Supplementary Figures

Supplementary Figure 1

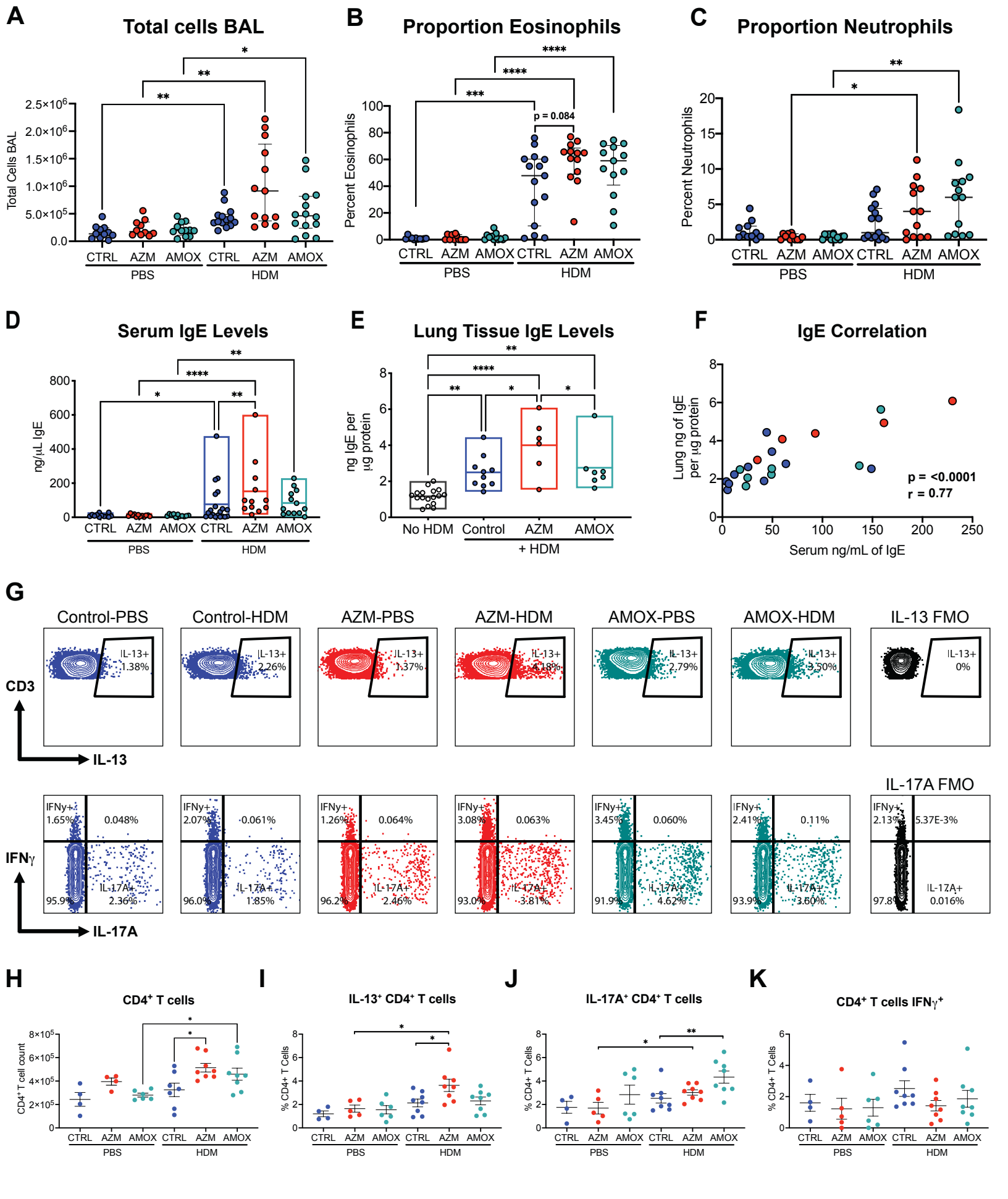
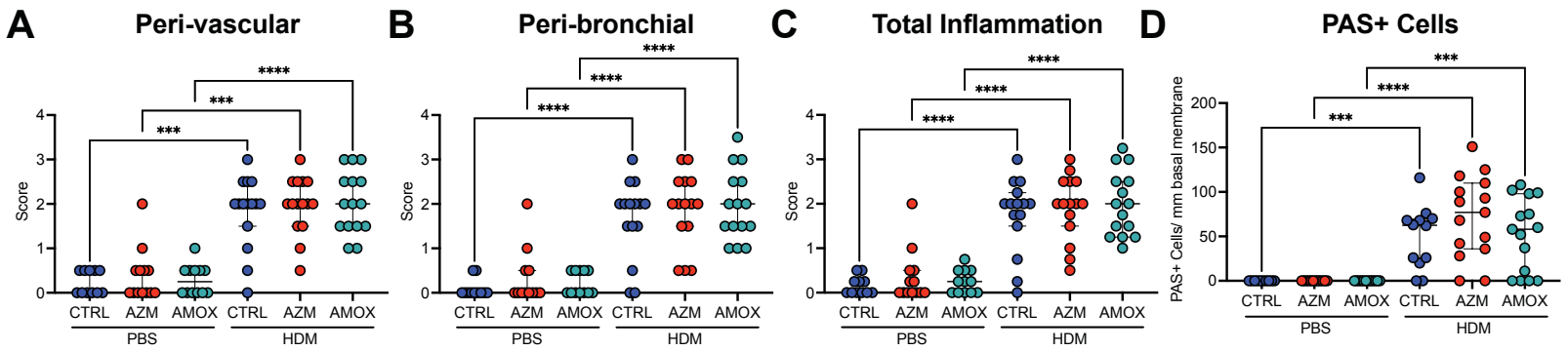
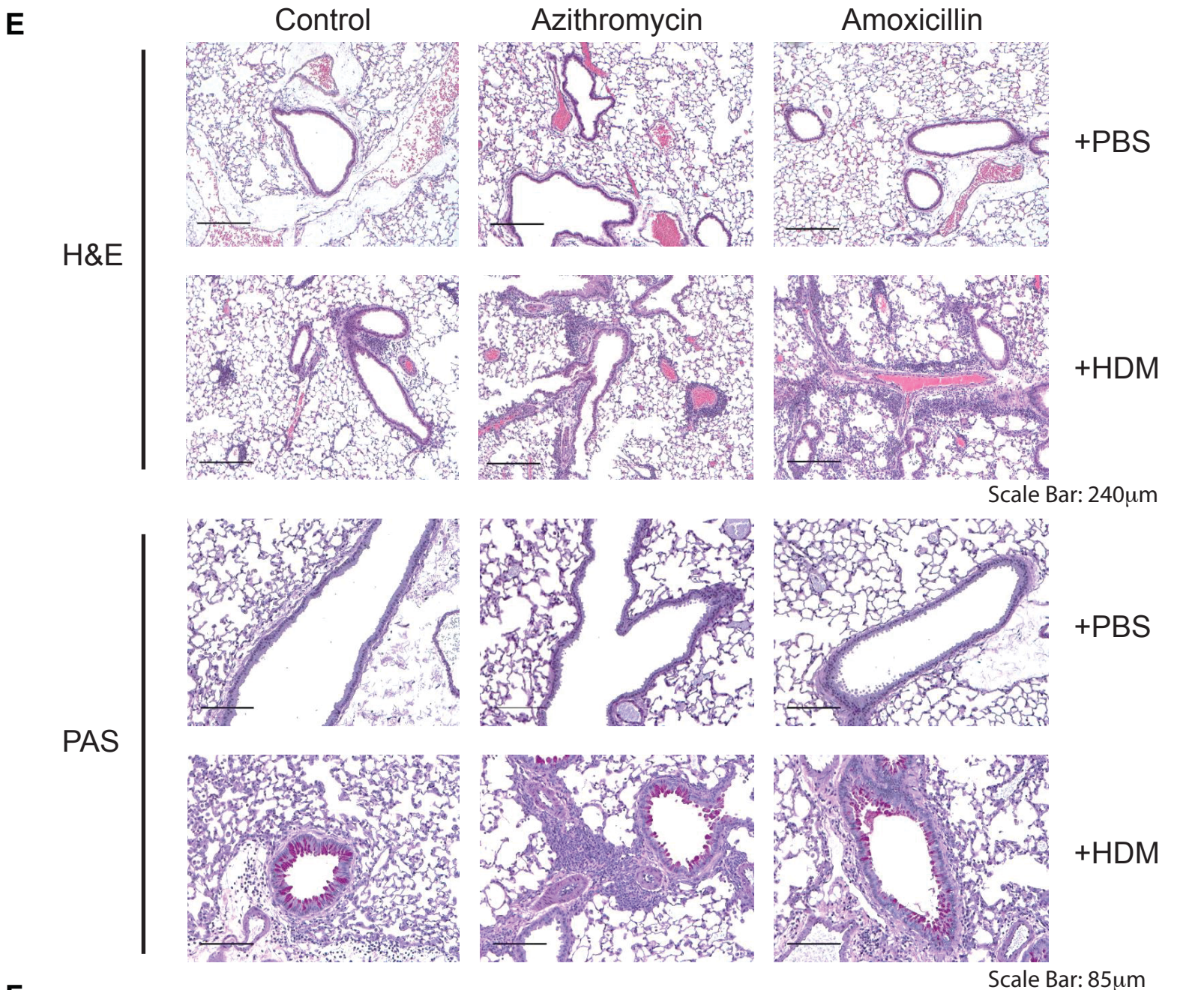


