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ORIGINAL ARTICLE

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Antibiotic use during pregnancy increases offspring asthma severity in a dose-dependent manner

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Abstract

Background: The use of antibiotics during pregnancy is associated with increased allergic asthma risk in the offspring, and given that approximately 25% of pregnant women are prescribed antibiotics, it is important to understand the mechanisms contributing to this phenomenon. Currently, there are no studies that directly test this association experimentally. Our objective was to develop a mouse model in which antibiotic treatment during pregnancy results in increased offspring asthma susceptibility.

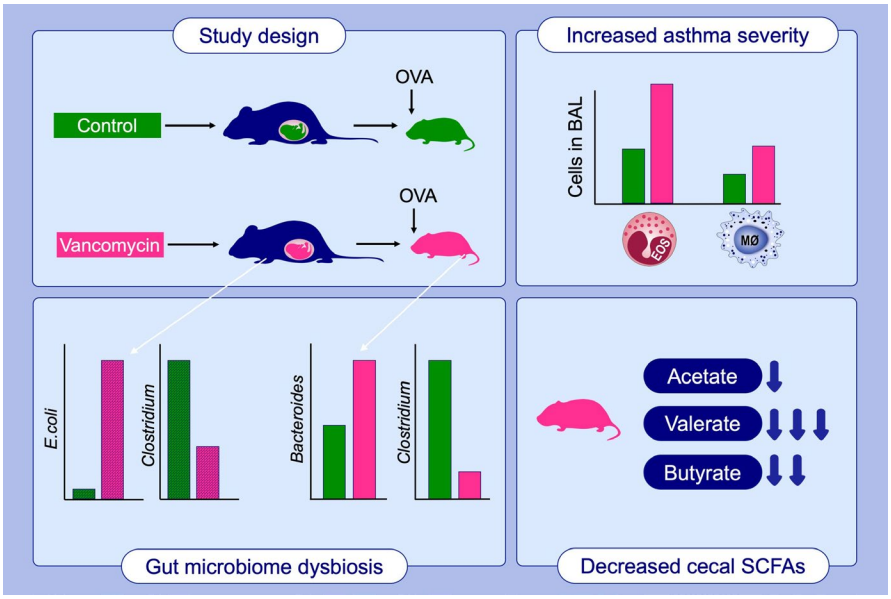
Methods: Pregnant mice were treated daily from gestation day 8-17 with an oral solution of the antibiotic vancomycin, and three concentrations were tested. At weaning, offspring were subjected to an adjuvant-free experimental asthma protocol using ovalbumin as an allergen. The composition of the gut microbiome was determined in mothers and offspring with samples collected from five different time points; short-chain fatty acids were also analyzed in allergic offspring.

Results: We found that maternal antibiotic treatment during pregnancy was associated with increased offspring asthma severity in a dose-dependent manner. Furthermore, maternal vancomycin treatment during pregnancy caused marked changes in the gut microbiome composition in both mothers and pups at several different time points. The increased asthma severity and intestinal microbiome changes in pups were also associated with significantly decreased cecal short-chain fatty acid concentrations.

Conclusion: Consistent with the "Developmental Origins Hypothesis," our results confirm that exposure to antibiotics during pregnancy shapes the neonatal intestinal environment and increases offspring allergic lung inflammation.

KEYWORDS

antibiotics, asthma, microbiome, pregnancy, short-chain fatty acid



GRAPHICAL ABSTRACT

We found that maternal antibiotic treatment during pregnancy was associated with increased offspring asthma severity in a dose dependent manner. Maternal vancomycin treatment during pregnancy caused marked changes in the gut microbiome composition in both mothers and pups at several different time points. The increased asthma severity and intestinal microbiome changes in pups were also associated with significantly decreased cecal short chain fatty acid concentrations.

Abbreviations: BAL, bronchoalveolar lavage; OVA, ovalbumin; SCFAs, short chain fatty acids.

1 | INTRODUCTION

Allergic asthma constitutes a morbidity of increasing prevalence and is the leading cause of chronic disease in children. During asthma exacerbation, inflammation of the respiratory epithelia manifests as airway hyperreactivity and breathing difficulties that can substantially reduce quality of life. Though it is not completely clear how atopy initially arises, it is commonly accepted that allergic asthma results from a failure to generate protective immunologic tolerance against particular aeroallergens. Pursuing this further, several lines of evidence suggest that the ability to generate tolerance is developed during the fetal and neonatal life stages.[1] The "Developmental Origins Hypothesis" proposes that environmental influences during these critical time points can alter disease susceptibility in early life.[2]

There is considerable evidence that the use of antibiotics during pregnancy contributes to increased asthma susceptibility in children.[3-6] During birth, the maternal vaginal, perineal, and fecal microbiome seed the initial intestinal colonization of the newborn.[7,8] Consequently, antibiotics taken during pregnancy have been shown to alter both the maternal and neonatal microbiomes in humans,[9] rats,[10] and mice.[11] The early life microbiome is crucial for the proper development of immune system functions, whereas microbial dysbiosis in early life is associated with altered neonatal immunity [11] and is thought to bias the maturing neonatal immune system toward a hypersensitive state.[2,12] Considering allergic airway diseases, studies demonstrate that germ-free mice displayed increased asthma severity compared to mice with a conventional commensal microbiome.[13] It was also shown that rescue of the severe asthma phenotype in germ-free mice was only possible by supplementation with commensal microbes during early life, but not in adulthood.[14] The neonatal microbiome is therefore critical for establishing immunity and mucosal tolerance.

In addition to contributing to immune system development in early life, intestinal microbes also facilitate the digestion of dietary fiber, resulting in the production of short-chain fatty acids (SCFAs). Acetate, propionate, and butyrate are the major metabolic by-products of bacterial fiber fermentation, and these SCFAs are associated with asthma susceptibility. Longitudinal human studies revealed that

increased levels of fecal butyrate and propionate in the first year of life were significantly associated with protection from asthma at 6 years of age,[15] whereas decreased fecal acetate concentrations at 3 months of age were associated with asthma diagnosis at 3 years of age.[16] Studies in mice showed that increased fecal SCFAs were associated with protection against airway inflammation,[17] whereas decreased SCFA concentrations were associated with more severe allergic airway diseases.[18] Direct supplementation of adult mice with SCFAs[17,19,20] also effectively protected from airway inflammation.

Antibiotics account for 80% of prescriptions given during pregnancy.[21] Given the strong association between maternal antibiotic use and offspring asthma susceptibility, it is increasingly important to understand the mechanisms behind this phenomenon. There are, however, no experimental in vivo models available to date that examine the effect of antibiotics given only during pregnancy on offspring asthma. In the present study, we therefore established a mouse model in which maternal exposure to vancomycin resulted in increased asthma severity in the offspring.

