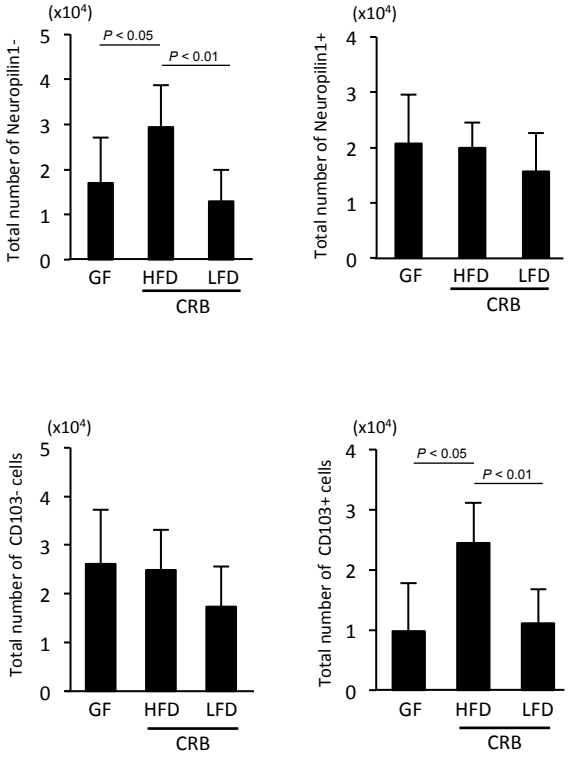
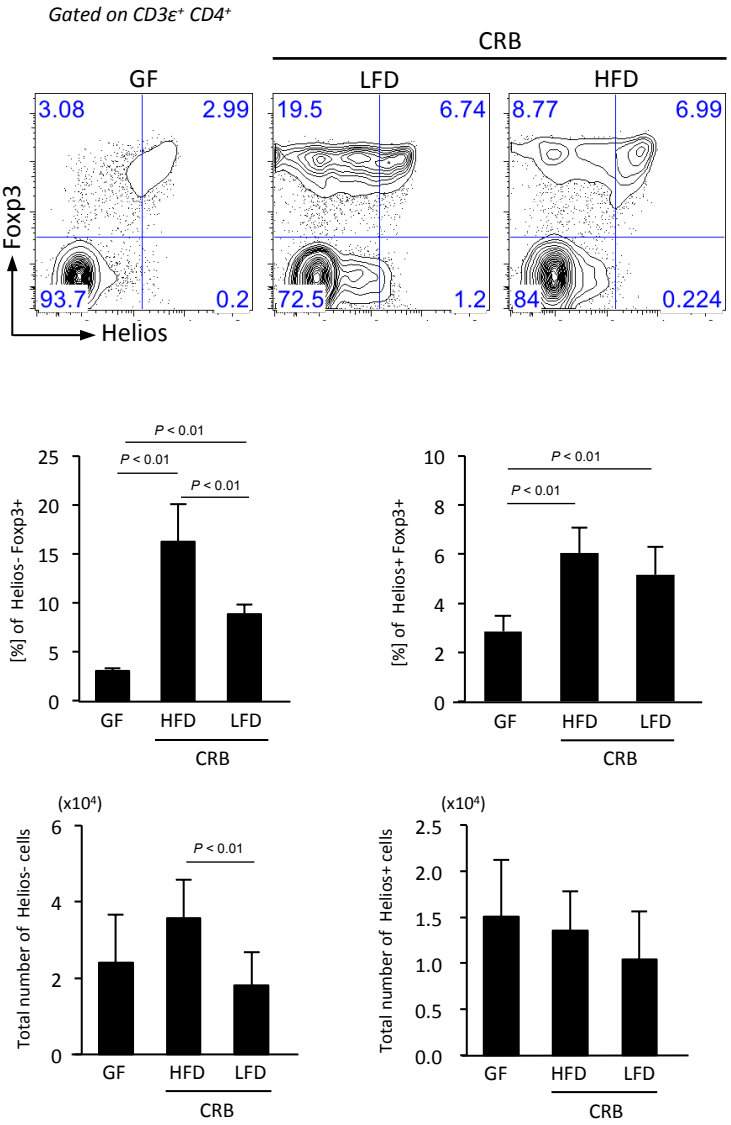
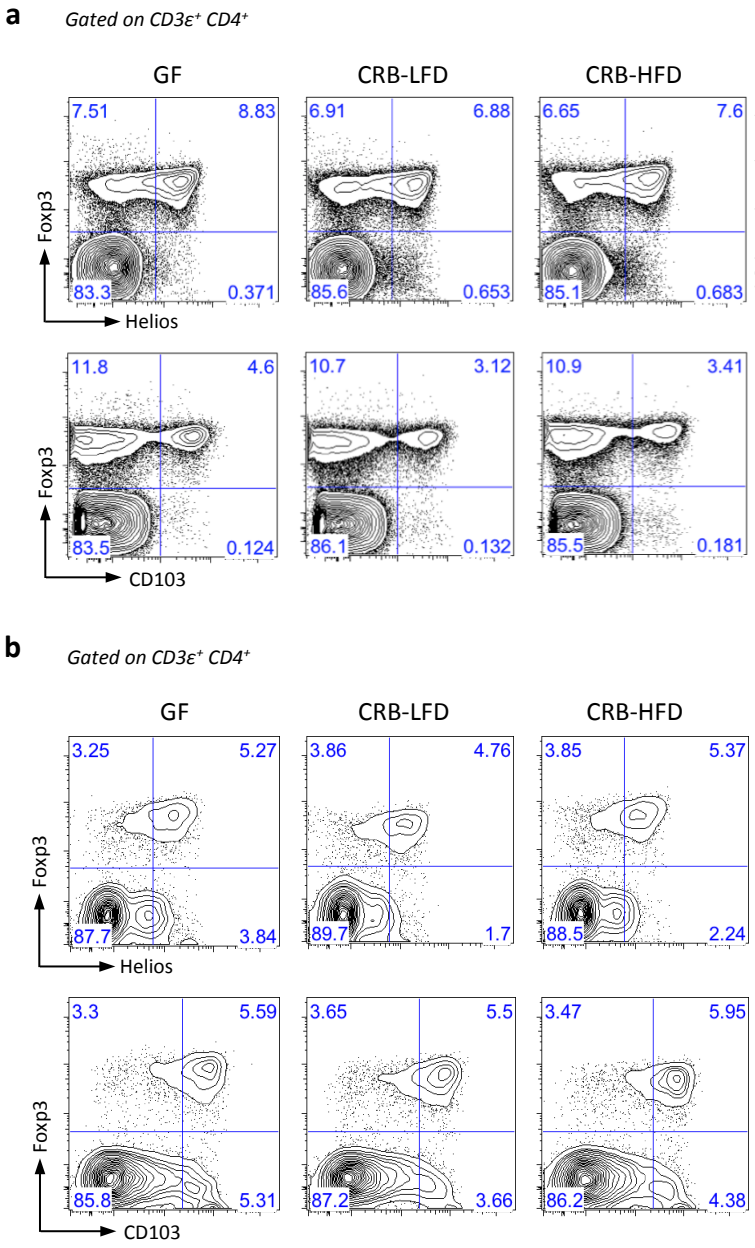
doi:10.1038/nature12721



