved in the regulation of immunity [34]. Recently, Kim et al. found that removing most gut microbiota by an antibiotic cocktail aggravates AD in an ovalbumin-induced mouse model of AD [35]. Here, we found two differential genera, including the increased *Lachnospiraceae unclassified* and decreased *Prevotellaceae unclassified*, in TMA induced AD model group. Our results are consistent with previous findings that *Lachnospiraceae* is enriched in human infants with eczema [36] but are inconsistent with that the abundance of family *Prevotellaceae* is increased in DNCB-induced AD mice [37]. On the other hand, compared with the vehicle-pretreated AD model group, four genera (*Bacteroides*, *Candidatus Saccharibacteria unclassified*, *Acetatifactor*, *Firmicutes unclassified*) significantly increased, and three genera (*Alistipes*, *Clostridiales unclassified*, *Butyricicoccus*) were significantly decreased in the AZI-pretreated AD model group. These data indicate that AZI pretreatment did not affect the relative abundance of the two gut bacterial genera (*Lachnospiraceae unclassified* and *Prevotellaceae unclassified*) which are directly associated with TMA induced AD; In other words, the effect of AZI on the compositions of gut microbiota was different from TMA. Of note, All the three AZI-decreased genera (*Alistipes*, *Clostridiales unclassified*, *Butyricicoccus*) were negatively correlated with the histopathological ear thickness, scratching frequency, skin IL-4 and IL-17A levels, as well as serum TNF- α and IL-6 levels, while the one AZI-increased genus (*Acetatifactor*) was positively correlated with these indexes. All these three AZI-decreased genera (*Alistipes*, *Clostridiales unclassified*, *Butyricicoccus*) were potential short-chain fatty acids (SCFAs) producing bacterial genera in the gut [38]. Besides, *Parabacteroides*, another SCFAs producing bacterial genera, were also not detected in all mice of the AZI-pretreatment group. Lower amounts of SCFAs have been found in fecal samples of children later developing eczema [39], and an increase of SCFAs may act as protective effects on eczema [40]. Thus, our results indicate AZI pretreatment may affect the SCFAs producing bacterial genera, which may contribute to the exacerbating effects of AZI pretreatment in AD.

SCFAs, mainly including acetate (C2), propionate (C3), and butyrate (C4), are metabolic by-products from the microbial fermentation of dietary fibers and resistant starch [41]. Increasing evidence showed that SCFAs can act as energy supply for colonocytes, improve gut barrier integrity, regulate the immune system, and influence inflammatory responses [42]. For example, SCFA propionate can protect against allergic inflammation (including reducing Th2 response) in the lung [43] by activation of GPR41 and can reduce IL-17 production by intestinal $\gamma\delta$ T cells by inhibition of histone deacetylase [44]; Acetate can inhibit LPS-induced TNF- α secretion from mononuclear cells by activating GPR43

[45]. In terms of our data, AZI pretreatment may attenuate these protective effects of SCFAs by reducing of SCFAs producing bacterial genera, which might consequently increase the level of serum and skin cytokines. Though we have no further data, our study at least gave some clues (such as intermediated by serum TNF α , serum IL-6, and/or SCFAs) for further studies to clarify the causality between reduction of gut SCFAs producing bacterial genera and aggravated AD in AZI pretreated mice. Except for AZI, other macrolides and other types of antibiotics may also result in the imbalance of gut microbiota and lower the SCFAs. For

example, it has been found the effect of cefdinir, another kind of cephalosporin antibiotic, on lowering the SCFAs was more pronounced than that of AZI [46]. Thus, further studies are also required to determine if other antibiotics act similarly deteriorating effects on AD.

Although our study provided direct and relatively solid pieces of evidence supporting AZI pretreatment deteriorated AD in mice, the causal association among gut microbiota, cytokines, and severity of AD still needed to be further addressed. Many further questions could be raised based on our data, some examples are as follows: (1) Could fecal

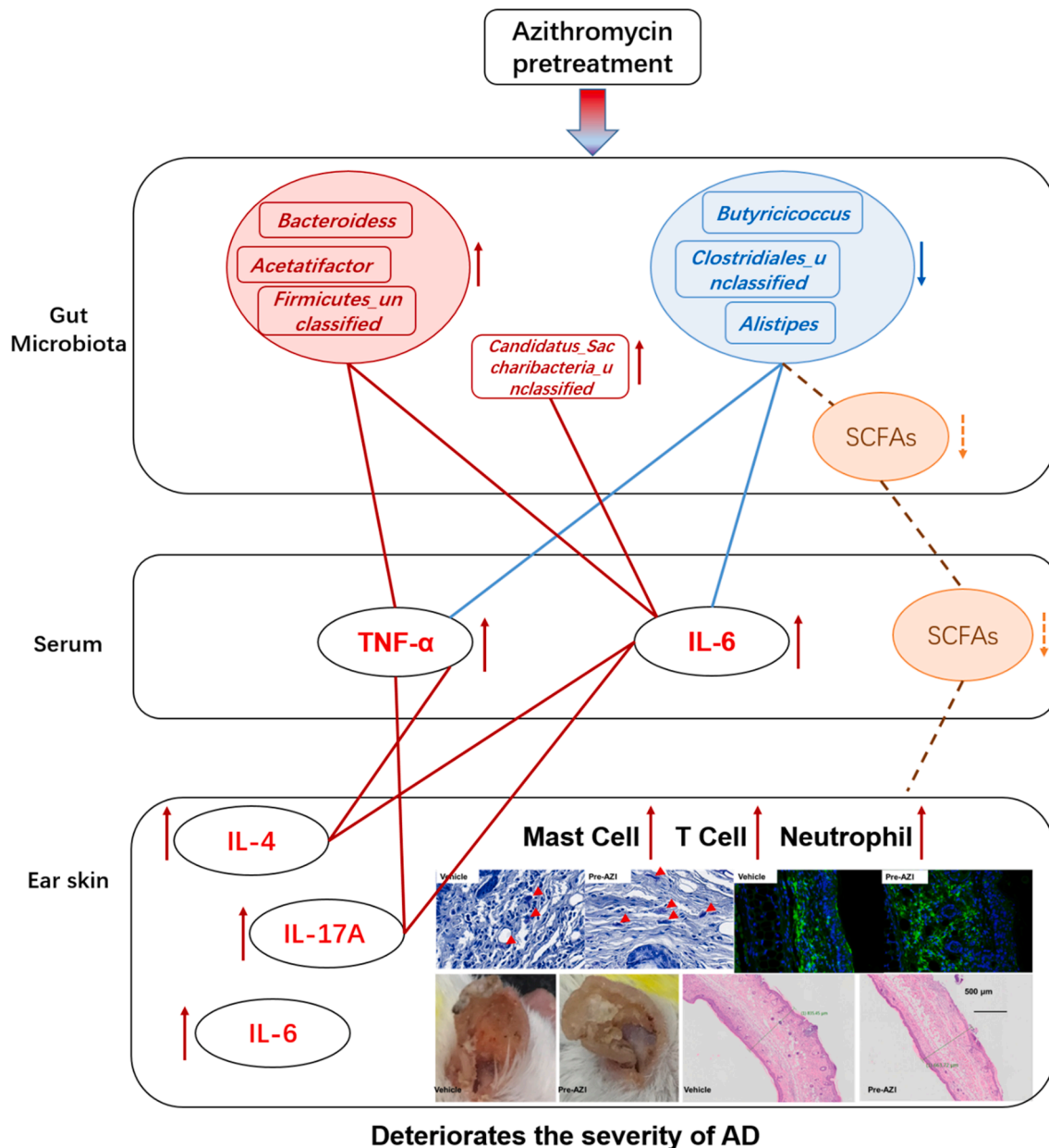


Fig. 9. Proposed mechanisms of AZI pretreatment deteriorating the development of the TMA-induced AD model in mice. AZI pretreatment deteriorates the severity of the TMA-induced AD model in mice, which was accompanied by an increased level of three skin cytokines (IL-4, IL-6, and IL-17A) and two serum cytokines (TNF- α and IL-6) as well as a perturbation in the gut microbiota. The relative abundance of three AZI-increased gut microbiota genera (*Bacteroides*, *Acetatifactor*, *Firmicutes_unclassified*) is positively correlated with the levels of serum TNF- α and IL-6; on the other hand, the relative abundance of the three AZI-decreased gut microbiota genera (*Alistipes*, *Clostridiales_unclassified*, *Butyricococcus*) is negatively correlated with the levels of serum TNF- α and IL-6. All these three AZI-decreased gut microbiota genera (*Alistipes*, *Clostridiales_unclassified*, *Butyricococcus*) were potential short-chain fatty acids (SCFAs) producing gut bacterial genera, which may have a protective effect on AD. Besides, the level of serum TNF- α and IL-6 is positively correlated with the ear thickness, skin IL-4 level, and skin IL-17A level in AD model mice; However, skin IL-6 level was independent on serum TNF- α and IL-6 levels. Of note, the changes of gut microbiota genera may be also correlated with some increased skin cytokines and other AD-like symptoms, which is not indicated here. The red text represents the increase caused by AZI pretreatment; the blue text represents the decrease caused by AZI pretreatment; the Orange text and dot line represent other factors that are probably reduced by AZI pretreatment.

microbiota transplantation, specific SCFAs producing bacteria transplantation, probiotics, or SCFAs related supplements ameliorate AD exacerbation of AZI pretreatment? (2) Is changes in gut microbiota preceding the exacerbation of AD induced by AZI pretreatment? (3) As systemic antibiotics may also influence skin microbiota [47]; is there any contribution of changes in skin microbiota? (4) What is the effect of posttreatment of AZI on developed AD? (5) What about other antibiotics or other skin allergies? Overall, our study just did initial preclinical research regarding the cornstalk between gut microbiota and skin allergy or inflammation. Further researches are still warranted.