2 | METHODS

2.1 | Animals

Twelve-week-old female BALB/c mice were obtained from Janvier Labs (Le Genest-Saint-Isle). Mice were kept under specific pathogen-free conditions and housed five animals per cage in a 12/12 hours light/dark cycle. Food and water were available ad libitum. All animal experiments were approved by local authorities (Landesamt für Gesundheit und Soziales; LAGeSo; registration number: G0158/18) and were performed in accordance with German and international guidelines.

2.2 | Experimental design

The presence of a vaginal plug after male and female co-habitation was denoted as gestation day (G)0. From G8 to G17, pregnant mice received a daily oral dose of the antibiotic vancomycin (Sigma-Aldrich) corresponding to 10, 20 or 40 mg/kg body weight. Vancomycin was specifically chosen because it is poorly absorbable, limiting the possibility that the antibiotic will directly affect the developing fetus, and allowing investigation of the downstream effects of maternal vancomycin use during pregnancy. Pregnant mice were given the antibiotic mixture (25 µL of vancomycin +15 µL Ora-Sweet syrup; Paddock Laboratories) through drops from a pipet; control animals were sham treated with water + syrup (Figure 1A, B). To reduce human error, we used a calibrated micropipette to administer this volume orally to each individual mouse. To ensure that the entirety of the volume was consumed, we visually confirmed after drug administration that each mouse had finished swallowing (5-10 seconds) before returning the animal to the cage (Figure 1C). Female offspring were weaned at 21 days of age, and experimental asthma was induced by subcutaneous sensitization using 10 µg ovalbumin (OVA VI—Sigma) in 200 µL PBS, once per week for 3 weeks. The asthma phenotype was induced by exposing the offspring for 20 minutes daily to a 1% OVA aerosol (OVA V—Sigma) for 3 days (Figure 1A, B). Twenty-four hours after the last OVA challenge, the asthma phenotype was assessed.[22]

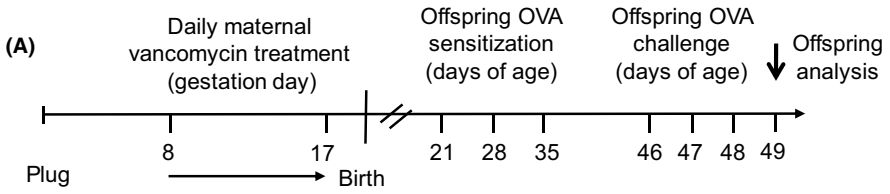
2.3 | Molecular analysis of maternal and offspring fecal microbiomes

2.3.1 | 16S rRNA qPCR

Fresh fecal pellets were immediately frozen and stored at −80°C. Maternal fecal samples were collected 14 days before mating, at G17, and at weaning. Offspring feces were collected at weaning (21 days of age) and after allergic asthma exacerbation (7 weeks of age). Fecal DNA was extracted and then quantified using Quant-iT PicoGreen reagent (Invitrogen) as described in.[23] qPCR was chosen to obtain a broad survey of the bacterial groups present, as well as to determine how maternal vancomycin treatment influenced the quantity of bacteria present in mothers and offspring. Briefly, numbers of 16S rRNA gene copies per ng DNA were quantified in each sample using species-, genera- or group-specific primers (TIB MOLBIOL) assessed by qRT-PCR. Primer sequences can be found in Table S1.[24] Total bacteria load was assessed, as well as the following bacterial groups: Gammaproteobacteria/Enterobacteriaceae (Enterobacteriaceae), *Bacteroides/Prevotella/Porphyromonas* (Bacteroidetes), Mouse Intestinal *Bacteroides*, *Clostridium leptum*, *Clostridium coccoides/Eubacterium rectale*, *Enterococcus*, *Lactobacillus*, and *Bifidobacterium*.

2.3.2 | 16S rRNA sequencing

DNA was extracted using the PowerSoil for KingFisher kit (MO Bio) following manufacturer's instructions. 16S rRNA V4 gene fragments were amplified using bar-coded primers as described in [25] with the following primer regions (5′-3′): fwd: GTGCCAGCMGCCGCGGTAA, rev: GGACTACHVGGGTWTCTAAT. Pooled PCR amplicons were diluted to 20 ng/mL and sequenced using MiSeq 2000 bi-directional Illumina sequencing and Cluster Kit v4 (Macrogen). Library preparation was done using TruSeq DNA Sample Prep v2 Kit (Illumina) with 100 ng of DNA per sample. The library was quantified and quality-checked using Qubit (Thermo Fisher Scientific). DNA extraction, amplification, and sequencing were performed by Microbiome Insights Inc. Sequence data were trimmed, quality-filtered, and clustered at 97% identity into operational taxonomic units (OTUs) using a modified MOTHUR standard operating procedure.[26] An average of 31 554 quality-filtered reads were generated per sample, and there was a total of 8631 OTUs. The OTUs were taxonomically annotated using the SILVA database.[27] Global community structure comparisons were made in an R environment using Phyloseq.[28]



(B)

Group	Maternal vancomycin treatment	Offspring allergy
1 (Control)	0 mg/kg (MC)	OVA
2	10 mg/kg (MV10)	OVA
3	20 mg/kg (MV20)	OVA
4	40 mg/kg (MV40)	OVA

