2021 Doctoral Thesis

Study on the Function of α-Defensin, Paneth cell-secreted Antimicrobial Peptide, as a Regulator of Intestinal Ecological System under Psychological Stress

（心理ストレス下における腸内エコロジーシステム制御因子としての

Paneth 細胞分泌抗菌ペプチドであるα-defensin 機能に関する研究）

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Chapter 1. General Introduction

Depression is a serious illness that is often persistent, recurrent, and the number of the patients has been growing worldwide, causing great social loss [1]. It has been known that multiple factors are involved in the onset of depression [2]. It has been reported that monoamine neurotransmitters such as serotonin, noradrenaline, and dopamine decrease in patients with depression, which contribute to the development of depression [3]. Genetic factors are also involved, showing that major depressive disorder is moderately hereditary, and SIRT1 has been identified as a risk-contributing locus [4]. In addition, psychological stress-induced cortisol secretion and subsequent impairment of feedback function via glucocorticoid receptors induce overactivation of the hypothalamic-pituitary-adrenal axis (HPA axis), leading to a decline in neuroplasticity due to a decrease in brain-derived neurotrophic factor (BDNF) [5]. Among them, psychological stress is considered an essential factor contributing to the progress of depression [6]. However, since mechanisms of depression are various, the overview of the disease process has been still unknown.

More than 1×10^{14} bacteria inhabit the human gut lumen and form intestinal microbiota in harmony with the host, contributing to the maintaining of intestinal homeostasis [7]. In the intestinal tract, the physical barrier of the mucosal epithelium and mucous layer prevents the invasion of bacteria into the host, and the secretion of antimicrobial peptides into the intestinal lumen suppresses the growth of pathogenic bacteria, resulting in maintaining normal intestinal microbiota. Dysbiosis has been considered to be involved with disruption of the systemic homeostasis and promotes many diseases including obesity, inflammatory bowel disease, type II diabetes, and nonalcoholic steatohepatitis [8-11]. It has been reported in recent years that the intestinal microbiota

and depression are related to each other. The intestinal microbiota has been known to affect the host immunity through their cell components such as lipopolysaccharide (LPS) or by producing metabolites including short-chain fatty acids and neurotransmitters including γ-aminobutyric acid (GABA) and serotonin, resulting in alteration of brain function associated with depression [12]. Besides, a positive correlation is confirmed between quality of life of patients with depression and the butyrate-producing microbiota or the synthetic potential of bacterial dopamine metabolites in a cohort study [13]. Furthermore, it has been reported that mice, transplanted depression patients' feces, show abnormalities in bacterial metabolism including production of tryptophan or short-chain fatty acids and further induces depression-like behavior characteristics [14]. Germ-free mice showed the excessive response via HPA axis when exposed to psychological stress [15]. It is known that dysbiosis and alterations of the microbial metabolites resulting in depression are induced by psychological stress [16]. However, underlying mechanisms that psychological stress causes the disruption of homeostasis in the microbial metabolites due to dysbiosis remains to be unidentified.

α-Defensin is an antimicrobial peptide with three disulfide bonds in a molecule consisting of 32 to 36 amino acids, and produced and secreted from Paneth cells located in the crypt of the small intestine. α-Defensin is responsible, in part, for innate enteric immunity [17-20] and plays a pivotal role in both elimination and symbiosis in the intestine by killing pathogenic bacteria while eliciting less bactericidal activities against commensal bacteria [21]. It has been reported that the active α-defensin knockout mice show changes in the composition of small intestinal microbiota [22], and oral α-defensin administration improves severe dysbiosis of graft-versus-host disease (GVHD) model mice [23], suggesting that α-defensin plays a critical role in

maintaining homeostasis of the intestinal microbiota. In addition, it has been known that Crohn's disease model mice show α-defensin abnormalities, leading to dysbiosis and disruption of the intestinal metabolism [24,25]. Dysbiosis caused by Paneth cell impairment with decreased α-defensin has been shown to relate with various diseases [26,27]. Thus, α-defensin secreted by Paneth cells may contribute to maintain systemic homeostasis by regulating the intestinal microbiota and their metabolites. However, relationships between Paneth cell α-defensin and dysbiosis or disruption of homeostasis in the intestinal metabolites in depression have been unclear.

Here, this study shows that a reduction of α-defensin due to psychological stress induces dysbiosis and subsequent disruption of homeostasis in microbial metabolites in chronic social defeat stress (CSDS) model mice, a model of depression induced by psychological stress, and that oral administration of α-defensin attenuates the observed imbalance of the intestinal microbiota and their metabolites. This study provides new insights into the mechanism of depression and further contributes to the discovery of prevention and therapeutic targets for depression.

Chapter 2. Materials and Methods

2.1 Animal

All animal experiments were approved by the Institutional Animal Care and Use Committee of the National University Corporation at Hokkaido University. All experiments were performed in accordance with Hokkaido University Regulations of Animal Experimentation. All animal experiments were also carried out in compliance with the ARRIVE guidelines. Male C57BL/6J (B6J) mice, male ICR mice and male retirement ICR (Stressor) mice (5 months old and older) were purchased from Charles River, Japan (Yokohama, Japan) and B6J mice and ICR mice were subjected to experiments at 7 weeks of age after acclimation and quarantine for more than one week. The bedding was PaperClean (Japan SLC Inc., Hamamatsu, Japan) and B6J mice had received a diet (CE-2, CLEA Japan, Tokyo, Japan) and drinking water ad libitum.

2.2 Experimental design of CSDS

CSDS load was partially modified from previous reports [16]. One stressor mouse and one B6J mouse were placed in same compartment of a cage divided into two compartments by a clear acrylic plate. Mice were brought into direct contact with each other. The direct contact time was counted from the time the stressor mice made contact, including covering or biting, and the direct contact time was reduced by 5 min on the first day and by 0.5 min thereafter. In other words, the direct contact time for the 10th time was 0.5 min. Direct contact was conducted at PM (13:00-17:00). After direct contact, B6J mice were transferred to a neighboring

compartment with stressor mice and subjected to olfactory and visual stress until the next direct contact (indirect contact). In the first experiment, five cycles of direct and indirect contact were conducted, followed by two days of indirect contact, followed by the remaining five cycles respectively (Fig. 1a). In the second experiment, 10 cycles of direct and indirect contact were conducted (Fig. 1b). Stressor mice were selected in advance in order of aggression from 15 tests counting the number of 3-minute bites to B6J mice performed three times a day for five days.

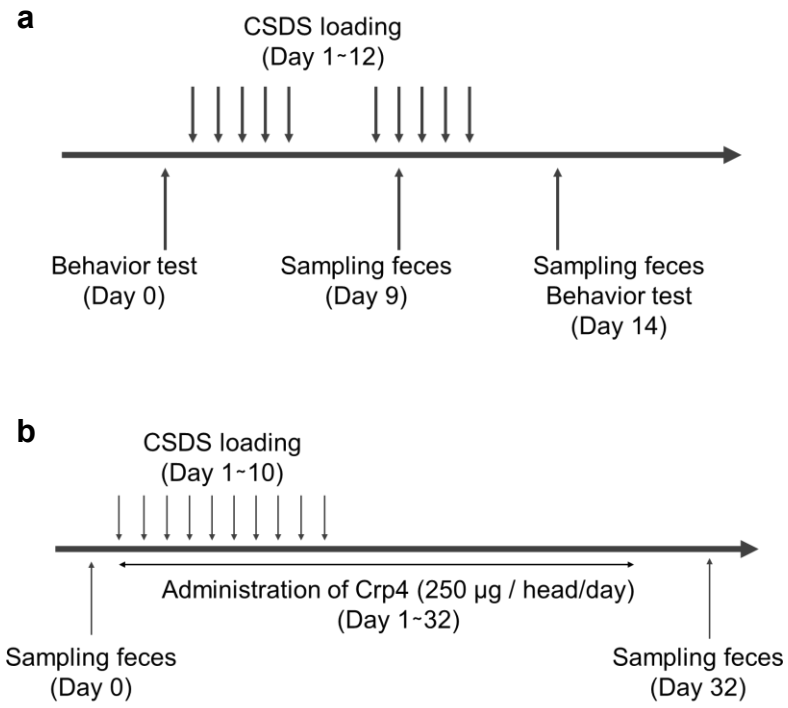


Figure 1. The experimental schedules of CSDS.

The schedules of (a) the first experiment and (b) the second experiment.

2.3 Behavior test

A cage (16.5 cm x 8.3 cm) was placed in a behavioral test box (50 cm x 50 cm), and the perimeter of the cage in the box was defined as the Social Interaction Zone (SI zone, cage perimeter 33.3 cm x 16.7 cm) and as both diagonal corners of the cage (Corner zone, 16.6 cm x 16.6 cm). B6J mice were placed in a box and allowed to explore freely for 5 min with ICR mice present in the cage. Behavioral analysis was conducted using the image analysis software HOLE BOARD (Muromachi KIKAI, Tokyo, Japan) to measure the interaction time, corner time and total traveling distance.

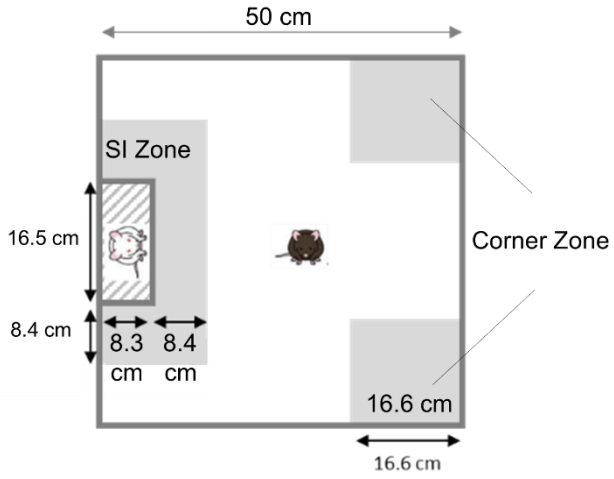


Figure 2. The box for behavior test.

2.4 Sampling feces

Fresh feces collection from B6J mice in cages was performed using wire mesh in the floor and stored frozen at -80℃. Feces during 24 hours were collected after cage replacement. Feces were collected at three time points on day 1, 9, and 14 in the first experiment (Fig. 1a) and at two time points on day 0 and 32 in the second

experiment (Fig. 1b).

2.5 Enzyme-Linked Immuno Sorbent Assay (ELISA)

Fecal α-defensin was measured as previously described [28,29]. Feces during 24 hours were air dried, pulverized using a bead beater-type homogenizer (Beads Crusher μT-12; TAITEC). Fecal extract was collected after mixing with PBS using a cortex mixer for 1 h and centrifugation at 20,000 g for 20 min, and levels of Crp1 and Crp4 were determined by sandwich ELISA. ELISA was conducted on the feces of B6J mice and the Crp1 antibodies detected Crp1-3 and 6, the Crp1 family. The Crp4 antibodies detected administered Crp4 since B6J mice do not genetically express Crp4.

2.6 Immunofluorescent analysis

The small intestine was harvested after mice were euthanized by isoflurane inhalation at day 14 (Fig. 1a). The small intestine from naïve and CSDS mice were fixed by 10% buffered formalin and embedded in paraffin and cut into 4 μm-thick sections. The sections were deparaffinized, rehydrated, and boiled for 20 min at 105°C in Dako REAL Target Retrieval Solution (pH 6, Agilent, Santa Clara, CA). After blocking in Block Ace (Dainippon Pharmaceutical, Osaka, Japan) containing 5% goat serum (Sigma-Aldrich, St. Louis, MO) at room temperature for 30 min, the sections were incubated with 1 μg/mL rat monoclonal anti-Crp1 (77-R63) at 4°C for overnight. Then, the sections were incubated with 5 μg/mL Alexa Fluor 488 goat anti-rat IgG H+L (Thermo Fisher Scientific, Waltham, MA) at room temperature for 1 h. After nucleus staining by 4', 6-

diamidino-2-phenylindole (DAPI, Thermo Fisher Scientific, Waltham, MA) for 5 min, the sections were embedded to the slide grass by RapiClear 1.52 (Sunjin Lab, Hsinchu, Taiwan). Fluorescent images were analyzed using confocal microscopy (A1, Nikon, Tokyo, Japan). The number of Paneth cells per crypt and Crp1 positive area of each crypt in binary images based on fluorescent intensity were analyzed in 5 crypts from the small intestine using NIS-Elements AR ver.5.11 (Nikon, Tokyo, Japan).