5. Conclusion

AZI pretreatment promoted the development of AD-like symptoms and enhanced the severity of developed AD-like symptoms in the TMA-induced mouse model of AD. AZI pretreatment promoted the infiltration of immune cells, elevated the levels of skin IL-4, IL-6, and IL-17A, increased serum TNF- α and IL-6, and induced a perturbation in the gut microbiota in the TMA-induced AD mouse model. The levels of most of these increased skin and serum cytokines were positively correlated to the TMA-induced AD-like symptoms but negatively associated with these decreased specific short-chain fatty acids producing bacterial genera, respectively. Thus, our study demonstrates that AZI pre-exposure deteriorates the TMA-induced AD-like symptoms in mice, which may be associated with the imbalances of gut microbiota and skin/serum cytokines. A proposed mechanism on the AD- deteriorating effect of AZI pretreatment based on our data was summarized in Fig. 9. Further studies are warranted to verify this phenomenon clinically and to study its underlying causal mechanisms.

CRedit authorship contribution statement

Huawei Zhao: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing – original draft, Writing – review & editing, Visualization, Project administration, Funding acquisition. **Jia Zhou:** Investigation, Methodology, Validation, Writing – review & editing. **Haimei Lu:** Investigation, Methodology, Validation, Writing – review & editing. **Anran Xi:** Investigation, Visualization. **Mengxian Luo:** Investigation, Visualization. **Keer Wang:** Investigation, Visualization. **Hongjie Lv:** Methodology, Investigation. **Huijuan Wang:** Validation, Writing – review & editing. **Ping Wang:** Validation, Methodology. **Jing Miao:** Writing – review & editing, Supervision, Project administration. **Zhenghao Xu:** Conceptualization, Methodology, Formal analysis, Resources, Data curation, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Author Contributions

Z.X., H.Z., and J.M. designed the study. H.Z., H.Lu, and A.X. performed the experiments and collected samples. H.Z., J.Z., M.L., and Z.X. analyzed the data. K.W., M.L., and A.X. performed toluidine blue staining and immunofluorescence staining as well as skin cytokine analysis for revision. H.Lv and P.W. identified neutrophil-like cells from HE staining images for revision. K.W. and H.Lv performed cell counting for revision. J.Z., K.W., H.W., and J.Z. were involved with the interpretation of data in the manuscript. H.Z. and Z.X. wrote the manuscript draft and did the manuscript revision in consultation with J.M., H.Lu, and J.Z.

Institutional Review Board Statement

Experiments were approved by Institutional Animal Care and Use Committee of Zhejiang Chinese Medical University (#ZSLL-2016-83).

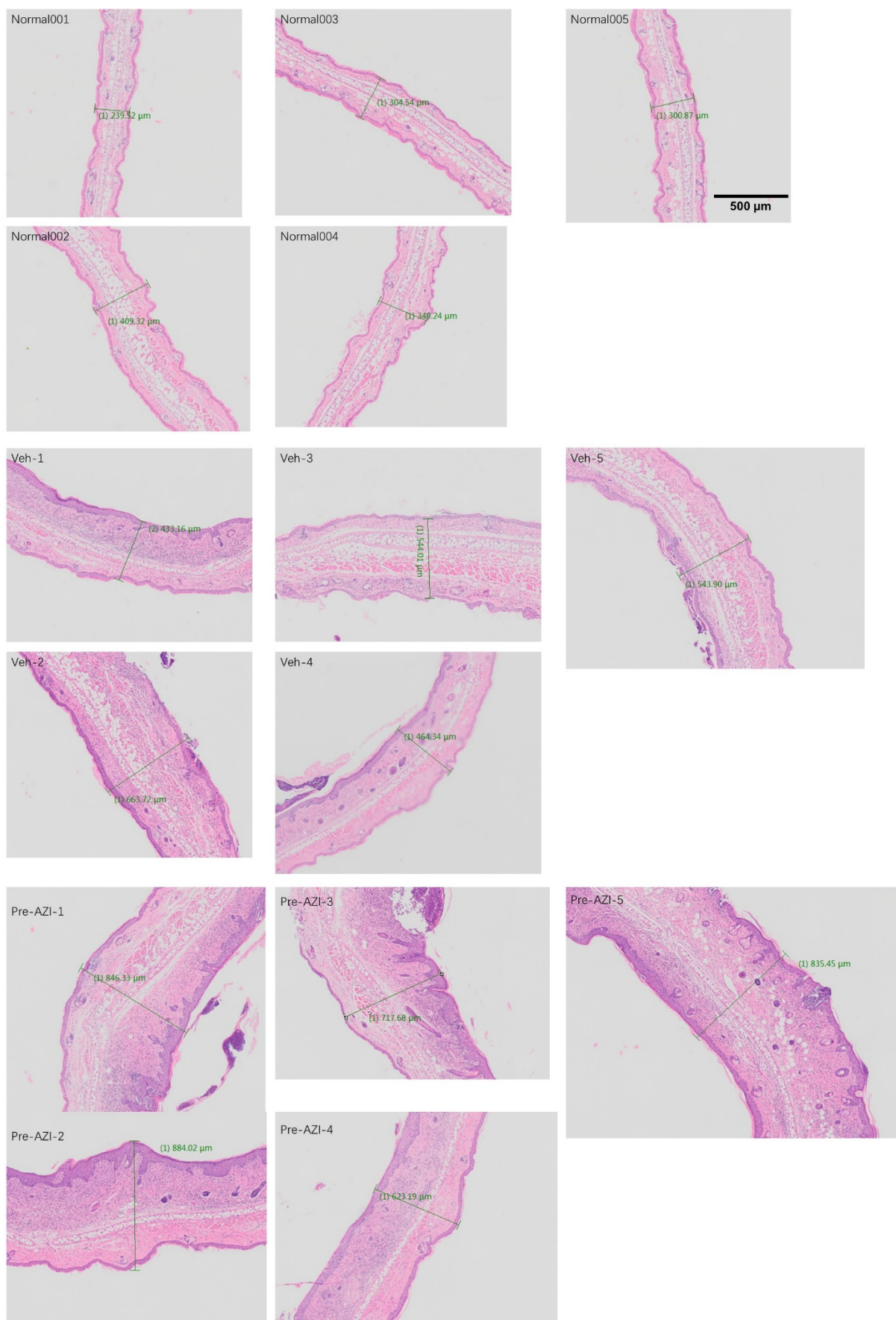
Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.intimp.2021.108388>.