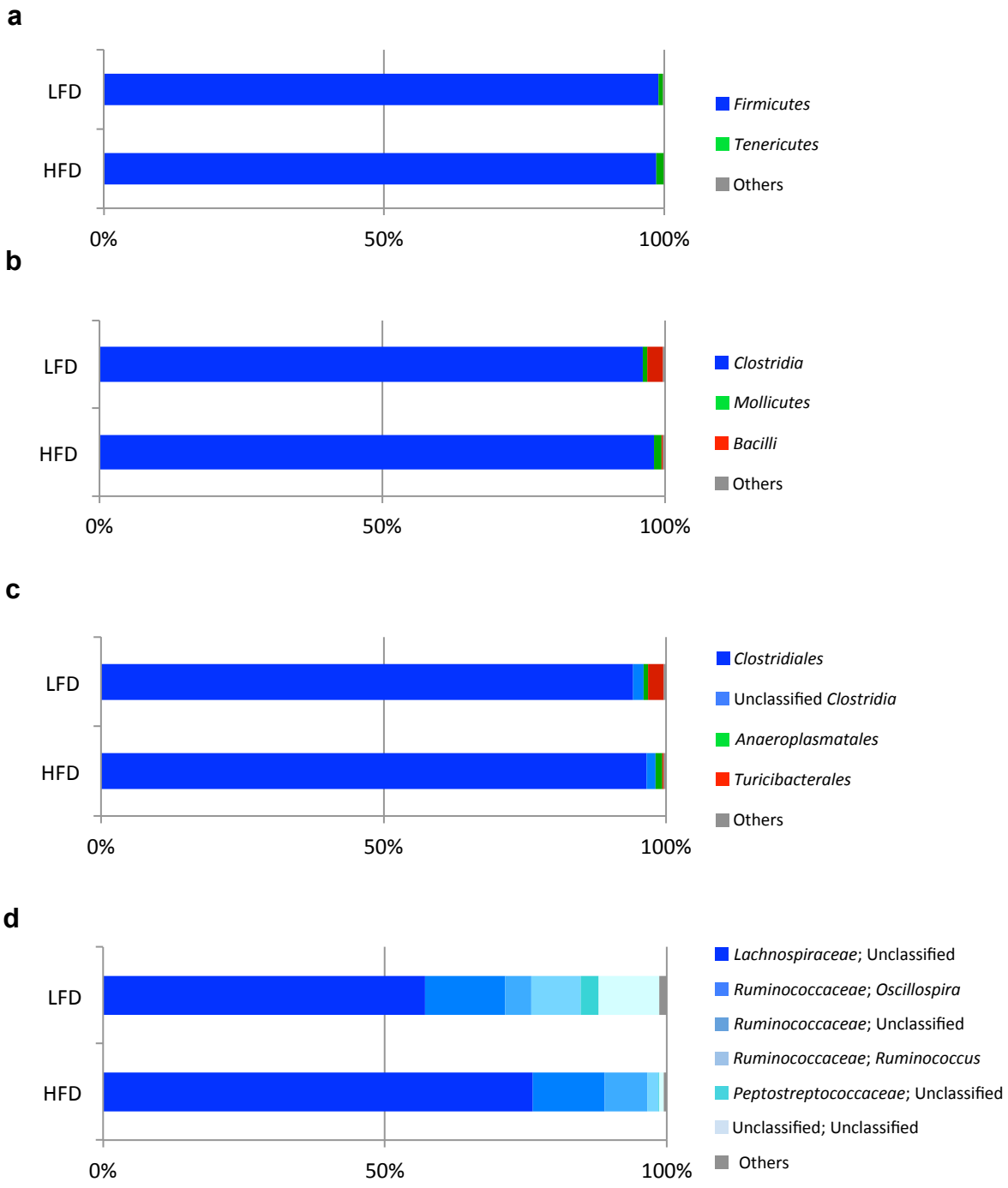
Supplementary Figure 1 | The number of total regulatory T cells in colonic lamina propria. Colonic lamina propria cells were prepared from GF and CRB mice fed with HFD and LFD. CD3ε $^{+}$CD4^{+} T cells were further analyzed for expression of Foxp3, Neuropilin1 and CD103. Data are representative of three independent experiments. The Error bars indicate s.d. (n = 10). *P* values were determined by the one-way ANOVA test followed by Tukey's test.



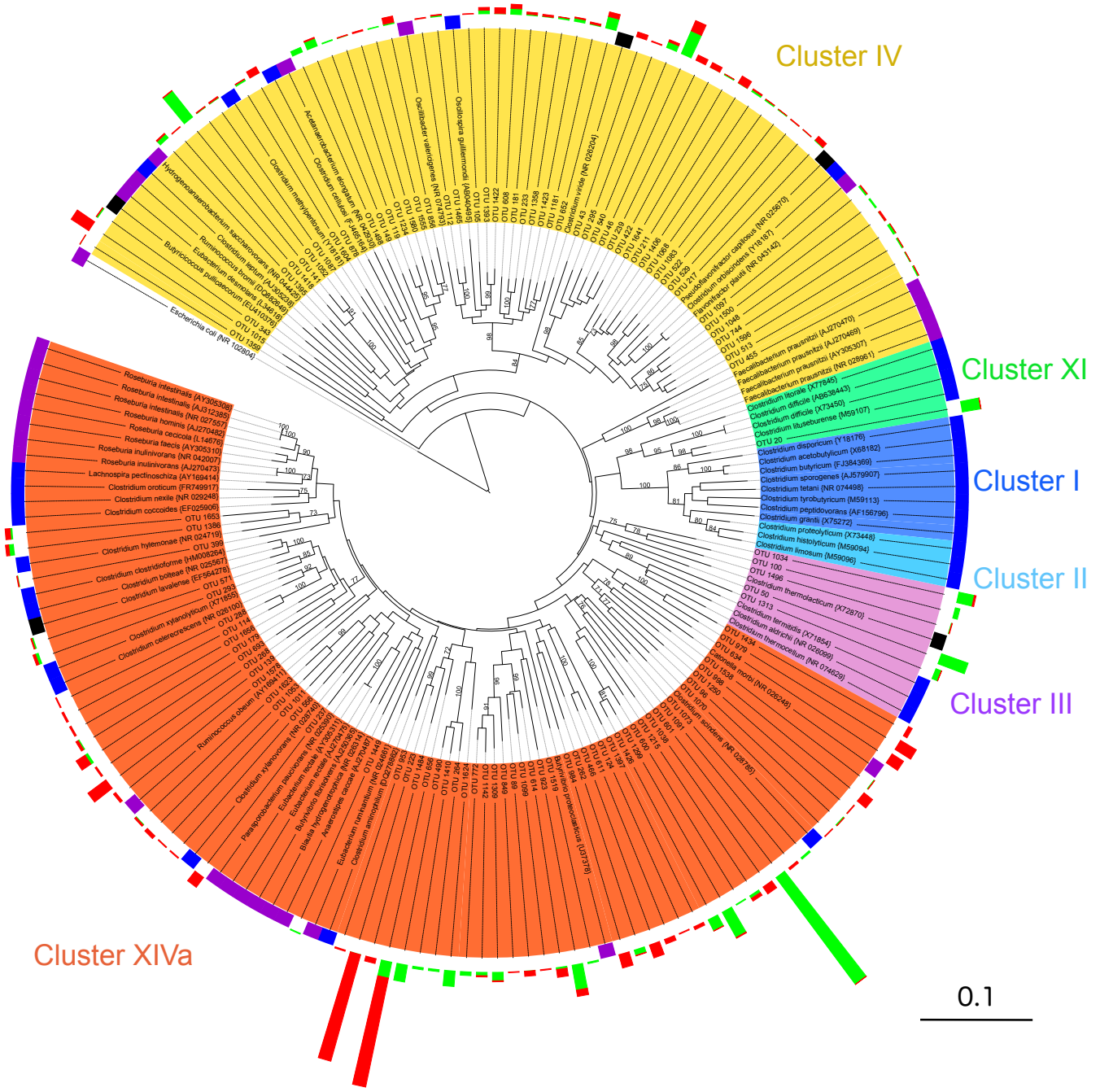
Supplementary Figure 2 | Helios expression in Foxp3⁺ cells in the colonic lamina propria. Colonic lamina propria cells were prepared from GF and CRB mice fed with HFD and LFD. CD3ε⁺CD4⁺ T cells were further analyzed for expression of Foxp3 and Helios. Data are representative of three independent experiments. The Error bars indicate s.d. (n = 10). *P* values were determined by the one-way ANOVA test followed by Tukey's test.