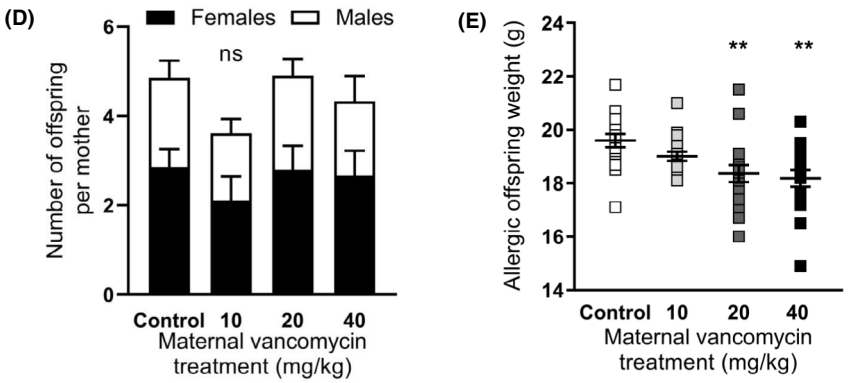


FIGURE 1 The effect of antibiotic use during pregnancy on asthma development in the offspring—Mouse model description and pregnancy effects. A, Pregnant mothers were treated daily from G8-G17 with an oral dose of 10, 20 or 40 mg/kg vancomycin plus Ora-Sweet syrup. Control mothers were treated orally with water and Ora-Sweet. At weaning, all offspring were subjected to an experimental asthma protocol with a subcutaneous sensitization to ovalbumin (OVA) at 21, 28, and 35 days of age, and asthma provocation by a 20 min daily aerosol challenge at 46, 47, and 48 days of age. B, Overview of the treatment for each group. C, Oral administration of vancomycin plus Ora-Sweet. The mouse is scruffed and presented the antibiotic via pipet, allowing the animal to voluntarily consume the drug-Ora-Sweet mixture. Untrained mice immediately learn this procedure and consume the entire mixture within 5-10 s of presentation. D, Litter size—Total offspring (entire bar), females (black bar) and males (white bar) born to mothers in each group. E, Weight of 7-wk-old allergic offspring that were prenatally exposed to maternal control or vancomycin during pregnancy. Data information: Means ± SEM are shown, control (n = 18), prenatal 10 mg/kg (n = 19), prenatal 20 mg/kg (n = 18), prenatal 40 mg/kg (n = 16). Results represent two independently performed experiments. Significance is represented by $^{**}P < .01$, one-way ANOVA with Dunnett's multiple comparisons test (10, 20, and 40 compared against control)

2.4 | Blood collection and antibody measurements

Blood samples were taken from the orbital sinus of terminally anaesthetized mice and serum was stored at −80°C. Total IgG, OVA-specific IgE and OVA-specific IgG1 were measured in the serum of OVA allergic offspring by an enzyme-linked immunosorbent assay (ELISA) (BD Bioscience).

2.5 | Bronchoalveolar lavage, cytospins, and bronchoalveolar lavage cytokine measurements

Using a tracheal cannula on euthanized offspring, Bronchoalveolar lavage (BAL) was performed once using 1 mL of 1× Complete Protease Inhibitor Cocktail (Roche). BAL supernatant was stored at −80°C, total leukocytes were counted with a Neubauer chamber, and cytospin preparations were made and stained with Diff-Quick (Merz & Dade). Two hundred cells were counted per cytospin by a researcher blinded to the sample names. For BAL cytokine measurements, IL-1α, IL-1β, IL2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-17A, Eotaxin, G-CSF, GM-CSF, IFNγ, KC, MCP-1, MIP-1α, MIP-1 β, RANTES, and TNF were measured using a mouse 23-plex cytokine assay (Bio-Rad), according to manufacturer's instructions.

2.6 | Lung collection and histology

Immediately after BAL, lungs were fixed with 4% formalin. 4 µm periodic acid-Schiff (PAS)-stained lung sections were microscopically analyzed using the new Computer Assisted Stereological Toolbox

(newCAST; Visiopharm) as described in,[29] by readers blinded to the study groups. For inflammation and goblet cell measurements, the software randomly selected 100 frames across multiple lung sections (40× objective), superimposed by a line grid and points. Point intercepts on an eosinophil or goblet cell were considered as a point of inflammation (PI) or point of goblet cell (PG), respectively. Line interceptions with the bronchial epithelium were considered a line of a goblet cell (LG) or line of a nongoblet cell (LnG), respectively. Line intercepts with blood vessel walls were labeled as line blood vessel (LV). Volume of inflammation (Vol. Inf), number of goblet cells (Nu. G), and volume of goblet cells (Vol. G) were calculated as follows:

$$\text{Vol. Inf} = (\Sigma PI) \times 9.395 / (\Sigma LG + \Sigma LV)$$

$$\text{Nu. G} = (\Sigma LG \times 100) / (\Sigma LG + \Sigma LnG)$$

$$\text{Vol. G} = (\Sigma PG \times 9.395) / (\Sigma LG + \Sigma LnG)$$

2.7 | Analysis of SCFA from allergic offspring cecum

Cecal acetate, propionate, butyrate, and valerate were measured with an HP 5890 series II gas chromatograph (Hewlett-Packard) equipped with an HP-20 M column and a flame ionization detector. Previously, flash-frozen cecal contents from allergic offspring were diluted and centrifuged, then the supernatant was mixed with 2-ethylbutyric acid as an internal standard, 0.36 M $HClO_4$ and 1 M NaOH. The mixture was lyophilized overnight, and the remnant was re-dissolved in a mixture of 400 µL of acetone and 5 M formic acid. After centrifugation, 1 mL of the supernatant was injected into the gas chromatograph.

2.8 | Statistical analysis

Samples were tested for normal distribution using a Shapiro-Wilk normality test. Parametric/nonparametric data were analyzed by one-way ANOVA/Kruskal-Wallis (with Dunnett's multiple comparison test) or Mann-Whitney U test in the case of two groups. Calculations were performed with GraphPad Prism 7 software. To confirm the robustness of the statistics with respect to outliers, we performed an outlier analysis for every model with Cook's distance using base R. In the presence of outliers, the initial analyses were repeated after extracting the respective values from the dataset. These results confirmed our initial analyses. Additionally, all models were estimated with robust ANOVA's using the WRS2 Package (version 0.10). Again, the analyses showed only small and expected differences to the initial analyses. Hence, we retained the initial results from the one-way ANOVA and Kruskal-Wallis tests. Pearson's correlation analysis and statistics were performed using the R package psych (version 1.8.12). Correlation figures were generated using ggplot2[30] and ColorBrewer (https://CRAN.R-project.org/package=RColorBrewer). Multiple comparisons were corrected using the Benjamini-Hochberg method.

3 | RESULTS

3.1 | Model description

To examine the effect of antibiotics taken during pregnancy on the development of offspring asthma, we established a mouse model in which daily treatment of mothers with the antibiotic compound vancomycin from gestation day G8 until G17 resulted in increased asthma severity in the offspring (Figure 1A). Mothers were treated with either 10, 20 or 40 mg vancomycin per kg body weight, corresponding to low, medium, and high vancomycin doses in humans, and as a prenatal control, mothers received sterile water (Figure 1B). Using a novel method adapted from,[31] we used a pipet to feed mice vancomycin mixed with Ora-Sweet, which is a medically approved syrup used to sweeten antibiotics for children.[32] In our experience, untrained, hand-held mice readily ate the entirety of the antibiotic mixture within a 10 second delivery time (Figure 1C). Offspring from all groups were subjected to an ovalbumin (OVA) allergic asthma protocol beginning at weaning (Figure 1A, B).