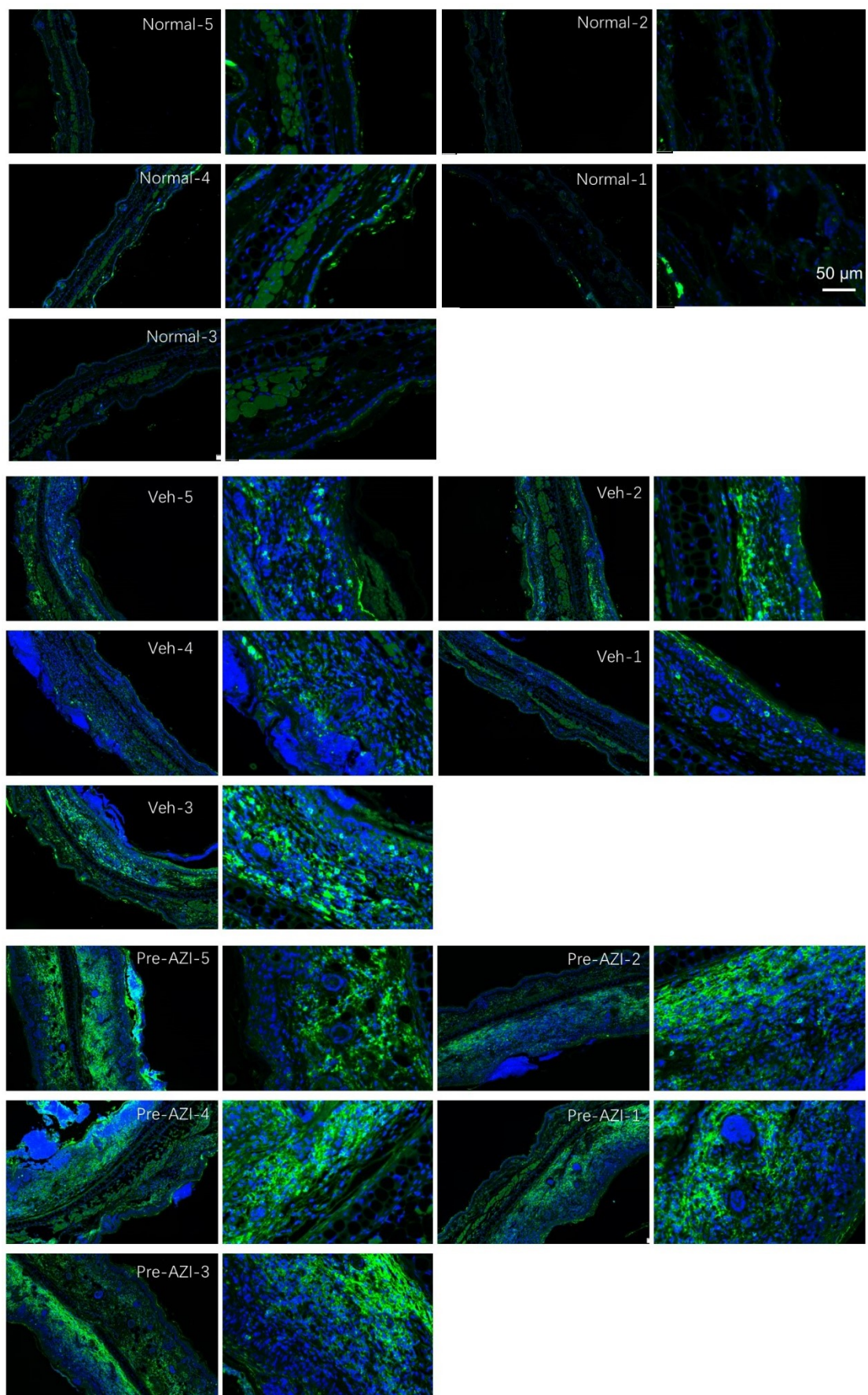
References

- [1] W.F. Davidson, D.Y.M. Leung, L.A. Beck, C.M. Berin, M. Boguniewicz, W.W. Busse, T.A. Chatila, R.S. Geha, J.E. Gern, E. Guttman-Yassky, A.D. Irvine, B.S. Kim, H. H. Kong, G. Lack, K.C. Nadeau, J. Schwaninger, A. Simpson, E.L. Simpson, J. M. Spengel, A. Togias, U. Wahn, R.A. Wood, J.A. Woodfolk, S.F. Ziegler, M. Plaut, Report from the national institute of allergy and infectious diseases workshop on “atopic dermatitis and the atopic march: mechanisms and interventions”, *J. Allergy Clin. Immunol.* 143 (3) (2019) 894–913.
- [2] S. Nutten, Atopic dermatitis: global epidemiology and risk factors, *Ann. Nutr. Metab.* 66 (Suppl 1) (2015) 8–16.
- [3] S.M. Langan, A.D. Irvine, S. Weidinger, Atopic dermatitis, *Lancet* 396 (10247) (2020) 345–360.
- [4] M. Olsson, R. Bajpai, L.W.Y. Wee, Y.W. Yew, M.J.A. Koh, S. Thng, J. Car, K. Jarbrink, The cost of childhood atopic dermatitis in a multi-ethnic Asian population: a cost-of-illness study, *British J. Dermatol.* 182 (5) (2020) 1245–1252.
- [5] A.S. Paller, J.M. Spengel, P. Mina-Osorio, A.D. Irvine, The atopic march and atopic multimorbidity: many trajectories, many pathways, *J. Allergy Clin. Immunol.* 143 (1) (2019) 46–55.
- [6] Y.S. Chang, M.K. Trivedi, A. Jha, Y.F. Lin, L. Dimaano, M.T. Garcia-Romero, Synbiotics for prevention and treatment of atopic dermatitis: a meta-analysis of randomized clinical trials, *JAMA Pediatr.* 170 (3) (2016) 236–242.
- [7] S. Wei, M.S. Mortensen, J. Stokholm, A.D. Brejnrod, J. Thorsen, M.A. Rasmussen, U. Trivedi, H. Bisgaard, S.J. Sorensen, Short- and long-term impacts of azithromycin treatment on the gut microbiota in children: a double-blind, randomized, placebo-controlled trial, *EBioMedicine* 38 (2018) 265–272.
- [8] A. Flavia Monteagudo Paz, J. Francisco Silvestre Salvador, N. Latorre Martínez, L. Cuesta Montero, F. Toledo Alberola, Toledo alberola, allergic contact dermatitis caused by azithromycin in an eye drop, *Contact Dermatitis* 64 (5) (2011) 300–301.
- [9] S. Milkovic-Kraus, J. Macan, B. Kanceljak-Macan, Occupational allergic contact dermatitis from azithromycin in pharmaceutical workers: a case series, *Contact Dermatitis* 56 (2) (2007) 99–102.
- [10] P. Mendes-Bastos, S. Bras, C. Amaro, J. Cardoso, Non-occupational allergic contact dermatitis caused by azithromycin in an eye solution, *Journal der Deutschen Dermatologischen Gesellschaft = Journal of the German Society of Dermatology : JDDG* 12(8) (2014) 729–30.
- [11] I.L. Chen, M.K. Tsai, H.W. Chung, H.M. Hsieh, Y.T. Huang, Y.C. Lin, C.H. Hung, The effects of antibiotic exposure on asthma in children with atopic dermatitis, *Sci. Rep.* 11 (1) (2021) 8526.