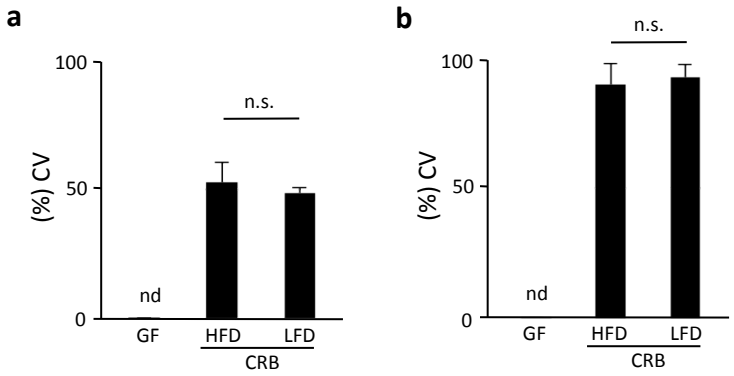
Supplementary Figure 3 | Flow cytometric analysis of lymphocytes in spleen and mesenteric lymph node (MLN).
MLN (**a**) and spleen (**b**) lymphocytes were prepared from GF and CRB mice fed with HFD and LFD. CD3ε$^+$CD4$^+$ T cells were further analyzed for expression of the indicated markers. The Error bars indicate s.d. (n = 5). Data are representative of three independent experiments.



Supplementary Figure 4 | 16S rRNA sequence-based analysis of the cecal microbiota of CRB-associated mice fed with LFD or HFD.
a-d, 16S rRNA gene was PCR amplified and sequenced on the 454 platform (n = 2). Phylum (**a**), class (**b**), order (**c**) and family; genus in *Clostridiales* (**d**) are shown.

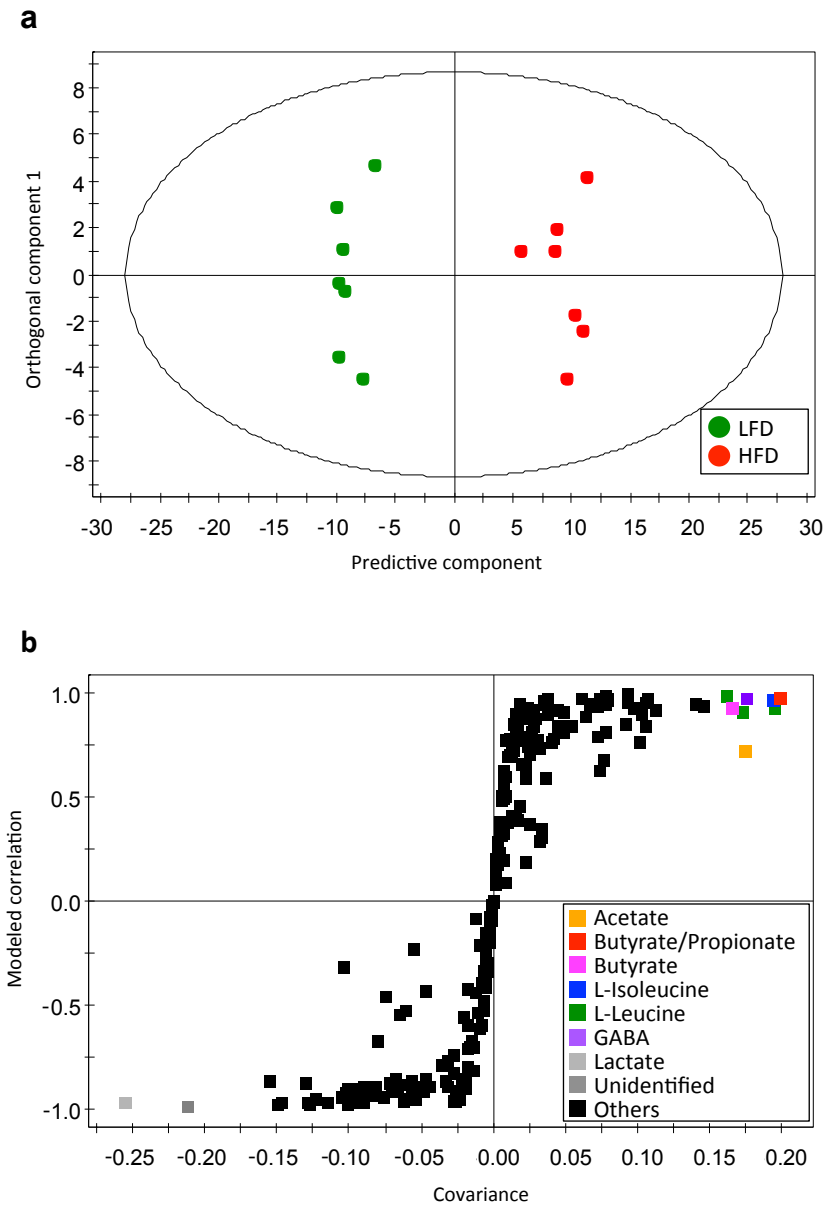


Supplementary Figure 5 | Phylogenetic distribution of operational taxonomic units (OTUs) obtained from 16S rRNA sequences in order *Clostriales* colonized in CRB mice.
12,000 filter-passed reads of 16S rRNA sequences in CRB mice were used for OTUs and a phylogenetic tree was constructed based on the CRB-derived 114 OTUs which were categorized into *Clostridiales* by RDP-classifier (v2.5) and 69 reference 16S rRNA sequences of *Clostridiales* obtained from the GenBank database. The 16S rRNA sequence of *Escherichia coli* was used as the outgroup. The phylogenetic tree was constructed based on the neighbor-joining method, with 100 bootstrap iterations (bootstrap values > 70 are shown on each branch). The scale bar indicates an estimated 0.1 change per nucleotide position. Colors configuring a doughnut shape represent the branches consisting of the distinct clusters of *Clostridiales*. The reference 16S rRNA sequences from spore forming, non-spore forming, and unknown spore forming capacity bacteria are shown in blue, purple and black, respectively. The outside red and green bars indicate the relative number of sequence reads in each OTU from CRB-HFD and CRB-LFD, respectively. Most of OTUs both in CRB-HFD and CRB-LFD were clustered into *Clostridium* clusters IV and XIVa.



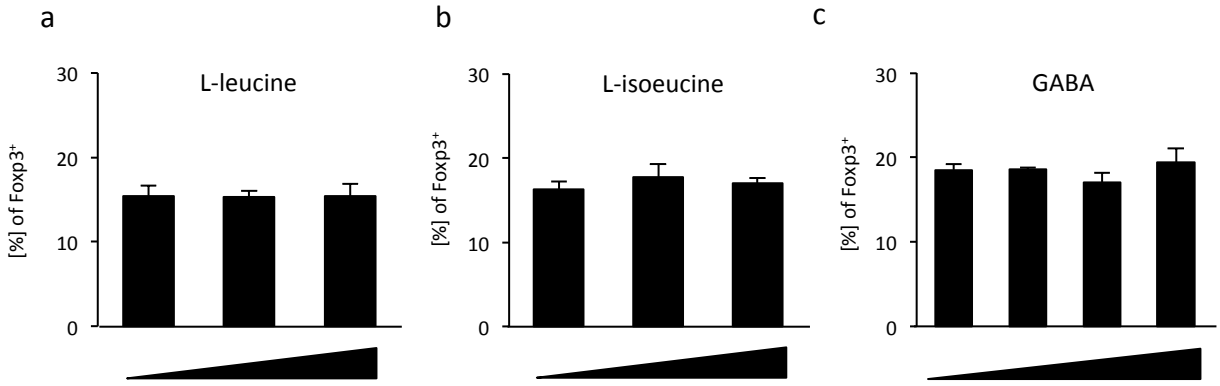
Supplementary Figure 6 | The number of total commensal microbes and *Clostridiales* in GF, and CRB mice fed with HFD and LFD.

a, b, Total number of commensal microbes (**a**) and *Clostridiales* (**b**) were detected by qPCR with 16S rRNA gene universal primers and *Clostridiales*-specific primers. The results were calculated as the quantity relative to the copy number detected in the feces of conventionalized mice (CV). Error bars indicate s.e.m. ($n = 5$). *P* values were determined using the Kruskal-Wallis test followed by the Scheffé test. n.s., not significantly different. n.d., not detected.