2.7 Real Time PCR

Total RNAs from small intestine were prepared using the RNeasy Mini Kit (Qiagen, Valencia, CA). RNA purity and concentration were measured using a NanoDrop 2000 Spectrophotometer (Thermo Fischer Scientific, Waltham, MA). 500 ng total RNA was reverse transcribed for 60 min at 42°C and 5 min at 85°C using SuperScript VILO MasterMix (Thermo Fischer Scientific, Waltham, MA). Real-time PCR was conducted using Roche LightCycler 96 system (Roche, Basel, Switzerland) with fluorescently labeled locked nucleic acid (LNA) probes from the Universal Probe Library (UPL). The PCR was performed three steps of 10 s at 95°C denature, 10 s at 60°C annealing, 1 s at 72°C extension for 45 cycles after a 10 min-pre-heat at 95°C. Expression of the tested gene was normalized relative to levels of hypoxanthine guanine phosphoribosyl transferase 1 (*HPRT-1*). The primer sequences are listed in Table 1.

Table 1 Primer sequences and Universal Probe Library numbers

Gene	Primer sequence (5'-3')	Universal Probe Library
Lyz1	F: GGCAAAACCCCAAGATCTAA	#46
	R: TCTCTCACCACCCTCTTTGC	
Olfm4	F: CTCCGGGAGGCACTTCTT	#102
	R: CTGTCCACAGACCCAGTGAA	
Atg16l1	F: CATCGCGGAGGAACTGAG	#34
	R: CTGACTTTTCCAGCAACTTGG	
LC3B	F: CCCCACCAAGATCCCAGT	#7
	R: CGCTCATGTTCACGTGGT	
CHOP	F: GCGACAGAGCCAGAATAACA	#91
	R: GATGCACTTCCTTCTGGAACA	
PERK	F: CCTTGGTTTCATCTAGCCTCA	#106
	R: ATCCAGGGAGGGGATGAT	
HPRT-1	F: TCCTCCTCAGACCGCTTTT	#95
	R: CCTGGTTCATCATCATCGGCTAATC	

F: Forward primer, R: Reverse primer.

2.8 α-Defensin administration

Recombinant Cryptdin-4 (mouse α-defensin) was manufactured and purified as previously described [30]. Cryptdin-4 was dissolved in ultrapure water and orally administered once daily from day 1 to day 32 at 250 μg/mouse, and then equal amounts of ultrapure water were orally administered to the naïve group and the CSDS group (Fig. 1b).

2.9 Measurement of the length of villi and the depth of crypt

10% buffered formalin-fixed small intestine from naïve and CSDS mice were embedded in paraffin and cut into 4 μm-thick sections. After deparaffinization and rehydration, the sections were stained with hematoxylin eosin (HE). HE images were obtained using a NanoZoomer scanner (Hamamatsu Photonics, Hamamatsu, Japan). The villus length and crypt depth were measured in 3 crypts of each mouse small intestine using NDP.view2 software (Hamamatsu Photonics, Hamamatsu, Japan).

2.10 DNA extraction and 16S rRNA sequencing

Genomic DNA was extracted from 100 mg of fresh fecal samples using the NucleoSpin Microbial DNA Kit (MACHEREY-NAGEL, Düren, Germany) following the manufacturer's protocol. Final DNA concentrations were measured by a NanoDrop 2000 spectrometer (Thermo Fischer Scientific) at 260 nm. 16S ribosomal RNA genes were amplified by PCR from each fecal DNA sample using universal primer set of Bakt 341F (5'-cctacgggnggcwgcag) and Bakt 805R (5'-gactachvgggtatctaatcc) which covers the V3-V4 variable region [24,31,32]. PCR amplification was conducted in 25-µl-volume reaction mixtures containing 12.5 ng of template DNA, 200 nM of each primer, and 1× KAPA HiFi Hot Start Ready Mix (Kapa Biosystems) under the following conditions: 95°C for 3 min, 25 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, followed by 72°C for 5 min. PCR products were purified by AMPure XP beads (Beckman Coulter) and sequencing adapters containing sample-specific 8-bp barcodes were added to the 3'- and 5'- ends by PCR using the Nextera XT Index Kit v2 Set B (Illumina)in 50 µl of reaction mixtures containing 5 µl of PCR amplicon, 5 µl of each indexing primer and 1× KAPA HiFi Hot Start Ready Mix under the following conditions: 95°C for 3 min, eight cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, followed by 72°C for 5 min. Each amplicon was purified, quantified by the Qubit dsDNA HS Assay Kit (Invitrogen), and then adjusted to 4 nM. Amplicons were pooled 4 µl and quantified by KAPA Library Quantification Kit LightCycler 480 qPCR Mix (Kapa Biosystems) and then diluted to 4 pM. The amplicon library was mixed with 5% equimolar PhiX Control v3 (Illumina) and sequenced on a MiSeq instrument using the MiSeq 600-cycle v3 kit (Illumina).

2.11 16S rRNA–based taxonomic analysis

Microbiome analysis was conducted using QIIME2 (version 2019.7), the open-source bioinformatics pipeline. Imported sequences were quality filtered and denoising into features by DADA2 plugin and remaining contigs were clustered into OTUs with 99% sequence similarity against the SILVA 128 reference database. To acquire taxonomic information for each OTU, representative sequences were aligned to MAFFT and assigned to its database for classification using a naïve-bayes classifier trained on 16S rRNA gene OTUs. β-diversity (unweighted UniFrac distance) was estimated by using QIIME2 workflow. Statistical significance of β-diversity was determined by PERMANOVA test in Qiime2 pipeline.

2.12 Measurement of fecal metabolites

50 mg of frozen feces were added at 0ºC into 1,500 μL of 50% acetonitrile/Milli-Q water containing internal standards (H3304-1002, Human Metabolome Technologies, Inc., Tsuruoka, Japan) to inactivate enzymes. Feces were homogenized three times for 120 sec at 1,500 rpm using a tissue homogenizer (Micro Smash MS100R, Tomy Digital Biology Co., Ltd., Tokyo, Japan) and then the homogenate was centrifuged for 5 min at 2,300 ×g and 4ºC. Subsequently, 800 μL of upper aqueous layer was filtered centrifugally by a Millipore 5-kDa cutoff filter for 120 min at 9,100 ×g and 4ºC in order to remove proteins. The filtrate was concentrated centrifugally and re-suspended in 50 μL of Milli-Q water for CE-MS analysis. Metabolome measurements were conducted through a facility service at Human Metabolome Technologies Inc., Tsuruoka, Japan. CE-TOFMS was performed using an Agilent CE Capillary Electrophoresis System equipped with an Agilent 6210

Time of Flight mass spectrometer, Agilent 1100 isocratic HPLC pump, Agilent G1603A CE-MS adapter kit, and Agilent G1607A CE-ESI-MS sprayer kit (Agilent Technologies, Waldbronn, Germany). The systems were controlled by Agilent G2201AA ChemStation software version B.03.01 for CE (Agilent Technologies, Waldbronn, Germany). The metabolites were analyzed by using a fused silica capillary (50 μm i.d. × 80 cm total length), with commercial electrophoresis buffer (Solution ID: H3301-1001 for cation analysis and H3302-1021 for anion analysis, Human Metabolome Technologies) as the electrolyte. The sample was injected in cation analysis and 25 sec (approximately 25 nL) in anion analysis at a pressure of 50 mbar for 10 sec (approximately 10 nL). The spectrometer was scanned from m/z 50 to 1,000 and other conditions were as in the described previously [33]. Peaks were extracted by automatic integration software MasterHands (Keio University, Tsuruoka, Japan) in order to obtain peak information including m/z, migration time for CE-TOFMS measurement (MT) and peak area [34]. Signal peaks corresponding to adduct ions, and other product ions of known metabolites were excluded, and remaining peaks were annotated with putative metabolites and their isotopic ions from the HMT metabolite database based on their MTs and m/z values determined by TOFMS. The tolerance range for the peak annotation was configured at ± 0.5 min for MT and ± 30 ppm for m/z. In addition, peak areas were normalized against those of the internal standards and then the resultant relative area values were further normalized by sample amount.

2.13 Statistical analysis

Statistical analysis was conducted by using JMP (version 14.0.0) software. Two groups were compared by the

Mann-Whitney U test and pairs T test and three group were compared by the Steel's test and Turkey's test. Correlation analysis was conducted by using the Pearson correlation coefficient. For all analyses, $P < 0.05$ was considered statistically significant.

Chapter 3. Results

3.1 CSDS load decreases α-defensin secretion

Analyzing behavior changes in the CSDS model showed that the interaction time significantly decreased in CSDS group (82.8 ± 20.3 sec.) compared to naïve group (148.5 ± 34.2 sec.) (Fig. 3a). In addition, the corner time increased from 53.1 ± 20.7 sec. to 74.6 ± 23.8 sec. ($p = 0.093$) (Fig. 3b), and the total traveling distance significantly reduced from 2654.8 ± 439.6 cm to 1490.7 ± 519.3 cm (Fig. 3c). These results indicated that a characteristic reduction in sociality was confirmed in the CSDS model.

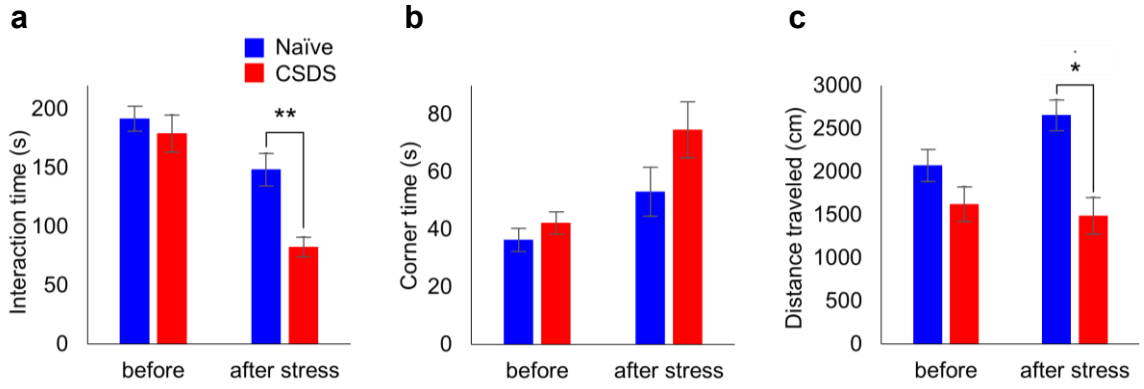


Figure 3. Decrease in sociality by CSDS.

(a) Staying time in SI zone. (b) Staying time in corner zone. (c) Total distance traveled. Data are expressed as the means ± SEM (n = 6 per each group). Mann–Whitney U tests were used to compare the data.

*, $P < 0.05$; **, $P < 0.01$.

Next, in order to analyze the relationship between the CSDS load and the amount of α-defensin secretion, fecal Crp1 of the CSDS (for 12 days) group was determined. The amounts of Crp1 on day 9 and day 14 of the CSDS group were reduced to 33% and 45%, respectively, compared to those on day 1 of the naïve group (Fig. 4, Table 2).

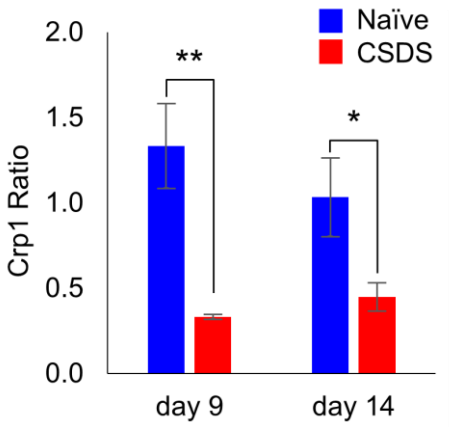


Figure4. Decrease in fecal cryptdin-1 by CSDS.

Fecal Crp1 protein ratio vs naïve group in day 1. Data are expressed as the means ± SEM (n = 6 per each group). Mann–Whitney U tests were used to compare the data. *, $P < 0.05$; **, $P < 0.01$.

Table 2 Cryptdin-1 concentration in feces

	Day 1		Day 9		Day 14	
	Average	SD	Average	SD	Average	SD
Naïve	440.1	246.3	587.6	267.1	455.5	248.2
CSDS	231.2	216.8	146.9	15.6	198.1	90.0

ng/mL, n = 6 per each group.

Furthermore, the small intestinal tissue was analyzed by immunofluorescent method and a significant decline in Paneth cell number and Crp1 positive granule area were confirmed in CSDS group compared to naïve group (Fig. 5a-c). These results were consistent with the reduction in fecal Crp1 in CSDS group (Fig. 4).