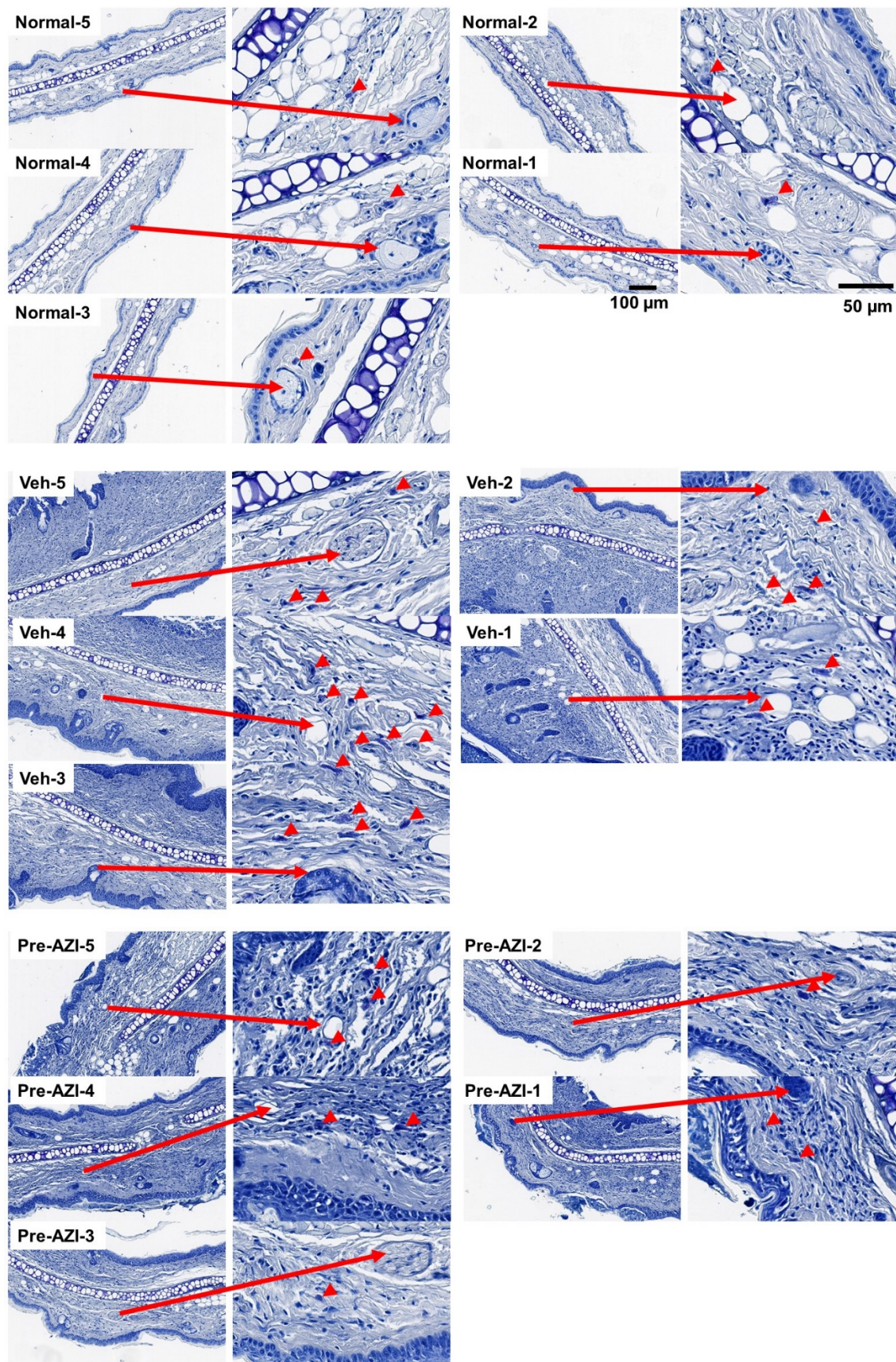
- [12] B.J. Parker, P.A. Wearsch, A.C.M. Veloo, A. Rodriguez-Palacios, The genus *Allestipes*: gut bacteria with emerging implications to inflammation, cancer, and mental health, *Front. Immunol.* 11 (2020) 906.
- [13] C. Schneider, W.-D. Döcke, T.M. Zollner, L. Röse, Chronic mouse model of TMA-induced contact hypersensitivity, *J. Invest. Dermatol.* 129 (4) (2009) 899–907.
- [14] B. Sur, S. Kang, M. Kim, S. Oh, Alleviation of atopic dermatitis lesions by a benzylidenacetophenone derivative via the MAPK signaling pathway, *Inflammation* 42 (3) (2019) 1093–1102.
- [15] N. Ikarashi, N. Fujitake, T. Togashi, N. Takayama, N. Fukuda, R. Kon, H. Sakai, J. Kamei, K. Sugiyama, Acacia polyphenol ameliorates atopic dermatitis in trimellitic anhydride-induced model mice via changes in the gut microbiota, *Food* 9 (6) (2020) 773, <https://doi.org/10.3390/foods9060773>.
- [16] X. Lin, T. Shao, L. Huang, X. Wen, M. Wang, C. Wen, Z. He, Simiao decoction alleviates gouty arthritis by modulating proinflammatory cytokines and the gut ecosystem, *Front. Pharmacol.* 11 (2020) 955.
- [17] X. Wen, Y. Lou, S. Song, Z. He, J. Chen, Z. Xie, X. Shi, C. Wen, T. Shao, Qu-Zhuo-Tong-Bi Decoction Alleviates Gouty Arthritis by Regulating Butyrate-Producing Bacteria in Mice, *Front. Pharmacol.* 11 (2020) 610556.
- [18] R. Nagpal, S. Wang, L.C. Solberg Woods, O. Seshie, S.T. Chung, C.A. Shively, T. C. Register, S. Craft, D.A. McClain, H. Yadav, Comparative microbiome signatures and short-chain fatty acids in mouse, rat, non-human primate, and human feces, *Front. Microbiol.* 9 (2018) 2897.
- [19] E. Bolyen, J.R. Rideout, M.R. Dillon, N.A. Bokulich, C.C. Abnet, G.A. Al-Ghalith, H. Alexander, E.J. Alm, M. Arumugam, F. Asnicar, Y. Bai, J.E. Bisanz, K. Bittinger, A. Brejnrod, C.J. Brislawn, C.T. Brown, B.J. Callahan, A.M. Caraballo-Rodriguez, J. Chase, E.K. Cope, R. Da Silva, C. Diener, P.C. Dorrestein, G.M. Douglas, D. M. Durall, C. Duvallet, C.F. Edwardson, M. Ernst, M. Estaki, J. Fouquier, J. M. Gault, S.M. Gibbons, D.L. Gibson, A. Gonzalez, K. Gorlick, J. Guo, B. Hillmann, S. Holmes, H. Holte, C. Huttenhower, G.A. Huttley, S. Janssen, A. K. Jarmusch, L. Jiang, B.D. Kaehler, K.B. Kang, C.R. Keefe, P. Keim, S.T. Kelley, D. Knights, I. Koester, T. Kosciolek, J. Kreps, M.G.I. Langille, J. Lee, R. Ley, Y. X. Liu, E. Loftfield, C. Lozupone, M. Maher, C. Marotz, B.D. Martin, D. McDonald, L.J. McIver, A.V. Melnik, J.L. Metcalf, S.C. Morgan, J.T. Morton, A.T. Naimey, J. A. Navas-Molina, L.F. Nothias, S.B. Orchanian, T. Pearson, S.L. Peoples, D. Petras, M.L. Preuss, E. Pruesse, L.B. Rasmussen, A. Rivers, M.S. Robeson 2nd, P. Rosenthal, N. Segata, M. Shaffer, A. Shiffer, R. Sinha, S.J. Song, J.R. Spear, A.D. Swafford, L. R. Thompson, P.J. Torres, P. Trinh, A. Tripathi, P.J. Turnbaugh, S. Ul-Hasan, J.J. J. van der Hooft, F. Vargas, Y. Vazquez-Baeza, E. Vogtmann, M. von Hippel, W. Walters, Y. Wan, M. Wang, J. Warren, K.C. Weber, C.H.D. Williamson, A. D. Willis, Z.Z. Xu, J.R. Zaneveld, Y. Zhang, Q. Zhu, R. Knight, J.G. Caporaso, Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2, *Nat. Biotechnol.* 37 (8) (2019) 852–857.
- [20] C. Quast, E. Pruesse, P. Yilmaz, J. Gerken, T. Schweer, P. Yarza, J. Peplies, F. O. Glöckner, The SILVA ribosomal RNA gene database project: improved data processing and web-based tools, *Nucleic Acids Res.* 41 (D1) (2013) D590–D596.
- [21] N.R. Coordinators, Database resources of the national center for biotechnology information, *Nucleic Acids Res.* 46 (D1) (2018) D8–D13.
- [22] D.F. Choy, D.K. Hsu, D. Seshasayee, M.A. Fung, Z. Modrusan, F. Martin, F.-T. Liu, J. R. Arron, Comparative transcriptomic analyses of atopic dermatitis and psoriasis reveal shared neutrophilic inflammation, *J. Allergy Clin. Immunol.* 130 (6) (2012) 1335–1343.e5.
- [23] T. Ando, W. Xiao, P. Gao, S. Namiranian, K. Matsumoto, Y. Tomimori, H. Hong, H. Yamashita, M. Kimura, J.-I. Kashiwakura, T. Hata, K. Izuhara, M. Gurish, A. Roers, N. Rafaeels, K. Barnes, C. Jamora, Y. Kawakami, T. Kawakami, Critical role for mast cell stat5 activity in skin inflammation, *Cell Rep.* 6 (2) (2014) 366–376.
- [24] S. Uttarkar, N.C. Bremilla, W.H. Boehncke, Regulatory cells in the skin: Pathophysiological role and potential targets for anti-inflammatory therapies, *J. Allergy Clin. Immunol.* 143 (4) (2019) 1302–1310.
- [25] S. Sumimoto, M. Kawai, Y. Kasajima, T. Hamamoto, Increased plasma tumour necrosis factor- α concentration in atopic dermatitis, *Arch. Dis. Child* 67 (3) (1992) 277–279.
- [26] S. Dom, J.H. Droste, M.A. Sariachvili, M.M. Hagendorens, E. Oostveen, C.H. Britts, W.J. Stevens, M.H. Wieringa, J.J. Weyler, Pre- and post-natal exposure to antibiotics and the development of eczema, recurrent wheezing and atopic sensitization in children up to the age of 4 years, *Clin. Exp. Allergy : J. British Soc. Allergy Clin. Immunol.* 40 (9) (2010) 1378–1387.
- [27] M. Mubanga, C. Lundholm, B.M. D'Onofrio, M. Stratmann, A. Hedman, C. Almqvist, Association of early life exposure to antibiotics with risk of atopic dermatitis in Sweden, *JAMA Network Open* 4 (4) (2021) e215245, <https://doi.org/10.1001/jamanetworkopen.2021.5245>.
- [28] T. Tsakok, T.M. McKeever, L. Yeo, C. Flohr, Does early life exposure to antibiotics increase the risk of eczema? a systematic review, *British J. Dermatol.* 169 (5) (2013) 983–991.
- [29] A. Otsuka, T. Nomura, P. Rerksnimitr, J.A. Seidel, T. Honda, K. Kabashima, The interplay between genetic and environmental factors in the pathogenesis of atopic dermatitis, *Immunol. Rev.* 278 (1) (2017) 246–262.
- [30] W.Z. Liu, M.J. He, L. Long, D.L. Mu, M.S. Xu, X. Xing, X. Zeng, G. Liao, H.X. Dan, Q. M. Chen, Interferon-gamma and interleukin-4 detected in serum and saliva from patients with oral lichen planus, *Int. J. Oral Sci.* 6 (1) (2014) 22–26.
- [31] C. Koga, K. Kabashima, N. Shiraishi, M. Kobayashi, Y. Tokura, Possible pathogenic role of Th17 cells for atopic dermatitis, *J. Invest. Dermatol.* 128 (11) (2008) 2625–2630.
- [32] M. Rincón, J. Anguita, T. Nakamura, E. Fikrig, R.A. Flavell, Interleukin (IL)-6 directs the differentiation of IL-4-producing CD4⁺ T cells, *J. Exp. Med.* 185 (3) (1997) 461–469.
- [33] H. Zhang, H. Yang, S. He, TNF Increases expression of IL-4 and PARs in mast cells, *Cell. Physiol. Biochem.* 26 (3) (2010) 327–336.
- [34] M.G. Rooks, W.S. Garrett, Gut microbiota, metabolites and host immunity, *Nat. Rev. Immunol.* 16 (6) (2016) 341–352.
- [35] H.J. Kim, S.H. Lee, S.J. Hong, Antibiotics-induced dysbiosis of intestinal microbiota aggravates atopic dermatitis in mice by altered short-chain fatty acids, *Allergy, Asthma & Immunol. Res.* 12 (1) (2020) 137–148.
- [36] H. Zheng, H. Liang, Y. Wang, M. Miao, T. Shi, F. Yang, E. Liu, W. Yuan, Z.-S. Ji, D.-K. Li, P.J. Planet, Altered gut microbiota composition associated with eczema in infants, *PLoS ONE* 11 (11) (2016) e0166026, <https://doi.org/10.1371/journal.pone.0166026>.
- [37] H.I. Kim, S.H. Hong, S.Y. Lee, J.M. Ku, M.J. Kim, S.-G. Ko, Gardenia jasminoides ameliorates antibiotic-associated aggravation of DNCB-induced atopic dermatitis by restoring the intestinal microbiome profile, *Nutrients* 13 (4) (2021) 1349, <https://doi.org/10.3390/nu13041349>.
- [38] C.T. Brown, A.G. Davis-Richardson, A. Giongo, K.A. Gano, D.B. Crabb, N. Mukherjee, G. Casella, J.C. Drew, J. Ilonen, M. Knip, H. Hyöty, R. Veijola, T. Simell, O. Simell, J. Neu, C.H. Wasserfall, D. Schatz, M.A. Atkinson, E. W. Triplett, R.M. Roop, Gut microbiome metagenomics analysis suggests a functional model for the development of autoimmunity for type 1 diabetes, *PLoS ONE* 6 (10) (2011) e25792, <https://doi.org/10.1371/journal.pone.0025792>.
- [39] H.K. Kim, N.B.M.M. Rutten, I. Besseling-van der Vaart, L.E.M. Niers, Y.H. Choi, G. T. Rijkers, S. van Hemert, Probiotic supplementation influences faecal short chain fatty acids in infants at high risk for eczema, *Beneficial Microb.* 6 (6) (2015) 783–790.
- [40] M. Gio-Batta, F. Sjöberg, K. Jonsson, M. Barman, A.C. Lundell, I. Adlerberth, B. Hesselmar, A.S. Sandberg, A.E. Wold, Fecal short chain fatty acids in children living on farms and a link between valeric acid and protection from eczema, *Sci. Rep.* 10 (1) (2020) 22449.
- [41] Floor, Hugenholtz, J. Adair, Mullaney, Michiel, Kleerebezem, Hauke, Smidt, D. Ian, Rosendale, Modulation of the microbial fermentation in the gut by fermentable carbohydrates, v. 2 (8) (2013–10).
- [42] Y. Xu, G. Naselli, E. Bandala-Sanchez, T. Thomas, L.C. Harrison, Y. Zhang, Regulation of immune cell function by short chain fatty acids, *Eur. J. Immunol.* 46 (2016) 321.
- [43] A. Trompette, E.S. Gollwitzer, K. Yadava, A.K. Sichelstiel, N. Sprenger, C. Ngom-Bru, C. Blanchard, T. Junt, L.P. Nicod, N.L. Harris, B.J. Marsland, Gut microbiota metabolism of dietary fiber influences allergic airway disease and hematopoiesis, *Nat. Med.* 20 (2) (2014) 159–166.
- [44] L. Dupraz, A. Magniez, N. Rolhion, M.L. Richard, G. Da Costa, S. Touch, C. Mayeur, J. Planchais, A. Agus, C. Danne, C. Michaudel, M. Spatz, F. Trottein, P. Langella, H. Sokol, M.L. Michel, Gut microbiota-derived short-chain fatty acids regulate IL-17 production by mouse and human intestinal $\gamma\delta$ T cells, *Cell. Rep.* 36 (1) (2021) 109332.
- [45] R. Masui, M. Sasaki, Y. Funaki, N. Ogasawara, M. Mizuno, A. Iida, S. Izawa, Y. Kondo, Y. Ito, Y. Tamura, K. Yamamoto, H. Noda, A. Tanabe, N. Okaniwa, Y. Yamaguchi, T. Iwamoto, K. Kasugai, G protein-coupled receptor 43 moderates gut inflammation through cytokine regulation from mononuclear cells, *Inflamm. Bowel Dis.* 19 (13) (2013) 2848–2856.
- [46] X.Y. Zhao, Z.Z. Jiang, F. Yang, Y. Wang, X.M. Gao, Y.F. Wang, X. Chai, G.X. Pan, Y. Zhu, Sensitive and simplified detection of antibiotic influence on the dynamic and versatile changes of fecal short-chain fatty acids, *PLoS ONE* 11 (12) (2016).
- [47] Y.R. Woo, S.H. Lee, S.H. Cho, J.D. Lee, H.S. Kim, Characterization and analysis of the skin microbiota in rosacea: impact of systemic antibiotics, *J. Clin. Med.* 9 (1) (2020).



