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# Supplementary Materials for

## Increasing breast milk betaine modulates *Akkermansia* abundance in mammalian neonates and improves long-term metabolic health

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**The PDF file includes:**



**Other Supplementary Material for this manuscript includes the following:**

(available at stm.sciencemag.org/cgi/content/full/13/587/eabb0322/DC1)

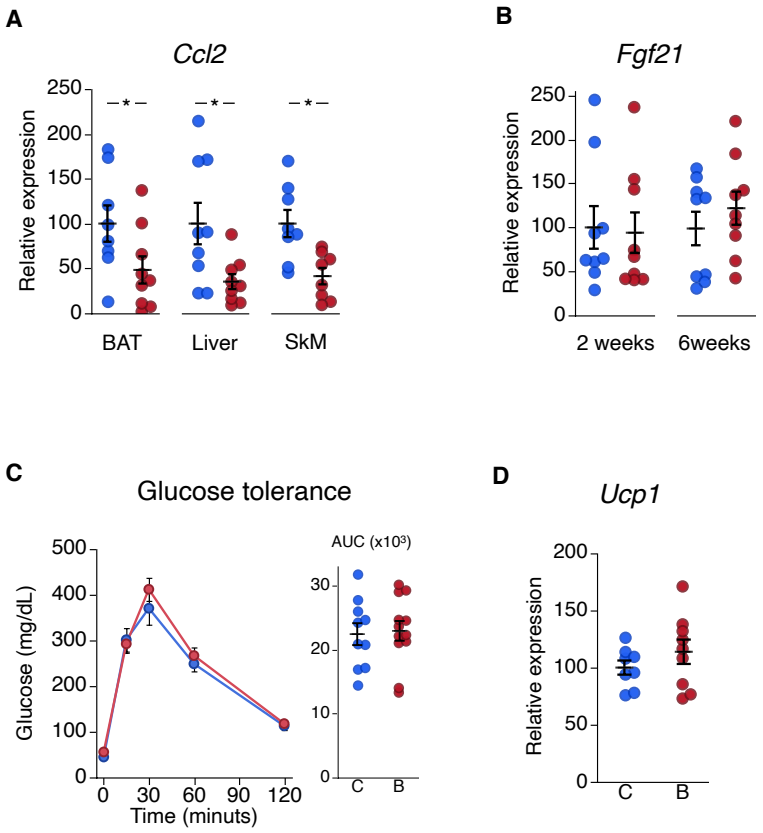


**Figure S1. Effect of maternal betaine administration on young mouse offspring.**

**A**) *Ccl2* mRNA levels in brown adipose (BAT, n=8-9 per group), liver (n=9 per group), and skeletal muscle (SkM) (n=8 per group) in 6-week-old offspring from control (C, blue color) and betaine-treated (B, red color) dams. **B**) *Fgf21* mRNA levels in liver from 2- and 6-week-old mice (n=9 per group). **C**) Glucose tolerance was measured in 8-week-old male offspring (n=10-12 per group). **D**) *Ucp1* mRNA expression in BAT from 6-week-old offspring (n=8-9 per group). Data are mean ± SEM. *, *t*-test $p<0.05$.