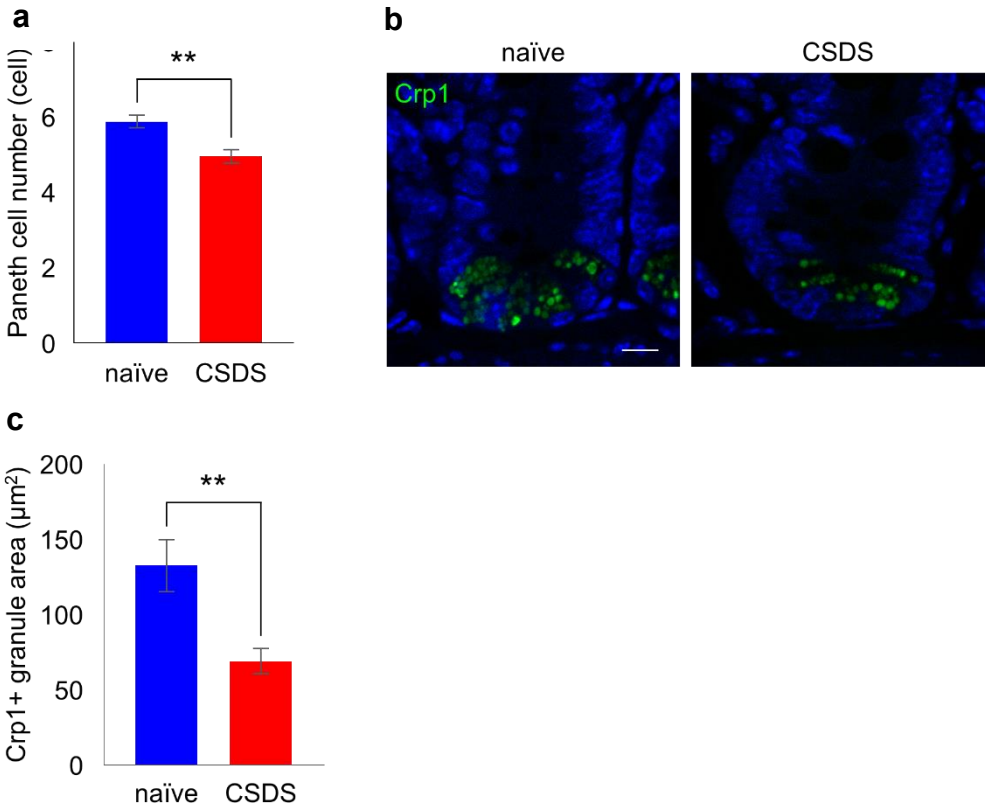


Figure 5. Decrease in Paneth cell number and cryptdin-1 expression in CSDS model.

(a) The number of Paneth cells per crypt. (b) Representative confocal images of Crp1 (green) with DAPI (blue) of crypt sections. Scale bars indicate 10 μm. (c) Crp1 positive granule area per crypt. Data are expressed as the means ± SEM (n = 6 per each group). Mann–Whitney U tests were used to compare the data. **, $P < 0.01$.

Next, the other aspects of Paneth cell function were analyzed in CSDS group. Using small intestinal tissues from CSDS group and naïve group, mRNA expressions of *lysozyme* (*Lyz1*) as an antimicrobial protein in Paneth cell, *Olfm4* as a crypt-base columnar stem cell marker, *Atg16l1* and *LC3B* as autophagy-related molecules, and *CHOP* and *PERK* as endoplasmic reticulum (ER) stress related molecules were tested and found that *CHOP* expression is significantly elevated in the CSDS compared to the naïve group, suggesting excessive ER stress in the CSDS (Fig. 6a-f). In contrast, no changes were found in all the other molecules tested.

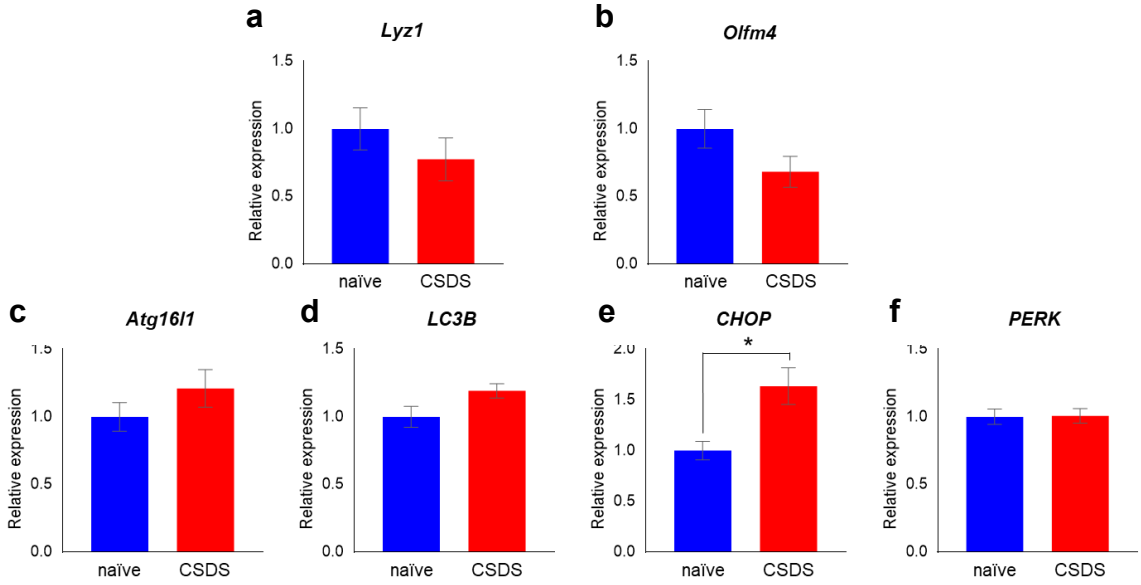


Figure 6. The gene expression levels in small intestine of naïve and CSDS model

Relative expression of mRNA of Paneth cell marker (a) *Lyz1*, Stem cell marker (b) *Olfm4*, Autophagy markers (c) *Atg16l1* and (d) *LC3B*, and ER stress markers (e) *CHOP* and (f) *PERK* were evaluated. Normalized to mean expression of *HPRT-1* and expressed as a fold change compared to naïve group. Data are expressed as the means ± SEM (n = 6 per each group). Mann–Whitney U tests were used to compare the data. *, $P < 0.05$.

In contrast, when the length of villi and depth of crypt in the small intestine were measured, no significant differences were found in CSDS group compared to naïve group (Fig. 7a-c).

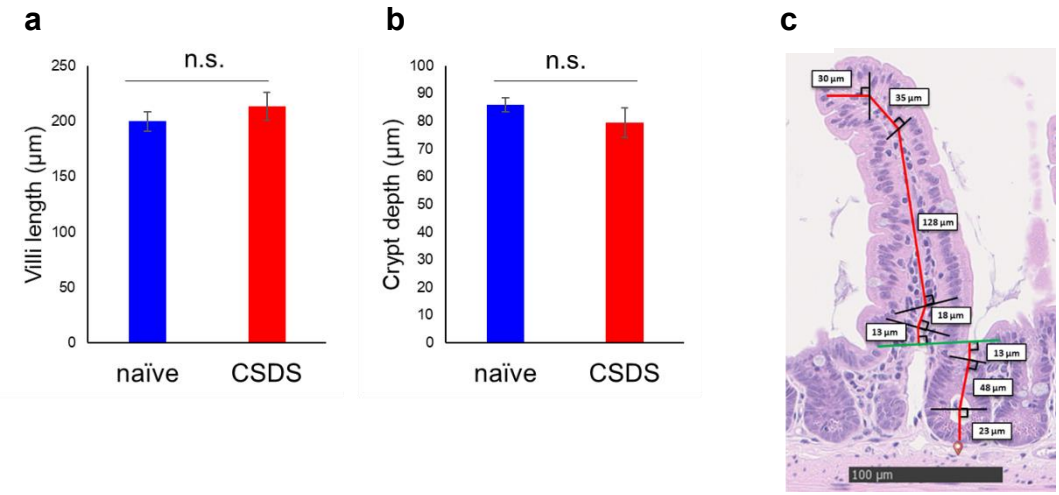


Figure 7. Villus length and crypt depth in the small intestine of naïve and CSDS model

(a) Villus length. (b) Crypt depth. (c) Representative hematoxylin and eosin (HE) staining image used for measurement of villus length and crypt depth. Scale bars indicate 100 μm. Data are expressed as the means ± SEM (n = 6 per each group). Mann–Whitney U tests were used to compare the data. n.s., not significant.

3.2 Dysbiosis is induced by α-defensin reduction due to CSDS load and recovered by α-defensin administration

Next, to clarify whether the intestinal microbiota changes due to CSDS and the changes depend on α-defensin decrease, experiment of Crp4 administration to rescue α-defensin was performed focusing on the initial phase under the short CSDS period. The three groups; naïve group, CSDS-loaded group (CSDS group), and CSDS-loaded plus administration of Crp4 group (Crp4 group) were analyzed. In the CSDS group, α-defensin in feces was reduced significantly compared to the naïve group (Fig. 8), consistent with data in the previous experiment (Fig. 4). In contrast, there was no significant difference between naïve group and Crp4 group, indicating α-defensin (Crps; Crp1 and Crp4) was rescued by oral Crp4 administration (Fig. 8).

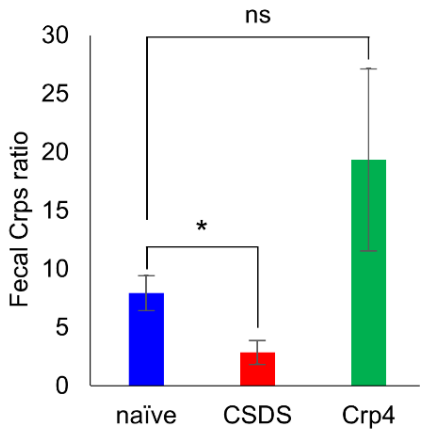
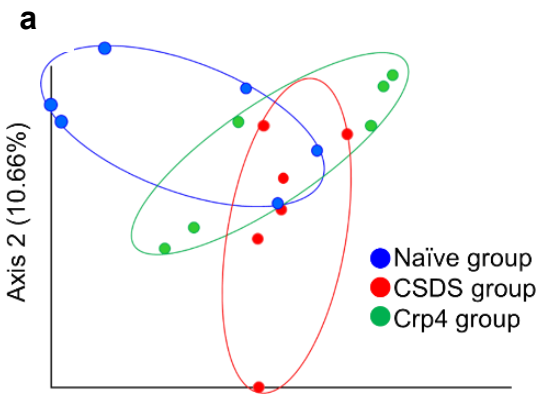


Figure 8. α-Defensin rescue by the administration of cryptdin-4.

Fecal Crp1+Crp4 (Crps) protein ratio in day 32 vs before CSDS. Data are expressed as the means ± SEM (n = 6 per each group). Steel's test was used to compare the data (vs naïve group). *, $P < 0.05$. n.s., not significant.

The intestinal microbiota of CSDS group and Crp4 group was analyzed and significant differences in β diversity were confirmed between naïve group and CSDS group ($p = 0.027$) and between CSDS group and Crp4 group ($p = 0.037$). In contrast, there was no difference between naïve group and Crp4 group (Fig. 9a,b), suggesting that dysbiosis caused by CSDS may be improved by administration of Crp4.



b

	naïve	CSDS	Crp4
naïve		0.027	0.081
CSDS			0.037
Crp4			

Figure 9. Change in the β-diversity of intestinal microbiota in the CSDS model by α-defensin administration.

(a) PCoA of β-diversity comparison. (b) The P-values of PERMANOVA test (n = 6 per each group).

Next, the Phylum level composition of intestinal microbiota was analyzed before and after CSDS in the three groups (Fig. 10, Table 6). *Bacteroidetes* significantly increased from 32 to 48% ($p = 0.04$) and *Firmicutes* significantly reduced from 65 to 48% ($p = 0.03$) in CSDS group. *Deferribacteres* did not change in CSDS group before and after CSDS loading, while reduced significantly in the naïve group from 0.25 to 0.05% ($p = 0.02$) and Crp4 group from 0.19 to 0.03% ($p = 0.03$). These data indicated that the characteristic changes of

Bacteroidetes, Firmicutes, and *Deferribacteres* due to CSDS loading were canceled by the administration of Crp4. *Actinobacteria* and *Proteobacteria* significantly increased in all groups after CSDS loading. *Tenericutes* significantly reduced only in naïve group from 0.3 to 0.1% ($p = 0.001$). There was no difference in *Verrucomicrobia* before and after CSDS loading in all groups. These results indicated that CSDS-related dysbiosis is partially rescued by the administration of Crp4 toward the intestinal microbiota in naïve group.