Figure S1: Evaluation of granulocyte recruitment to airways, IgE responses, and lung $CD4^+$ T cell cytokine expression in the lungs of antibiotic-exposed and antigen-challenged mice.

Mice received early-life treatment with azithromycin, amoxicillin, or not—control, between P5-9 by direct oral administration; dams were unexposed. For each independent experiment, there were at least 2 or 3 litters per treatment group. At P46, mice were intranasally sensitized with 1μg of HDM antigen or PBS as a negative control for HDM sensitization. One week later, mice received three separate intranasal challenges with 10μg of HDM antigen, or again with PBS as a negative control. All mice were sacrificed 3 days after the last challenge at P60 (see schematic in **A**). BAL was collected at sacrifice and evaluated for **B.** total cell counts by trypan blue staining, or after BAL samples were cytocentrifuged and differentially stained to determine proportion of **C.** eosinophils, or **D.** neutrophils. **E, F** IgE in mouse serum and lung tissue extract were quantitated by ELISA. Serum is from three independent experiments, and the lung tissue samples from two independent experiments. The same trends were found in all experiments for BAL cytology and IgE quantitation. **G.** At P60, lungs were harvested for flow cytometric analysis (see Methods). **A**. Representative flow cytometry plots and **B.** abundance and frequency of lung $CD4^+$ T cells, and frequency of IL-13^+, IL-$17A^+$, and IFNγ^+ $CD4^+$ T cells following stimulation with PMA/Ionomycin from two independent studies. Significance testing used a nonparametric one-way ANOVA ***p<0.05; **p<0.01; ***p<0.001, ****p<0.0001.**