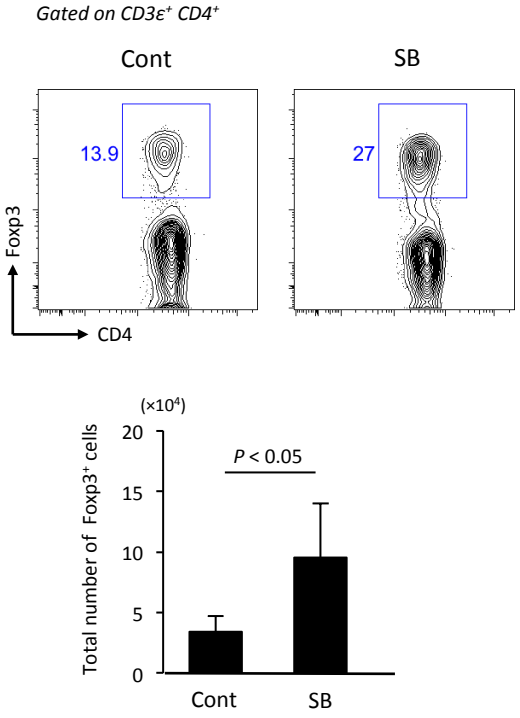
Supplementary Figure 7 | Orthogonal partial least squares discriminate analysis (OPLS-DA) on the cecal metabolome data of CRB-LFD and CRB-HFD mice.

a, Cross-validated score plots from OPLS-DA of ^{1}H-NMR data of CRB-LFD and CRB-HFD murine cecum (n = 7). The model resulted in one predictive and one orthogonal (1+1) components with the cross-validated predictive ability $Q^2(Y)$ = 0.93 and the total explained variance $R^2(X)$ = 0.79. The ellipse denotes the 95% significance limit of the model, as defined by Hotelling's *t*-test. **b,** S-plot for predictive component from OPLS-DA of ^{1}H-NMR data of CRB-LFD and CRB-HFD murine cecum. The S-plot visualizes the variable influence in a model and corresponds to combining the contribution or magnitude (covariance) with the effect and reliability (correlation) for the model variables with respect to model component scores. The highlighted signals (|covariance| > 0.16) in the S-plot have high contribution and reliability for class separation between CRB-LFD and CRB-HFD mice.

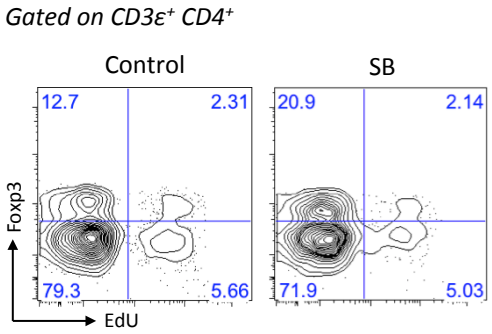


Supplementary Figure 8 | Leucine, Isoleucine and GABA have no effect on the induction of Foxp3$^+$ cells

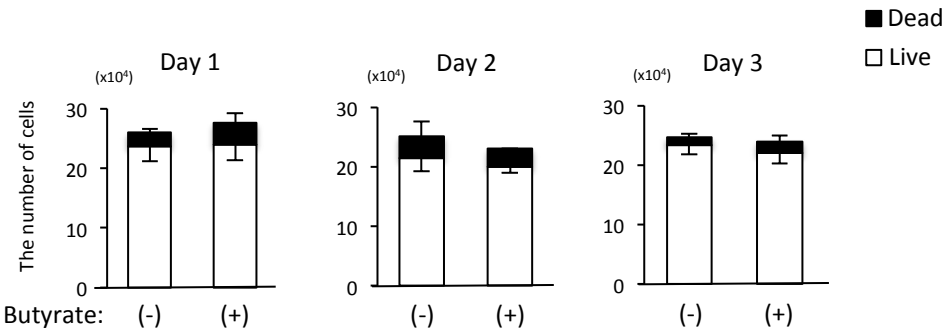
a, b, c, Naïve CD4$^+$ T cells were cultured under Treg-inducing conditions *in vitro*. The cells were cultured in RPMI 1640 medium, which contains 0.38 mM each of L-leucine and L-isoleucine, that was supplemented with L-leucine (0, 0,12 and 0.62 mM) (**a**), L-isoleucine (0, 0,12 and 0.62 mM) (**b**) or GABA (0, 0,001, 0.01 and 0.1 mM) (**c**). Cells were further analyzed for expression of Foxp3. The Error bars indicate s.d. (n = 3).



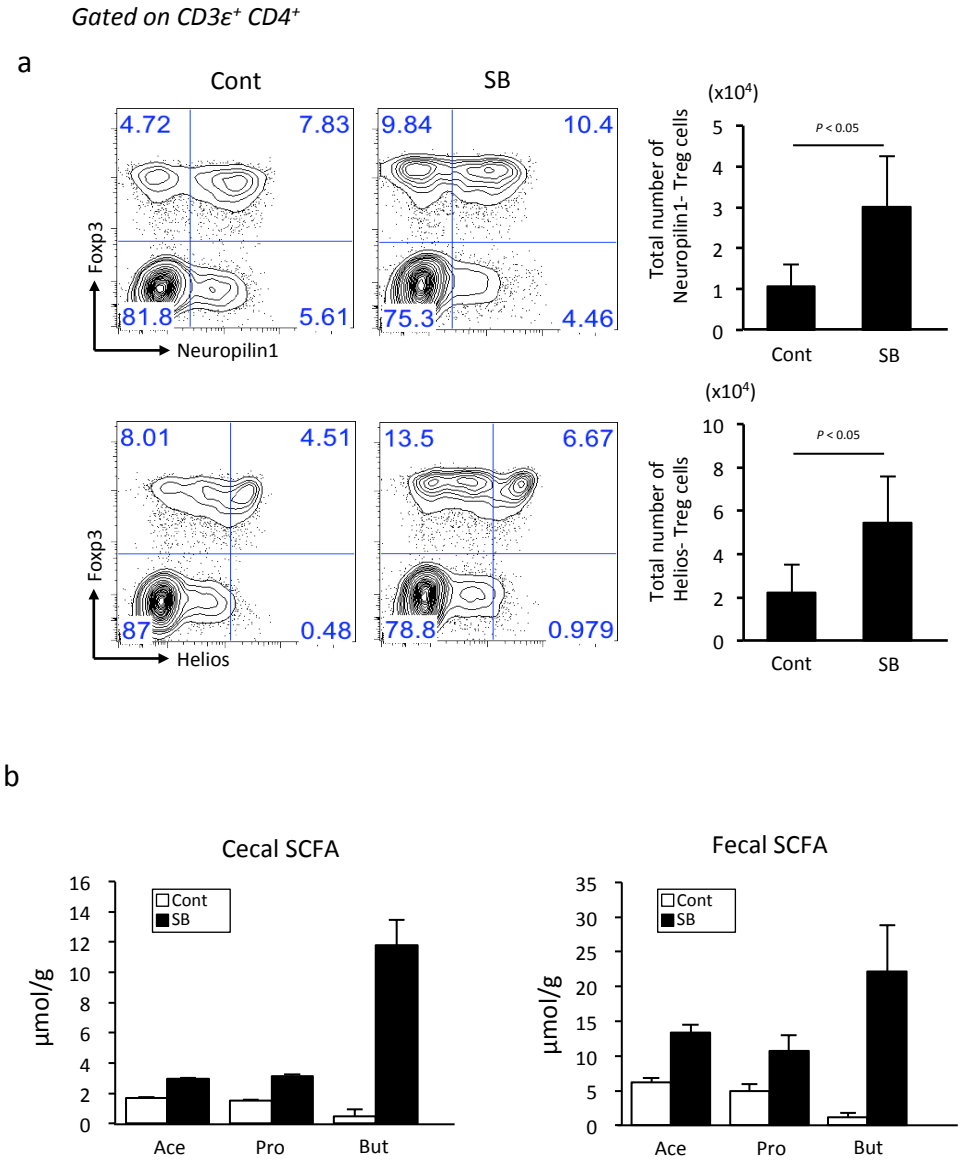
Supplementary Figure 9 | The number of total Foxp3$^+$ cells in colonic lamina propria of C57BL/6 mice fed with HAMS or HAMSB. Colonic lamina propria cells were prepared from C57BL/6 mice fed with HAMS (Cont) or HAMSB (SB). CD3ε$^+$CD4$^+$ T cells were further analyzed for expression of Foxp3. Data are representative of three independent experiments. The Error bars indicate s.d. (n = 10). *P* values were determined by the non-parametrical Mann-Whitney U test