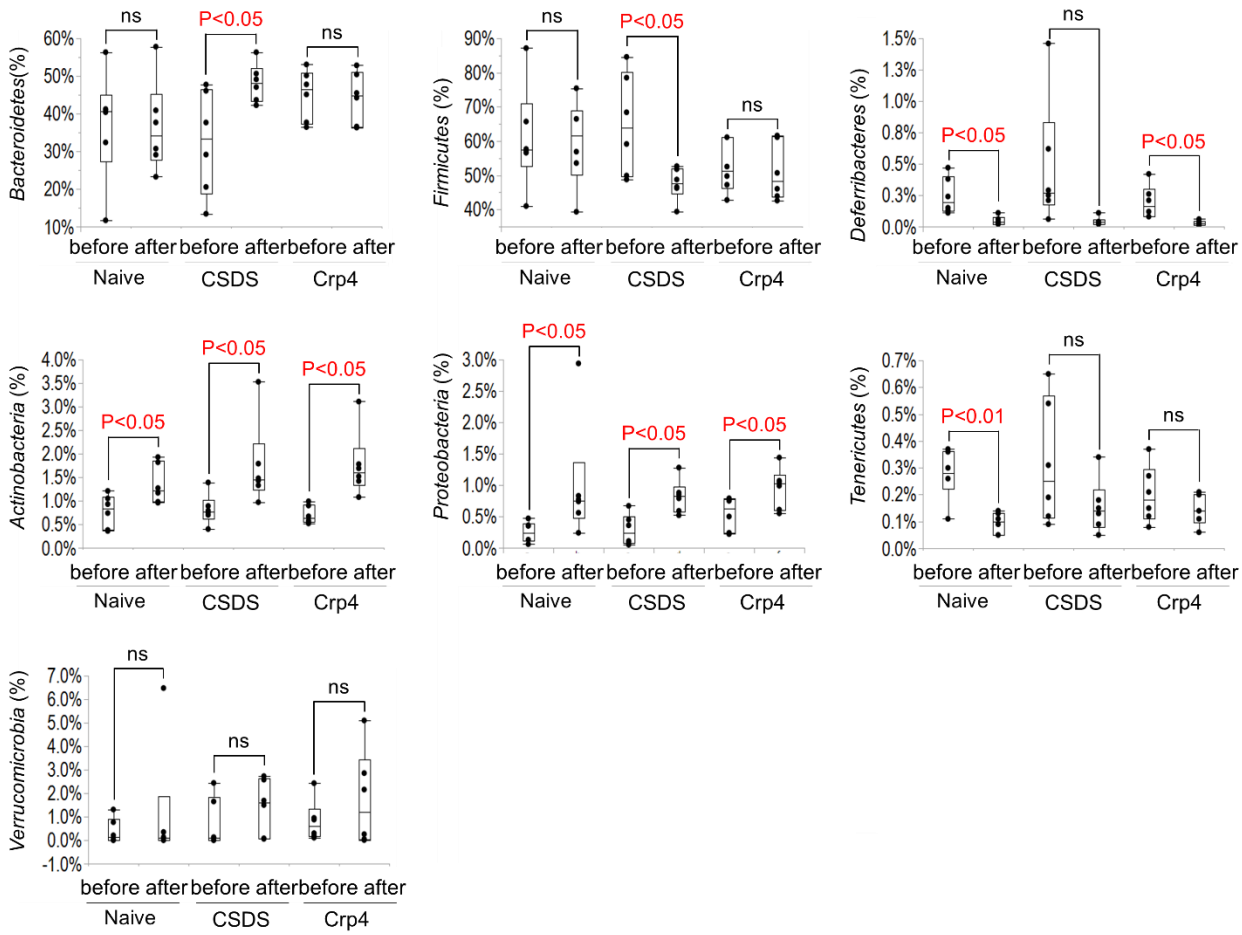


Figure 10. Changes in the composition of intestinal microbiota in the CSDS model by α-defensin administration.

Changes in compositions of microbiota at the phylum level before or after CSDS (shown only >0.1% abundance, n = 6 per each group). Pairs student's t test was used to compare the data. n.s., not significant.

Furthermore, the intestinal microbiota which changed significantly triggered by α-defensin increase or decrease was determined by performing correlation analysis between the amount of fecal α-defensin and the composition of intestinal microbiota. Positive correlation with α-defensin was confirmed in *Ruminococcaceae* ($r = 0.493$, $p = 0.038$), *Allobaculum* ($r = 0.795$, $p < 0.0001$), *Sutterella* ($r = 0.535$, $p = 0.022$), and *Akkermansia* ($r = 0.612$, $p = 0.007$), while *Erysipelotrichaceae* ($r = -0.475$, $p = 0.046$) showed negative correlation (Table 3, Fig. 11). Taken together, these results including the rescue experiment clarified that α-defensin decrease due to CSDS causes dysbiosis at least partially.

Table 3. Correlation between fecal Cryptdins and microbiota at genus level

phylum	class	order	family	genus	r	p value
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Actinomycetaceae*	*Actinomyces*	0.000	1.000
			Corynebacteriaceae	*Corynebacterium*	0.000	1.000
			Microbacteriaceae	*Microbacterium*	0.000	1.000
			Nocardiaceae	*Rhodococcus*	0.000	1.000
		Bifidobacteriales	*Bifidobacteriaceae*	*Bifidobacterium*	-0.102	0.688
	Coriobacteriia	*Coriobacteriales*	*Coriobacteriaceae*	*unknown*	0.096	0.704
			Coriobacteriaceae	*unknown*	0.070	0.784
			Coriobacteriaceae	*Adlercreutzia*	-0.017	0.947
Aquificae	*Aquificae*	*Aquificales*	*Aquificaceae*	*Hydrogenobacter*	0.000	1.000
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Bacteroidaceae*	*Bacteroides*	0.170	0.499
			Porphyromonadaceae	*Parabacteroides*	0.115	0.650
			Rikenellaceae	*unknown*	-0.154	0.542
			S24-7	*unknown*	0.020	0.936
			Paraprevotellaceae	*Prevotella*	-0.116	0.646
Cyanobacteria	*Chloroplast*	*Streptophyta*	*unknown*	*unknown*	0.000	1.000
Deferribacteres	*Deferribacteres*	*Deferribacterales*	*Deferribacteraceae*	*Mucispirillum*	-0.346	0.160
Firmicutes	*unknown*	*unknown*	*unknown*	*unknown*	0.000	1.000
	Bacilli	*unknown*	*unknown*	*unknown*	0.000	1.000
		Bacillales	*Planococcaceae*	*Sporosarcina*	0.000	1.000
			Staphylococcaceae	*Jeotgalicoccus*	0.000	1.000
			Staphylococcaceae	*Staphylococcus*	-0.324	0.189
		Lactobacillales	*Enterococcaceae*	*Enterococcus*	0.000	1.000
			Lactobacillaceae	*unknown*	-0.219	0.383
			Lactobacillaceae	*Lactobacillus*	-0.350	0.154
			Leuconostocaceae	*unknown*	0.000	1.000
			Leuconostocaceae	*Weissella*	0.000	1.000
			Streptococcaceae	*Streptococcus*	-0.435	0.071
		Turicibacterales	*Turicibacteraceae*	*Turicibacter*	-0.076	0.765
	Clostridia	*unknown*	*unknown*	*unknown*	-0.237	0.343
		Clostridiales	*unknown*	*unknown*	-0.251	0.316
			unknown	*unknown*	0.153	0.546
			Christensenellaceae	*unknown*	-0.005	0.986
			Clostridiaceae	*Candidatus Arthromitus*	-0.168	0.504
			Clostridiaceae	*Clostridium*	0.295	0.234
			Dehalobacteriaceae	*Dehalobacterium*	-0.216	0.390
			Eubacteriaceae	*Anaerofustis*	-0.016	0.950
			Lachnospiraceae	*unknown*	-0.328	0.184
			Lachnospiraceae	*unknown*	-0.283	0.256
			Lachnospiraceae	*Clostridium*	-0.109	0.668
			Lachnospiraceae	*Coprococcus*	-0.116	0.648
			Lachnospiraceae	*Dorea*	-0.154	0.542
			Lachnospiraceae	*Roseburia*	-0.203	0.420
			Lachnospiraceae	*Ruminococcus*	-0.122	0.631
			Peptococcaceae	*unknown*	0.000	1.000
			Peptococcaceae	*unknown*	-0.212	0.399
			Peptococcaceae	*rc4-4*	0.044	0.861
			Ruminococcaceae	*unknown*	-0.308	0.213
			Ruminococcaceae	*unknown*	0.493	0.038*
			Ruminococcaceae	*Anaerotruncus*	-0.349	0.156
			Ruminococcaceae	*Butyricicoccus*	-0.238	0.341
			Ruminococcaceae	*Gemmiger*	-0.343	0.164
			Ruminococcaceae	*Oscillospira*	-0.351	0.153
			Ruminococcaceae	*Ruminococcus*	-0.037	0.884
			Mogibacteriaceae	*unknown*	-0.223	0.373
	Erysipelotrichi	*Erysipelotrichales*	*Erysipelotrichaceae*	*unknown*	-0.475	0.046*
			Erysipelotrichaceae	*unknown*	-0.151	0.551
			Erysipelotrichaceae	*Allobaculum*	0.795	<.0001**
			Erysipelotrichaceae	*Clostridium*	-0.144	0.569
			Erysipelotrichaceae	*Coprobacillus*	-0.250	0.317
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Methylobacteriaceae*	*Methylobacterium*	0.000	1.000
		Rickettsiales	*mitochondria*	*unknown*	0.000	1.000
	Betaproteobacteria	*Burkholderiales*	*Alcaligenaceae*	*Sutterella*	0.535	0.022*
			Oxalobacteraceae	*Herbaspirillum*	0.000	1.000
	Deltaproteobacteria	*Desulfovibrionales*	*Desulfovibrionaceae*	*Desulfovibrio*	-0.137	0.588
	Gammaproteobacteria	*Enterobacteriales*	*Enterobacteriaceae*	*unknown*	0.196	0.436
		Pseudomonadales	*Moraxellaceae*	*Acinetobacter*	0.000	1.000
TM7	*TM7-3*	*CW040*	*F16*	*unknown*	-0.154	0.542
Tenericutes	*Mollicutes*	*Anaeroplasmatales*	*Anaeroplasmataceae*	*Anaeroplasma*	-0.462	0.053
		RF39	*unknown*	*unknown*	-0.100	0.695
Verrucomicrobia	*Verrucomicrobiae*	*Verrucomicrobiales*	*Verrucomicrobiaceae*	*Akkermansia*	0.612	0.007**
unknown	*unknown*	*unknown*	*unknown*	*unknown*	-0.246	0.325

*p < 0.05, **p < 0.01

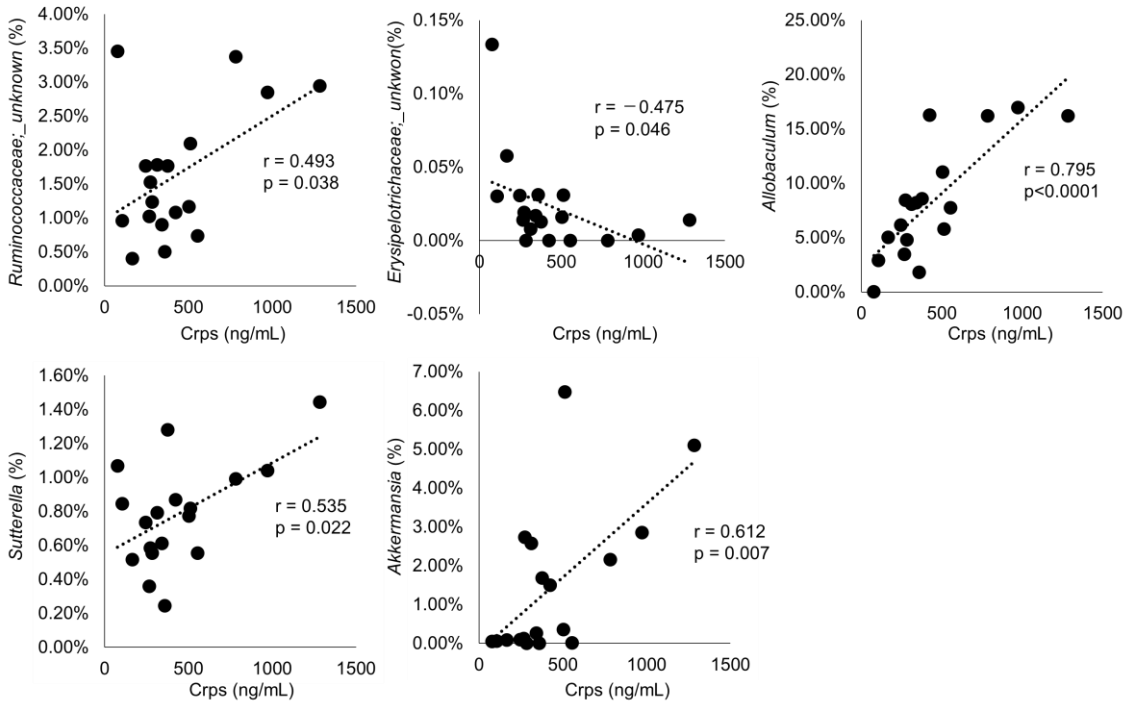


Figure 11. Correlation analysis between Cryptdins and microbiota at genus level. Only pairs with significant correlations are shown in the graph.