3.2 | Maternal treatment with a high antibiotic concentration increased miscarriages and reduced offspring weight

To assess the effect of maternal treatment with antibiotics on pregnancy outcome, we recorded litter sizes, miscarriage rates, offspring sex and adult offspring weight. Of the term pregnancies, the mean litter size was 4.5 pups and no differences were observed in litter size or pup sex between groups (Figure 1D). In mice with confirmed pregnancy (more than 3.5 grams of weight gained from G0-G13),[33] we observed increased miscarriages, that is, abortion of all pups, with increasing antibiotic concentration. Mothers treated with 20 mg/kg vancomycin had a 9% miscarriage rate, whereas in mice with 40 mg/kg the number rose to 30%. Control mothers and those exposed to 10 mg/kg vancomycin had no observed miscarriages. In addition to miscarriage, prenatal exposure to increasing antibiotic concentrations was also associated with decreased offspring weight in a dose-dependent manner. Seven-week-old allergic offspring from mothers treated with 20 mg/kg and 40 mg/kg vancomycin weighed significantly less than allergic offspring from control mothers (Figure 1E).

3.3 | Maternal antibiotic treatment during pregnancy increased offspring asthma severity in a dose-dependent manner

To determine how antibiotic use during pregnancy impacts offspring asthma severity, we induced experimental allergic asthma in offspring derived from control- and antibiotic-treated mothers. As expected, serum from all allergic offspring contained OVA-specific IgE and OVA-specific IgG1, though no differences were observed in these antibodies or total IgG concentrations

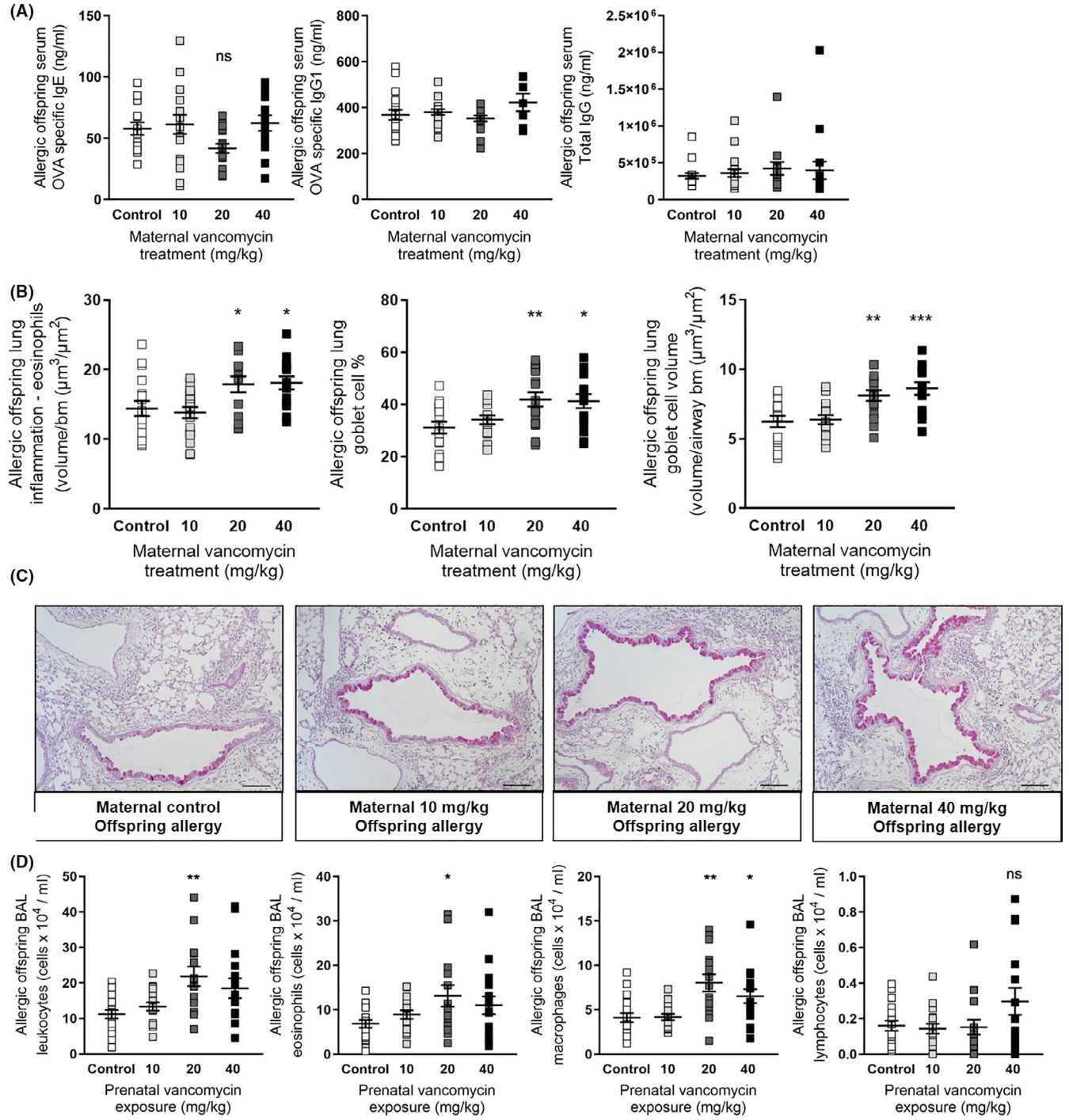


FIGURE 2 Maternal antibiotic treatment during pregnancy results in increased asthma severity in the offspring. A, Serum antibody titers from OVA allergic offspring: OVA-specific IgE, OVA-specific IgG1 and Total IgG. B, Allergic offspring lung histology quantification: eosinophilic inflammation, goblet cell percentage, goblet mucous volume. C, Allergic offspring lung histology images. Scale bar = 100 µm. D, Bronchoalveolar lavage (BAL) cell counts from allergic offspring: leukocytes, eosinophils, macrophages, and lymphocytes. Data information: Means ± SEM are shown, control (n = 18), prenatal 10 mg/kg (n = 19), prenatal 20 mg/kg (n = 18), prenatal 40 mg/kg (n = 16). Results represent two independently performed experiments. Significance is represented by $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, one-way ANOVA or Kruskal-Wallis test with Dunnett's multiple comparisons test (10, 20, and 40 compared against control)

between maternal control (MC) and maternal vancomycin (MV) allergic offspring groups (Figure 2A). Analysis of allergic offspring inflammation revealed increasing asthma severity in correspondence with increasing maternal vancomycin concentrations. Histopathological quantification of pulmonary inflammation and mucous production revealed that allergic offspring from

TABLE 1 Bronchoalveolar lavage from allergic offspring displays decreased cytokine concentrations with increasing prenatal antibiotic exposure