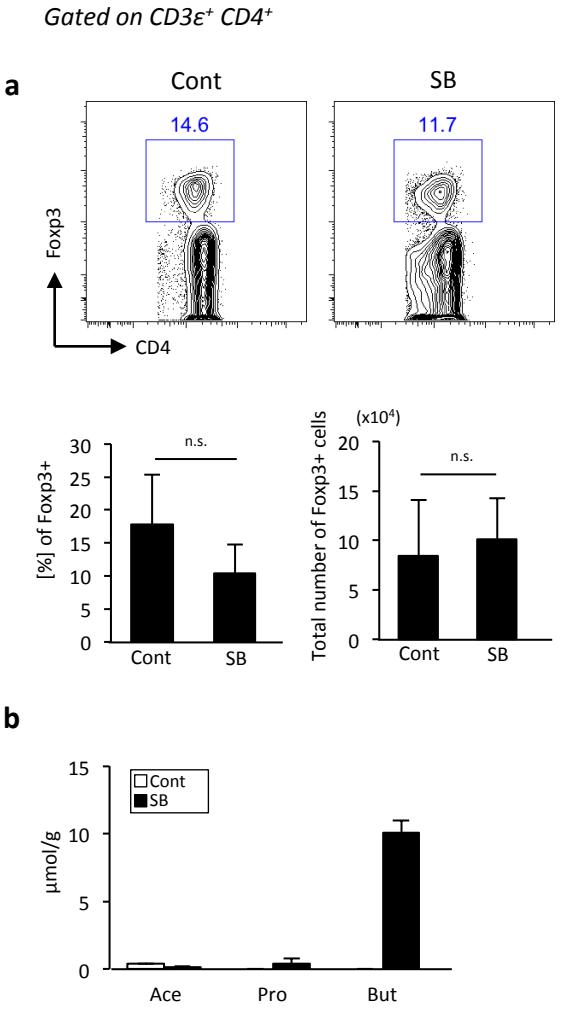
Supplementary Figure 10 | Butyrate has no effect on proliferation of Treg cells in colonic lamina propria. a, Mice fed with HAMS (Control) or HAMSB (SB) were treated with EdU, a nucleoside analog incorporated into DNA during synthesis phase of cell cycle, for 2 days before the analysis. Colonic lamina propria cells were prepared from those mice and CD3ε $^{+}$CD4^{+} T cells were further analyzed for expression of Foxp3 and EdU. The Error bars indicate s.d. (n = 5).



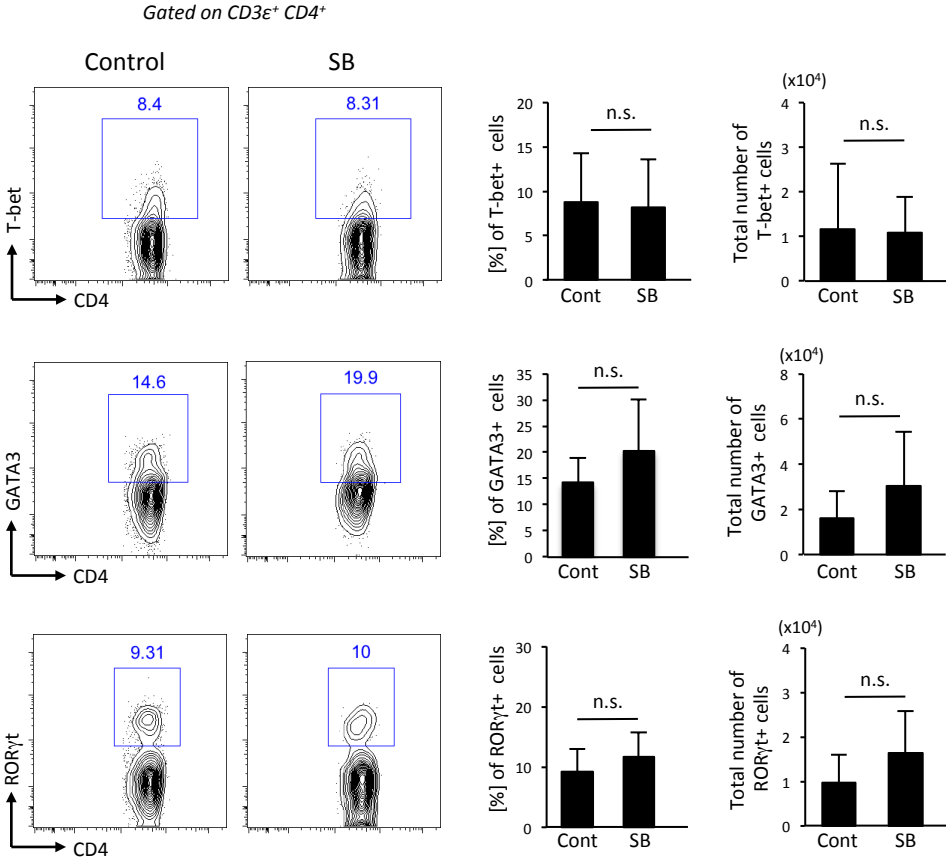
Supplementary Figure 11 | Butyrate has no effect on cell survival under Treg-inducing conditions *in vitro.*
The number of total cells under Treg-inducing condition cultures in the absence or presence of butyrate. Cells were cultured as described in Fig.3a. The viability was evaluated by Trypan blue dye exclusion test. The Error bars indicate s.d. (n = 5).



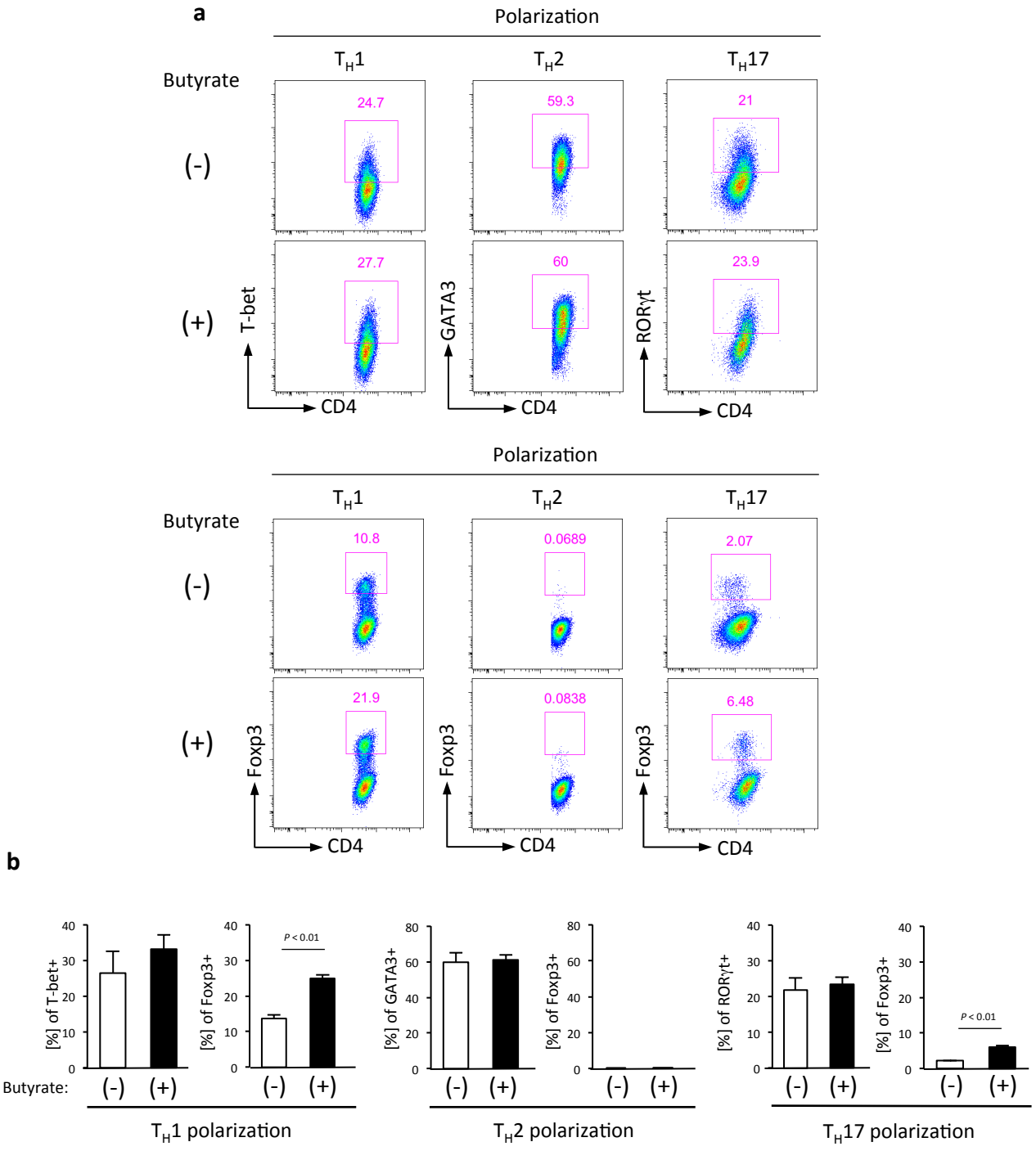
Supplementary Figure 12 | Intestinal butyrate induces the accumulation of Neuropilin- and Helios- subsets of Treg cells in mice colonized with *Bacteroides thetaiotaomicron*. GF mice were colonized with *B. thetaiotaomicron*. *B. thetaiotaomicron*-associated mice were fed with HAMS (Cont) or HAMSB (SB) for 4 weeks. **a,** Colonic lamina propria cells were prepared and CD3ε$^+$CD4$^+$-gated cells were further analyzed for expression of Foxp3, Neuropilin and Helios. *P* values were determined by the non-parametrical Mann-Whitney U test. **b,** Cecal and fecal amount of short-chain fatty acids from *B. thetaiotaomicron*-associated mice fed with HAMS or HAMSB. Ace, acetate; Pro, propionate; But, butyrate. The Error bars indicate s.d. (n = 5).