Horizontal axis shows fecal Cryptdins concentrations after CSDS (day 32) in each mouse (n = 6 per each group).

3.3 The decrease of α-defensin due to CSDS disrupts fecal metabolites via dysbiosis, and the disruption is recovered by α-defensin administration

Next, to clarify whether intestinal metabolites changes due to dysbiosis in the CSDS model and the changes depend on α-defensin decrease, the intestinal metabolites were analyzed in CSDS group and Crp4 group.

Fecal metabolites were measured simultaneously by CE-TOFMS and 322 candidate compounds were identified (Table 7). In order to clarify metabolites in feces triggered by α-defensin increase or decrease, correlation analyses between α-defensin and the intestinal metabolites were conducted. There were thirty-four metabolites positively correlated and five metabolites negatively correlated with Crps such as amino-acids and vitamins (Table 4). Sixteen amino acids or their metabolites and derivatives such as glutamic acid ($r = 0.49$, $p = 0.040$), lysine ($r = 0.58$, $p = 0.012$), and 3-amino butyric acid ($r = 0.47$, $p = 0.049$), alanine ($r = 0.53$, $p = 0.025$), allo-threonine ($r = 0.57$, $p = 0.014$), citrulline ($r = 0.49$, $p = 0.041$), isoleucine ($r = 0.49$, $p = 0.038$), methionine ($r = 0.51$, $p = 0.030$), threonine ($r = 0.52$, $p = 0.028$), tyrosine ($r = 0.50$, $p = 0.036$), β-alanine ($r = 0.61$, $p = 0.007$), N- acetyl glutamic acid ($r = 0.56$, $p = 0.017$), carnitine ($r = 0.57$, $p = 0.013$), isoglutamic acid ($r = 0.50$, $p = 0.033$), N-acetyllysine ($r = 0.60$, $p = 0.008$), and N5-ethylglutamine ($r = 0.57$, $p = 0.013$) showed a positive correlation. Five vitamins or their derivatives such as nicotinic acid ($r = 0.47$, $p = 0.049$), pantothenic acid ($r = 0.60$, $p = 0.009$), pyridoxamine ($r = 0.66$, $p = 0.003$), pyridoxamine 5-phosphate ($r = 0.50$, $p = 0.035$), and thiamine phosphate ($r = 0.61$, $p = 0.007$) showed a positive correlation. In addition, the other 13 metabolites which showed a positive correlation were uracil ($r = 0.51$, $p = 0.032$), 1H-imidazole-4-propionic acid ($r = 0.61$, $p = 0.007$), and 2-hydroxypyridine ($r = 0.53$, $p = 0.025$), 4-methyl-2-oxovaleric acid or 3-methyl-2-oxovaleric acid ($r = 0.47$, $p = 0.047$), 4-methyl-5-thiazoleethanol ($r = 0.63$, $p = 0.005$), 5-oxo-2-tetrahydrofurancarboxylic acid ($r = 0.53$, $p = 0.023$), ethanolamine ($r = 0.54$, $p = 0.022$), fumaric acid ($r = 0.56$, $p = 0.015$), hexanoic acid ($r = 0.48$, $p = 0.045$), loperamide ($r = 0.52$, $p = 0.026$), malic acid ($r = 0.58$, $p = 0.012$), orotic acid ($r = 0.53$, $p = 0.023$), and succinic acid ($r = 0.50$, $p = 0.035$). In contrast, five

metabolites showed negative correlation with α-defensin including 4-guanidinobutyric acid ($r = -0.54$, $p = 0.021$), cytidine ($r = -0.48$, $p = 0.044$), kynurenic acid ($r = -0.55$, $p = 0.018$), N-methylproline ($r = -0.58$, $p = 0.011$), and sinapic acid ($r = -0.51$, $p = 0.032$). These results indicated that the amount of intestinal α-defensin affects some specific intestinal metabolites.

Table 4. Significant correlation between fecal Cryptdins and intestinal metabolites

category	metabolites	r	p value
Amino acids and derivatives	Glutamic acid	0.49	0.040*
	Lysine	0.58	0.012*
	3-Aminobutyric acid	0.47	0.049*
	Alanine	0.53	0.025*
	allo-Threonine	0.57	0.014*
	Citrulline	0.49	0.041*
	Isoleucine	0.49	0.038*
	Methionine	0.51	0.030*
	Threonine	0.52	0.028*
	Tyrosine	0.50	0.036*
	β-Alanine	0.61	0.007**
	N-Acetylglutamic acid	0.56	0.017*
	Carnitine	0.57	0.013*
	Isoglutamic acid	0.50	0.033*
	N-Acetyllysine	0.60	0.008**
	N5-Ethylglutamine	0.57	0.013*
Vitamins and derivatives	Nicotinic acid	0.47	0.049*
	Pantothenic acid	0.60	0.009**
	Pyridoxamine	0.66	0.003**
	Pyridoxamine 5'-phosphate	0.50	0.035*
	Thiamine phosphate	0.61	0.007**
Others	Uracil	0.51	0.032*
	1H-Imidazole-4-propionic acid	0.61	0.007**
	2-Hydroxypyridine	0.53	0.025*
	4-Methyl-2-oxovaleric acid or 3-Methyl-2-oxovaleric acid	0.47	0.047*
	4-Methyl-5-thiazoleethanol	0.63	0.005**
	5-Oxo-2-tetrahydrofurancarboxylic acid	0.53	0.023*
	Ethanolamine	0.54	0.022*
	Fumaric acid	0.56	0.015*
	Hexanoic acid	0.48	0.045*
	Loperamide	0.52	0.026*
	Malic acid	0.58	0.012*
	Orotic acid	0.53	0.023*
	Succinic acid	0.50	0.035*
	4-Guanidinobutyric acid	-0.54	0.021*
	Cytidine	-0.48	0.044*
	Kynurenic acid	-0.55	0.018*
	N-Methylproline	-0.58	0.011*
	Sinapic acid	-0.51	0.032*

*$p < 0.05$, **$p < 0.01$

Next, correlation analyses between the intestinal microbiota in Fig. 11 and 322 metabolites were performed to clarify the metabolites affected by α-defensin-induced dysbiosis. There were seventy-nine metabolites positively or negatively correlated with at least one of five intestinal microbiota (Table 5). Among them, twenty-two metabolites including pyridoxamine and β-alanine were identified as metabolites correlated positively or negatively with α-defensin in Table 4, indicating that dysbiosis induced by α-defensin abnormalities correlates with the specific intestinal metabolites.

Table. 5 Correlation between metabolites and microbiota which correlate with Cryptdins

