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PII: S0091-6749(20)30184-6

DOI: <https://doi.org/10.1016/j.jaci.2020.01.044>

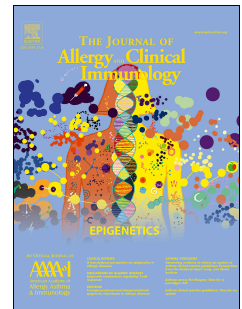
Reference: YMAI 14412

To appear in: *Journal of Allergy and Clinical Immunology*

Received Date: 5 October 2019

Revised Date: 19 January 2020

Accepted Date: 22 January 2020



Please cite this article as: Park H-K, Choi Y, Lee D-H, Kim S, Lee J-M, Choi SW, Lee H-R, Rho M, Park H-S, Altered gut microbiota by azithromycin attenuates airway inflammation in allergic asthma, *Journal of Allergy and Clinical Immunology* (2020), doi: <https://doi.org/10.1016/j.jaci.2020.01.044>.

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Altered gut microbiota by azithromycin attenuates airway inflammation in allergic asthma

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Conflicts of Interest

The authors declare no competing financial interest.

Acknowledgments

This study was supported by a grant from the Korean Health Technology R & D Project, Ministry of Health & Welfare, Republic of Korea (HI16C0992). This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2018R1D1A1B07050941).

Author contributions

Han-Ki Park and Youngwoo Choi designed and performed the experiments and wrote the manuscript; Dong-Hyun Lee performed the experiments; Sae Won Choi revised the manuscript; Sujeong Kim and Jong-Myung Lee helped design the experiments; MiNa Ro analyzed metagenomic data; Hae-Sim Park supervised all the processes.

Capsule Summary

Allergic inflammation in the asthmatic airway may change gut microbiota; azithromycin treatment could alter gut microbiota (especially increased proportion of Clostridiales), reducing airway inflammation.

Key words: Allergic asthma, gut microbiota, *clostridiales*, acetate, airway hyperresponsiveness, Antibiotics

Altered gut microbiota by azithromycin attenuates airway inflammation in allergic asthma

To the Editor:

Recent epidemiological and experimental studies have demonstrated that the dysbiosis of gut microbiota is linked to airway disease through modification of the immune response, which is called the gut-lung axis. In allergic asthma, altered gut microbiota of fetal age in mice has been reported to augment allergic sensitization.^{1,2} Moreover, epidemiological studies have shown that the use of antibiotics and a modern lifestyle in infancy increase the prevalence of asthma.³ However, despite the emerging evidence of the gut-lung axis, there is still a lack of insight into the relationship between gut microbiota and allergic airway inflammation.

Antibiotics induce changes in gut microbiota even after a short period of 1 to 2 weeks, inducing the “dysbiosis” effect or the “eubiotic” effect depending on the class of antibiotics.⁴ Azithromycin has been discussed as a candidate treatment for airway inflammatory disease.⁵ Although antibiotics affect gut microbiota, studies on how azithromycin affects gut microbiota in patients with uncontrolled airway inflammation are scarce. The present study aimed to evaluate whether changes in gut microbiota could modulate Th2-driven airway inflammation in allergic asthma regardless of allergen sensitization. To confirm our hypothesis, we compared the effects of 2 antibiotics (having different mechanisms, cefixime vs azithromycin) in ovalbumin-induced (OVA) asthma mouse model during the OVA challenge period (Fig 1, A).

The present study confirmed markedly elevated airway hyperresponsiveness (AHR) and

Th2-airway inflammation markers (eosinophilia and IL-4/IL-5/L-13) in bronchial alveolar lavage fluid (BALF) of the OVA-challenged mice with no treatment of antibiotics (OVA-none group) compared with the control group (Fig E1). Then, we analyzed bacterial diversity and composition in BALF and feces by 16s metagenome analysis. **As shown in previous study,¹ airway inflammation induces dysbiosis in the gut microbiota without altering the airway microbiome (Fig 1, B–E and Fig E2).**

To induce changes in gut microbiota in allergic asthma mice, cefixime or azithromycin was orally administered during the challenge period. Greater reductions in AHR and Th2-inflammation markers in BALF were noted in the azithromycin-treated group than in the cefixime-treated and OVA-none group (Fig E1), implicating that azithromycin may have a function against AHR and airway inflammation in allergic asthma. To identify mechanisms by which gut microbiota affects airway inflammation, this study further analyzed the microbiome in BALF and feces. **In feces**, cefixime and azithromycin treatment reduced the bacterial load (Fig. 1, B), but not diversity (Fig. 1, C and D). The gut microbial composition was significantly altered in each group (Fig 1, E and Fig E2). Bacteroidaceae, which was the dominant gut microbiota in the control and OVA-none groups, was almost completely eliminated by both cefixime and azithromycin. However, Clostridiales such as Ruminococcaceae and Lachnospiraceae was significantly increased in the azithromycin-treated group, while no significant difference was noted in the cefixime-treated group (Fig 1, F and Fig E4) Even without OVA sensitization, azithromycin-induced changes in gut microbiota were maintained. (Fig E5).

Many commensal clostridia, especially *Clostridium* clusters IV and XIVa, have been reported to have anti-inflammatory effects through the production of short chain fatty acids (SCFA). Recent studies have reported that SCFA may reduce allergic airway inflammation by

limiting eosinophil trafficking and survival.^{6,7} The present study demonstrated that clostridiales was significantly elevated in allergic mice after azithromycin treatment, so we assessed alterations in the fecal levels of SCFA. The propionate level was significantly decreased in the cefixime group (not in the azithromycin group) compared to the OVA-treated group; the acetate level was higher in the azithromycin group than in the OVA-treated group. The total SCFA level in the azithromycin group tended to increase than in the OVA-treated group, although it was not statistically significant (Fig. 1, G). To evaluate the function of acetate in the lungs, acetate was orally administered to allergic mice. As a result, AHR and Th2 cytokines levels in BALF were reduced. In addition, lung T cells isolated from the acetate-treated mice released less Th2 cytokines than those from the OVA-treated mice (Fig. E6).

To further validate our hypothesis, fecal microbiota transplantation (FMT) was performed (Fig 2, A). When the allergic mice received FMT, body weight was not significantly changed (Fig 2, B); however, significantly reduced AHR as well as decreased eosinophil counts and Th2 cytokines in BALF were observed (Fig 2, C–E). Moreover, airway epithelium thickness and mucus production were also reduced after FMT (Fig 2, F), indicating that gut microbiota could modulate airway inflammation in asthma exacerbation.