Supplementary Figure 2





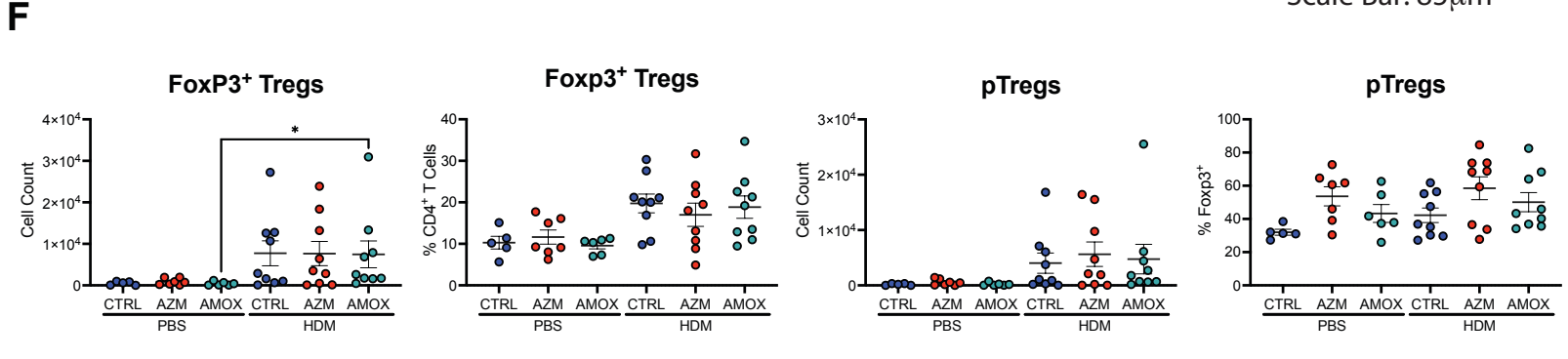


Figure S2. Lung histological scoring in mice differing in antibiotic exposure, then sensitized and challenged with either HDM or PBS.

Lung tissue sections were stained with hematoxylin and eosin (H&E) or Periodic acid-Schiff (PAS) and evaluated blindly for: **A.** peri-bronchial inflammation. **B.** peri-vascular inflammation, **C.** total inflammation, **D.** PAS^{+} cells. Scores are reported for each antibiotic-exposure group, according to PBS or HDM sensitization and challenge (n = 11-15 mice per group from three independent experiments). All comparisons between the respective scores for those challenged with PBS or HDM were significant ($p<0.0001$). **E.** Representative images of lung H&E (100x magnification) and PAS (200x magnification) staining. **F.** Lung tissue Treg and pTreg cell counts and frequencies at P60 both with and without HDM sensitization and challenge from two independent experiments that showed the same trend in both studies. *$p<0.05$; **$p<0.01$; ***$p<0.001$, ****$p<0.0001$; nonparametric one-way ANOVA.