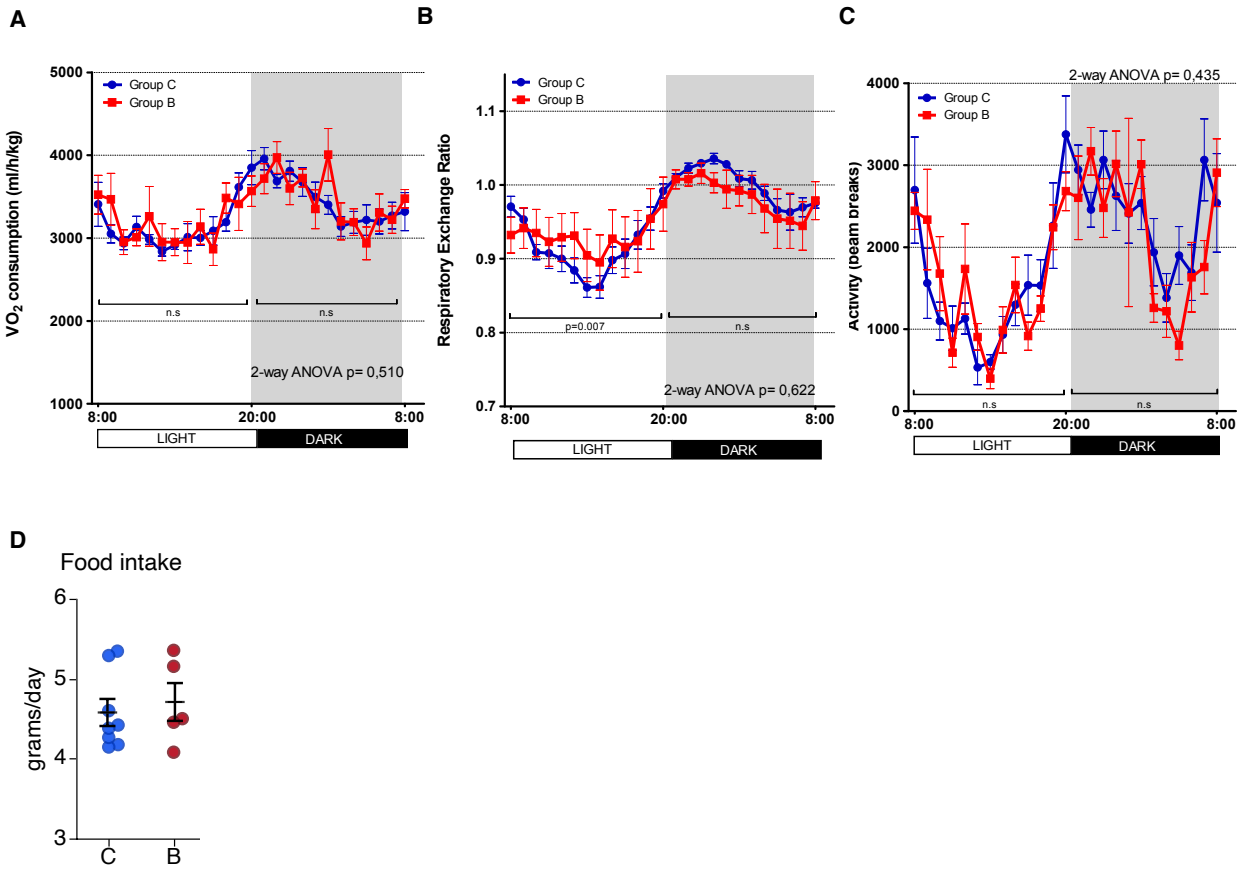


**Figure S2. Effects of maternal betaine administration on mouse offspring energy homeostasis.**

Six-week-old offspring from control (C, blue color, n=8) and betaine-treated (B, red color, n=5) dams were examined in metabolic chambers for 24 hours. **A**) Oxygen consumption, **B**) respiratory exchange ratio. **C**) physical activity, and **D**) food intake were measured. Data are mean ± SEM. Differences between groups were assessed by 2-way ANOVA (A-C).