The current study demonstrated that allergic airway inflammation can be attenuated by controlling gut microbiota in a murine allergic asthma model. The use of antibiotics during the maturation of gut microbiota may affect allergen sensitization. However, in 6 to 8 weeks old mice that we used in our experiment, allergen sensitization was not affected since the gut microbiota has already matured.⁸ In addition, we administered antibiotics only during the challenge period, but not during the sensitization period which differed from previous studies' models that induced gut microbiota changes during the sensitization period.^{1,8} Another

previous studies reported that the lower respiratory tract microbiota is also associated with asthma inflammation. However, our study revealed that the airway microbiome is too small in terms of biomass to have a substantial effect in airway inflammation. These results suggest that the effects of antibiotics on the respiratory tract with not respiratory infections or pathogen colonization may not be significant, while the gut microbiome and its products are more important in controlling airway inflammation.

In this study, it was suggested that commensal *Clostridium* is important for explaining mechanisms underlying attenuation of airway inflammation by gut microbiota. Unlike young children in whom *Lactobacillus* and *Bifidobacterium* are crucial commensal floras, commensal *Clostridium*, *Bacteroides*, *Prevotella* have been shown to be the most numerous bacteria in the intestine of healthy adults.⁹ Our study showed that *Lactobacillus* disappeared in the control group after azithromycin administration (Fig E3), which indicates inconsistent findings on azithromycin effects in attenuating airway inflammation and acute exacerbation of asthma, and that the early use of macrolide in children is significantly associated with the development of asthma. The difference in the role of gut microbiome between the development of allergic asthma and the control of allergic airway inflammation could suggest a clinical implication that probiotics and prebiotics for airway inflammation after the onset of asthma may be different compared early childhood.

Increases in SCFA may be involved in mechanisms by which commensal *Clostridium* reduces allergic inflammation. SCFA was significantly increased in the azithromycin group than the cefixime group. However, SCFA except acetate were not significantly increased compared to the OVA-none group. Further research is needed to determine other mechanisms by which commensal *Clostridium* reduces allergic inflammation. In addition, since asthma in humans is a combination of more complex mechanisms and various asthma phenotypes,

studies on the relationship between changes in gut microbiota and asthma symptoms by azithromycin in humans are necessary.

In conclusion, we demonstrated that allergic inflammation in the asthmatic airway may change gut microbiota and that administration of azithromycin to allergic asthmatics could alter gut microbial composition (especially, the increased proportion of Clostridiales) and reduce airway inflammation, suggesting the close interaction between airway inflammation and gut microbiota in allergic asthma controls.

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FIGURE LEGEND

FIG 1. Alteration of gut and lung microbiota in mice. **A**, Experimental schedule. **B**, Bacterial loads **C**, Operational taxonomic unit (OTU). **D**, Shannon diversity index. **E**, Principal coordinates analysis of BALF (left panel) and feces (right panel). **F**, Relative abundance of Clostridiales in feces. **G**, Levels of short-chain fatty acid (SCFA) in fecal content. Data are represented as means $\pm$ SD, * $P < .05$, ** $P < .01$, and *** $P < .001$ were obtained by one-way ANOVA with Bonferroni's *post hoc* test. n.s., not significant.

FIG 2. Effect of fecal transfer from azithromycin-treated mice to allergic mice. **A**, Experimental protocol. **B**, Change in body weight. **C**, Airway hyperresponsiveness. **D**, Differential cell number in BALF. **E**, Concentration of BALF cytokines. **F**, Lung histology stained with H&E (upper panel) or PAS (lower panel). Scale bar, 100 μ m. Data are represented as means $\pm$ SD, $n = 5$. * $P < 0.05$, ** $P < .01$, and *** $P < .001$ were obtained by one-way ANOVA with Bonferroni's *post hoc* test. n.s., not significant.

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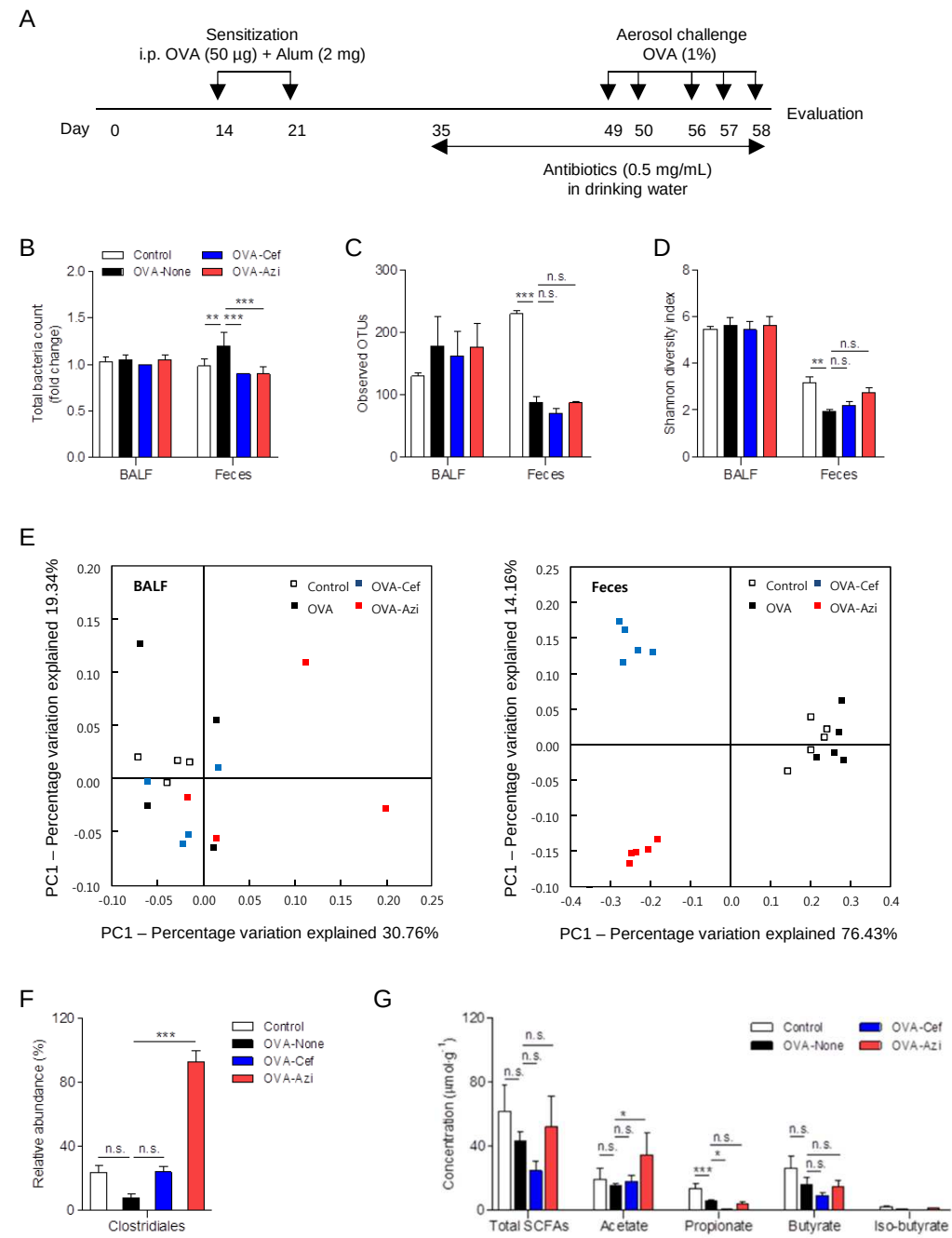