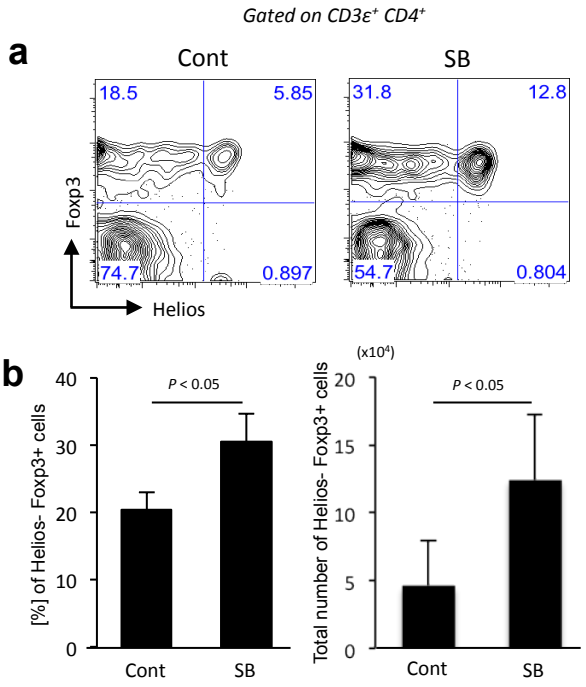
Supplementary Figure 13 | Butyrate has no effect on Foxp3$^+$ induction in colonic lamina propria of GF mice fed with HAMS or HAMSB. a, Colonic lamina propria cells were prepared from GF mice fed with HAMS (Cont) or HAMSB (SB). CD3ε$^+$CD4$^+$ T cells were further analyzed for expression of Foxp3. **b**, Amount of cecal short-chain fatty acids from GF mice fed with HAMS or HAMSB. Ace, acetate; Pro, propionate; But, butyrate. Data are representative of three independent experiments. n.s., not statistically significant. The Error bars indicate s.d. (n = 5).



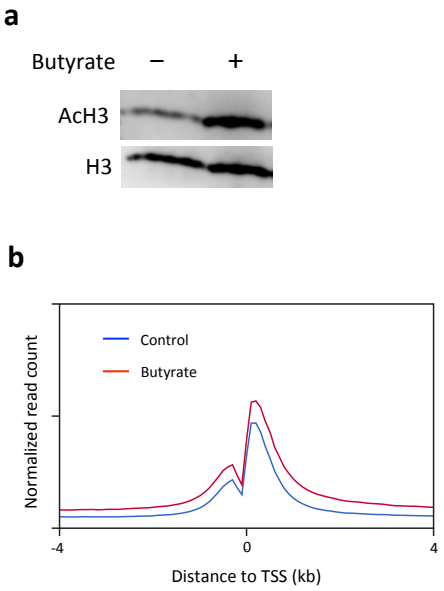
Supplementary Figure 14 | Intestinal butyrate has no effect on the accumulation of T-bet$^+$, GATA3$^+$ and RORγt$^+$ T cells. Colonic lamina propria cells were prepared from SPF C57BL/6 mice fed with HAMS (Control or Cont) or HAMSB (SB). CD3ε$^+$CD4$^+$ T cells were further analyzed for expressions of T-bet, GATA3, and RORγt, the lineage-specific transcription factors of T_H1, T_H2, and T_H17, respectively. The Error bars indicate s.d. (n = 10). *P*-values were calculated by Mann-Whitney U test. Data are representative of three independent experiments.



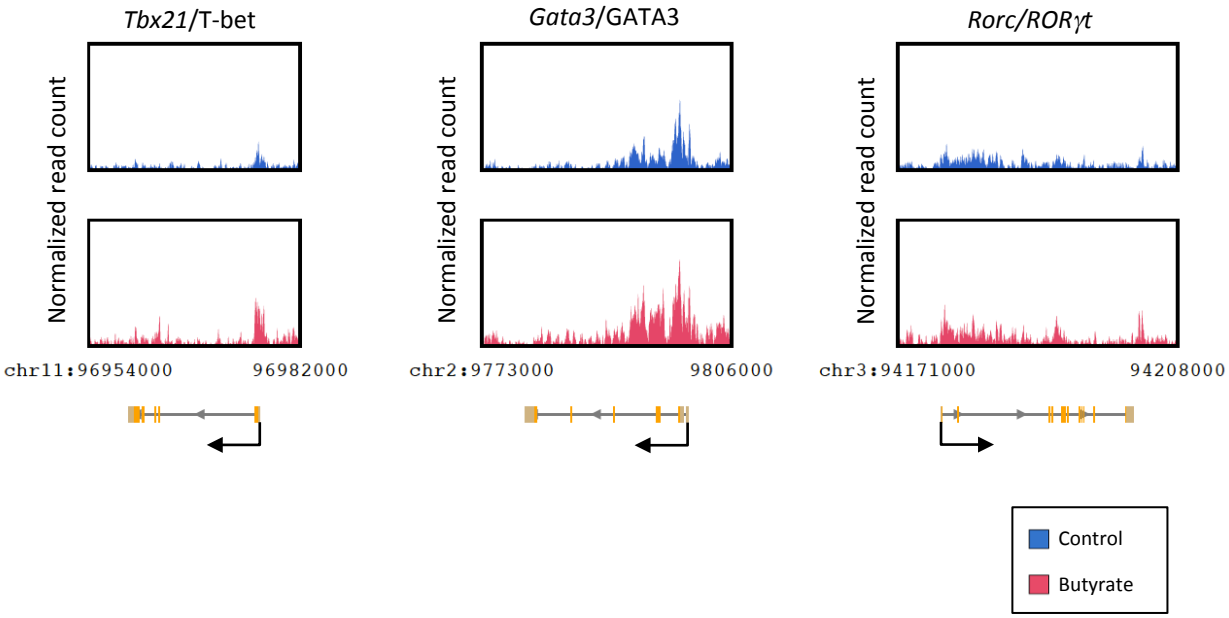
Supplementary Figure 15 | Butyrate increases the frequency of Foxp3$^+$ T cells under T_H1 and T_H17 polarizing conditions. a, b, Naïve CD4$^+$ T cells were stimulated with immobilized anti-CD3 and soluble anti-CD28 antibodies. Cells were cultured in the presence or absence of butyrate and either with rIL-12 (10 ng/mL) plus anti-IL-4 (10 mg/mL) for T_H1 polarization, with rIL-4 (10 ng/mL) plus anti-IFNγ (10 mg/mL) for T_H2 polarization or with rIL-6 (10 ng/mL), rhTGFβ (0.2 ng/mL), rIL-1β (10 ng/mL), anti-IFNγ plus anti-IL-4 for T_H17 polarization. rIL-2 (10 ng/mL) was added to culture medium 2 days later. Cells were further analyzed for expression of Foxp3, T-bet, Gata3 and RORγt on day 4. Representative FACS plots gated on CD3ε$^+$CD4$^+$ are shown in **c**. The Error bars indicate s.d. (n = 3). *P*-values were calculated by Mann-Whitney U test. Data are representative of three independent experiments.