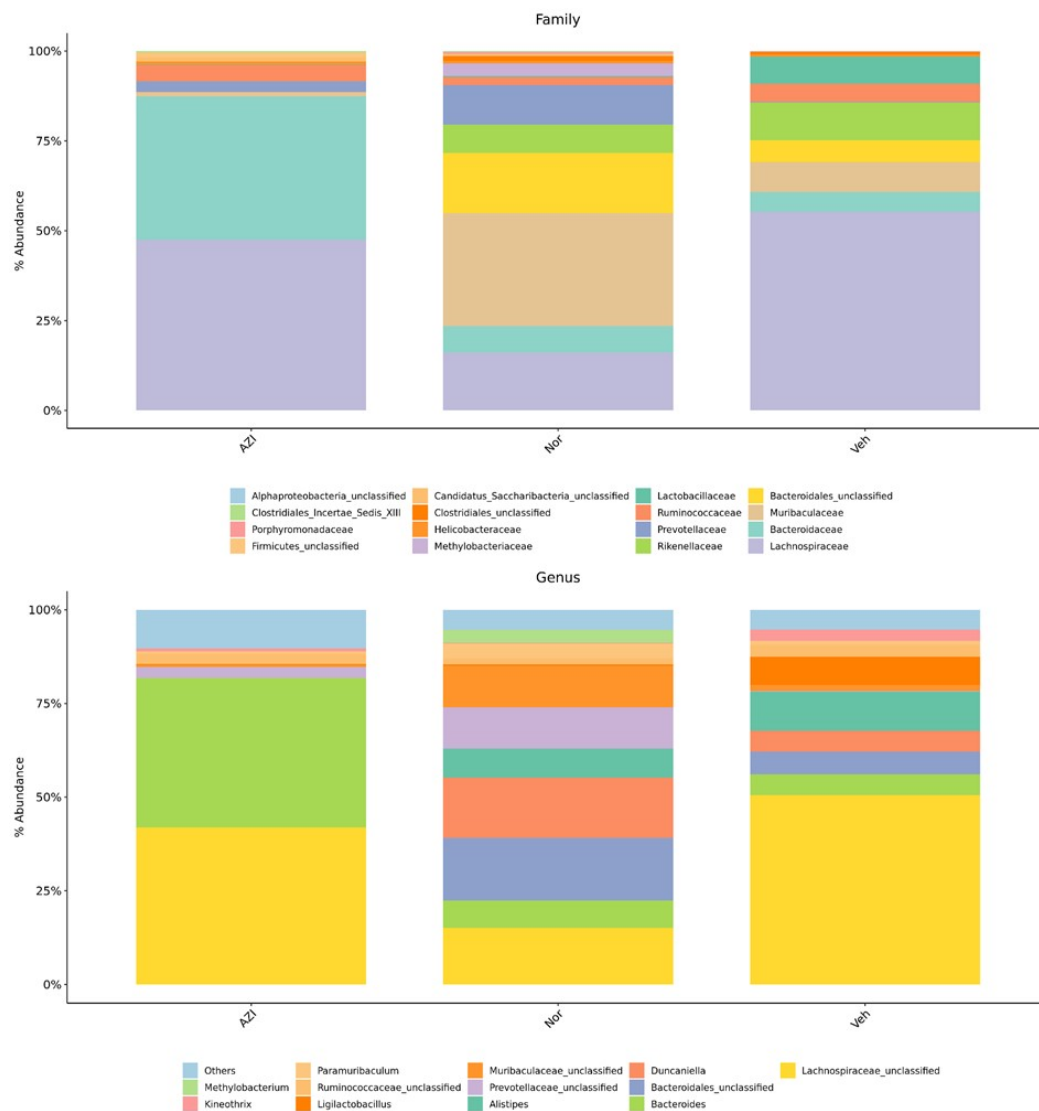
Supplementary Figure 1. HE staining of ear sections



Supplementary Figure 2. CD3 immunofluorescence staining of ear sections



Supplementary Figure 3. Toluidine blue staining for ear sections.



Supplementary Figure 4. Pre-treatment AZI affected the profiles of microbiota composition in TMA-induced AD mice. (A) microbial community structure of each group at the Family level; (B) Microbial community structure of each group at the Genus level. Nor: normal healthy mice; Veh: pretreatment of vehicle; Pre-AZI: pretreatment of AZI. N=5 for each group.