metabolites	*Ruminococcaceae_ unknown*		*Erysipelotrichaceae_ unknown*		*Erysipelotrichaceae_ Allobaculum*		*Alcaligenaceae_ Sutterella*		*Verrucomicrobiaceae _Akkermansia*	
	r	p value	r	p value	r	p value	r	p value	r	p value
1H-Imidazole-4-propionic acid	0.250	0.317	-0.408	0.093	0.510	0.031*	0.097	0.701	0.133	0.598
2-Hydroxypyridine	0.555	0.017*	-0.247	0.324	0.581	0.011*	0.278	0.264	0.204	0.417
4-Guanidinobutyric acid	-0.296	0.233	0.175	0.488	-0.512	0.030*	-0.445	0.065	-0.373	0.128
5-Oxo-2-tetrahydrofurancarboxylic acid	0.528	0.024*	-0.238	0.342	0.573	0.013*	0.277	0.267	0.210	0.404
Alanine	0.214	0.395	-0.375	0.125	0.496	0.036*	0.032	0.900	0.210	0.404
Carnitine	0.074	0.772	-0.368	0.133	0.590	0.010*	-0.024	0.925	0.254	0.310
Citrulline	0.407	0.094	-0.151	0.549	0.485	0.042*	0.139	0.582	0.246	0.325
Ethanolamine	0.112	0.658	-0.219	0.384	0.134	0.597	0.165	0.514	0.550	0.018*
Fumaric acid	-0.032	0.900	-0.374	0.126	0.469	0.050*	0.330	0.182	0.326	0.187
Isoleucine	0.206	0.412	-0.360	0.142	0.497	0.036*	0.014	0.955	0.291	0.242
Kynurenic acid	-0.425	0.079	0.441	0.067	-0.531	0.023*	-0.271	0.277	-0.667	0.003**
Loperamide	0.560	0.016*	-0.248	0.322	0.581	0.012*	0.277	0.265	0.202	0.422
N-Acetyllysine	0.407	0.094	-0.263	0.292	0.541	0.020*	0.218	0.385	0.306	0.217
N-Methylproline	-0.211	0.401	0.305	0.218	-0.630	0.005**	-0.330	0.181	-0.500	0.035*
Orotic acid	0.545	0.019*	-0.244	0.330	0.579	0.012*	0.278	0.264	0.207	0.410
Pyridoxamine	0.523	0.026*	-0.058	0.818	0.311	0.210	0.271	0.277	0.353	0.151
Pyridoxamine 5'-phosphate	0.495	0.037*	-0.086	0.735	0.320	0.195	0.144	0.568	0.337	0.172
Sinapic acid	-0.282	0.257	0.331	0.180	-0.522	0.026*	-0.304	0.220	-0.340	0.167
Succinic acid	0.038	0.882	-0.463	0.053	0.508	0.032*	0.140	0.580	0.166	0.510
Tyrosine	0.221	0.378	-0.369	0.132	0.531	0.023*	0.050	0.845	0.234	0.350
β-Alanine	0.485	0.041*	-0.232	0.355	0.495	0.037*	0.197	0.435	0.226	0.367
3-Aminobutyric acid	0.563	0.015*	0.015	0.952	0.327	0.185	0.178	0.481	0.107	0.674
1,3-Diaminopropane	0.488	0.040*	0.296	0.233	0.172	0.495	0.533	0.023*	0.342	0.165
2' or 5'-Deoxyadenosine	0.081	0.750	0.548	0.019*	-0.234	0.350	0.234	0.349	0.020	0.937
2'-Deoxycytidine	0.033	0.897	0.723	0.001**	-0.318	0.199	0.078	0.758	-0.255	0.307
2,4-Diaminobutyric acid	0.232	0.354	0.030	0.906	-0.070	0.784	0.023	0.928	0.557	0.016*
2,6-Diaminopimelic acid	0.566	0.014*	0.327	0.185	-0.019	0.939	0.374	0.126	-0.066	0.794
2-Aminoethylphosphonic acid	0.013	0.961	0.311	0.209	-0.478	0.045*	-0.130	0.606	-0.061	0.810
2-Deoxyribose 1-phosphate	0.368	0.133	0.561	0.016*	-0.070	0.782	0.205	0.414	0.001	0.998
2-Hydroxy-4-methylvaleric acid	-0.062	0.808	-0.575	0.013*	0.295	0.234	-0.202	0.421	-0.045	0.860
2-Hydroxyisobutyric acid	0.118	0.640	0.062	0.807	-0.115	0.650	0.029	0.910	0.655	0.003**
2-Hydroxyvaleric acid	-0.065	0.799	-0.503	0.033*	0.271	0.277	-0.174	0.491	-0.066	0.796
3'-AMP	0.120	0.635	0.276	0.267	-0.193	0.443	0.001	0.996	0.588	0.010*
3' or 2'-CMP	0.099	0.696	0.216	0.389	-0.204	0.417	-0.058	0.820	0.517	0.028*
3-Phosphoglyceric acid	0.118	0.640	0.062	0.807	-0.115	0.650	0.029	0.910	0.655	0.003**
5-Hydroxyindoleacetic acid	-0.470	0.049*	-0.183	0.468	-0.179	0.478	-0.240	0.337	-0.261	0.295
AMP	0.068	0.790	-0.195	0.437	0.272	0.275	0.356	0.147	0.471	0.049*
Arginine	0.131	0.605	-0.361	0.141	0.648	0.004**	0.168	0.506	0.217	0.386
Ascorbate 2-glucoside	0.090	0.723	0.491	0.039*	-0.429	0.076	0.009	0.972	-0.003	0.989
Azelaic acid	-0.288	0.247	-0.498	0.036*	-0.061	0.811	-0.377	0.123	-0.278	0.264
CMP	0.043	0.866	-0.208	0.407	0.303	0.222	0.379	0.121	0.474	0.047*
dAMP	0.153	0.544	-0.002	0.995	0.213	0.397	0.256	0.306	0.515	0.029*
Diphenylcarbazide	0.532	0.023*	0.429	0.076	-0.128	0.614	0.400	0.100	0.056	0.827
Ethyl glucuronide	0.201	0.423	0.568	0.014*	-0.517	0.028*	-0.091	0.719	-0.073	0.774
Fructose 6-phosphate	0.223	0.374	-0.176	0.485	0.318	0.199	0.054	0.833	0.470	0.049*
Glutamine	0.242	0.334	-0.287	0.248	0.613	0.007**	0.246	0.325	0.388	0.112
Glucose 6-phosphate	0.215	0.392	-0.029	0.911	0.186	0.461	0.108	0.670	0.616	0.007**
Gly-Asp	-0.179	0.478	-0.535	0.022*	0.492	0.038*	0.191	0.449	0.096	0.706
Gly-Gly	-0.161	0.525	-0.397	0.103	0.482	0.043*	0.193	0.444	0.083	0.744
Gly-Leu	0.095	0.709	-0.364	0.138	0.472	0.048*	0.068	0.788	0.099	0.696
Glyceric acid	-0.519	0.027*	-0.237	0.344	-0.397	0.103	-0.417	0.085	-0.155	0.540
Homovanillic acid	-0.032	0.901	-0.595	0.009**	0.313	0.206	-0.222	0.377	0.011	0.965
Hydroxyproline	-0.146	0.564	-0.629	0.005**	0.231	0.357	-0.088	0.728	0.042	0.869
IsovalerylalanineN-Acetylleucine	-0.121	0.632	-0.655	0.003**	0.394	0.106	-0.095	0.708	0.152	0.547
Lactic acid	-0.282	0.256	-0.611	0.007**	0.150	0.553	-0.271	0.277	-0.061	0.810
N,N-Dimethylhistidine	0.366	0.135	-0.366	0.136	0.480	0.044*	0.364	0.137	0.549	0.018*
N-Acetylasparagine	-0.509	0.031*	0.048	0.849	-0.457	0.056	-0.301	0.225	-0.362	0.141
N-Acetylglucosamine 6-phosphate	0.474	0.047*	0.877	<.0001**	-0.389	0.111	0.233	0.352	-0.182	0.469
N-Acetylglucosylamine	0.028	0.912	0.572	0.013*	-0.138	0.585	0.042	0.869	-0.214	0.394
N-Acetylmuramic acid	0.668	0.002**	0.072	0.778	0.157	0.534	0.293	0.238	0.255	0.307
p-Hydroxymandelic acid	-0.411	0.090	-0.181	0.473	-0.428	0.077	-0.540	0.021*	-0.152	0.547
Phenylalanine	0.138	0.586	-0.407	0.094	0.530	0.024*	0.028	0.913	0.207	0.410
Picolinic acid	-0.404	0.096	0.032	0.899	-0.479	0.044*	-0.502	0.034*	-0.328	0.183
Pimelic acid	-0.082	0.748	-0.486	0.041*	0.082	0.746	-0.247	0.324	-0.063	0.804
Proline	-0.372	0.129	-0.525	0.025*	0.297	0.232	-0.172	0.495	0.103	0.684
Putrescine	-0.062	0.808	-0.187	0.457	0.136	0.591	0.483	0.042*	0.244	0.330
Saccharopine	0.427	0.077	0.268	0.282	-0.017	0.946	0.255	0.308	0.473	0.047*
Sebacic acid	-0.233	0.353	-0.472	0.048*	-0.037	0.884	-0.389	0.111	-0.049	0.846
Sedoheptulose 7-phosphate	0.237	0.345	-0.056	0.827	0.194	0.440	0.089	0.726	0.509	0.031*
Syringic acid	-0.566	0.014*	-0.294	0.237	-0.075	0.767	-0.618	0.006**	-0.485	0.041*
Thiamine diphosphate	0.492	0.038*	0.009	0.971	0.217	0.387	0.150	0.554	0.375	0.125
Thymidine	0.372	0.128	0.649	0.004**	-0.024	0.924	0.267	0.284	-0.074	0.771
Tryptophan	-0.110	0.665	-0.342	0.165	0.475	0.046*	0.031	0.904	0.071	0.781
Tryptamine	0.194	0.440	0.536	0.022*	-0.310	0.210	0.048	0.851	-0.269	0.280
UMP	0.214	0.394	-0.078	0.758	0.298	0.230	0.353	0.150	0.777	<.0001**
Undecanoic acid	0.476	0.046*	0.031	0.903	0.115	0.650	0.107	0.672	0.229	0.360
Urocanic acid	0.551	0.018*	0.093	0.714	0.294	0.237	0.125	0.621	0.076	0.764
Valine	0.082	0.747	-0.395	0.105	0.471	0.048*	-0.042	0.869	0.175	0.489
γ-Glu-Val-Gly	0.070	0.782	0.736	0.001**	-0.531	0.024*	0.038	0.881	-0.004	0.989

Blue: metabolites correlated with Crps, red: *p < 0.05, **p < 0.01

Finally, to determine the causal relationship between α-defensin decrease and changes of the intestinal metabolites observed in the CSDS model, differences in metabolites among each group were analyzed when α-defensin was administered. In the metabolites shown in Table 4, those showed significant differences between any of the three groups were summarized, then the metabolites listed in Table 5 were shown in Fig. 12a and remaining metabolites in Table 5 were shown in Fig. 12b. The metabolites significantly increased in Crp4 group compared to CSDS group were pyridoxamine ($p = 0.002$), pyridoxamine-5 phosphate ($p = 0.027$), β-alanine ($p = 0.032$), 3-aminobutyric acid, ($p = 0.008$), 1H-imidazole propionic acid ($p = 0.044$), pantothenic acid ($p = 0.019$), and thiamine-phosphoric acid ($p = 0.044$) (Fig. 12a, b). Lysine tended to decrease in CSDS group ($p = 0.066$), whereas significantly increased in Crp4 group ($p = 0.023$). Glutamic acid and uracil were significantly reduced in CSDS group ($p = 0.038$, $p = 0.029$), whereas significantly increased in Crp4 group ($p = 0.019$, $p = 0.014$) (Fig. 12b). On the other hand, there was no significant difference between naïve group and Crp4 group for all those metabolites, indicating that the intestinal metabolites correlated with α-defensin are recovered to the same extent in naïve group by administration of α-defensin.

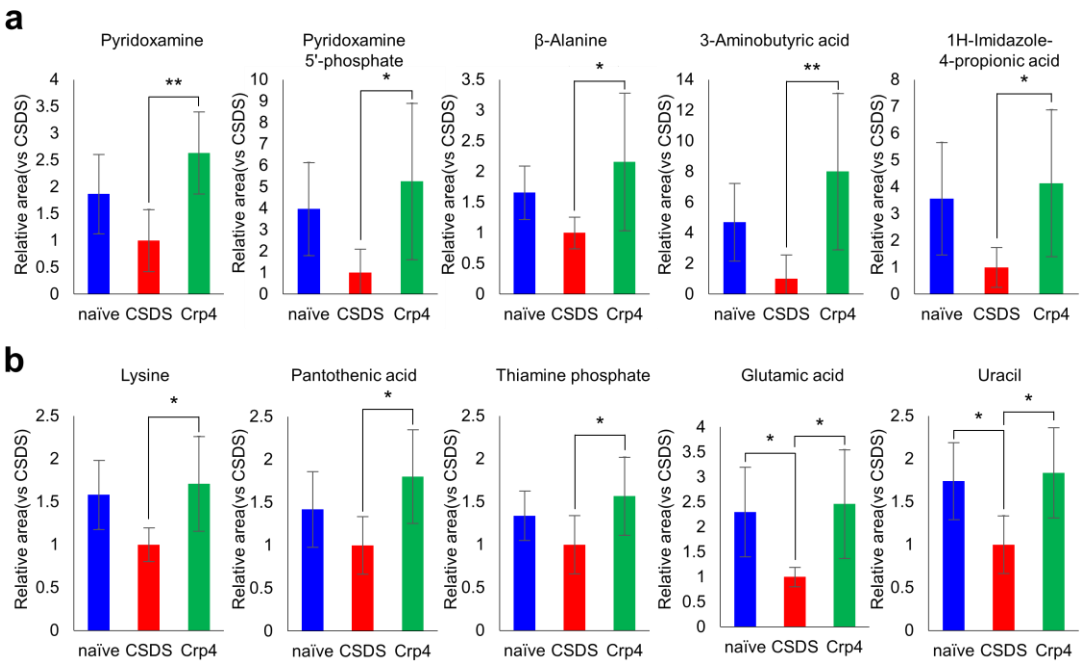


Figure 12. Changes in metabolites in feces correlated with Cryptdins in each group.

(a) Metabolites significantly correlated with Cryptdins and microbiota (blue metabolites in Table 5). (b) Metabolites significantly correlated with Cryptdins (Metabolites in Table 4 other than those shown in (a). Data are expressed as the means ± SEM (n = 6 per each group). Tukey's tests were used to compare the data. *, $P < 0.05$; **, $P < 0.01$.

In addition, there were metabolites with significant differences in both naïve group and Crp4 group compared to CSDS group, although no correlation was confirmed with α-defensin (Fig. 13). Among these, three metabolites which significantly decreased in CSDS group were N6-acetyllysine, penicillamine, threo-β-methylaspartic acid, and the decrease was significantly attenuated by administration of α-defensin. Conversely, seven metabolites which significantly increased in CSDS group were cadaverine, glucaric acid,

ferulic acid, mevalonic acid, digalacturonic acid, myo-inositol 2-phosphate, and p-aminophenol or m-aminophenol, and the increase was suppressed by administration of α-defensin.

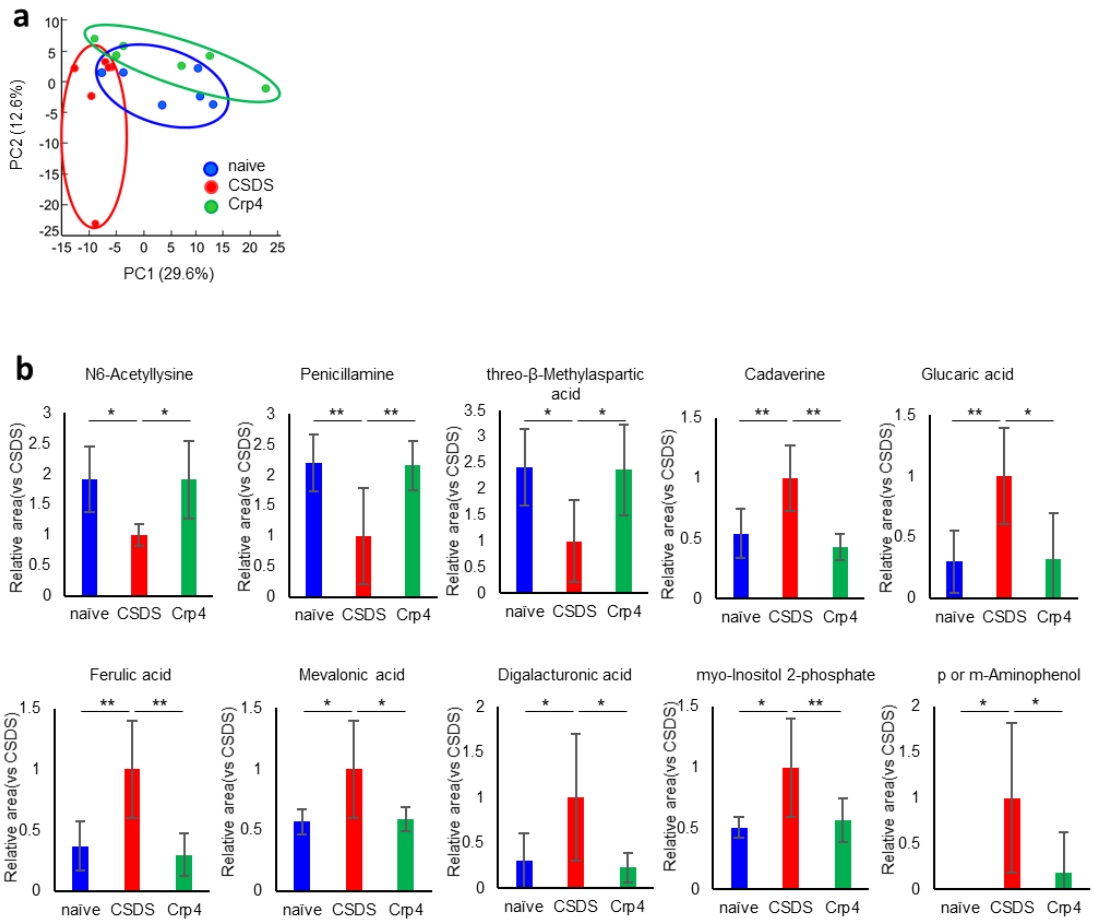


Figure 13. Changes in fecal metabolites induced by α-defensin administration. Only metabolites not significantly correlated with α-defensin are shown in the graph.