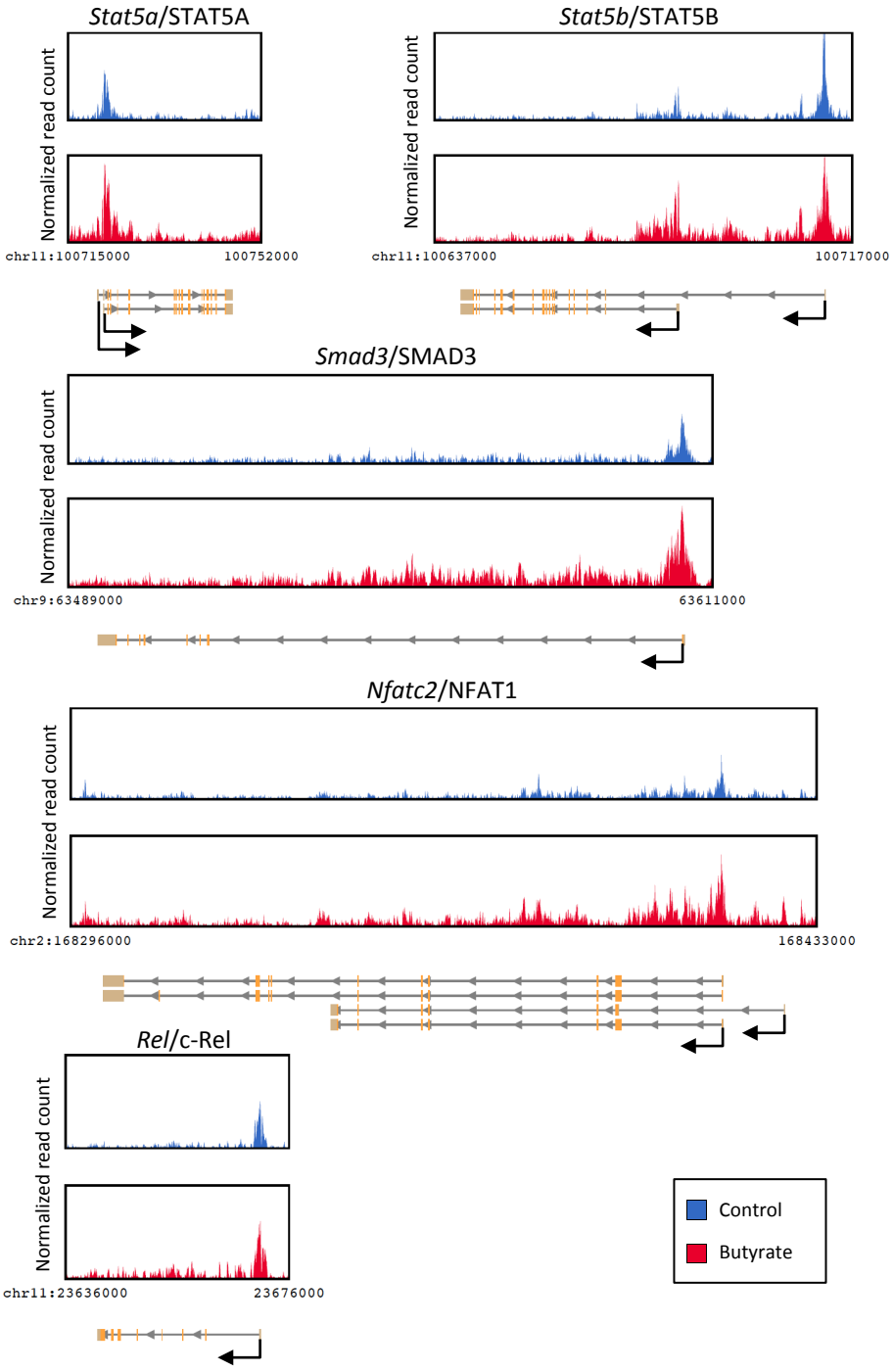
Supplementary Figure 16 | Induction of colonic Treg cells by intestinal butyrate in SPF *Myd88*$^{-/-}$*Ticam1*$^{-/-}$ mice.
a-b, SPF *Myd88*$^{-/-}$*Ticam1*$^{-/-}$ mice were fed with HAMS (Cont) or HAMSB (SB). Representative FACS plots gated on CD3ε^{+}CD4^{+} are shown in **a**. The frequency and total number of Helios^{-} Foxp3^{+} cells among colonic CD4^{+} T cells are shown in **b**. Data are representative of two independent experiments. The Error bars indicate s.d. (n = 4). P-values were calculated by Mann-Whitney U test.



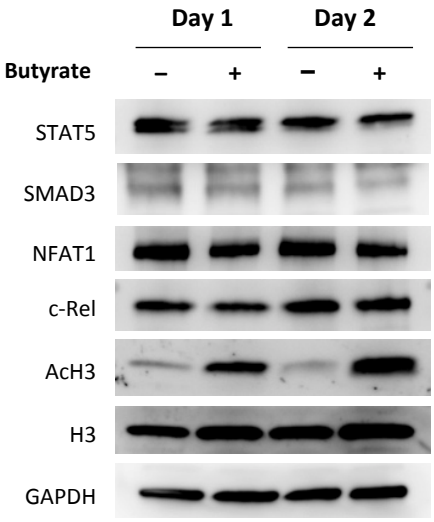
Supplementary Fig. 17 | Genome-wide histone H3 acetylation status after butyrate exposure. Naïve CD4$^+$ T cells were cultured under Treg-inducing conditions in the presence or absence (control) of butyrate for 1 day. **a**, Total and acetylated histone H3 (AcH3) were measured by Western blot analysis of total cell lysates. **b**, ChIP-Seq analysis was performed using anti-AcH3 antibody. The average values of AcH3 status around the transcription start site (TSS) over total genes are shown.