FIG 1.

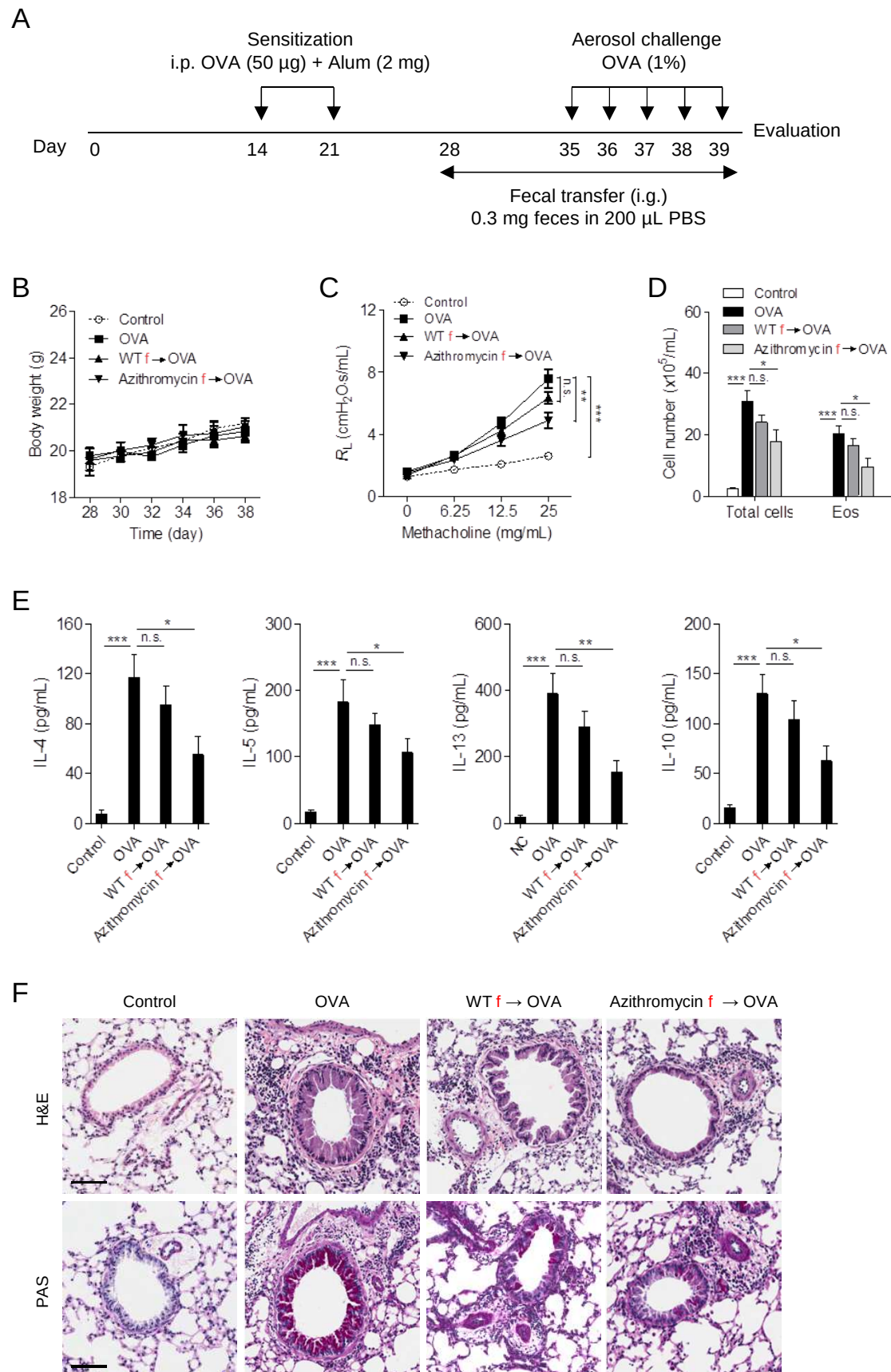


FIG 2.