Supplementary Figure 3

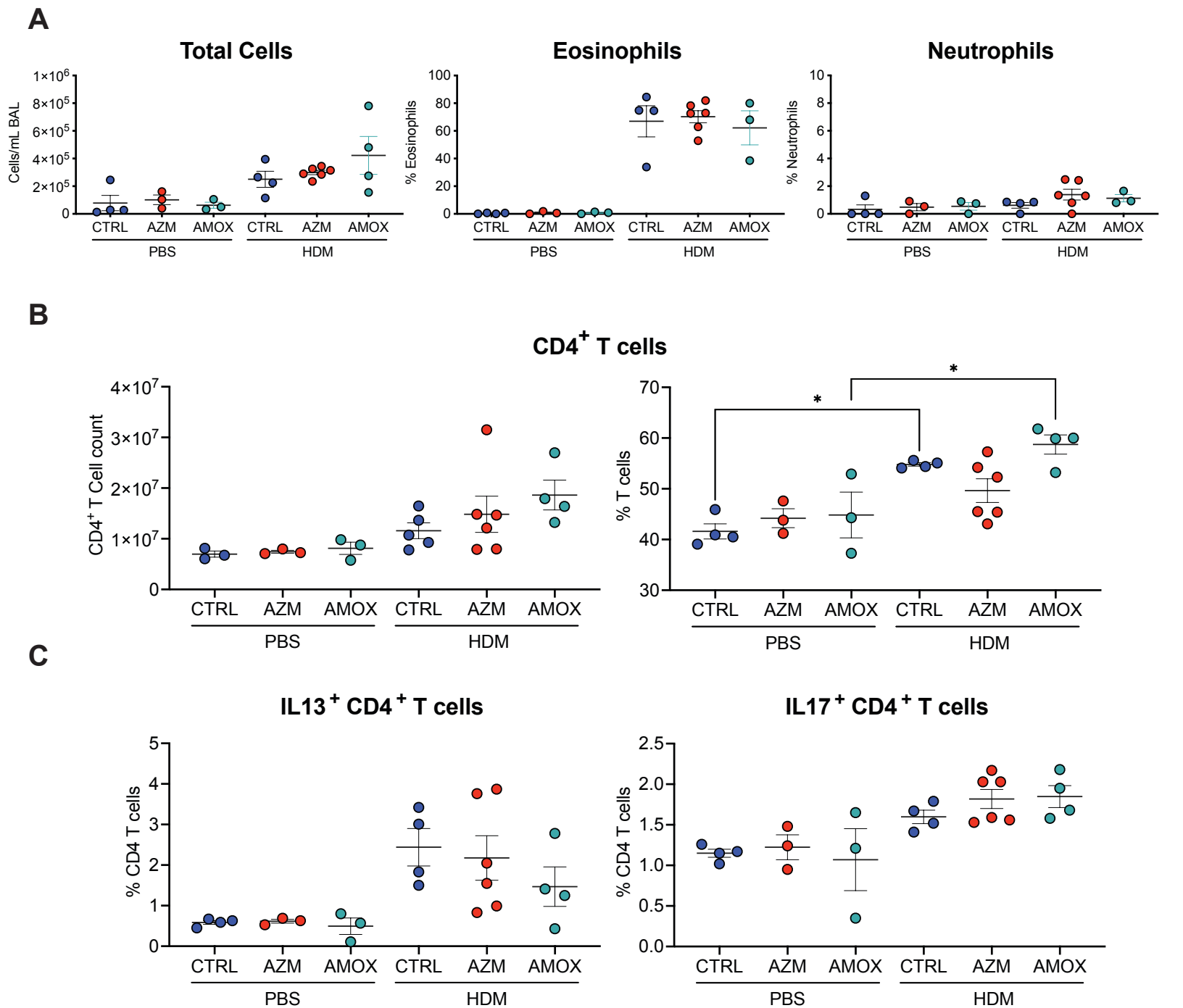


Figure S3: The allergy-enhancing effects of antibiotic exposure require the presence of a microbiota.

Germ-free mice were exposed to antibiotics, or not, during early-life and then subject to HDM sensitization and challenge (or not, PBS group). At sacrifice, BAL was collected from all mice (n= 3-4/PBS group, n= 4-7/HDM group). **A.** Cells were counted to calculate total cells/mL in the lavage and then differentially stained to detect: the proportions of eosinophils and neutrophils. Lung tissue also was mechanically and enzymatically digested and stimulated *ex vivo* using PMA and ionomycin. Flow cytometric analysis examined: **B.** the total count and proportions of $CD4^+$ T cells in the lung; **C.** IL-13-secreting $CD4^+$ cells and IL-17A-secreting $CD4^+$ cells among lung $CD4^+$ T cells. *$p<0.05$; nonparametric one-way ANOVA.

Supplementary Figure 4

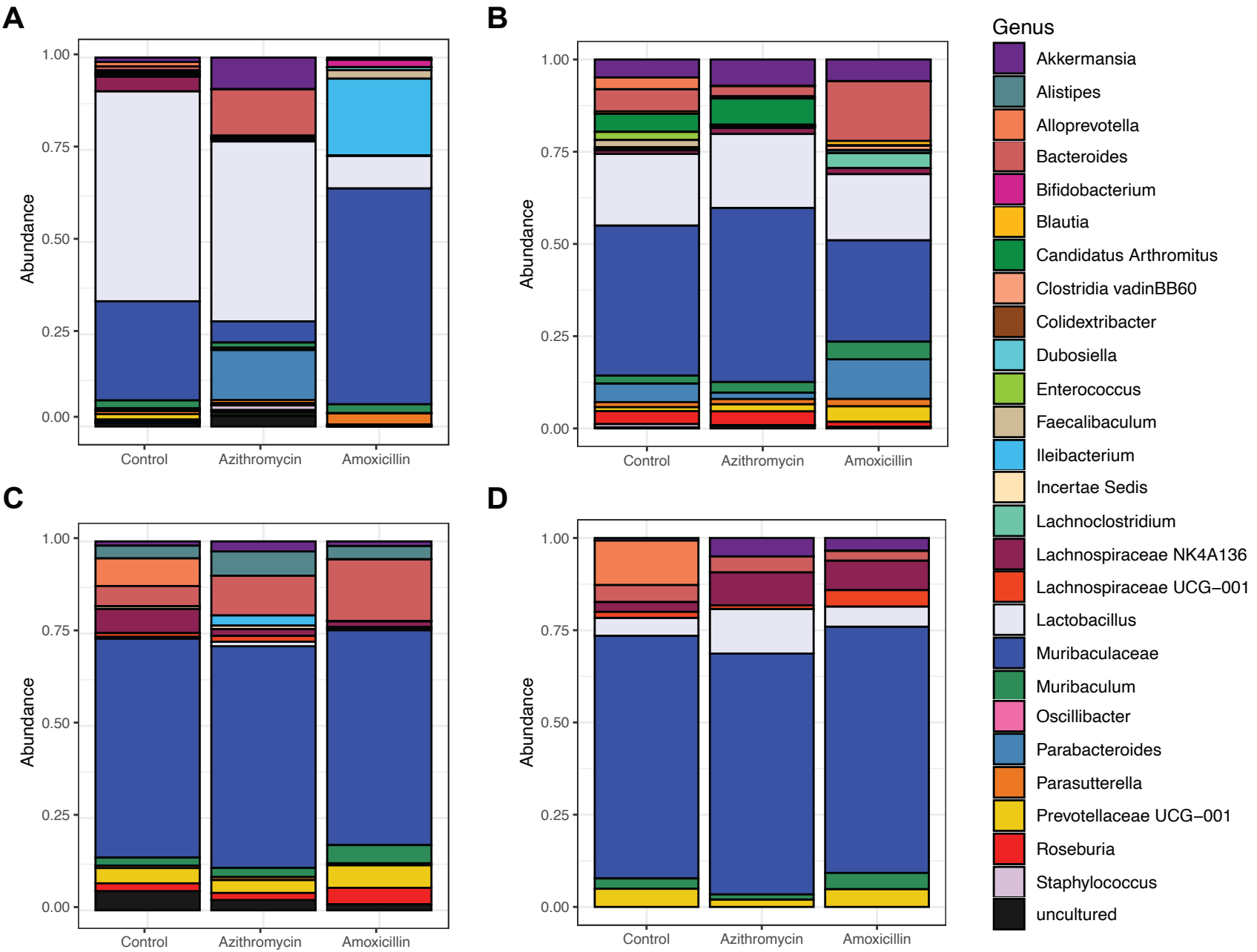
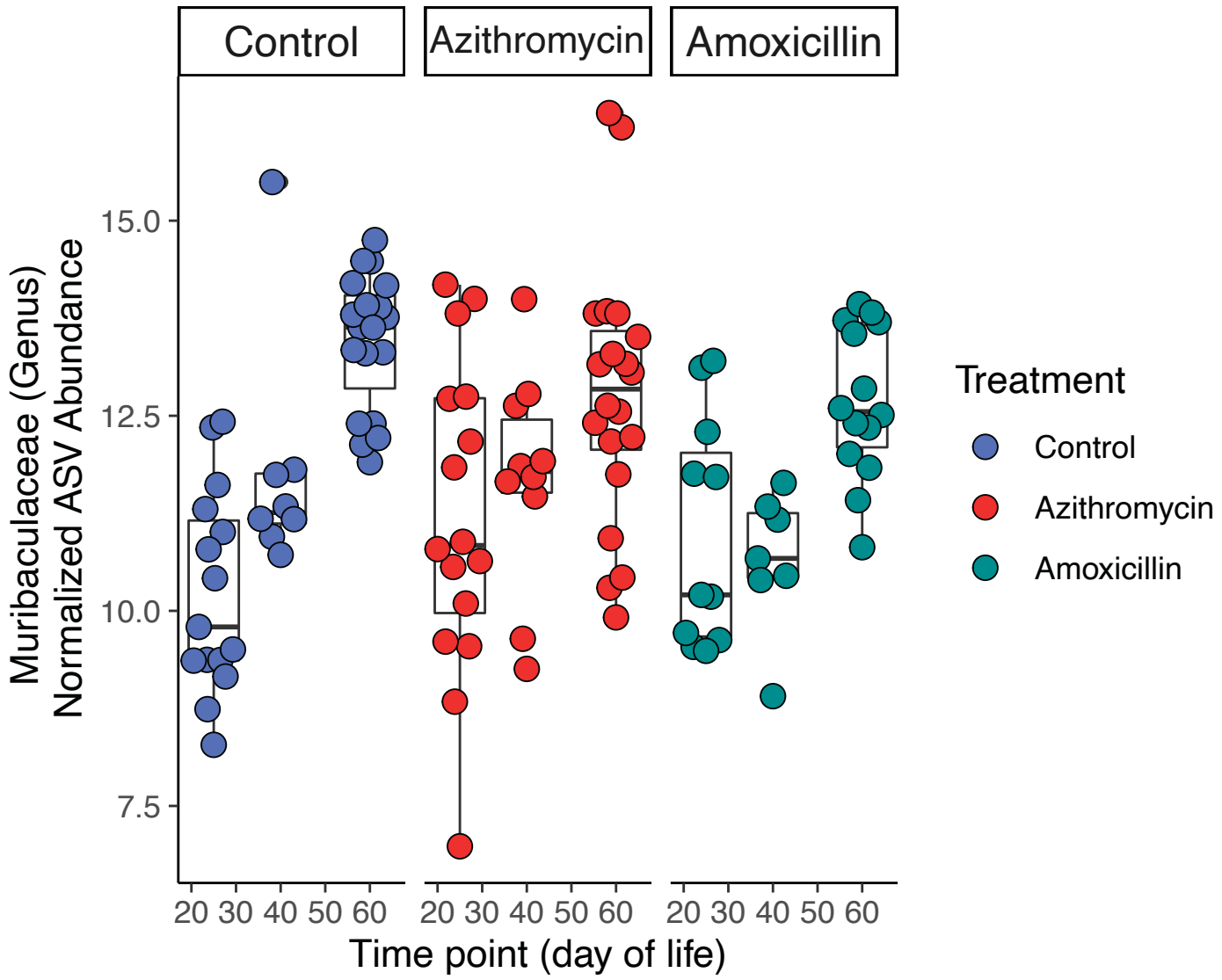