	Maternal control	Maternal 10 mg/kg	Maternal 20 mg/kg	Maternal 40 mg/kg
	n = 18	n = 18	n = 13	n = 16
Concentration (pg/mL)	Mean (SEM)	Mean (SEM)	Mean (SEM)	Mean (SEM)
IL-1α	7.2 (0.4)	8.5 (0.9)	9.0 (0.8)	9.0 (1.0)
IL-1β	48.0 (4.9)	36.1 (4.0)	**22.0 (5.3)****	**25.4 (5.6)***
IL-2	164.8 (18.1)	6.4 (0.4)	6.6 (0.4)	6.8 (0.7)
IL-4	165.4 (17.5)	142.9 (16.4)	**82.9 (16.9)***	145.0 (4.2)
IL-5	164.8 (18.1)	146.2 (11.4)	**103.2 (17.2)***	130.7 (13.4)
IL-6	9.7 (2.9)	10.3 (1.6)	9.7 (1.6)	8.2 (1.4)
IL-9	42.0 (6.0)	**20.9 (5.5)***	26.4 (7.8)	**9.7 (4.4)*****
IL-12p40	52.6 (4.2)	43.4 (2.8)	40.1 (3.8)	44.4 (4.2)
IL-12p70	12.4 (1.0)	11.1 (0.7)	**7.7 (0.8)****	**9.1 (1.3)***
IL-13	111.1 (13.4)	83.6 (9.3)	81.5 (12.1)	92.2 (9.6)
Eotaxin	161.6 (7.2)	141.0 (7.0)	135.6 (11.2)	**113.0 (16.9)****
G-CSF	8.4 (0.8)	7.9 (0.9)	**5.3 (0.9)***	**5.5 (0.9)***
GM-CSF	10.0 (0.6)	8.5 (0.3)	**6.5 (0.6)*****	**3.3 (6.5)*****
KC	213.2 (27.5)	230.8 (29.1)	240.7 (34.7)	207.3 (26.5)
MCP-1	28.9 (5.9)	16.2 (2.8)	**4.7 (3.0)*****	**6.1 (2.7)*****
MIP-1α	9.8 (0.9)	9.6 (0.6)	**5.1 (0.1)*****	7.7 (0.9)
MIP-1β	4.3 (0.4)	3.8 (0.2)	**2.4 (0.3)*****	**2.6 (0.2)*****
RANTES	6.5 (0.4)	5.8 (0.2)	**4.7 (0.3)****	**5.3 (0.4)***
TNFα	20.9 (1.3)	19.6 (1.0)	17.1 (1.9)	22.6 (4.7)

Note: Data information: Means ± SEM are shown, control (n = 18), prenatal 10 mg/kg (n = 19), prenatal 20 mg/kg (n = 18), prenatal 40 mg/kg (n = 16). Results represent two independently performed experiments. Significance is represented by $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$ one-way ANOVA or Kruskal-Wallis test with Dunnett's multiple comparisons test (10, 20, and 40 compared against control).

mothers treated with 20 or 40 mg/kg vancomycin (henceforth referred to as MV20 and MV40 allergic offspring, respectively) displayed significantly increased airway and vessel eosinophilic inflammatory responses as well as increased goblet cell numbers and mucous volume when compared to allergic offspring from control mothers (Figure 2B, C).

In addition to tissue inflammation, total leukocyte influx into the bronchoalveolar lavage (BAL) fluid was significantly increased in MV20 and MV40 allergic offspring; this result was largely due to increased numbers of eosinophils and macrophages (Figure 2D). In the BAL, increased allergic lung inflammation correlated with significantly lower concentrations of 12 of the 23 measured BAL cytokines, namely IL-1β, IL-4, IL-5, IL-9 IL-10, IL-12p70, eotaxin, G-CSF, GM-CSF, MCP1, MIP-1α and RANTES (Table 1). Finally, correlation analysis showed that increasing vancomycin concentration during pregnancy correlated with significantly increased asthma severity in the offspring (Figure S1A-D). In summary, based on significant increases in lung inflammation, mucous production, and BAL cell infiltrates, as well as a correlated decrease in BAL cytokines in MV20 and MV40 allergic offspring, we thereby show that maternal antibiotic treatment during pregnancy was associated with increased severity of offspring allergic asthma.

3.4 | Antibiotic treatment during pregnancy altered the maternal and offspring intestinal microbiomes

To correlate increased MV offspring asthma severity with changes in gut microbial communities, we quantified the changing fecal microbiome composition of mothers and their offspring using qPCR. At G17, following antibiotic treatment, we observed a significant increase in total bacterial load in all mothers that were treated with vancomycin in comparison to control G17 mothers (Figure 3A). At weaning (23 days after the last antibiotic treatment), total bacterial loads of vancomycin-treated mothers returned to control levels; however, all MV offspring had significantly increased fecal bacterial numbers, both at weaning and as allergic adults. The increased bacterial load in MV mothers corresponded to greatly increased numbers (up to Log 4) of bacteria in the Enterobacteriaceae in all vancomycin-treated groups (Figure 3B). qPCR additionally revealed that microbes in the Bacteroidetes were also increased (Log 2) in G17 maternal feces in the MV10 and MV20 groups (Figure 3C). Again, members of this group returned to control levels in the mothers at weaning, whereas they remained elevated in offspring from all MV groups, both at weaning and as allergic adults (Figure 3C). Mouse