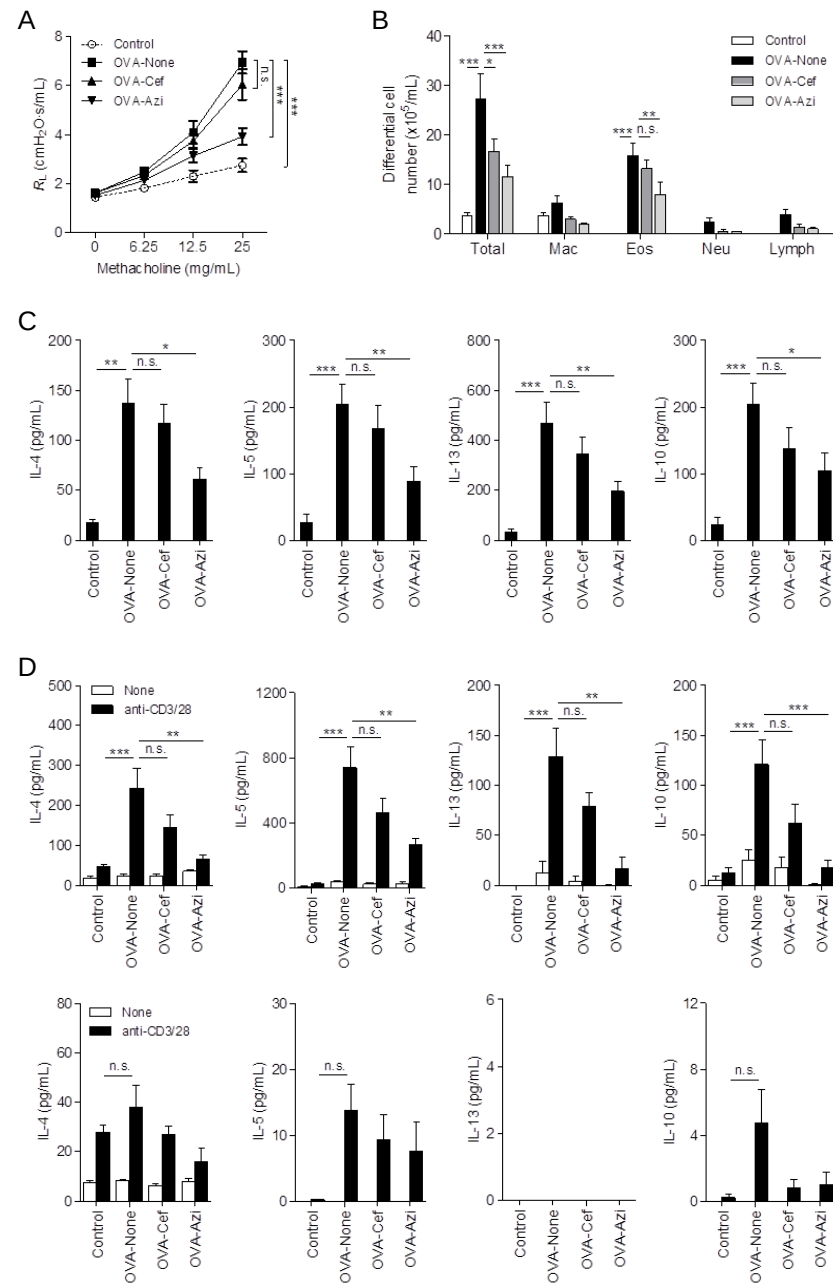
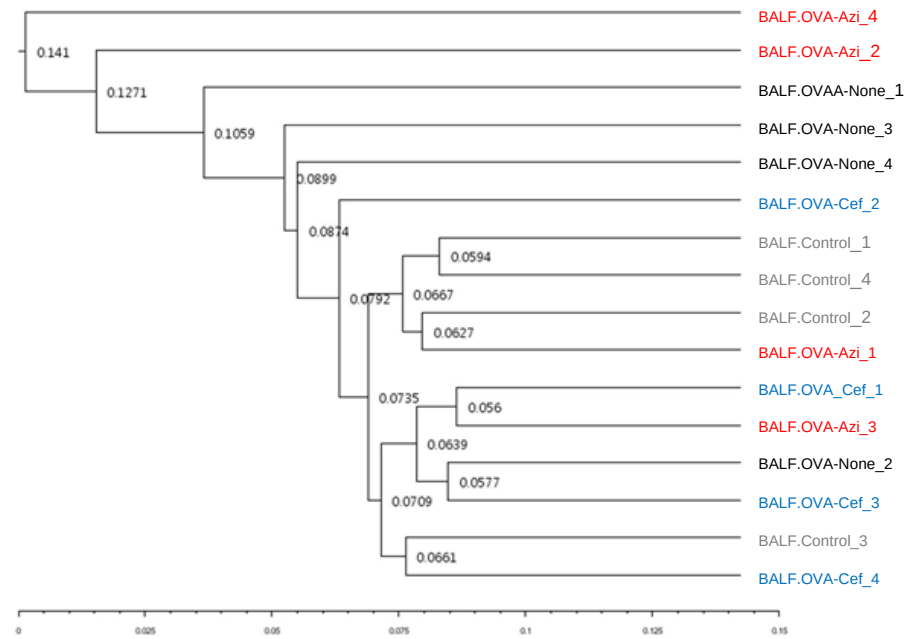


FIG E1.

A



B

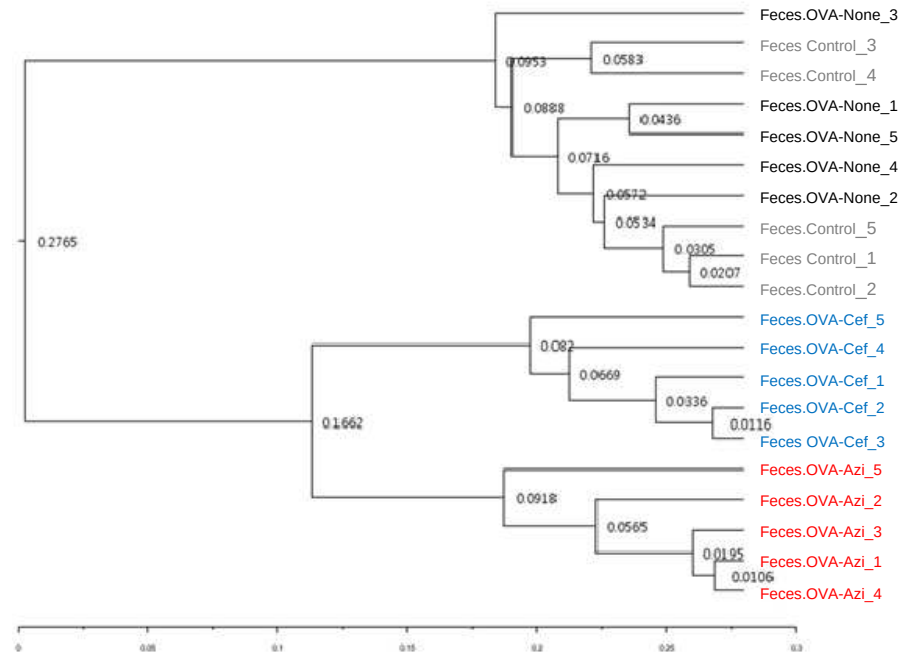


FIG E2.