Figure S4: Taxonomic abundances at P60, according to antibiotic-exposure group. Samples of: **A.** gastric, **B.** ileal, and **C.** cecal tissue, and **D.** fecal samples were obtained at sacrifice. After 16S rRNA sequencing, relative taxonomic abundance of the 50 most abundant ASVs were determined for mice in each antibiotic exposure group and data collapsed to the genus level.

Supplementary Figure 5



Fixed effects:	Std. Error	df	t value	Pr(>|t|)
TreatmentAzithromycin	0.83428	108.98708	2.355	0.0203 *
TreatmentAmoxicillin	0.91919	108.83704	1.386	0.1685
TimePoint	0.01261	89.67033	7.389	7.38e-11 ***
TreatmentAzithromycin:TimePoint	0.01752	87.61824	-2.475	0.0153 *
TreatmentAmoxicillin:TimePoint .	0.01927	86.78812	-1.757	0.0825

Number of fecal samples: 120
Number of mice: 44

Significance codes:
'***' 0.001
'**' 0.01
'*' 0.05

Figure S5: Changes in *Muribaculaceae* over time, with respect to antibiotic-exposure. The normalized ASV abundance of *Muribaculaceae* over time is shown for azithromycin or amoxicillin-exposed and control mice. A mixed linear effect model was used to determine statistical significance and the interaction of antibiotic treatment and day of life (time point). All comparisons were with the control group. The results indicate that azithromycin significantly differed from control with respect to *Muribaculaceae* abundance (p-value = 0.02), that the change in abundance over time was significant for all groups (p-value = 7.38×10^{-11}), and that the change over time in the control and azithromycin mice was significantly different (p-value = 0.015).