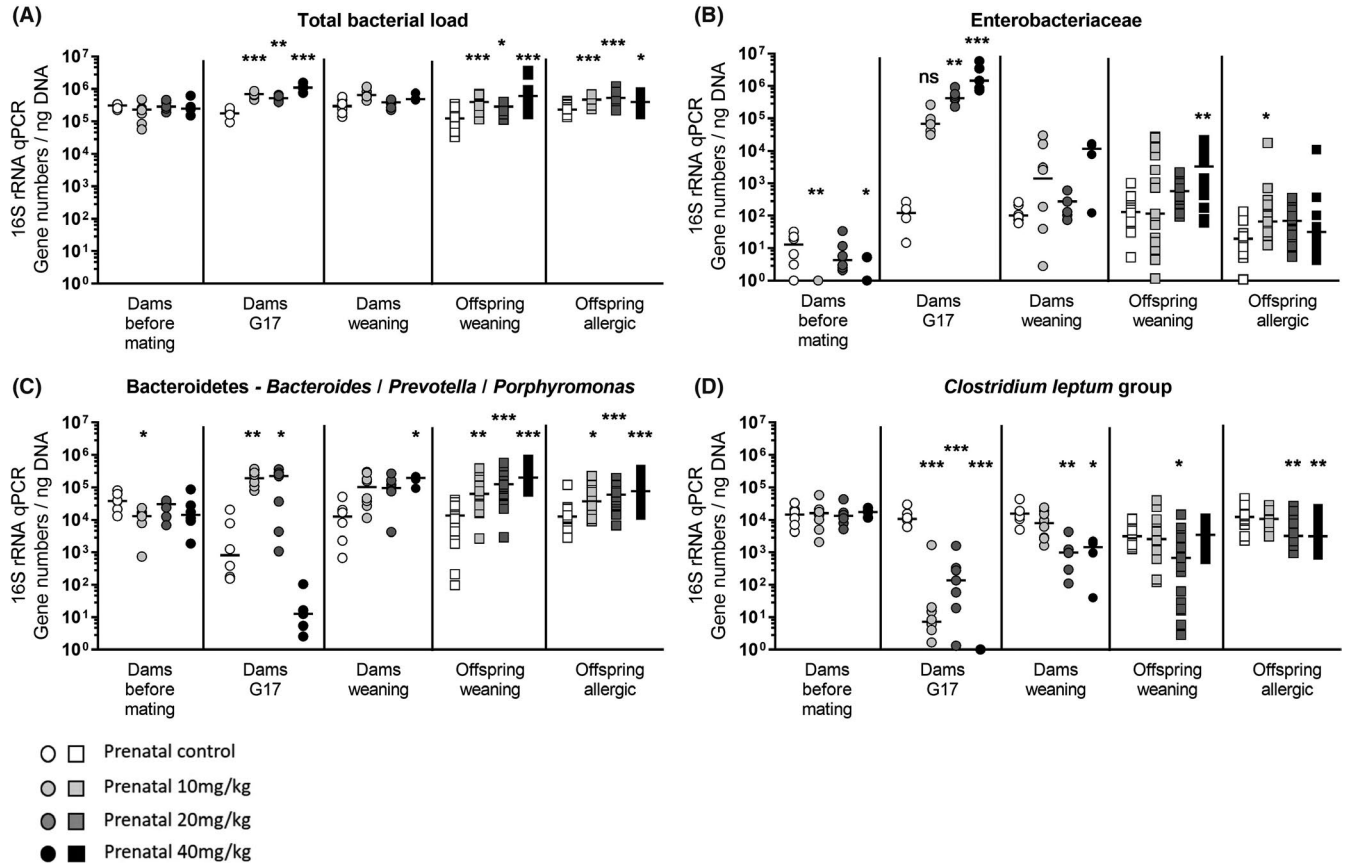


FIGURE 3 qPCR analysis of the fecal microbiome from mothers and offspring. Broad bacterial clades in the maternal and offspring fecal microbiome were quantified in all treatment groups using taxa-specific 16S rRNA qPCR. A, Total eubacterial load, B, γ-proteobacteria—Enterobacteriaceae, C, Bacteroidetes—*Bacteroides*, *Prevotella*, and *Porphyromonas*, D, *Clostridium leptum* group. Data information: Medians (black bars) are shown, *Maternal*: control (n = 6), 10 mg/kg, (n = 8), 20 mg/kg (n = 7), 40 mg/kg (n = 6). *Offspring*: from control mothers (n = 18), from 10 mg/kg vancomycin-treated mothers (n = 19), from 20 mg/kg vancomycin-treated mothers (n = 18), from 40 mg/kg vancomycin-treated mothers (n = 16). Results represent two independently performed experiments. Significance is represented by $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, one-way ANOVA or Kruskal-Wallis test with Dunnett's multiple comparisons test (10, 20, and 40 compared against control)

Intestinal *Bacteroides* (MIB) followed a similar pattern but with less pronounced differences between groups (Figure S2A).

Considering gram-positive bacteria, qPCR analysis revealed an expected decrease in the fecal *Clostridium leptum* group in all vancomycin-treated mothers at G17 (up to Log 4 decrease) (Figure 3D). A similar trend was observed in the *Clostridium coccoides/Eubacterium Rectale* group (Figure S2B). *Clostridium leptum* remained significantly decreased in both mothers and offspring at weaning and in the adult allergic offspring (Log1) (Figure 3D). Finally, *Lactobacillus* concentrations did not show any major differences between control and vancomycin-treated mothers or pups (Figure S2C).

To expand on the qPCR data, we chose the group with the greatest increase in asthma severity (MV20), to examine the maternal and offspring fecal microbiome by 16S amplicon sequencing. 16S amplicon analysis confirmed that members of the Enterobacteriaceae increased in relative abundance at G17 in MV20 maternal feces (Figure 4A). One of the most differentially abundant operational taxonomic units (OTUs) at G17 in MV20 maternal feces was identified as *Escherichia coli*, a member of the Enterobacteriaceae (Figure 4B). 16S amplicon analysis also allowed us to infer the contributions of particular taxa to the Bacteroidetes qPCR results. In MV20 mothers at G17, OTUs corresponding to unclassified Bacteroidetes had elevated relative abundances (Figure 4C), whereas in MV20 offspring at weaning and at adulthood, different OTUs belonging to the genus *Bacteroides* had increased relative abundances (Figure 4D). The genera, *Prevotella* and *Porphyromonas*, did not appear to contribute to the increase in Bacteroidetes, as the relative abundance of the former actually decreased in MV20 mothers and offspring at all time points measured after vancomycin treatment (Figure 4D); the latter did not make up a substantial proportion of sequencing reads (<0.001%).

16S amplicon analysis of *Clostridium* confirmed our observations in the MV20 groups, with the G17 time point, as well as mothers and offspring at weaning, having significantly lower relative abundances of OTUs classified as members of the order, Clostridiales. In summary, these data confirm that the increases in total fecal bacterial numbers in vancomycin-treated mothers at G17 and in their offspring at weaning and adulthood is likely due to a bloom of