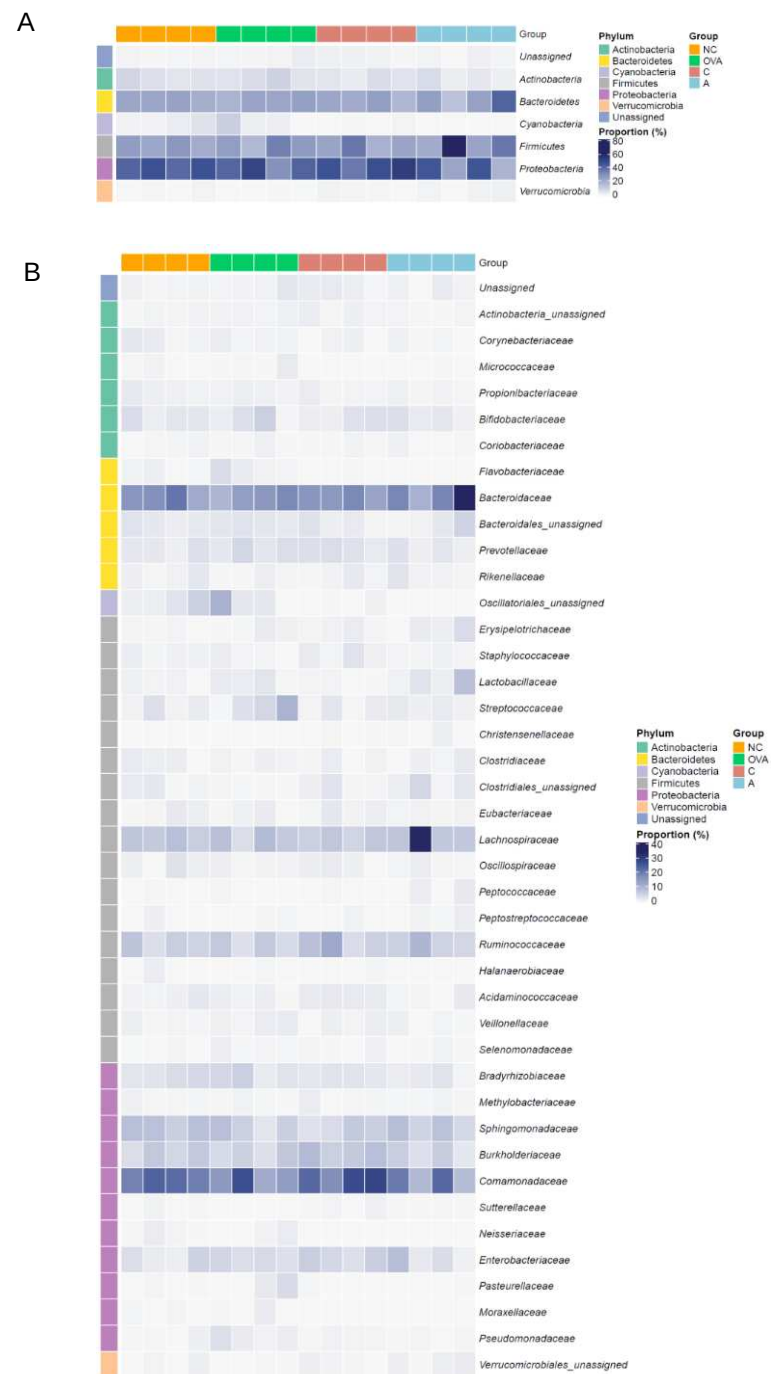


FIG E3.

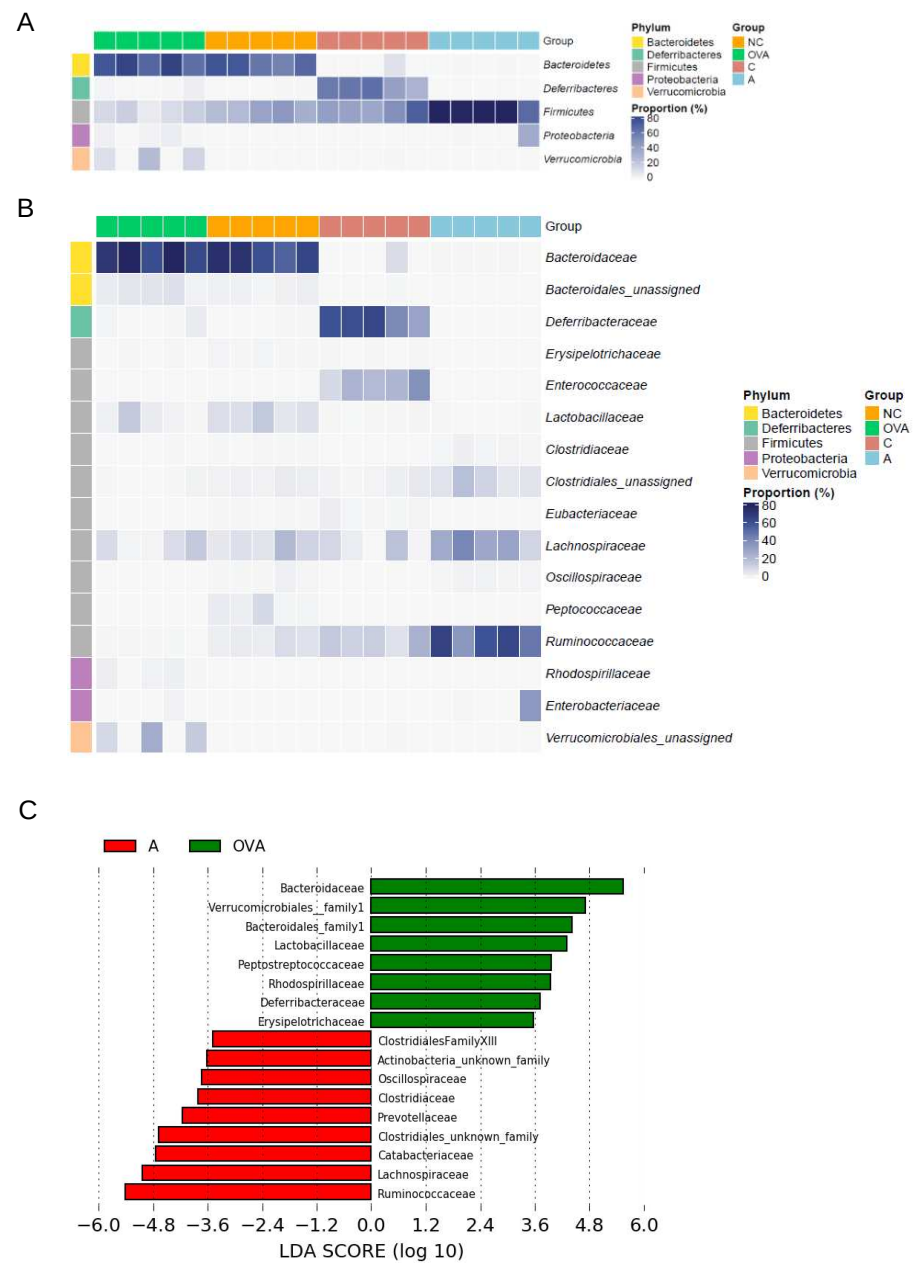


FIG E4.