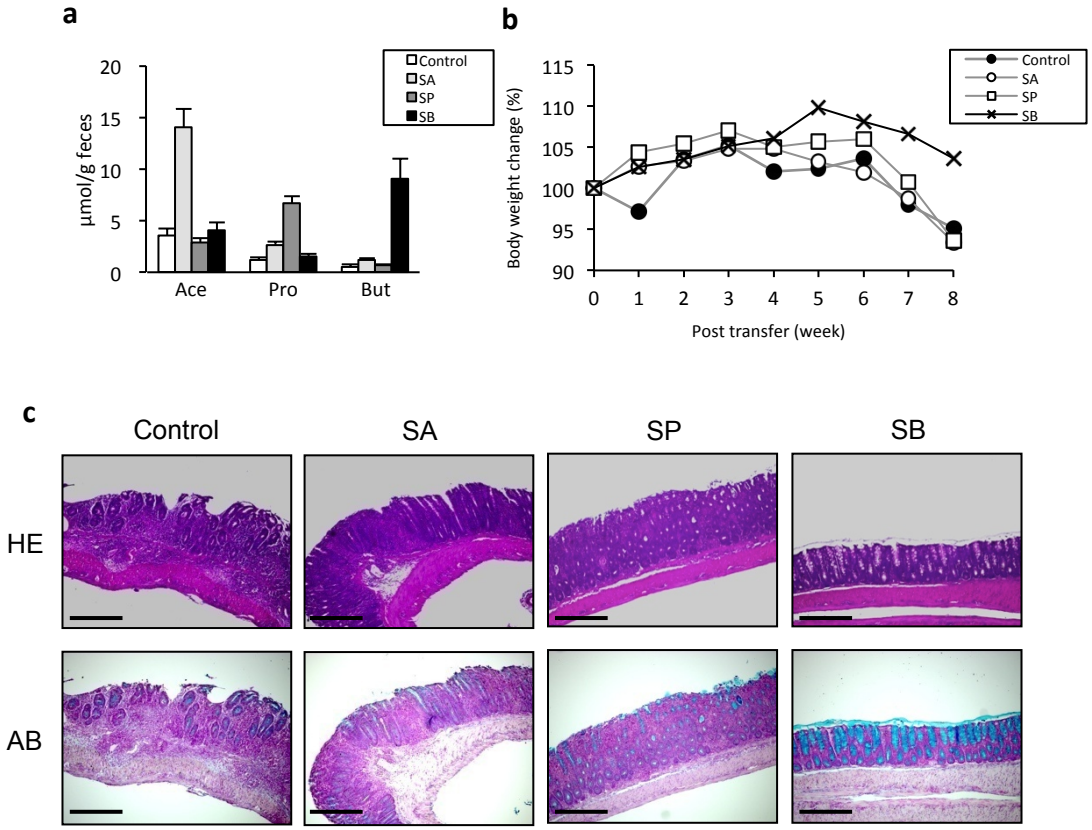
Supplementary Figure 18 | Histone acetylation status of *Tbx21*, *Gata3* and *Rorc*.
Naïve $CD4^+$ T cells were cultured under Treg-inducing conditions in the presence or absence (control) of butyrate for 1 day. Histone acetylation in the genes encoding master molecules for effector T cells was analyzed by ChIP-sequencing using anti-acetylated histone H3.



Supplementary Figure 19 | Histone acetylation status of *Stat5, Smad3, Nfatc2 and Rel*.
Naïve CD4$^+$ T cells were cultured under Treg-inducing conditions in the presence or absence (control) of butyrate for 1 day. Histone acetylation in the genes encoding transcriptional regulators controlling *Foxp3* expression was analyzed by ChIP-sequencing using anti-acetylated histone H3.

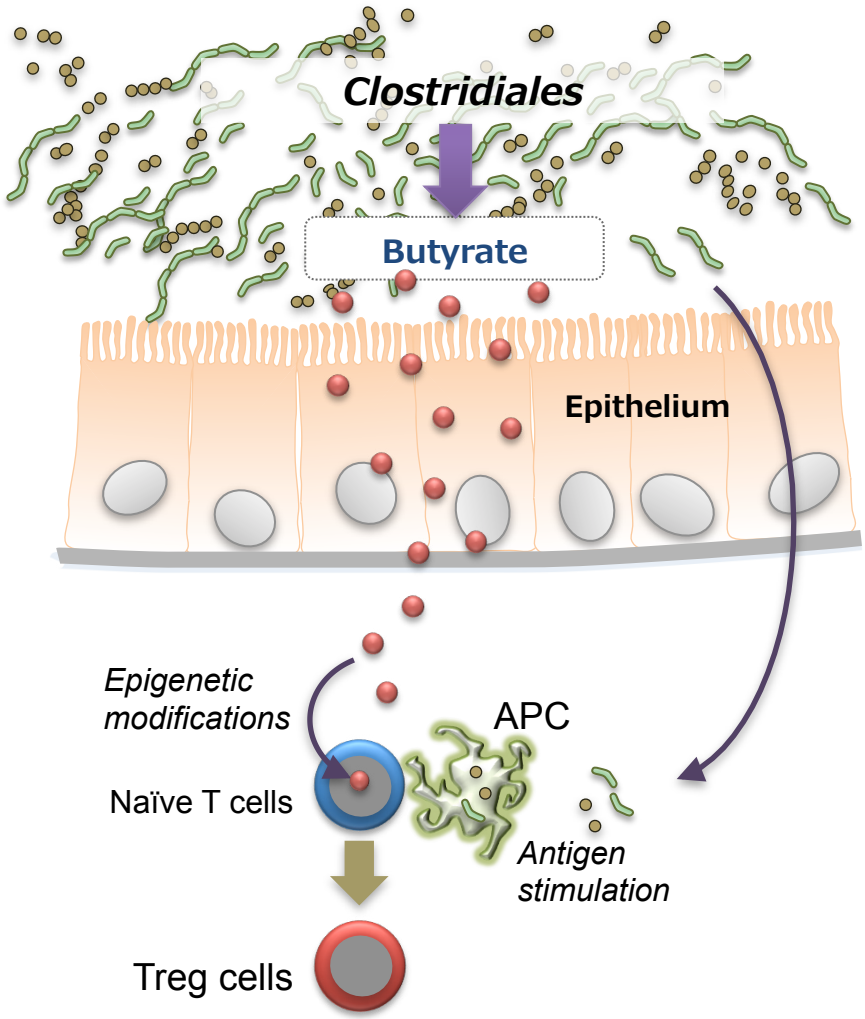


Supplementary Figure 20 | Expression of STAT5, SMAD3, NFAT1, and c-Rel.
Naïve $CD4^+$ T cells were cultured under Treg-inducing conditions in the absence or presence of butyrate. Total cell lysates were subjected to western blotting analysis with antibodies described in Methods. H3 and AcH3 indicate histone H3 and acetylated histone H3, respectively. Data are representative of three independent experiments.



Supplementary Figure 21 | Intestinal butyrate, but not acetate or propionate, ameliorates T-cell-dependent experimental colitis.

a-f, Experimental colitis was induced by adoptive transfer of $CD4^{+}CD45RB^{hi}$ T cells in $Rag1^{-/-}$ mice fed with HAMS (Control), HAMSA (SA), HAMSP (SP) or HAMSB (SB). Fecal amount of SCFAs (**a**), body weight change (**b**), and representative colonic specimens stained with hematoxylin-eosin (HE) and Alcian blue (AB) (**c**) are shown. The Error bars indicate s.d. (n = 5). Scale bars: 200 µm (**c**). All data are representative of two independent experiments.



Supplementary Figure 22 | A schematic diagram of epigenetic modifications by commensal microbial fermentation product.
Clostridiales abundantly produce butyrate, which promotes histone H3 lysine acetylation in *Foxp3* gene locus of $CD4^+$ T cells, and eventually facilitates induction of Treg cells. APC: antigen-presenting cells.

Supplementary Table 1. Key observed cecal metabolites most influential in discriminating between CRB-LFD and CRB-HFD mice in the S-plot shown in Supplementary Figure 7.

^{1}H Chemical shift (ppm)	Metabolite	Covariance	Modeled correlation	Normalized signal intensitiy (a.u.)		Fold change (HFD/LFD)	*P* value
				CRB-LFD	CRB-HFD		
2.13	Butyrate/Propionate	0.199	0.973	11.3±0.2	18.4±0.6	1.62	<0.001
0.96	L-Leucine	0.196	0.926	21.8±0.7	28.8±0.8	1.32	<0.001
1.00	L-Isoleucine	0.195	0.962	9.6±0.4	16.3±0.7	1.71	<0.001
3.01	GABA	0.176	0.975	8.1±0.2	13.7±0.3	1.70	<0.001
1.89	Acetate	0.174	0.722	19.2±1.3	26.8±1.5	1.39	0.003
3.74	L-Leucine	0.174	0.908	17.4±0.4	23.3±0.7	1.34	<0.001
1.53	Butyrate	0.166	0.924	11.3±0.3	16.7±0.5	1.47	<0.001
1.73	L-Leucine	0.162	0.979	0.8±0.3	15.5±0.3	1.44	<0.001
0.84	Unidentified	−0.211	−0.991	14.5±0.3	6.7±0.5	0.45	<0.001
1.29	Lactate	−0.258	−0.962	20.4±1.2	8.3±0.1	0.40	<0.001

The normalized signal intensity of each metabolie is shown as the mean±SE (n = 7)