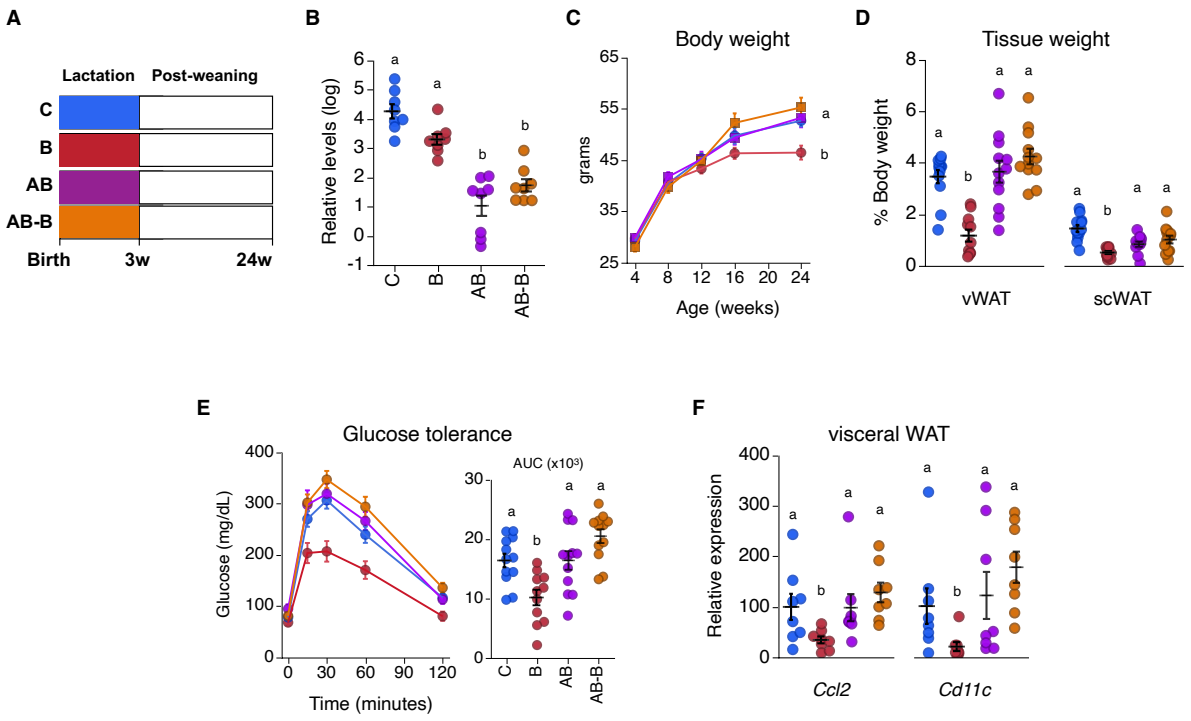


**Figure S3. Effects of maternal antibiotic co-administration on offspring long-term metabolic health in mice.** Dams were treated with betaine (B group, red), ampicillin (1g/L) and neomycin (0.5 g/L) (AB group, purple), antibiotics and betaine (AB-B group, orange) in the drinking water, or with no supplement (C group, blue) during lactation and offspring monitored until adulthood. **A**) Schematic representation of the experimental design. **B**) A subset of mice was sacrificed at 2 weeks of age and bacterial DNA extracted from cecal samples; levels of bacterial 16S gene as a measure of microbial content was determined by qPCR (oligos 5'-ACTCCTACGGGAGGCAGCAG-3' and 5'-ATTACCGCGGCTGCTGG-3') and expressed as log-transformed relative levels per gram of cecal sample (n=8 per group). **C**) Body weight and **D**) WAT weight at sacrifice (n=11-12 per group). **E**) Glucose tolerance at 22 weeks of age (n=11-12 per group). **F**) mRNA expression of vWAT immune markers (n=8 per group). Data are mean ± SEM. Different letters indicate statistically significant differences after one-way ANOVA and post-hoc Tukey test ($p<0.05$).