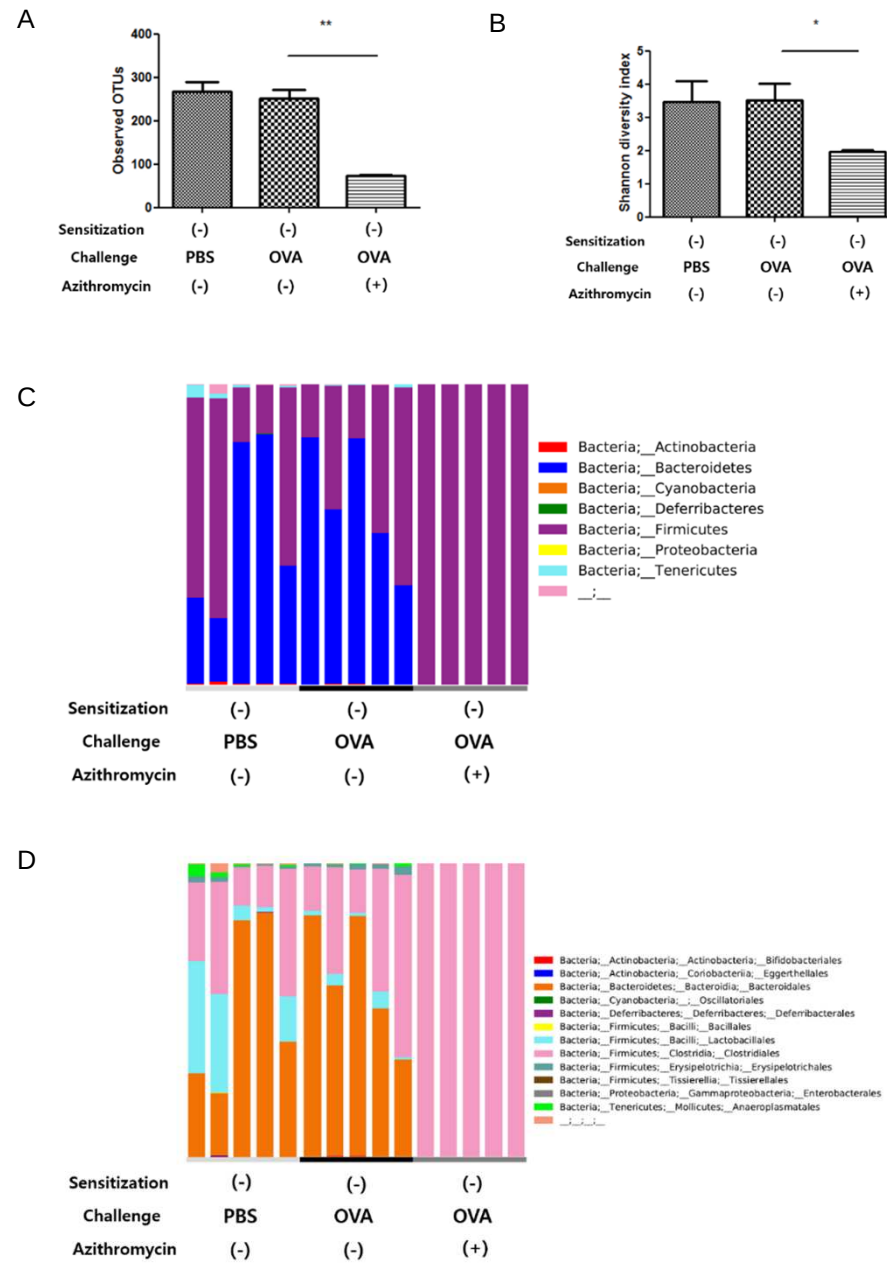


FIG E5.