	Thickness	Scratching	Skin IL-4	Skin IFN-γ	Skin IL-4/IFN-γ	Skin IL-2	Skin TNF-α	Skin IL-6	Skin IL-17	Skin IL-10	Serum IL-4	Serum IFN-γ	Serum IL-4/IFN-γ	Serum IL-2	Serum TNF-α	Serum IL-6	Serum IL-17	Serum IL-10	Bacteroidales_unclassified	Duncanella	Muribaculum	Parabacteroides	Ligilactobacillus	Muribaculaceae_unclassified	Alistipes	Clostridiales_unclassified	Bacteroides	Butyrivibrio	Candidatus_Saccharibacteria_unclassified	Acetatifactor	Firmicutes_unclassified	Lachnospiraceae_unclassified	Prevotellaceae_unclassified
Thickness	6.259	1.094	1.515	0.555	1.515	0.257	0.412	0.369	1.198	0.021	0.348	0.369	0.166	0.067	3.966	1.826	0.289	0.606	2.782	2.171	0.652	1.141	0.805	0.245	1.829	2.415	1.22	1.317	0.91	1.64	1.227	0.328	0.412
Scratching	1.094	6.259	2.431	0.199	1.22	0.137	0.135	1.264	1.433	0.514	0.555	0.166	0.555	0.046	1.016	1.573	0.217	0.555	1.829	2.079	1.249	2.079	0.889	0.414	2.293	1.548	0.842	2.003	3.476	1.869	1.576	0.289	0.661
Skin IL-4	1.515	2.431	6.259	0.875	2.796	0.064	0.199	0.875	1.864	0.003	0.348	0.15	0.348	0.162	1.573	2.057	0.05	0.529	2.293	2.661	1.447	2.394	2.184	0.348	2.782	1.579	1.359	2.293	2.327	1.995	1.775	0.091	0.718
Skin IFN-γ	0.555	0.199	0.875	6.259	2.327	0.092	0.81	0	0.516	0.021	0.166	0.328	0.369	0.672	0.529	1.054	0.529	0.289	0.359	0.668	0.3	0.236	0.645	0.114	0.555	0.338	0.091	0.113	0.15	0.576	0.113	0.15	0.024
Skin IL-4/IFN-γ	1.515	1.22	2.796	2.327	6.259	0.078	0.58	0.348	1.335	0.046	0.183	0.15	0.348	0.24	1.409	2.431	0.063	0.199	1.467	2.293	0.98	1.273	1.68	0.249	2.079	1.248	0.748	1.017	1.094	1.47	1.017	0.135	0.481
Skin IL-2	0.257	0.137	0.064	0.092	0.078	6.259	1.155	0.051	0.501	1.744	0.238	0.122	0.169	0.003	0.186	0.081	1.035	0.566	0.148	0.132	0.233	0.069	0.429	0.274	0.185	0.507	0.064	0.04	0.038	0.003	0.04	0.186	0.038
Skin TNF-α	0.412	0.135	0.199	0.81	0.58	1.155	6.259	0.135	0.238	0.837	0.135	0	0.217	0.304	0.434	0.779	0.606	0.199	0.668	0.13	0.408	0.028	0.081	0.069	0.04	0.01	0.012	0.025	0.037	0.51	0.113	0.434	0.12
Skin IL-6	0.369	1.264	0.875	0	0.348	0.051	0.135	6.259	1.94	0.046	1.134	0.05	0.308	0.116	0.748	0.944	0.977	0.39	1.096	0.903	1.595	1.707	0.525	0.814	0.978	1.142	1.515	1.829	1.978	1.705	1.775	0.105	1.759
Skin IL-17	1.198	1.433	1.864	0.516	1.335	0.501	0.238	1.94	5.782	0.281	0.54	0.025	0.078	0.003	1.486	1.433	0.186	0.275	2.186	2.186	1.109	1.854	1.301	0.587	2.186	2.245	1.542	1.795	1.864	1.722	2.42	0.078	1.433
Skin IL-10	0.021	0.514	0.003	0.021	0.046	1.744	0.837	0.046	0.281	6.259	0.06	0.054	0.277	0.042	0.156	0.14	0.514	0.205	0.023	0.102	0.01	0.109	0.569	0.076	0.05	0.135	0.477	0.169	0.156	0.014	0.071	0.514	0.128
Serum IL-4	0.348	0.555	0.348	0.166	0.183	0.238	0.135	1.134	0.54	0.05	6.259	0.199	0.108	1.82	0.661	1.134	1.359	1.22	0.453	0.729	2.146	1.417	0.602	2.031	0.798	0.764	1.409	1.096	1.176	1.102	1.273	0.412	2.543
Serum IFN-γ	0.369	0.166	0.15	0.328	0.15	0.122	0	0.05	0.025	0.054	0.199	6.259	3.273	0.453	0.412	0.077	0.875	1.359	0.316	0.13	0.053	0.163	0.277	0.317	0	0.003	0.27	0.04	0.135	0.121	0.383	1.054	0.308
Serum IL-4/IFN-γ	0.166	0.555	0.348	0.369	0.348	0.169	0.217	0.308	0.078	0.277	0.105	3.273	6.259	0.212	0.183	0.457	0.328	0.661	0.082	0.097	0.381	0.475	0.356	0.035	0.236	0.17	0.063	0.256	0.481	0.437	0.068	0.944	0
Serum IL-2	0.067	0.046	0.162	0.672	0.24	0.003	0.304	0.116	0.003	0.042	1.82	0.453	0.212	6.259	0.003	0.101	1.089	0.889	0.057	0.15	0.468	0.321	0.27	1.674	0.203	0.12	0.398	0.126	0.188	0.021	0.459	0.179	1.24
Serum TNF-α	3.966	1.016	1.573	0.528	1.409	0.186	0.434	0.748	1.486	0.156	0.661	0.412	0.183	0.003	6.259	2.142	0.39	0.81	2.782	2.003	1.187	1.467	0.923	0.381	1.707	2.31	1.978	1.775	1.134	2.31	1.576	0.718	0.779
Serum IL-6	1.826	1.573	2.057	1.054	2.431	0.051	0.779	0.944	1.433	0.14	1.134	0.077	0.457	0.101	2.142	6.259	0.328	0.505	1.637	2.536	2.059	2.171	1.379	0.75	2.394	2.031	1.409	1.707	1.826	3.216	1.467	0.234	1.134
Serum IL-17	0.289	0.217	0.05	0.529	0.063	1.035	0.606	0.077	0.186	0.514	1.359	0.875	0.328	1.089	0.39	0.328	6.259	2.543	0.199	0.199	0.722	0.336	0.028	0.881	0.163	0.063	0.58	0.383	0.308	0.267	0.429	0.718	0.555
Serum IL-10	0.606	0.555	0.529	0.289	0.199	0.566	0.199	0.39	0.275	0.205	1.22	1.359	0.661	0.889	0.81	0.505	2.543	6.259	0.829	0.729	1.032	0.765	0.399	1.12	0.637	0.317	1.264	0.903	0.748	0.485	1.363	1.016	1.134
Bacteroidales_unclassified	2.782	1.829	2.293	0.359	1.467	0.149	0.068	1.096	2.186	0.023	0.453	0.316	0.082	0.057	2.782	1.637	0.199	0.829	4.481	2.868	0.964	1.84	1.11	0.564	2.688	2.572	1.829	2.093	1.637	1.785	2.173	0.256	0.903
Duncanella	2.171	2.079	2.661	0.668	2.293	0.132	0.13	0.903	2.186	0.102	0.729	0.13	0.097	0.16	2.003	2.536	0.199	0.729	2.868	4.481	1.231	2.043	1.587	0.887	3.782	2.572	1.417	1.84	2.003	1.785	2.173	0.068	1.227
Muribaculum	0.652	1.249	1.447	0.3	0.98	0.233	0.408	1.595	1.109	0.01	2.146	0.053	0.381	0.468	1.187	2.059	0.722	1.032	0.964	1.231	3.401	2.447	1.33	1	1.281	0.983	1.954	2.079	2.255	2.123	1.447	0.535	1.954
Parabacteroides	1.141	2.079	2.394	0.236	1.273	0.069	0.025	1.707	1.854	0.109	1.417	0.163	0.475	0.321	1.467	2.171	0.336	0.765	1.84	2.043	2.447	4.481	2.193	1.086	2.688	1.998	2.394	3.334	3.481	2.459	2.173	0.13	1.707
Ligilactobacillus	0.805	0.889	2.184	0.645	1.60	0.429	0.081	0.525	1.301	0.569	0.602	0.277	0.356	0.27	0.923	1.379	0.628	0.399	1.11	1.587	1.33	2.193	6.259	0.772	1.934	1.228	1.56	1.653	1.282	1.069	1.433	0.205	1.07
Muribaculaceae_unclassified	0.249	0.414	0.348	0.114	0.249	0.274	0.069	0.814	0.587	0.075	2.031	0.317	0.035	1.674	0.381	0.75	0.881	1.12	0.564	0.887	1	1.086	0.772	5.18	0.95	0.688	1.292	0.807	0.689	0.448	1.504	0.338	2.64
Alistipes	1.829	2.293	2.782	0.555	2.079	0.185	0.04	0.978	2.186	0.05	0.798	0	0.236	0.203	1.707	2.394	0.163	0.637	2.688	3.782	1.281	2.688	1.934	0.95	4.481	2.655	1.467	1.992	2.293	1.785	2.173	0	1.273
Clostridiales_unclassified	2.415	1.548	1.579	0.338	1.248	0.507	0.01	1.142	2.245	0.135	0.764	0.003	0.17	0.12	2.31	2.031	0.063	0.317	2.572	2.572	0.983	1.998	1.228	0.688	2.655	5.18	1.579	1.929	1.604	2.043	1.785	0	1.005
Bacteroides	1.22	0.842	1.359	0.091	0.748	0.064	0.012	1.515	1.542	0.477	1.409	0.27	0.063	0.398	1.978	1.409	0.58	1.264	1.829	1.417	1.954	2.394	1.56	1.292	1.467	1.579	6.259	3.003	1.409	1.731	2.661	1.016	2.057
Butyrivibrio	1.317	2.003	2.293	0.113	1.017	0.04	0.025	1.829	1.795	0.169	1.096	0.04	0.256	0.126	1.775	1.707	0.383	0.903	2.093	1.84	2.079	3.334	1.653	0.807	1.992	1.929	3.003	4.481	2.782	2.459	2.173	0.274	1.363
Candidatus_Saccharibacteria_unclassified	0.91	3.476	2.327	0.18	1.094	0.035	0.037	1.978	1.864	0.156	1.176	0.135	0.481	0.188	1.134	1.826	0.308	0.748	1.637	2.003	2.255	3.481	1.282	0.689	2.293	1.604	1.409	2.782	6.259	2.479	2.171	0.05	1.359
Acetatifactor	1.64	1.869	1.995	0.576	1.47	0.003	0.51	1.705	1.722	0.014	1.102	0.121	0.437	0.021	2.31	3.216	0.267	0.485	1.785	1.785	2.123	2.459	1.069	0.448	1.785	2.043	1.731	2.459	2.479	5.18	1.567	0.24	1.063
Firmicutes_unclassified	1.227	1.576	1.775	0.113	1.017	0.04	0.113	1.775	2.42	0.071	1.273	0.383	0.068	0.459	1.576	1.467	0.429	1.363	2.173	2.173	1.447	2.173	1.433	1.504	2.173	1.785	2.661	2.173	2.171	1.567	4.481	0.383	2.536
Lachnospiraceae_unclassified	0.328	0.289	0.091	0.15	0.135	0.186	0.434	0.105	0.078	0.514	0.412	1.054	0.944	0.179	0.718	0.234	0.718	0.106	0.256	0.068	0.535	0.13	0.205	0.338	0	0	1.016	0.274	0.05	0.24	0.383	6.259	0.529
Prevotellaceae_unclassified	0.412	0.661	0.718	0.024	0.481	0.038	0.12	1.759	1.433	0.125	2.543	0.308	0	1.24	0.779	1.134	0.555	1.134	0.903	1.227	1.954	1.707	1.07	2.64	1.273	1.005	2.057	1.363	1.359	1.063	2.536	0.529	6.259

Supplementary Figure 5. The correlation P-value matrix.