Supplementary Figure 6

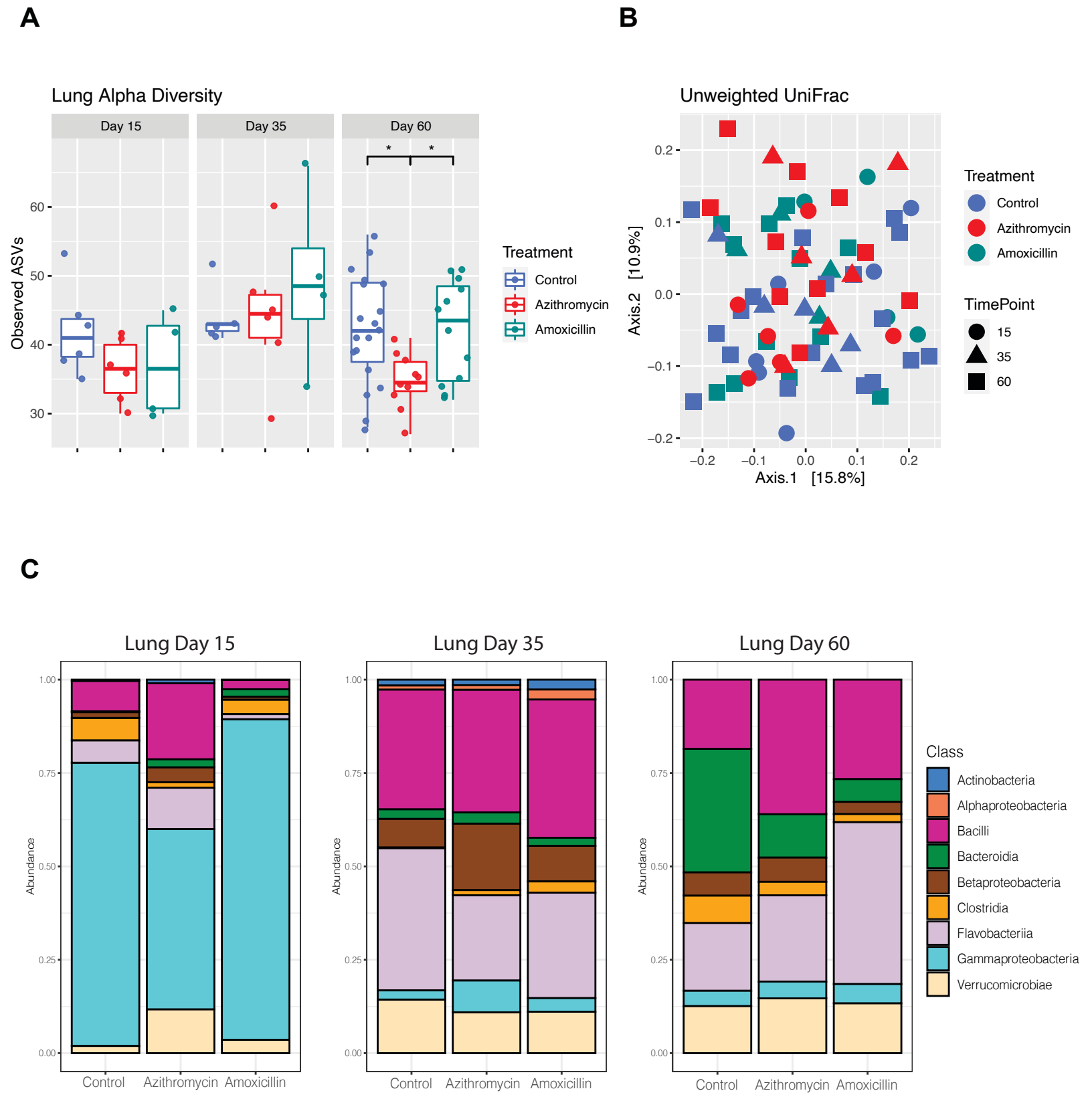


Figure S6: Low impact of the early-life antibiotic exposure on lung microbiota. Mice were sacrificed at P15, P35, or P60, as shown in **Figure 1A**, and lung tissue obtained (n = 72 mice across all timepoints). After 16S rRNA sequencing, **A.** Alpha-diversity, measured as observed ASVs, was determined in each lung sample, by treatment group. *$p < 0.05$; Wilcox test. **B.** In unweighted UniFrac analysis, samples are color-coded by antibiotic-exposure group. **C.** Relative taxonomic abundance at the class level. Taxonomic plots represent the grouped average of the extracted lung microbiota, according to treatment group and day of life.