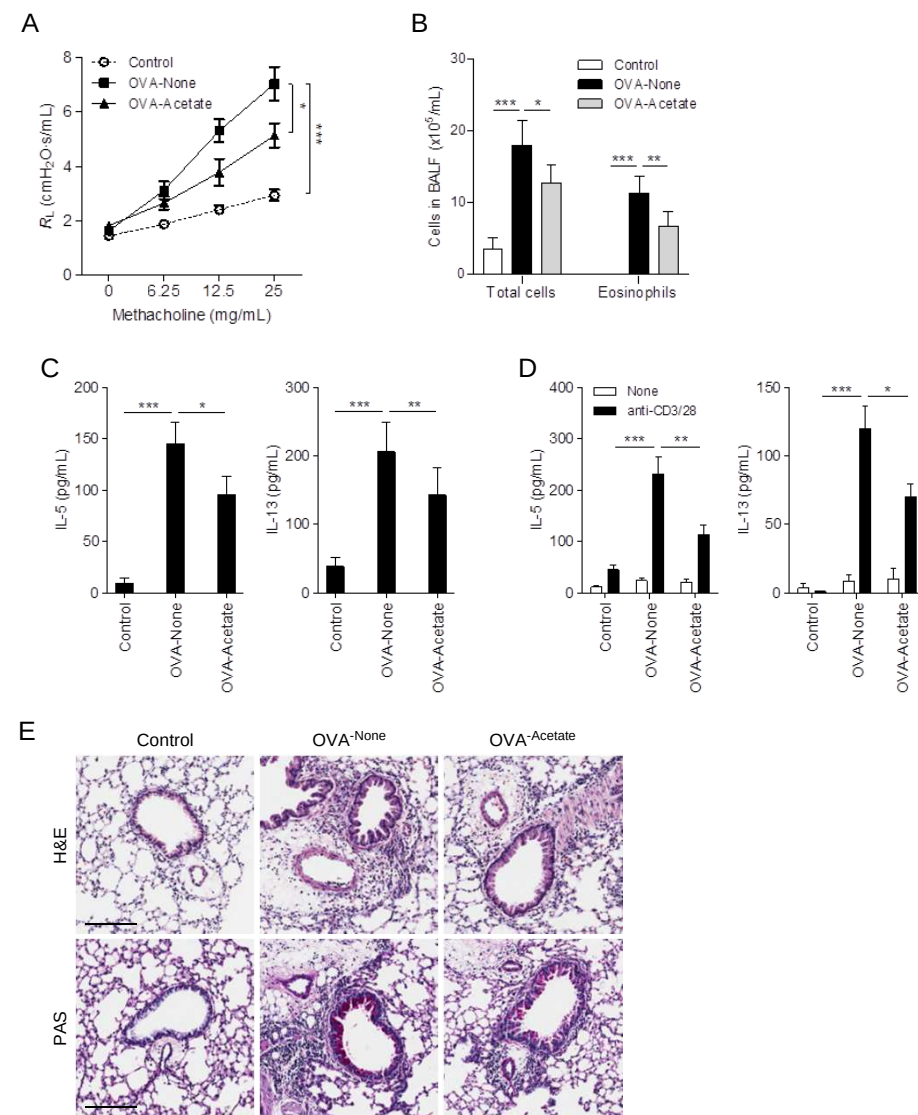


FIG E6.

Supplement

METHODS

Mice

Six-week-old female BALB/c mice (Jackson Laboratory, Bar Harbor, ME, USA) were used under specific pathogen-free conditions. To induce asthma, mice were intraperitoneally sensitized with 50 μ g of ovalbumin (OVA) (Sigma-Aldrich, St. Louis, MO, USA) with 2 mg of alum (Thermo Fisher Scientific, Waltham, CA, USA) and challenged with 1% ovalbumin in saline using a nebulizer (OVA-treated group), while phosphate-buffered saline (PBS; Sigma-Aldrich) was applied in the control group. After sensitization, mice received drinking water containing azithromycin (0.5 mg/mL), cefixime (0.5 mg/mL) or water alone for 2 weeks prior to challenge and until the end of the challenge period. To evaluate the effect of SCFAs on airway inflammation in OVA-induced asthma mice, 100 mM acetate (Sigma-Aldrich) in drinking water was administered for 2 weeks during the challenge period. Fecal transplant was performed on the basis of an established protocol.¹ To prepare enough feces for daily FMT, two groups of mice (wild type and azithromycin-treated mice for two weeks) were maintained as donors. Feces were collected from each group every day, and 3mg fecal pellets were re-suspended in 2 mL PBS. To collect fecal supernatant, the solution was centrifuged at 1500 rpm for 5 min. Then FMT recipients were administered by oral gavage (200 μ L fecal supernatant/mouse) from day 28 to 39 within 10 min to minimize changes in microbial contents.

Airway resistance measurement

Airway resistance to inhaled methacholine (Sigma-Aldrich) was measured as described previously.² Briefly, the mice were connected to a computer-controlled, small-animal

ventilator and were ventilated with a tidal volume of 10 mL kg^{-1} at a frequency of 150 breaths per minute. Each mouse was injected with aerosol methacholine at increasing concentrations (0, 6.25, 12.5 and 25 mg/mL), then the peak airway responses to the inhaled methacholine were recorded.

Bronchoalveolar lavage fluid (BALF) cells and cytokines

Isolated cells from BALF were quantified using a hemocytometer. These cells were classified as macrophages, neutrophils or lymphocytes using Wright–Giemsa staining. BALF cytokines were measured using ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

T-cell stimulation

Lung or spleen T cells were isolated using immunomagnetic cell sorting (Miltenyi Biotec Inc., Auburn, CA, USA) according to the manufacturer's recommendations. These cells (5×10^5) were seeded on 24-well plate (TPP, Trasadingen, Switzerland) and stimulated with anti-CD3/CD28 antibodies (1 $\mu\text{g/mL}$ each; eBioscience, San Diego, CA, USA) for 15 hours. IFN- γ , IL-4, IL-5, IL-13 and IL-10 in the culture supernatant were measured using ELISA kits (R&D Systems) according to the manufacturer's instructions.

Lung histology

Lungs were fixed in 4% formalin, embedded in paraffin and cut into 5- μm sections. These sections were stained with hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS). Tissues were observed using an Aperio ImageScope (Leica Microsystems, Wetzlar, Germany) or ImageJ (National Institutes of Health, Bethesda, MD, USA).

Metagenomic DNA extraction and pyrosequencing

Extraction of bacterial DNA from BALF and feces samples was performed by using a PowerMax Soil DNA Isolation Kit (MO BIO Laboratories Inc., San Diego, CA, USA). Each sequenced sample was prepared according to the Illumina 16S Metagenomic Sequencing Library protocols to amplify the V3 and V4 regions (519F-806R). The DNA quality was measured by PicoGreen and Nanodrop. Input gDNA (10ng) was PCR-amplified. The barcoded fusion primer sequences used for amplification were as follows: 319F: 5' CCTACGGGNGGCWGCAG 3', 806R: 5' GACTACHVGGGTATCTAATCC 3'. The final purified product was then quantified using qPCR according to the qPCR Quantification Protocol Guide (KAPA Library Quantification kits for Illumina Sequencing platforms) and qualified using the LabChip GX HT DNA High Sensitivity Kit (PerkinElmer, MA, USA). Paired-end (2×300 bp) sequencing was performed by MacroGen using the MiSeq™ platform (Illumina, San Diego, CA, USA).