(a) Principal component analysis of intestinal metabolites. (b) relative ratio of intestinal metabolites to CSDS group which are significantly changed between naïve group and CSDS group, and between CSDS group and Crp4 group respectively. Data are expressed as the means ± SEM (n = 6 per each group). Tukey's tests were used to compare the data. *, $P < 0.05$; **, $P < 0.01$.

Table 6 All the data of abundance of microbiota（%）in each group

Phylum	Class	Order	Family	Genus	naive				CSDS				Crp4			
					before		after		before		after		before		after	
					Mean.	SD	Mean.	SD	Mean.	SD	Mean.	SD	Mean.	SD	Mean.	SD
unknown					0.02	0.02	0.01	0.01	0.03	0.02	0.01	0.01	0.06	0.08	0.02	0.02
Actinobacteria					0.78	0.35	1.36	0.42	0.82	0.32	1.75	0.91	0.70	0.20	1.77	0.70
Aquificae					0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bacteroidetes					37.16	14.71	36.57	12.15	32.44	13.87	48.19	5.10	45.05	6.73	44.33	6.87
Cyanobacteria					0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Deferribacteres					0.25	0.15	0.05	0.03	0.48	0.51	0.04	0.03	0.19	0.13	0.03	0.02
Firmicutes					60.87	15.18	59.72	12.66	64.90	14.85	47.58	4.79	52.44	7.46	51.02	8.49
Proteobacteria					0.25	0.17	1.01	0.97	0.29	0.25	0.82	0.27	0.55	0.26	0.95	0.33
TM7					0.00	0.00	0.01	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Tenericutes					0.28	0.10	0.09	0.04	0.32	0.23	0.16	0.10	0.20	0.11	0.14	0.06
Verrucomicrobia					0.39	0.54	1.18	2.60	0.72	1.06	1.44	1.16	0.81	0.87	1.74	2.04
unknown	*unknown*				0.02	0.02	0.01	0.01	0.03	0.02	0.01	0.01	0.06	0.08	0.02	0.02
Actinobacteria	*Actinobacteria*				0.00	0.01	0.18	0.43	0.00	0.00	0.47	1.08	0.00	0.00	0.00	0.00
Actinobacteria	*Coriobacteriia*				0.78	0.36	1.18	0.35	0.82	0.33	1.29	0.35	0.70	0.20	1.77	0.70
Aquificae	*Aquificae*				0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bacteroidetes	*Bacteroidia*				37.16	14.71	36.57	12.15	32.44	13.87	48.19	5.10	45.05	6.73	44.33	6.87
Cyanobacteria	*Chloroplast*				0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Deferribacteres	*Deferribacteres*				0.25	0.15	0.05	0.03	0.48	0.51	0.04	0.03	0.19	0.13	0.03	0.02
Firmicutes	*unknown*				0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Firmicutes	*Bacilli*				14.42	9.57	3.04	3.60	21.61	14.05	7.38	5.79	10.63	6.18	4.17	2.04
Firmicutes	*Clostridia*				43.67	20.26	50.80	12.24	40.77	16.43	31.66	8.68	37.21	7.14	35.65	10.85
Firmicutes	*Erysipelotrichi*				2.78	3.46	5.88	3.16	2.52	2.20	8.54	4.42	4.60	2.69	11.20	6.60
Proteobacteria	*Alphaproteobacteria*				0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Proteobacteria	*Betaproteobacteria*				0.25	0.16	0.58	0.24	0.28	0.25	0.81	0.27	0.53	0.29	0.95	0.33
Proteobacteria	*Deltaproteobacteria*				0.00	0.00	0.43	1.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Proteobacteria	*Gammaproteobacteria*				0.00	0.01	0.00	0.00	0.01	0.01	0.00	0.00	0.02	0.04	0.00	0.01
TM7	*TM7-3*				0.00	0.00	0.01	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Tenericutes	*Mollicutes*				0.28	0.10	0.09	0.04	0.32	0.23	0.16	0.10	0.20	0.11	0.14	0.06
Verrucomicrobia	*Verrucomicrobiae*				0.39	0.54	1.18	2.60	0.72	1.06	1.44	1.16	0.81	0.87	1.74	2.04
unknown	*unknown*	*unknown*			0.02	0.02	0.01	0.01	0.03	0.02	0.01	0.01	0.06	0.08	0.02	0.02
Actinobacteria	*Actinobacteria*	*Actinomycetales*			0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Actinobacteria	*Actinobacteria*	*Bifidobacteriales*			0.00	0.00	0.18	0.43	0.00	0.00	0.47	1.08	0.00	0.00	0.00	0.00
Actinobacteria	*Coriobacteriia*	*Coriobacteriales*			0.78	0.36	1.18	0.35	0.82	0.33	1.29	0.35	0.70	0.20	1.77	0.70
Aquificae	*Aquificae*	*Aquificales*			0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bacteroidetes	*Bacteroidia*	*Bacteroidales*			37.16	14.71	36.57	12.15	32.44	13.87	48.19	5.10	45.05	6.73	44.33	6.87
Cyanobacteria	*Chloroplast*	*Streptophyta*			0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Deferribacteres	*Deferribacteres*	*Deferribacterales*			0.25	0.15	0.05	0.03	0.48	0.51	0.04	0.03	0.19	0.13	0.03	0.02
Firmicutes	*unknown*	*unknown*			0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Firmicutes	*Bacilli*	*unknown*			0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Firmicutes	*Bacilli*	*Bacillales*			0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Firmicutes	*Bacilli*	*Lactobacillales*			14.42	9.57	3.04	3.60	21.37	14.13	7.38	5.79	10.52	6.34	3.73	2.03
Firmicutes	*Bacilli*	*Turicibacterales*			0.00	0.00	0.00	0.00	0.24	0.42	0.00	0.00	0.11	0.24	0.43	1.06
Firmicutes	*Clostridia*	*unknown*			0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00
Firmicutes	*Clostridia*	*Clostridiales*			43.67	20.26	50.80	12.23	40.76	16.42	31.66	8.68	37.21	7.14	35.65	10.85
Firmicutes	*Erysipelotrichi*	*Erysipelotrichales*			2.78	3.46	5.88	3.16	2.52	2.20	8.54	4.42	4.60	2.69	11.20	6.60
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*			0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Proteobacteria	*Alphaproteobacteria*	*Rickettsiales*			0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*			0.25	0.16	0.58	0.24	0.28	0.25	0.81	0.27	0.53	0.29	0.95	0.33
Proteobacteria	*Deltaproteobacteria*	*Desulfovibrionales*			0.00	0.00	0.43	1.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Proteobacteria	*Gammaproteobacteria*	*Enterobacteriales*			0.00	0.01	0.00	0.00	0.01	0.01	0.00	0.00	0.02	0.04	0.00	0.01
Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*			0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
TM7	*TM7-3*	*CW040*			0.00	0.00	0.01	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Tenericutes	*Mollicutes*	*Anaeroplasmatales*			0.01	0.01	0.02	0.03	0.06	0.13	0.03	0.05	0.01	0.01	0.02	0.03
Tenericutes	*Mollicutes*	*RF39*			0.26	0.11	0.08	0.03	0.26	0.21	0.13	0.06	0.19	0.10	0.13	0.05
Verrucomicrobia	*Verrucomicrobiae*	*Verrucomicrobiales*			0.39	0.54	1.18	2.60	0.72	1.06	1.44	1.16	0.81	0.87	1.74	2.04
unknown	*unknown*	*unknown*	*unknown*		0.02	0.02	0.01	0.01	0.03	0.02	0.01	0.01	0.06	0.08	0.02	0.02
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Actinomycetaceae*		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Corynebacteriaceae*		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Phylum	Class	Order	Family	Genus	naive				CSDS				Crp4			
					before		after		before		after		before		after	
					Mean.	SD	Mean.	SD	Mean.	SD	Mean.	SD	Mean.	SD	Mean.	SD
Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae		0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Actinobacteria	Actinobacteria	Actinomycetales	Nocardiaceae		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae		0.00	0.00	0.18	0.43	0.00	0.00	0.47	1.08	0.00	0.00	0.00	0.00
Actinobacteria	Coriobacteriia	Coriobacteriales	Coriobacteriaceae		0.78	0.36	1.18	0.35	0.82	0.33	1.29	0.35	0.70	0.20	1.77	0.70
Aquificae	Aquificae	Aquificales	Aquificaceae		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae		1.91	0.56	0.39	0.44	1.31	0.70	0.48	0.22	2.05	0.61	0.67	0.36
Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae		0.92	0.26	0.63	0.25	0.74	0.39	0.71	0.40	1.16	0.53	0.64	0.16
Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae		0.00	0.00	0.19	0.45	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bacteroidetes	Bacteroidia	Bacteroidales	S24-7		34.33	14.62	34.30	10.79	30.38	13.18	46.34	4.92	41.84	6.34	43.01	6.77
Bacteroidetes	Bacteroidia	Bacteroidales	[Paraprevotellaceae]		0.00	0.00	1.07	1.22	0.00	0.00	0.66	1.23	0.00	0.00	0.00	0.00
Cyanobacteria	Chloroplast	Streptophyta	unknown		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Deferribacteres	Deferribacteres	Deferribacterales	Deferribacteraceae		0.25	0.15	0.05	0.03	0.48	0.51	0.04	0.03	0.19	0.13	0.03	0.02
Firmicutes	unknown	unknown	unknown		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Firmicutes	Bacilli	unknown	unknown		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Firmicutes	Bacilli	Bacillales	Planococcaceae		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Firmicutes	Bacilli	Bacillales	Staphylococcaceae		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Firmicutes	Bacilli	Lactobacillales	Enterococcaceae		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00
Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae		14.42	9.57	3.03	3.60	21.37	14.13	7.37	5.78	10.52	6.34	3.72	2.01
Firmicutes	Bacilli	Lactobacillales	Leuconostocaceae		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Firmicutes	Bacilli	Lactobacillales	Streptococcaceae		0.00	0.00	0.01	0.01	0.00	0.00	0.01	0.01	0.00	0.00	0.01	0.02
Firmicutes	Bacilli	Turicibacterales	Turicibacteraceae		0.00	0.00	0.00	0.00	0.24	0.42	0.00	0.00	0.11	0.24	0.43	1.06
Firmicutes	Clostridia	unknown	unknown		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00
Firmicutes	Clostridia	Clostridiales	unknown		7.23	5.44	12.62	5.23	6.18	2.83	6.20	2.42	6.02	2.46	5.07	1.74
Firmicutes	Clostridia	Clostridiales	unknown		4.45	0.98	4.97	1.54	5.43	3.26	3.92	1.00	4.08	1.88	4.46	0.90
Firmicutes	Clostridia	Clostridiales	Christensenellaceae		0.02	0.01	0.03	0.02	0.01	0.01	0.03	0.01	0.01	0.01	0.02	0.01
Firmicutes	Clostridia	Clostridiales	Clostridiaceae		1.20	0.75	0.12	0.11	1.62	0.63	0.25	0.11	1.76	0.59	0.32	0.25
Firmicutes	Clostridia	Clostridiales	Dehalobacteriaceae		0.18	0.09	0.41	0.22	0.15	0.14	0.17	0.13	0.20	0.07	0.23	0.16
Firmicutes	Clostridia	Clostridiales	Eubacteriaceae		0.01	0.01	0.01	0.01	0.00	0.00	0.00	0.01	0.01	0.01	0.00	0.00
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae		20.52	10.62	21.68	6.49	18.56	8.27	13.29	5.84	16.29	5.98	15.96	7.52
Firmicutes	Clostridia	Clostridiales	Peptococcaceae		0.87	0.55	0.89	0.56	1.16	0.53	1.52	0.38	0.91	0.46	1.92	0.67
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		9.07	4.31	9.89	3.00	7.53	3.42	6.16	2.08	7.80	1.74	7.48	1.73
Firmicutes	Clostridia	Clostridiales	[Mogibacteriaceae]		0.13	0.04	0.19	0.04	0.13	0.09	0.12	0.02	0.12	0.05	0.17	0.06
Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae		2.78	3.46	5.88	3.16	2.52	2.20	8.54	4.42	4.60	2.69	11.20	6.60
Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Proteobacteria	Alphaproteobacteria	Rickettsiales	mitochondria		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae		0.24	0.16	0.58	0.24	0.28	0.25	0.81	0.27	0.53	0.29	0.95	0.33
Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae		0.00	0.00	0.43	1.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae		0.00	0.01	0.00	0.00	0.01	0.01	0.00	0.00	0.02	0.04	0.00	0.01
Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
TM7	TM7-3	CW040	F16		0.00	0.00	0.01	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Tenericutes	Mollicutes	Anaeroplasmatales	Anaeroplasmataceae		0.01	0.01	0.02	0.03	0.06	0.13	0.03	0.05	0.01	0.01	0.02	0.03
Tenericutes	Mollicutes	RF39	unknown		0.26	0.11	0.08	0.03	0.26	0.21	0.13	0.06	0.19	0.10	0.13	0.05
Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae		0.39	0.54	1.18	2.60	0.72	1.06	1.44	1.16	0.81	0.87	1.74	2.04
unknown	unknown	unknown	unknown	unknown	0.02	0.02	0.01	0.01	0.03	0.02	0.01	0.01	0.06	0.08	0.02	0.02
Actinobacteria	Actinobacteria	Actinomycetales	Actinomycetaceae	Actinomyces	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Actinobacteria	Actinobacteria	Actinomycetales	Corynebacteriaceae	Corynebacterium	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Microbacterium	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Actinobacteria	Actinobacteria	Actinomycetales	Nocardiaceae	Rhodococcus	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	Bifidobacterium	0.00	0.00	0.18	0.43	0.00	0.00	0.47	1.08	0.00	0.00	0.00	0.00
Actinobacteria	Coriobacteriia	Coriobacteriales	Coriobacteriaceae	unknown	0.07	0.02	0.04	0.04	0.05	0.08	0.04	0.06	0.07	0.05	0.04	0.03
Actinobacteria	Coriobacteriia	Coriobacteriales	Coriobacteriaceae	unknown	0.07	0.06	0.25	0.18	0.09	0.06	0.27	0.35	0.08	0.05	0.13	0.13
Actinobacteria	Coriobacteriia	Coriobacteriales	Coriobacteriaceae	Adlercreutzia	0.64	0.31	0.89	0.22	0.68	0.35	0.98	0.21	0.54	0.15	1.59	0.77
Aquificae	Aquificae	Aquificales	Aquificaceae	Hydrogenobacter	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	1.91	0.56	0.39	0.44	1.31	0.70	0.48	0.22	2.05	0.61	0.67	0.36
Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Parabacteroides	0.92	0.26	0.63	0.25	0.74	0.39	0.71	0.40	1.16	0.53	0.64	0.16
Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	unknown	0.00	0.00	0.19	0.45	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bacteroidetes	Bacteroidia	Bacteroidales	S24-7	unknown	34.33	14.62	34.30	10.79	30.38	13.18	46.34	4.92	41.84	6.34	43.01	6.77