Supplementary Figure 7

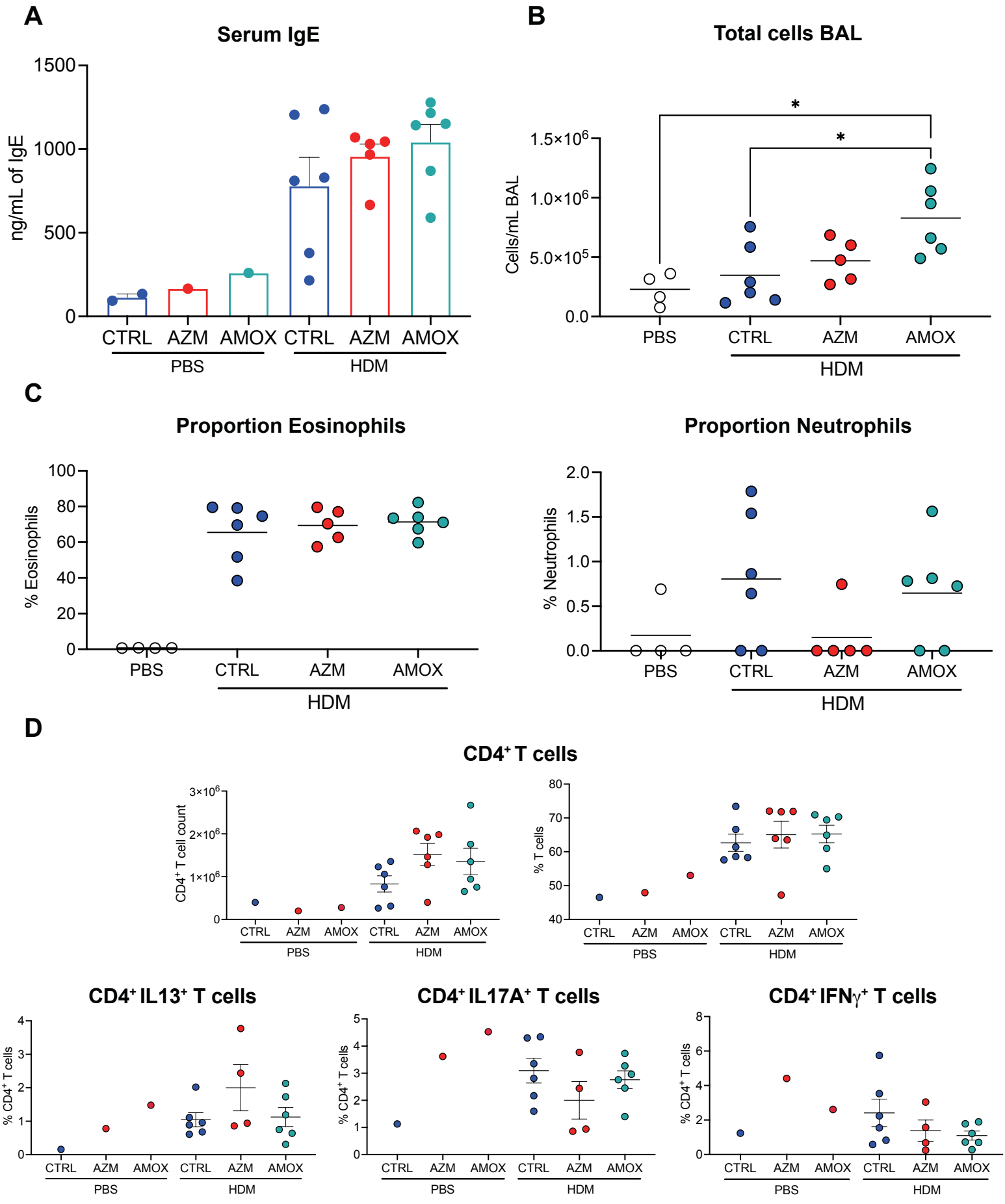


Figure S7: Phenotypes observed when adult mice were conventionalized. Adult (6-8-week-old) germ-free mice were conventionalized with the fecal microbiota of antibiotic- or control-exposed SPF mice (see **Figure 4A**). At sacrifice at day 60: **A.** Serum IgE levels were determined by IgE-specific ELISA **B.** BAL was evaluated for total cell count by trypan blue staining and after BAL samples were cytocentrifuged and differentially stained, for proportion of **C.** eosinophils, and neutrophils. Lung single cell suspensions were isolated, stimulated *ex vivo* using PMA and ionomycin, and flow cytometric analysis examined: **D.** absolute count and proportions of $CD4^+$ T cells; and frequencies of IL-13, Il-17A, and IFNγ producing $CD4^+$ T cells, in a single experiment. *$p<0.05$, nonparametric one-way ANOVA.