Processing of sequences and bioinformatics analysis

After sequencing of MiSeq raw data, the FASTQ file was created using Real Time Analysis (RTA) and bcl2fastq (v2.20.0.422). The paired-end data for each sample were assembled into a single sequence using FLASH (v1.2.11).^{3,4} The resulting sequence was passed into CD-HIT-out,⁵ an operational taxonomic unit (OTU) analysis program based on CD-HIT-EST, to remove sequences of low quality, ambiguous sequences and chimera sequences, and form species-level OTU via clustering. The representative sequence of each OTU was performed by BLASTN (v.2.4.0)⁶ on the reference DB (NCBI 16S Microbial), and the taxonomic assignment was performed with the organism information of the subject having the highest similarity. If the query coverage of the best hit that matched the DB was less than 85% and the identity of the matched domain was less than 85%, the taxonomy was considered not

defined. A variety of microbial community comparisons were performed using QIIME (v1.9)⁷. Shannon Index and Inversed Simpson Index were obtained to confirm the species diversity and uniformity of microbial communities in environmental samples. Alpha diversity information was confirmed by the Rarefaction curve and the Chao1 value. Based on the Weighted UniFrac Distance, beta diversity between samples (information on diversity among samples in the comparison group) was obtained, and the relationship between samples was visualized through PCoA and UPGMA trees.

Quantitative real-time PCR

The total amount of gut bacteria in fecal samples was determined by quantitative real-time PCR for 16S rRNA genes as previously described.⁸ The 16S rRNA gene was amplified with the primers 340F (5'-TCC TAC GGG AGG CAG CAG-3') and 518R (5-ATT ACC GCG GCT GCT GG-3').

SCFA analysis

The concentration of SCFAs was determined by gas chromatography-mass spectrometry (GC-MS) as previously described⁹ with some modification. In brief, 300 μ L of distilled water was added to freeze-dried cecal samples (20 mg), and the samples were homogenized using a Mixermill (frequency 1/s: 30, 30 seconds, 2 times, Tissue Lyser) for 1 minute. The samples were sonicated for 10 minutes at room temperature and were centrifuged at 3500 rpm for 10 minutes. For the analysis of acetate,¹⁰ an aliquot of aqueous extracts (10 μ L) were transferred to a 2-mL glass vial, and 250 μ L of acetone were subsequently added to the vial. These solutions were reacted with 172 mM pentafluorobenzyl bromide (PFBBBr) in acetone (100 μ L) at 60°C for 30 minutes and were allowed to cool down at room temperature for 15 minutes. After derivatization, phase separation was achieved by adding *n*-hexane (500 μ L) and

distilled water (250 μ L). Then, the upper phase was transferred to vials for GC-MS analysis. For the analysis of propionate, butyrate and isobutyrate,^{9,11} aliquots of aqueous extracts (50 μ L) were transferred to a 4 mL-glass vial, and 0.5 M phosphate buffer (pH 10, 250 μ L) and 0.1 M tetrabutylammonium hydrogen sulfate (TBAHS, 500 μ L) were subsequently added to the vial. After vortexing, these solutions were reacted with 5% PFBBBr in dichloromethane (500 μ L) at 80°C for 30 minutes under vigorous agitation along with magnetic stirring. After derivatization, the lower organic phase was dried under a gentle stream of nitrogen. Dried samples were reconstituted with ethyl acetate (100 μ L) and analyzed with GC-MS. The SCFAs were analyzed using Shimadzu a QP-2010 Ultra gas chromatograph-mass spectrometer (Shimadzu Corp., Kyoto, Japan) in electron impact ionization mode (70 eV). Chromatographic separation of SCFAs was accomplished using an Rtx-5 Sil MS column (30 m, 0.25 mm i.d., 0.25 μ m film thickness; Restek, Bellefonte, PA, USA). The sample (1 μ L) was injected in splitless mode. The temperature of injector, MS interface and ion source were 280°C. The column oven temperature was programmed as follows: 40°C for 1 minute, 40°C/min until 60°C for 3 minutes, 25°C/min until 210°C for 5 minutes and 40°C/min until 315°C for 3 minutes 250°C for 1 minute. Helium was used as a carrier gas at a constant flow rate of 1.2 mL min⁻¹ through the column. Quantitative determination was performed using single ion monitoring mode at m/z 240, 254, 268 and 268 for acetate, propionate, butyrate and isobutyrate, respectively.

Statistical analysis

Statistical analyses were performed using SPSS software version 20.0 (IBM Corp., Armonk, NY, USA). GraphPad Prism 5.0 software (GraphPad Inc., San Diego, CA, USA) was used to produce graphs.

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FIGURE LEGEND

FIG E1. Effect of antibiotics in allergic mice. **A**, Airway hyperresponsiveness. **B**, Differential cell number in BALF. **C**, Concentration of BALF cytokines. **D**, Levels of type 2 cytokines released from lung T cells (upper panel) or spleen T cells (lower panel). Data are represented as means $\pm$ SD, $n = 6$. $*P < .05$, $**P < .01$, and $***P < .001$ were obtained by one-way ANOVA with Bonferroni's *post hoc* test. n.s., not significant.

FIG E2. UPGMA tree of bacterial composition between groups in **A**, BALF and **B**, feces.

FIG E3. The heat map showing the core bacterial microbiome and the relative frequencies in BALF. The taxa shown in the figure are those that exist at least 1% in at least one sample. **A**, Phylum **B**, Family. NC: negative control, OVA: OVA-treated group, C: Cefixime-treated group, A: Azithromycin-treated group.

FIG E4. The heat map showing the core bacterial microbiome and the relative frequencies in Feces. The taxa shown in the figure are those that exist at least 1% in at least one sample. **A**, Phylum **B**, Family **C**, Linear discriminant analysis (LDA) scores between the OVA group and the azithromycin-treated group. NC: negative control, OVA: OVA-treated group, C: Cefixime-treated group, A: Azithromycin-treated group

FIG E5. Even without OVA sensitization and induction of eosinophilic inflammation of the airways, azithromycin-induced changes in gut microbiota are maintained. **A**, Operational

taxonomic unit (OTU). **B**, Shannon diversity index, **C**, Composition of Phylum level, **D**, Composition of Order level. Data are represented as means $\pm$ SD, * $P < .05$, and ** $P < .01$ were obtained by one-way ANOVA with Bonferroni's post hoc test.

FIG E6. Acetate attenuates airway inflammation in the lungs of mice. **A**, Airway hyperresponsiveness. **B**, Total cell and eosinophil numbers. **C**, BALF cytokines. **D**, Levels of IL-5 and IL-13 released from isolated T cells. Data are represented as means $\pm$ SD, $n = 5$. * $P < .05$, ** $P < .01$, and *** $P < .001$ were obtained by one-way ANOVA with Bonferroni's *post hoc* test. n.s., not significant. **E**, Lung histology stained with H&E (upper panel) or PAS (lower panel). Scale bar, 200 μm