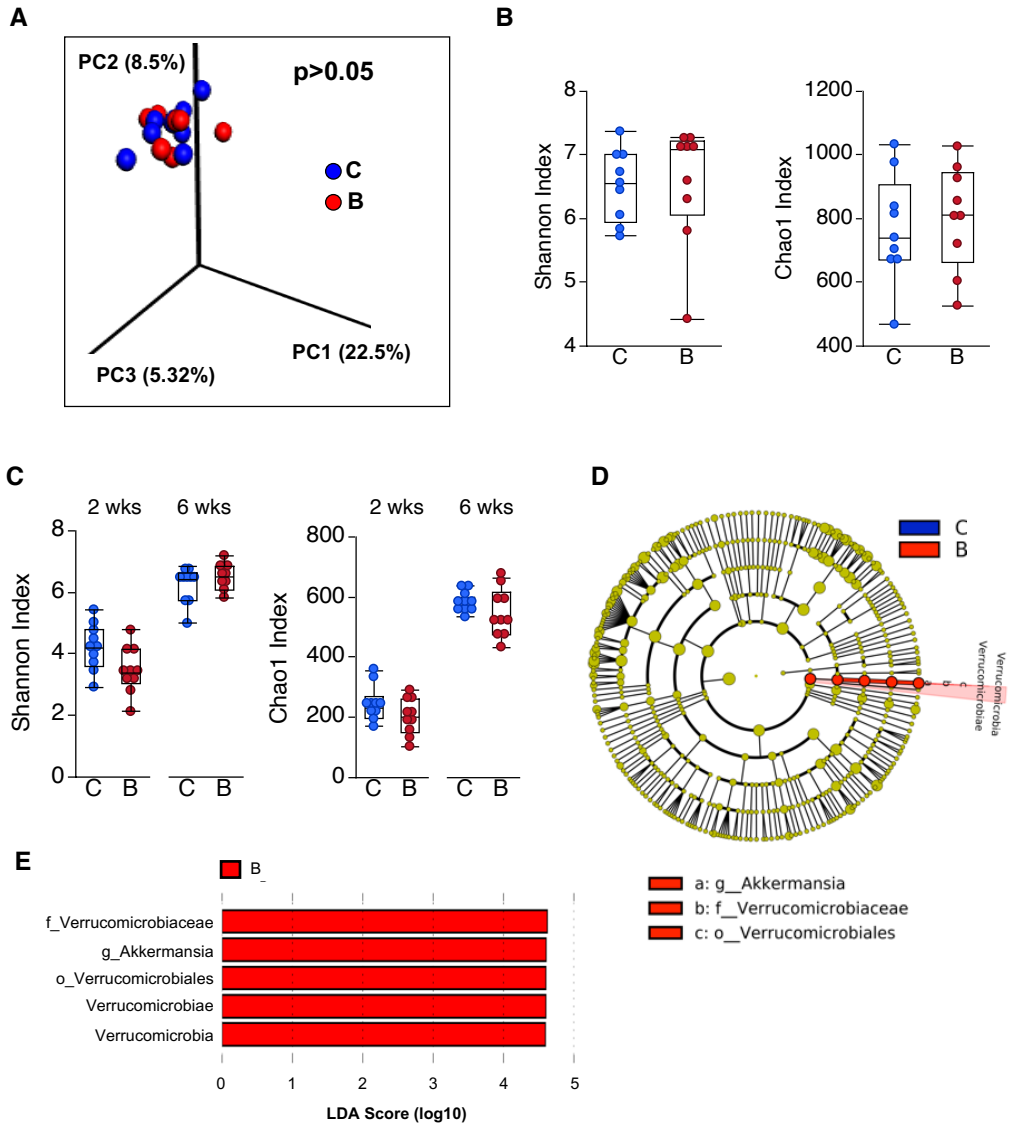


**Figure S4. Effect of betaine administration on the maternal and offspring gut microbiome in mice.** Betaine-treated (B group, red color, n=8) or control (C group, blue color, n=8) dams and offspring were sacrificed at day 14 after delivery, and at 6 weeks of age (n=10 per group). **A)** Principal coordinate analysis of unweighted UniFrac distances of cecal samples; p value assessed by adonis test. **B**) Diversity Shannon and Chao1 indices from C and B dams. **C**) Diversity Shannon and Chao1 indices from offspring at 2 and 6 weeks of age. **D**) Cladogram and **E**) LDA scores arising from LEfSe analysis in 2-week-old pups from betaine-treated and control groups.

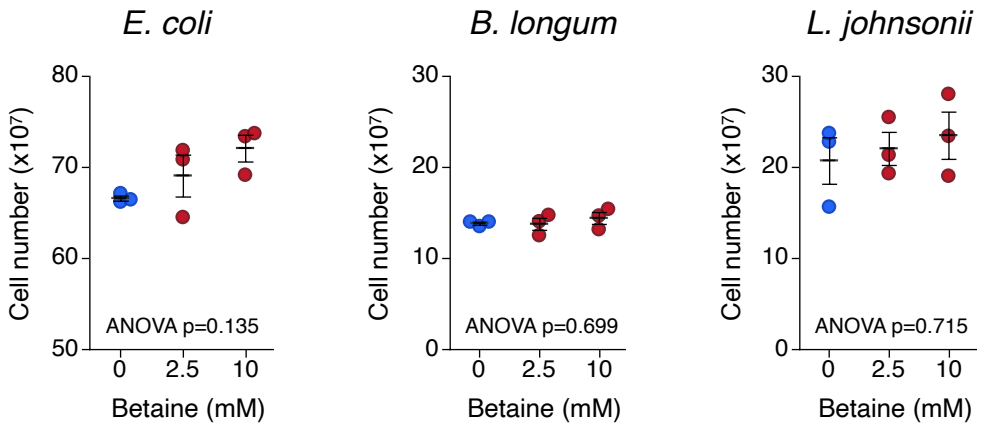


**Figure S5. Effect of betaine on bacterial growth in vitro.**

*Escherichia coli*, *Bifidobacterium longum* and *Lactobacillus johnsonii* were grown in vitro in the absence (blue color) or presence (red color) of 2.5 mM or 10 mM betaine in the media until reaching the stationary phase (n=3 per group). Cell number for the indicated bacterial species was determined based on optical density (600 nm) after 8h, 12h, or 15h of growth, respectively. Data are mean ± SEM. One-way ANOVA was applied to assess differences between groups.