		Thickness	Scratching	Skin IL-4	Skin IFN-γ	Skin IL-4/IFN-γ	Skin IL-2	Skin TNF-α	Skin IL-6	Skin IL-17	Skin IL-10	Serum IL-4	Serum IFN-γ	Serum IL-4/IFN-γ	Serum IL-2	Serum TNF-α	Serum IL-6	Serum IL-17	Serum IL-10	Bacteroidales_unclassified	Duncanella	Muribaculum	Parabacteroides	Ligilactobacillus	Muribaculaceae_unclassified	Alistipes	Clostridiales_unclassified	Bacteroides	Butyrivibrio	Candidatus_Saccharibacteria_unclassified	Acetatifactor	Firmicutes_unclassified	Lachnospiraceae_unclassified	Prevotellaceae_unclassified
	Thickness	1	0.588	0.697	-0.38	0.697	0.215	-0.31	0.285	0.62	0.024	0.273	0.285	-0.15	-0.07	0.952	0.758	0.236	0.406	-0.89	-0.82	-0.42	-0.6	-0.48	-0.21	-0.77	-0.84	0.624	-0.65	0.527	0.725	0.627	-0.26	0.309
	Scratching	0.588	1	0.842	-0.18	0.624	-0.13	-0.13	0.636	0.681	-0.36	0.382	-0.15	0.382	-0.05	0.564	0.709	0.188	0.382	-0.77	-0.81	-0.62	-0.81	-0.52	-0.31	-0.83	-0.71	0.503	-0.8	0.927	0.769	0.718	0.236	0.43
	Skin IL-4	0.697	0.842	1	-0.52	0.878	0.067	-0.18	0.515	0.767	0.006	0.273	-0.14	0.273	-0.15	0.709	0.794	0.555	0.37	-0.83	-0.87	-0.68	-0.85	-0.81	-0.27	-0.89	-0.71	0.661	-0.83	0.83	0.788	0.756	-0.09	0.455
	Skin IFN-γ	-0.38	-0.18	-0.52	1	-0.83	-0.09	0.491	0.006	-0.36	-0.02	0.152	0.261	-0.28	0.432	-0.37	-0.58	0.37	0.236	0.278	0.433	0.24	0.2	0.419	-0.11	0.381	0.263	-0.09	0.11	-0.14	-0.39	-0.11	0.139	-0.03
	Skin IL-4/IFN-γ	0.697	0.624	0.878	-0.83	1	0.08	-0.39	0.273	0.656	-0.05	0.164	-0.14	0.273	-0.2	0.673	0.842	-0.07	0.176	-0.69	-0.83	-0.55	-0.64	-0.73	-0.21	-0.81	-0.63	0.467	-0.56	0.588	0.688	0.562	-0.13	0.345
	Skin IL-2	0.215	-0.13	0.067	-0.09	0.08	1	0.607	-0.06	0.354	0.745	-0.2	-0.12	-0.15	-0.01	0.166	-0.06	-0.57	-0.39	-0.14	-0.12	0.194	-0.07	-0.31	0.222	-0.16	-0.35	0.067	-0.05	-0.04	-0.01	0.046	0.166	-0.04
	Skin TNF-α	-0.31	-0.13	-0.18	0.491	-0.39	0.607	1	0.127	0.202	0.498	-0.13	-0.01	-0.19	0.243	-0.32	-0.48	-0.41	-0.18	0.071	0.123	0.302	0.032	-0.08	-0.07	0.045	-0.01	-0.02	0.032	-0.04	-0.36	0.11	0.321	0.115
	Skin IL-6	0.285	0.636	0.515	0.006	0.273	-0.06	0.127	1	0.779	0.049	0.6	-0.05	0.248	0.109	0.467	0.539	0.079	0.297	-0.59	-0.52	-0.71	-0.74	-0.36	-0.49	-0.55	-0.6	0.697	-0.77	0.782	0.738	0.756	-0.1	0.746
	Skin IL-17	0.62	0.681	0.767	-0.36	0.656	0.354	0.202	0.779	1	0.228	0.374	0.031	0.08	0.006	0.693	0.681	-0.17	0.227	-0.83	-0.83	-0.59	-0.78	-0.65	-0.39	-0.83	-0.83	0.706	-0.77	0.767	0.747	0.857	-0.08	0.681
	Skin IL-10	0.024	-0.36	0.006	-0.02	-0.05	0.745	0.498	0.049	0.228	1	-0.06	-0.05	-0.22	0.043	0.14	-0.13	-0.36	-0.18	-0.03	0.097	-0.01	-0.1	-0.38	0.072	0.052	-0.12	0.34	-0.15	-0.14	-0.02	0.071	-0.36	0.116
	Serum IL-4	0.273	0.382	0.273	0.152	0.164	-0.2	-0.13	0.6	0.374	-0.06	1	0.176	0.103	0.754	0.43	0.6	0.661	0.624	-0.33	-0.46	-0.82	-0.68	-0.4	-0.79	-0.48	-0.47	0.673	-0.59	0.612	0.588	0.64	-0.31	0.855
	Serum IFN-γ	0.285	-0.15	-0.14	0.261	-0.14	-0.12	-0.01	-0.05	0.031	-0.05	0.176	1	-0.92	0.328	0.309	-0.08	0.515	0.661	-0.25	-0.12	0.055	0.149	0.225	-0.25	-0.01	0.224	0.045	-0.13	-0.11	0.291	-0.58	0.248	
	Serum IL-4/IFN-γ	-0.15	0.382	0.273	-0.28	0.273	-0.15	-0.19	0.248	0.08	-0.22	0.103	-0.92	1	-0.18	-0.16	0.333	-0.26	-0.43	0.084	-0.1	-0.29	-0.34	-0.27	-0.04	-0.2	-0.15	-0.07	-0.21	0.345	0.319	-0.07	0.539	-0.01
	Serum IL-2	-0.07	-0.05	-0.15	0.432	-0.2	-0.01	0.243	0.109	0.006	0.043	0.754	0.328	-0.18	1	-0.01	0.097	0.584	0.517	0.058	-0.14	-0.33	-0.25	-0.22	-0.73	-0.18	-0.11	0.298	-0.12	0.164	-0.02	0.331	-0.16	0.626
	Serum TNF-α	0.952	0.564	0.709	-0.37	0.673	0.166	-0.32	0.467	0.693	0.14	0.43	0.309	-0.16	-0.01	1	0.806	0.297	0.491	-0.89	-0.8	-0.61	-0.69	-0.53	-0.29	-0.74	-0.83	0.782	-0.76	0.6	0.632	0.718	-0.45	0.479
	Serum IL-6	0.758	0.709	0.794	-0.58	0.842	-0.06	-0.48	0.539	0.681	-0.13	0.6	-0.08	0.333	0.097	0.806	1	0.261	0.358	-0.73	-0.86	-0.81	-0.82	-0.66	-0.46	-0.85	-0.79	0.673	-0.74	0.758	0.913	0.692	-0.2	0.6
	Serum IL-17	0.236	0.188	0.055	0.37	-0.07	-0.57	-0.41	0.079	-0.17	-0.36	0.661	0.515	-0.26	0.584	0.297	0.261	1	0.855	-0.17	-0.17	-0.45	-0.27	-0.03	-0.51	-0.15	-0.06	0.394	-0.29	0.248	0.219	0.317	-0.45	0.382
	Serum IL-10	0.406	0.382	0.37	0.236	0.176	-0.39	-0.18	0.297	0.227	-0.18	0.624	0.661	-0.43	0.517	0.491	0.358	0.855	1	-0.5	-0.46	-0.56	-0.47	-0.3	-0.59	-0.42	-0.25	0.636	-0.52	0.467	0.344	0.666	-0.56	0.6
	Bacteroidales_unclassified	-0.89	-0.77	-0.83	0.278	-0.69	-0.14	0.071	-0.59	-0.83	-0.03	-0.33	-0.25	0.084	0.058	-0.89	-0.73	-0.17	-0.5	1	0.903	0.534	0.766	0.59	0.38	0.876	0.874	-0.77	0.834	-0.73	-0.77	-0.86	0.213	-0.52
	Duncanella	-0.82	-0.81	-0.87	0.433	-0.83	-0.12	0.123	-0.52	-0.83	0.097	-0.46	-0.12	-0.1	-0.14	-0.8	-0.86	-0.17	-0.46	0.903	1	0.629	0.821	0.72	0.514	0.886	0.874	-0.68	0.766	-0.8	-0.77	-0.86	0.071	-0.63
	Muribaculum	-0.42	-0.62	-0.68	0.24	-0.55	0.194	0.302	-0.71	-0.59	-0.01	-0.82	0.055	-0.29	-0.33	-0.61	-0.81	-0.45	-0.56	0.534	0.629	1	0.87	0.653	0.559	0.651	0.545	-0.78	0.812	-0.83	-0.85	-0.73	0.37	-0.78
	Parabacteroides	-0.6	-0.81	-0.85	0.2	-0.64	-0.07	0.032	-0.74	-0.78	-0.1	-0.68	0.149	-0.34	-0.25	-0.69	-0.82	-0.27	-0.47	0.766	0.821	0.87	1	0.824	0.58	0.876	0.807	-0.85	0.959	-0.92	-0.87	-0.86	0.123	-0.74
	Ligilactobacillus	-0.49	-0.52	-0.81	0.419	-0.73	-0.31	-0.08	-0.36	-0.65	-0.38	-0.4	0.225	-0.27	-0.22	-0.53	-0.66	-0.03	-0.3	0.59	0.72	0.653	0.824	1	0.47	0.785	0.624	-0.71	0.733	-0.64	-0.58	-0.68	0.176	-0.58
	Muribaculaceae_unclassified	-0.21	-0.31	-0.27	-0.11	-0.21	0.222	-0.07	-0.49	-0.39	0.072	-0.79	-0.25	-0.04	-0.73	-0.29	-0.46	-0.51	-0.59	0.38	0.514	0.559	0.58	0.47	1	0.54	0.435	-0.64	0.487	-0.44	-0.32	-0.7	0.263	-0.87
	Alistipes	-0.77	-0.83	-0.89	0.381	-0.81	-0.16	0.045	-0.55	-0.83	0.052	-0.48	-0.01	-0.2	-0.18	-0.74	-0.85	-0.15	-0.42	0.876	0.986	0.651	0.876	0.785	0.54	1	0.887	-0.69	0.807	-0.83	-0.77	-0.86	-0.01	-0.64
	Clostridiales_unclassified	-0.84	-0.71	-0.71	0.263	-0.63	-0.35	-0.01	-0.6	-0.83	-0.12	-0.47	-0.01	-0.15	-0.11	-0.83	-0.79	-0.06	-0.25	0.874	0.874	0.545	0.807	0.624	0.435	0.887	1	-0.71	0.794	-0.72	-0.81	-0.77	0	-0.56
	Bacteroides	0.624	0.503	0.661	-0.09	0.467	0.067	-0.02	0.697	0.706	0.34	0.673	0.224	-0.07	0.298	0.782	0.673	0.394	0.636	-0.77	-0.68	-0.78	-0.85	-0.71	-0.64	-0.69	-0.71	1	-0.9	0.673	0.744	0.873	-0.56	0.794
	Butyrivibrio	-0.65	-0.8	-0.83	0.11	-0.56	-0.05	0.032	-0.77	-0.77	-0.15	-0.59	0.045	-0.21	-0.12	-0.76	-0.74	-0.29	-0.52	0.834	0.766	0.812	0.959	0.733	0.487	0.807	0.794	-0.9	1	-0.89	-0.87	-0.86	0.226	-0.67
	Candidatus_Saccharibacteria_unclassified	0.527	0.927	0.83	-0.14	0.588	-0.04	-0.04	0.782	0.767	-0.14	0.612	-0.13	0.345	0.164	0.6	0.758	0.248	0.467	-0.73	-0.8	-0.83	-0.92	-0.64	-0.44	-0.83	-0.72	0.673	-0.89	1	0.85	0.821	0.055	0.661
	Acetatifactor	0.725	0.769	0.788	-0.39	0.688	-0.01	-0.36	0.738	0.747	-0.02	0.588	-0.11	0.319	-0.02	0.832	0.913	0.219	0.344	-0.77	-0.77	-0.85	-0.87	-0.58	-0.32	-0.77	-0.81	0.744	-0.87	0.85	1	0.714	-0.2	0.575
	Firmicutes_unclassified	0.627	0.718	0.756	-0.11	0.562	0.046	0.11	0.756	0.857	0.071	0.64	0.291	-0.07	0.331	0.718	0.692	0.317	0.666	-0.86	-0.86	-0.73	-0.86	-0.68	-0.7	-0.86	-0.77	0.873	-0.86	0.821	0.714	1	-0.29	0.86
	Lachnospiraceae_unclassified	-0.26	0.236	-0.09	0.139	-0.13	0.166	0.321	-0.1	-0.08	-0.36	-0.31	-0.58	0.539	-0.16	-0.45	-0.2	-0.45	-0.56	0.213	0.071	0.37	0.123	0.176	0.263	-0.01	0	-0.56	0.226	0.055	-0.2	-0.29	1	-0.37
	Prevotellaceae_unclassified	0.309	0.43	0.455	-0.03	0.345	-0.04	0.115	0.745	0.681	0.116	0.855	0.248	-0.01	0.626	0.479	0.6	0.382	0.6	-0.52	-0.63	-0.78	-0.74	-0.58	-0.87	-0.64	-0.56	0.794	-0.67	0.661	0.575	0.86	-0.37	1