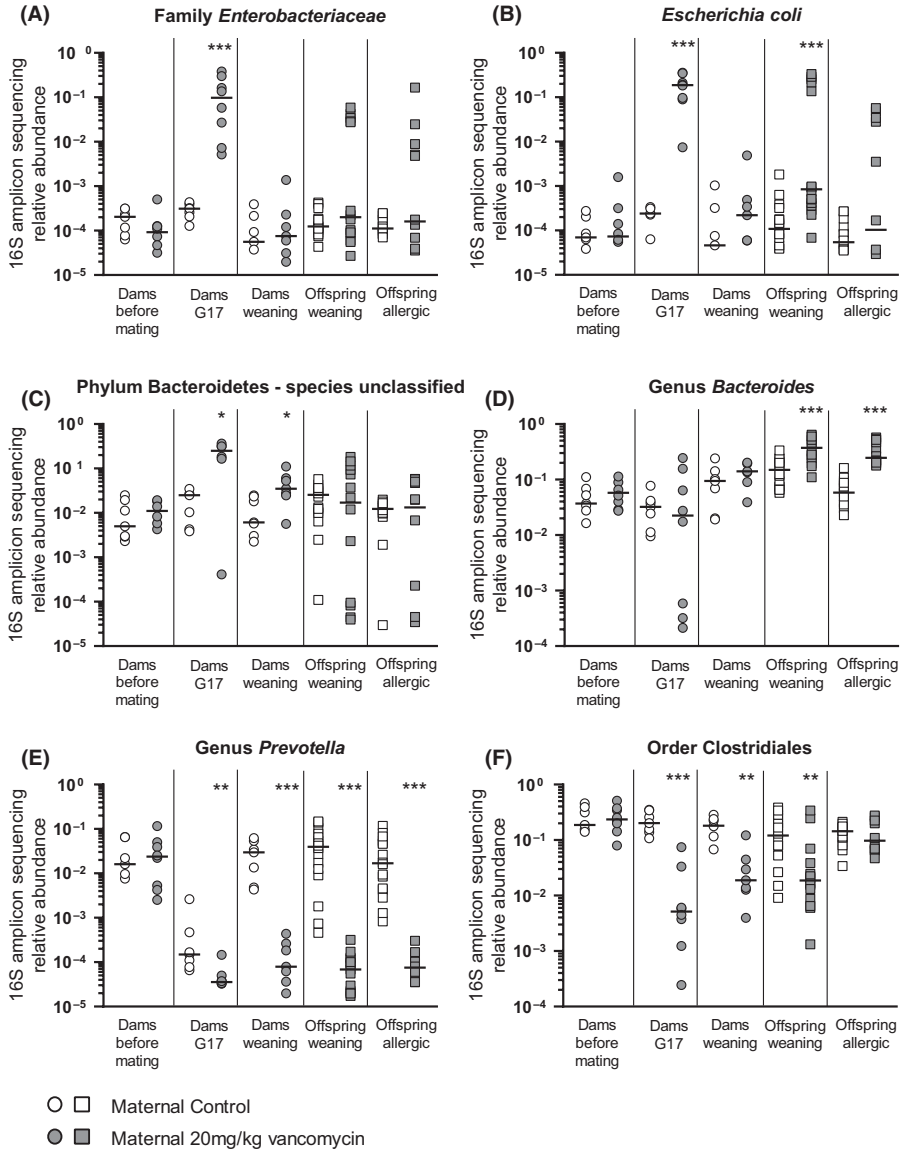


FIGURE 4 16S rRNA amplicon sequencing analysis of the fecal microbiome from MV20 mothers and offspring. Relative abundance of A, Enterobacteriaceae, B, *Escherichia coli*, C, an unclassified species of Bacteroidetes, D, *Bacteroides*, E, *Prevotella*, F, Clostridiales. Data information: Medians (black bars) are shown. *Maternal*: control (n = 6), 10 mg/kg, (n = 8), 20 mg/kg (n = 7), 40 mg/kg (n = 6). *Offspring*: from control mothers (n = 18), from 10 mg/kg vancomycin-treated mothers (n = 19), from 20 mg/kg vancomycin-treated mothers (n = 18), from 40 mg/kg vancomycin-treated mothers (n = 16). Results represent two independently performed experiments. Significance is represented by $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$. A Mann-Whitney U test was performed (control vs 20 mg/kg)

gram-negative *E coli* as well as bacteria in the phylum Bacteroidetes. These increases were accompanied by comparable decreases in members from the order Clostridiales.

3.5 | SCFAs are significantly decreased in the cecum of MV allergic offspring

Due to the associations between the microbiome, SCFA production, and asthma susceptibility, we also measured SCFAs in the cecum of MC and MV allergic offspring. In both humans[16] and mice,[18] decreased levels of fecal SCFAs are associated with increased asthma susceptibility and severity. In agreement with these studies, we found that increased asthma severity in MV allergic offspring was associated with significant decreases in cecal SCFAs. Correlation analyses further indicated that increasing vancomycin dose during pregnancy (Figure S3A-D), as well as increasing total bacterial numbers at G17 (Figure S4A-D), were correlated with decreased SCFA concentrations in the offspring. As shown in Figure 5A and B, all MV allergic offspring had significantly decreased concentrations of cecal acetate and valerate, whereas cecal propionate was only decreased in the MV40 group (Figure 5C). Cecal butyrate was decreased in both the MV20 and MV40 allergic offspring groups (Figure 5D), of which MV20 displayed the most severe asthma phenotype. Decreased