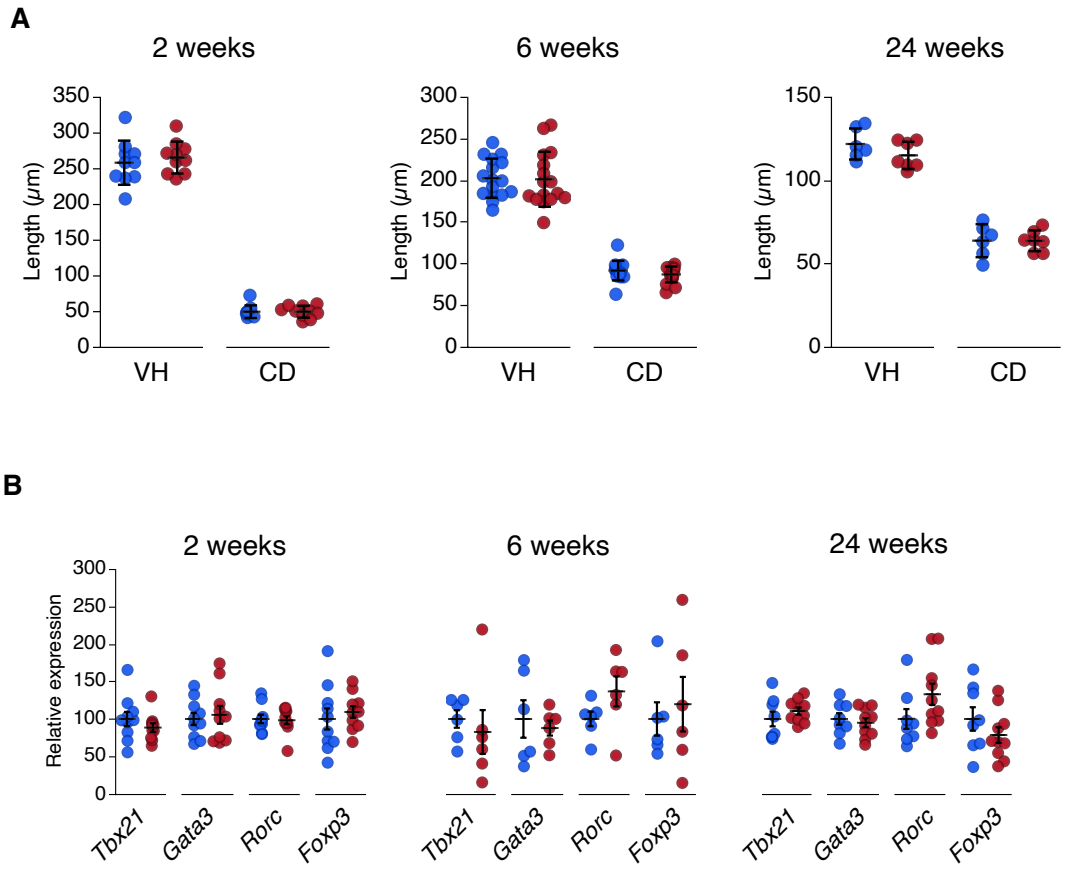


**Figure S6. Effect of maternal betaine supplementation on mouse ileum histology and gene expression.**

**A**) Ileal vili height (VH) and crypt depth (CD) in ileum sections from 2- (n=10 per group), 6- (n=16 per group), and 24-week-old (n=6-7 per group) control (blue circles) and betaine-treated mice (red circles). **B**) Ileal mRNA levels of T cell differentiation markers *Tbx21*, *Gata3*, *Rorc*, and *Foxp* at 2 weeks (n=10 per group), 6 weeks (n=6 per group), and 24 weeks of age (n=8-10 per group). Data are shown as mean ± SEM. Group differences were assessed by Student's *t*-test.

| | Cohort I | | Cohort II | |
|---|---|---|---|---|
| | **B (CI 95%)** | ***P* value** | **B (CI 95%)** | ***P* value** |
| **Change in body length z score** | | | | |
| Birth - 1 month | 0.01 (-0.57, 0.58) | 0.982 | 0.22 (-0.07, 0.51) | 0.138 |
| Birth - 12 months | --- | | -0.04 (-0.37, 0.30) | 0.826 |
| **Change in head circumference (cm)** | | | | |
| Birth - 1 month | -0.01 (-0.67, 0.65) | 0.973 | -0.14 (-0.51, 0.24) | 0.478 |
| Birth - 12 months | --- | | -0.37 (-0.91, 0.15) | 0.162 |