Supplementary Figure 6. The correlation r-value matrix.

Supplementary Table 1. Details about statistical tests

Figures	Method	t, F, df value	P value	Post hoc method	Remarks
Figure 1B	Two-way ANOVA	F (2, 18) = 3.582	0.049	LSD	
Figure 1C	Student's t-test	t=2.656, df=10	0.0241	NA	
Figure 2C	One-way ANOVA	F (2, 12) = 33.76	<0.0001	Tukey	
Figure 2D	One-way ANOVA	F (2, 12) = 189.2	<0.0001	Tukey	
Figure 2E	Student's t-test			NA	
Figure 3D	One-way ANOVA	F (2, 12) = 72.13	<0.0001	Tukey	
Figure 4D	One-way ANOVA	F (2, 12) = 33.34	<0.0001	Tukey	
Figure 5A	Mann Whitney test	Mann-Whitney U=0	0.0079	NA	
Figure 5B	Student's t-test	t=0.3339, df=8	0.7471	NA	
Figure 5C	Mann Whitney test	Mann-Whitney U=2	0.0317	NA	
Figure 5D	Student's t-test	t=0.06435, df=8	0.9503	NA	
Figure 5E	Student's t-test	t=0.4482, df=8	0.6659	NA	
Figure 5F	Student's t-test	t=2.752, df=8	0.025	NA	
Figure 5G	Student's t-test	t=4.626, df=8	0.0017	NA	
Figure 5H	Student's t-test	t=0.09125, df=8	0.9295	NA	
Figure 6A	Student's t-test	t=1.776, df=10	0.1061	NA	
Figure 6B	Student's t-test	t=0.3682, df=10	0.7204	NA	
Figure 6C	Student's t-test	t=0.1432, df=10	0.889	NA	
Figure 6D	Student's t-test	t=0.7756, df=10	0.4559	NA	
Figure 6E	Student's t-test	t=4.794, df=10	0.0007	NA	
Figure 6F	Mann Whitney test	Mann-Whitney U=3	0.0152	NA	
Figure 6G	Student's t-test	t=1.350, df=10	0.2068	NA	
Figure 6H	Student's t-test	t=1.263, df=10	0.2353	NA	
Figure 7A	One-way ANOVA	F (2, 12) = 4.051	0.0452	Tukey	
Figure 7B	One-way ANOVA	F (2, 12) = 7.141	0.0091	Tukey	
	Kruskal-Wallis test	11.22	0.0002	Dunn	Bacteroidales_unclassified
	Kruskal-Wallis test	11.59	<0.0001	Dunn	Duncaniella
	Kruskal-Wallis test	10.65	0.0007	Dunn	Muribaculum
	Kruskal-Wallis test	10.49	0.0005	Dunn	Parabacteroides
	Kruskal-Wallis test	6.992	0.021	Dunn	Ligilactobacillus
	Kruskal-Wallis test	10.69	0.0003	Dunn	Muribaculaceae_unclassified
	Kruskal-Wallis test	9.976	0.0015	Dunn	Alistipes
	Kruskal-Wallis test	9.673	0.0019	Dunn	Clostridiales_unclassified
	Kruskal-Wallis test	9.5	0.0021	Dunn	Bacteroides
	Kruskal-Wallis test	7.418	0.0139	Dunn	Butyricoccus
	Kruskal-Wallis test	10.16	0.0007	Dunn	Candidatus_Saccharibacteria_unclassified
	Kruskal-Wallis test	7.632	0.0143	Dunn	Acetatifactor

Figure 8	Kruskal-Wallis test	8.889	0.0054	Dunn	Firmicutes_unclassified
	Kruskal-Wallis test	6.32	0.0333	Dunn	Lachnospiraceae_unclassified
	Kruskal-Wallis test	7.74	0.0123	Dunn	Prevotellaceae_unclassified
	Spearman's rank correlation	NA	NA	NA	Supplementary Figures 5 and 6