Supplementary Figure 8

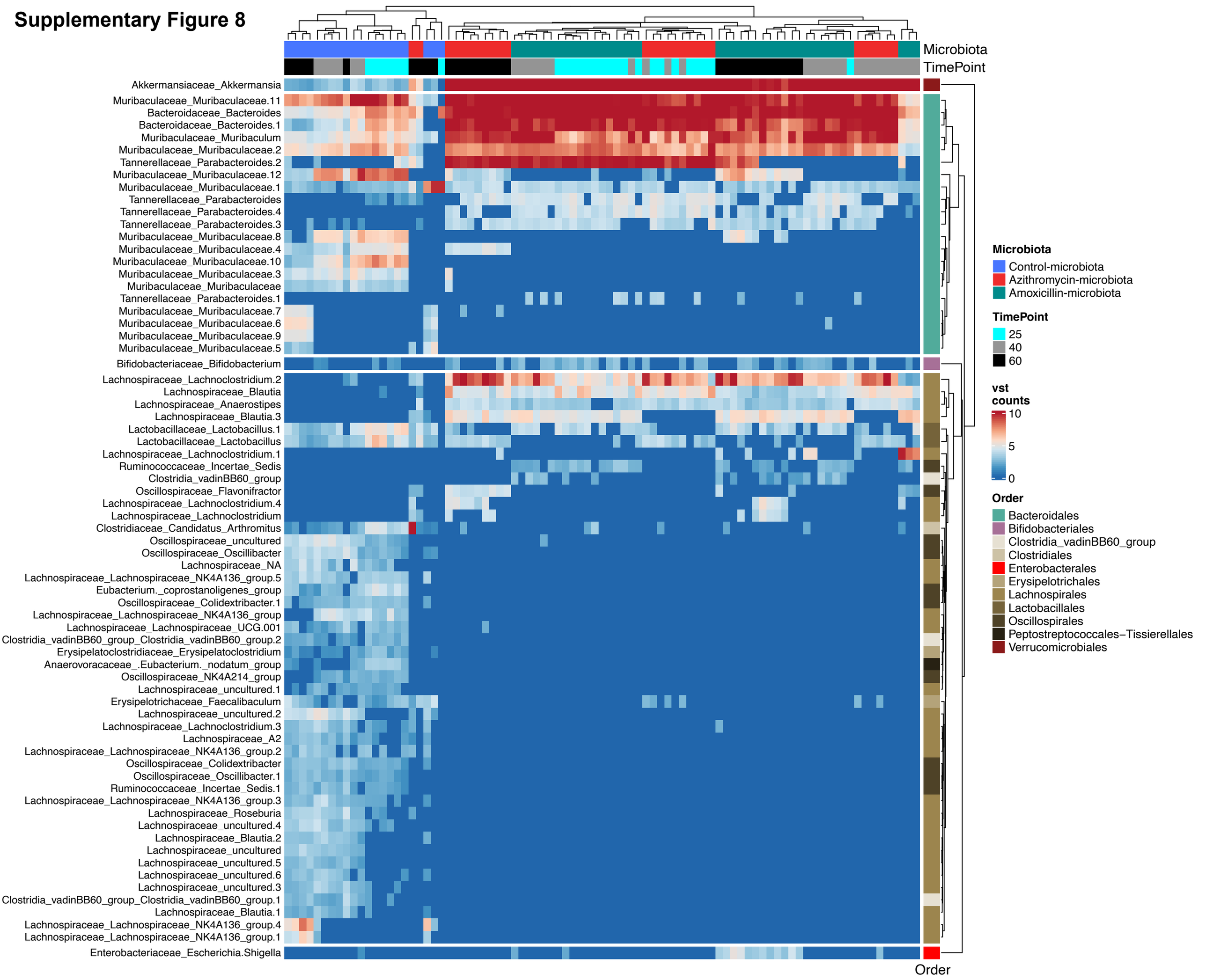


Figure S8: Identifying significantly altered ASVs between mice that were raised with control or antibiotic-perturbed microbiota. Unsupervised hierarchical clustering of differentially abundant ASVs from 87 fecal samples (columns) from 37 individual mice across multiple timepoints, identified using DESeq2, with FDR < 0.01. Samples are color-coded by treatment, day of life, and body site. Rows indicate statistically significant taxa (n = 70), taxa annotation at the genus level (species level information also included when available, annotated as: genus_species).

Supplementary Table 1: Differentially Abundant ASVs at P25 with consistent results in both experiments

log2 Fold Change SPF	Adjusted p value SPF	log2 Fold Change Transfer	Adjusted p value Transfer	Phylum	Class	Order	Family	Genus
11.6	1.57E-10	6.1	4.89E-07	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Candidatus Arthromitus
-6.6	1.48E-02	-9.1	2.29E-149	Verrucomicrobiota	Verrucomicrobiae	Verrucomicrobiales	Akkermansiaceae	Akkermansia
6.9	2.34E-02	8.2	4.96E-12	Firmicutes	Clostridia	Oscillospirales	[Eubacterium] coprostanoligenes group	[Eubacterium] coprostanoligenes group
8.8	7.66E-02	10.3	1.58E-21	Bacteroidota	Bacteroidia	Bacteroidales	Muribaculaceae	Muribaculaceae
11.5	1.37E-18	12.0	1.77E-32	Bacteroidota	Bacteroidia	Bacteroidales	Muribaculaceae	Muribaculaceae
33.8	8.33E-18	8.4	9.48E-06	Bacteroidota	Bacteroidia	Bacteroidales	Muribaculaceae	Muribaculaceae

Supplemental Table 1: Differentially abundant ASVs at P25 with consistent results in both experiments. From the experiments outlined in **Figure 1A** (in SPF mice) and **Figure 3A** (Transfer mice), ASVs that were differentially abundant at P25 between control and azithromycin-exposed mice and mice exposed to control or azithromycin-perturbed microbiota mice were identified. This table shows those ASVs that were significantly different in both experiments. The $\log_2$ fold-change between control and azithromycin mice is reported for both experiments (positive values indicate enrichment in the control mice, negative values indicate enrichment in the azithromycin mice). The FDR-adjusted p-value is reported for each ASV from both experiments.

Supplementary Table 2: Differentially Abundant ASVs at P60 with consistent results in both experiments

log2 Fold Change SPF	Adjusted p value SPF	log2 Fold Change Transfer	Adjusted p value Transfer	Phylum	Class	Order	Family	Genus
33.5	3.30E-53	8.8	3.84E-02	Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	Lachnospiraceae NK4A136 group
3.7	2.39E-02	4.9	9.16E-02	Bacteroidota	Bacteroidia	Bacteroidales	Muribaculaceae	Muribaculaceae
13.1	5.99E-37	3.3	3.44E-02	Bacteroidota	Bacteroidia	Bacteroidales	Muribaculaceae	Muribaculaceae
10.6	5.75E-15	10.7	1.12E-04	Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	Lachnospiraceae NK4A136 group
-20.8	1.05E-07	-8.1	1.28E-03	Bacteroidota	Bacteroidia	Bacteroidales	Tannerellaceae	Parabacteroides
6.5	1.33E-02	6.5	9.69E-02	Firmicutes	Clostridia	Clostridia vadinBB60 group	Clostridia vadinBB60 group	Clostridia vadinBB60 group
4.7	8.70E-02	6.3	7.27E-02	Firmicutes	Clostridia	Oscillospirales	Oscillospiraceae	Oscillibacter

Supplemental Table 2: Differentially abundant ASVs at P60 with consistent results in both experiments. From the experiments outlined in **Figure 1A** (in SPF mice) and **Figure 3A** (Transfer mice), ASVs that were differentially abundant at P60 between control and azithromycin-exposed mice, and mice exposed to the control of azithromycin-perturbed microbiota mice were identified. This table shows those ASVs that were significantly different in both experiments. The $\log_2$ fold change between control and azithromycin mice is reported for both experiments (positive values indicate enrichment in the control mice, negative values indicate enrichment in the azithromycin mice). The FDR-adjusted p-value is reported for each ASV from both experiments.