**Table S1. No association between milk betaine concentration and change in human infant body length z score and head circumference.** Least-square regression modelling was applied to assess the correlation between milk betaine level (independent variable) and body length z score change from birth to 1 month or 12 months (dependent variable). Model was adjusted for gestational age, pre-pregnancy BMI, gestational weight gain, birth method, and body length z score at birth (for change in body length) or head circumference at birth (for change in head circumference). B, size effect estimate from the regression model; CI, confidence interval.

| | | *Akkermansia muciniphila* | | Chi-square |
|---|---|---|---|---|
| | | **Absent** | **Present** | **p value** |
| **1 month (n=83)** | Low betaine | 32 (38.6 %) | 10 (12.1 %) | 0.840 |
| | High betaine | 32 (38.6 %) | 9 (10.8 %) | |
| **12 months (n=91)** | Low betaine | 28 (30.8 %) | 18 (19.8 %) | 0.348 |
| | High betaine | 23 (25.3 %) | 22 (23.5 %) | |

**Table S2. Prevalence of *A. muciniphila* in human infants exposed to low and high breast milk betaine content.** Infants were categorized into Low and High betaine groups based on the median value of breast milk betaine concentration (4.1 $\mu$M). Presence of *A. muciniphila* was determined in fecal samples from 1-month (n=83) and 12-month-old infants (n=91) by qPCR. Data are shown as n (%) of subjects with either absence or presence of *A. muciniphila* in fecal samples. Chi-square test was applied to detect differences in prevalence among low and high betaine groups at 1 month or 12 months of age.

| Gene | Forward | Reverse |
|---|---|---|
| ***Hprt*** | 5'-GCCCCAAAATGGTTAAGGTTG-3' | 5'-GTCAAGGGCATATCCAACAAC-3' |
| ***Muc2*** | 5'-CTGACCAAGAGCGAACACAA-3' | 5'-CATGACTGGAAGCAACTGGA-3' |
| ***Ocln*** | 5'-ATGTCCGGCCGATGCTCTC-3' | 5'-CTTTGGCTGCTCTTGGGTCTGTAT-3' |
| ***Zo2*** | 5'-CTAGACCCCCAGAGCCCCAGAAA-3' | 5'-TCGCAGGAGTCCACGCATACAAG-3' |
| ***Zo1*** | 5'-TTTTGACAGGGGGAGTGG-3' | 5'-TGCTGCAGAGCTCAAAGTTCAAG-3' |
| ***Ccl2*** | 5'-CAAGATGATCCCAATGAGTAG-3' | 5'-TTGGTGACAAAAACTACAGC-3' |
| ***Tlr4*** | 5'-GCCTCCCTGGCTCCTGGCTA-3' | 5'-CAGGGACTTTGCTGAGTTTCTGATCCA-3' |
| ***Cd11c*** | 5'-AGTCTGTTGGGTTCTGTAAG-3' | 5'-ACAGTTCTGTTATGACATGC-3' |
| ***Fgf21*** | 5'-AGCTCTCTATGGATCGCCTCACTT-3' | 5'-ACACATTGTAACCGTCCTCCAGCA-3' |
| ***Ucp1*** | 5'-CAAATCAGCTTTGCCTCACTC-3' | 5'-ACACCTCCAGTCATTAAGCC-3' |
| ***Tbx21*** | 5'-ACGTCTTTACTTTCCAAGAG-3' | 5'-GTACATGGACTCAAAGTTCTC-3' |
| ***Gata3*** | 5'-TATTAACAGACCCCTGACTATG-3' | 5'-CACCTTTTTGCACTTTTTCG-3' |
| ***Rorc*** | 5'-CTGTGTTTTTCTGAGGATGAG-3' | 5'-GCAGAGATGATGATGGAAAG-3' |
| ***Foxp3*** | 5'-AATAGTTCCTTCCCAGAGTTC-3' | 5'-GGTAGATTTCATTGAGTGTCC-3' |
| ***A. muciniphila*** | 5'-CAGCACGTGAAGGTGGGGAC-3' | 5'- CCTTGCGGTTGGCTTCAGAT-3' |

**Table S3. Primer sequences for qPCR analyses.**