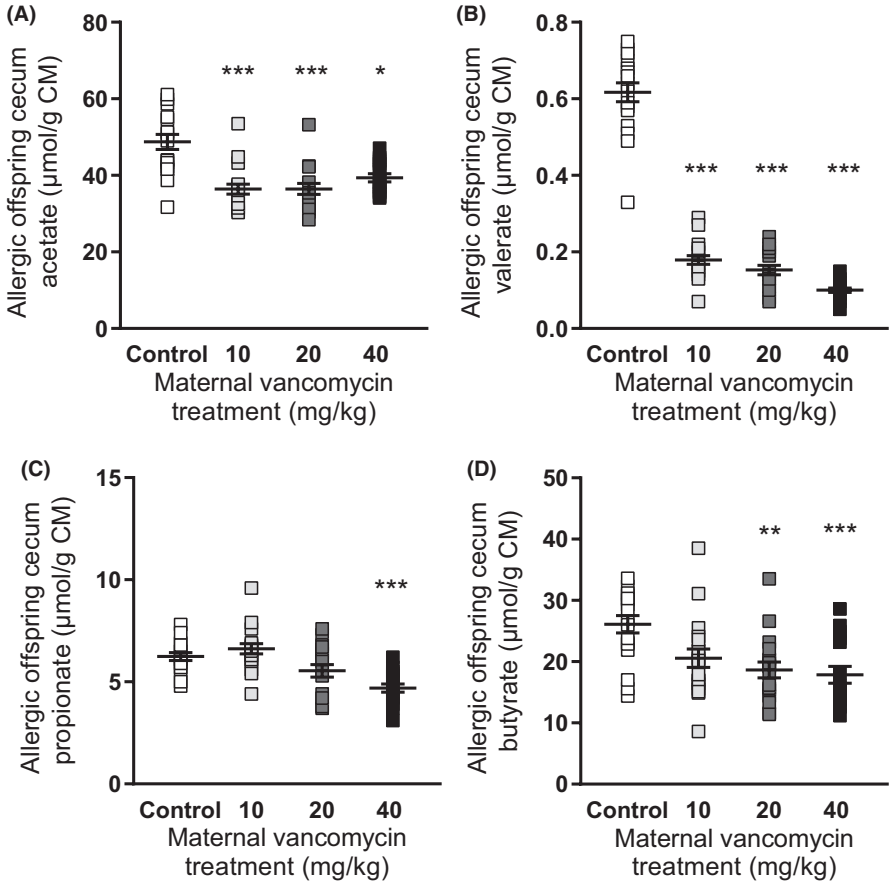


FIGURE 5 Short-chain fatty acids are decreased in the cecum of allergic offspring whose mothers were treated with vancomycin during pregnancy. Short-chain fatty acid concentrations in the cecum of allergic offspring. A, Acetate, B, valerate, C, propionate, D, butyrate. Data information: Means ± SEM are shown, control (n = 18), prenatal 10 mg/kg (n = 19), prenatal 20 mg/kg (n = 18), prenatal 40 mg/kg (n = 16). Results represent two independently performed experiments. Significance is represented by $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$one-way ANOVA or Kruskal-Wallis test with Dunnett's multiple comparisons test (10, 20, and 40 compared against control)

cecal butyrate concentrations in these groups were also associated with significantly decreased *Clostridium leptum* gene numbers in the feces (Figure 3D).

4 | DISCUSSION

We have designed the first mouse model in which maternal treatment with antibiotics only during pregnancy resulted in increased asthma severity in the offspring. Our model is unique in that the offspring were not exposed directly to the antibiotic. Other groups have demonstrated that prenatal plus neonatal exposure to antibiotics resulted in increased offspring asthma severity; however, these models also treated the offspring with antibiotic from birth until allergy induction.[34,35] Russell et al (2013) tested the effect of vancomycin given only during pregnancy on offspring asthma development in mice, but found no difference between MV and MC allergic offspring.[36] We propose that the difference observed between studies reflects the mode of vancomycin delivery. Delivery of medication individually to mice with a pipet is an excellent alternative to delivering antibiotics by gavage or via water bottles. Gavage is very stressful for pregnant mice, while water bottle distribution of antibiotics is limited by the facts that (a) individual mouse dosage cannot be measured precisely and (b) antibiotic may not reach the minimum inhibitory concentration due to the small-dose, high-frequency manner of water bottle delivery.[37]

Maternal vancomycin treatment during pregnancy increased offspring asthma severity in a dose-dependent manner. This is in line with several human studies that show higher asthma risk in children whose mothers were prescribed antibiotics several times during pregnancy.[3] MV allergic offspring displayed significantly augmented inflammation in the lung tissue and BAL. Regarding BAL cytokine levels, which are often reported to increase with increasing asthma severity, we expected that animals with higher inflammation would also display increased type 2 cytokines. To our surprise, increased airway inflammation was associated with significantly lower concentrations of 12 of the 23 measured BAL cytokines. The type 2 cytokines IL-4 and IL-5 were among the cytokines decreased in the MV20 and MV40 allergic offspring; IL-13, however, did not show any difference between groups. Since the decreased allergic offspring BAL cytokine concentrations were correlated with increasing maternal vancomycin concentration, this strongly reduces the possibility of random error. Additionally, we have also observed this phenomenon using an unrelated prenatal risk model in our laboratory (unpublished information). At the moment, we unfortunately cannot offer a firm explanation as to *why* decreased cytokine levels were observed. This topic requires specifically designed future studies to examine this question in further detail.

Maternal vancomycin treatment during pregnancy was associated with microbial dysbiosis that was passed on to the offspring. Both taxa-specific 16S rRNA qPCR and universal bacterial primer

16S rRNA amplicon sequencing were used to analyze the intestinal microbiota of both mothers and offspring at several different time points. The two methods are complementary, with the former quantifying the density of targeted populations and the latter providing relative abundances of most bacterial taxa. At G17, maternal vancomycin treatment was associated with substantial (Log 4) increases in Enterobacteriaceae and unidentified Bacteroidetes. Given that vancomycin mainly targets gram-positive bacteria,[38] this bloom of bacteria is likely due to the availability of new intestinal ecological niches. Accordingly, at G17 we also observed log 4 decreases in maternal Clostridia. The maternal microbiota is thought to contribute directly to the neonatal microbiome composition due to transfer of bacteria during birth[7] and, in mice, through consumption of maternal feces by neonates in early life.[39] Our analysis of the fecal microbiota from the offspring supports this notion. Pups and adult allergic offspring from vancomycin-treated mothers had an increased bacterial load that was contributed to by Enterobacteriaceae as well as *Bacteroidetes*. Additionally, qPCR and amplicon sequencing confirmed that Clostridiales was also decreased in offspring at weaning.

In both humans [16] and mice,[18] changes to the microbiome are associated with decreased levels of fecal SCFAs and increased asthma susceptibility. Our study also demonstrated that MV allergic offspring had significantly decreased cecal SCFA concentrations compared to their MC allergic counterparts. Further analysis showed that both maternal vancomycin concentration and maternal total bacterial load at G17 were significantly associated with decreased SCFA concentrations in allergic offspring, with butyrate being the most strongly impacted. Studies have revealed the importance of this SCFA in allergic asthma, demonstrating that butyrate supplementation during asthma sensitization of adult mice resulted in protection against airway inflammation.[19] Additionally, mice treated with vancomycin since birth could be rescued from a severe asthma phenotype by supplementation with butyrate during vancomycin treatment.[40] Butyrate acts as an inflammatory regulator, and several studies have implicated butyrate in protection against asthma via FoxP3 induction on regulatory T cells and subsequent suppression of inflammatory Th9 cells in the allergic lung.[19]

4.1 | Conclusion

We have established the first mouse model in which maternal antibiotic treatment during pregnancy increases offspring asthma severity. Increased asthma severity was accompanied by maternal and offspring microbial dysbiosis, as well as decreases in offspring cecal acetate, butyrate, and valerate concentrations. Our model is ideal for mechanistic analysis of how antibiotics given during pregnancy can alter offspring immune system development and subsequent disease susceptibility. Future analyses will examine SCFA production in concert with fetal and neonatal development to better understand the effects of antibiotic use during pregnancy at critical developmental time points.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

MA performed the mouse work. MA and ES analyzed the allergic offspring samples. MMH and SB performed qPCR microbiome analysis and co-edited paper. AC and WWM performed 16S rRNA amplicon analysis. MB performed the short-chain fatty acid analysis, RK TMC, and AOY analyzed lung histology. MLC and AC conceptualized the study and interpreted the data. AC performed correlation analyses. AW performed statistical outlier analysis and robust ANOVAs. MLC provided funding, performed mouse work, supervised all stages of the study and wrote the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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