Phylum	Class	Order	Family	Genus	naive				CSDS				Crp4			
					before		after		before		after		before		after	
					Mean.	SD	Mean.	SD	Mean.	SD	Mean.	SD	Mean.	SD	Mean.	SD
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*[Paraprevotellaceae]*	*[Prevotella]*	0.00	0.00	1.07	1.22	0.00	0.00	0.66	1.23	0.00	0.00	0.00	0.00
Cyanobacteria	*Chloroplast*	*Streptophyta*	*unknown*	*unknown*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Deferribacteres	*Deferribacteres*	*Deferribacterales*	*Deferribacteraceae*	*Mucispirillum*	0.25	0.15	0.05	0.03	0.48	0.51	0.04	0.03	0.19	0.13	0.03	0.02
Firmicutes	*unknown*	*unknown*	*unknown*	*unknown*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Firmicutes	*Bacilli*	*unknown*	*unknown*	*unknown*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Firmicutes	*Bacilli*	*Bacillales*	*Planococcaceae*	*Sporosarcina*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Firmicutes	*Bacilli*	*Bacillales*	*Staphylococcaceae*	*Jeotgalicoccus*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Firmicutes	*Bacilli*	*Bacillales*	*Staphylococcaceae*	*Staphylococcus*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Firmicutes	*Bacilli*	*Lactobacillales*	*Enterococcaceae*	*Enterococcus*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00
Firmicutes	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	*unknown*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Firmicutes	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	*Lactobacillus*	14.42	9.57	3.03	3.60	21.37	14.13	7.37	5.78	10.52	6.34	3.72	2.01
Firmicutes	*Bacilli*	*Lactobacillales*	*Leuconostocaceae*	*unknown*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Firmicutes	*Bacilli*	*Lactobacillales*	*Leuconostocaceae*	*Weissella*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Firmicutes	*Bacilli*	*Lactobacillales*	*Streptococcaceae*	*Streptococcus*	0.00	0.00	0.01	0.01	0.00	0.00	0.01	0.01	0.00	0.00	0.01	0.02
Firmicutes	*Bacilli*	*Turicibacterales*	*Turicibacteraceae*	*Turicibacter*	0.00	0.00	0.00	0.00	0.24	0.42	0.00	0.00	0.11	0.24	0.43	1.06
Firmicutes	*Clostridia*	*unknown*	*unknown*	*unknown*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00
Firmicutes	*Clostridia*	*Clostridiales*	*unknown*	*unknown*	7.23	5.44	12.62	5.23	6.18	2.83	6.20	2.42	6.02	2.46	5.07	1.74
Firmicutes	*Clostridia*	*Clostridiales*	*unknown*	*unknown*	4.45	0.98	4.97	1.54	5.43	3.26	3.92	1.00	4.08	1.88	4.46	0.90
Firmicutes	*Clostridia*	*Clostridiales*	*Christensenellaceae*	*unknown*	0.02	0.01	0.03	0.02	0.01	0.01	0.03	0.01	0.01	0.01	0.02	0.01
Firmicutes	*Clostridia*	*Clostridiales*	*Clostridiaceae*	*andidatus Arthromitu*	1.14	0.74	0.10	0.08	1.60	0.64	0.23	0.10	1.68	0.54	0.25	0.27
Firmicutes	*Clostridia*	*Clostridiales*	*Clostridiaceae*	*Clostridium*	0.06	0.04	0.03	0.04	0.02	0.02	0.01	0.01	0.07	0.08	0.07	0.07
Firmicutes	*Clostridia*	*Clostridiales*	*Dehalobacteriaceae*	*Dehalobacterium*	0.18	0.09	0.41	0.22	0.15	0.14	0.17	0.13	0.20	0.07	0.23	0.16
Firmicutes	*Clostridia*	*Clostridiales*	*Eubacteriaceae*	*Anaerofustis*	0.01	0.01	0.01	0.01	0.00	0.00	0.00	0.01	0.01	0.01	0.00	0.00
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*unknown*	16.24	8.90	13.85	4.18	15.32	7.31	9.96	3.97	12.71	6.15	12.12	5.58
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*unknown*	1.31	1.04	2.32	1.08	1.03	0.69	0.99	0.73	1.13	0.66	1.20	0.93
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Clostridium*	0.27	0.52	0.16	0.21	0.11	0.15	0.06	0.04	0.08	0.07	0.09	0.09
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Coprococcus*	0.68	0.45	1.80	1.38	0.92	0.47	1.17	0.93	0.49	0.31	0.87	0.39
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Dorea*	0.01	0.01	0.03	0.04	0.01	0.02	0.02	0.03	0.02	0.02	0.03	0.03
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Roseburia*	0.12	0.11	0.23	0.34	0.12	0.14	0.17	0.16	0.10	0.15	0.16	0.19
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*[Ruminococcus]*	1.89	0.99	3.28	1.26	1.05	0.74	0.91	0.53	1.76	1.07	1.50	0.93
Firmicutes	*Clostridia*	*Clostridiales*	*Peptococcaceae*	*unknown*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Firmicutes	*Clostridia*	*Clostridiales*	*Peptococcaceae*	*unknown*	0.02	0.02	0.07	0.07	0.04	0.02	0.05	0.03	0.03	0.02	0.05	0.03
Firmicutes	*Clostridia*	*Clostridiales*	*Peptococcaceae*	*rc4-4*	0.85	0.55	0.82	0.59	1.12	0.52	1.48	0.39	0.88	0.46	1.87	0.67
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*unknown*	0.96	0.58	0.93	0.54	0.69	0.59	0.43	0.17	0.55	0.12	0.39	0.18
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*unknown*	0.73	0.33	1.30	0.56	0.68	0.25	1.25	0.54	1.00	0.49	2.38	1.23
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*Anaerotruncus*	0.15	0.09	0.29	0.12	0.16	0.12	0.12	0.08	0.11	0.07	0.11	0.08
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*Butyricicoccus*	0.32	0.29	0.58	0.58	0.15	0.17	0.21	0.17	0.27	0.19	0.16	0.12
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*Gemmiger*	0.48	0.60	0.05	0.04	0.14	0.15	0.04	0.02	0.12	0.03	0.04	0.02
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*Oscillospira*	4.80	2.84	5.31	2.64	4.19	2.33	3.21	1.51	3.86	1.26	3.05	1.77
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*Ruminococcus*	1.63	1.11	1.43	0.39	1.52	0.95	0.90	0.36	1.88	1.12	1.37	1.12
Firmicutes	*Clostridia*	*Clostridiales*	*[Mogibacteriaceae]*	*unknown*	0.13	0.04	0.19	0.04	0.13	0.09	0.12	0.02	0.12	0.05	0.17	0.06
Firmicutes	*Erysipelotrichi*	*Erysipelotrichales*	*Erysipelotrichaceae*	*unknown*	0.01	0.03	0.02	0.01	0.06	0.05	0.02	0.02	0.07	0.03	0.03	0.05
Firmicutes	*Erysipelotrichi*	*Erysipelotrichales*	*Erysipelotrichaceae*	*unknown*	1.10	0.84	0.29	0.33	0.95	0.37	0.24	0.20	1.57	0.71	0.20	0.12
Firmicutes	*Erysipelotrichi*	*Erysipelotrichales*	*Erysipelotrichaceae*	*Allobaculum*	1.23	2.48	5.51	3.15	1.25	2.06	8.21	4.55	2.45	2.05	10.89	6.76
Firmicutes	*Erysipelotrichi*	*Erysipelotrichales*	*Erysipelotrichaceae*	*Clostridium*	0.28	0.34	0.04	0.06	0.14	0.11	0.05	0.06	0.22	0.19	0.05	0.04
Firmicutes	*Erysipelotrichi*	*Erysipelotrichales*	*Erysipelotrichaceae*	*Coprobacillus*	0.15	0.16	0.02	0.03	0.12	0.06	0.01	0.02	0.29	0.30	0.03	0.04
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Methylobacteriaceae*	*Methylobacterium*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Proteobacteria	*Alphaproteobacteria*	*Rickettsiales*	*mitochondria*	*unknown*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Alcaligenaceae*	*Sutterella*	0.24	0.16	0.58	0.24	0.28	0.25	0.81	0.27	0.53	0.29	0.95	0.33
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Oxalobacteraceae*	*Herbaspirillum*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Proteobacteria	*Deltaproteobacteria*	*Desulfovibrionales*	*Desulfovibrionaceae*	*Desulfovibrio*	0.00	0.00	0.43	1.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Proteobacteria	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*unknown*	0.00	0.01	0.00	0.00	0.01	0.01	0.00	0.00	0.02	0.04	0.00	0.01
Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Acinetobacter*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
TM7	*TM7-3*	*CW040*	*F16*	*unknown*	0.00	0.00	0.01	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Tenericutes	*Mollicutes*	*Anaeroplasmatales*	*Anaeroplasmataceae*	*Anaeroplasma*	0.01	0.01	0.02	0.03	0.06	0.13	0.03	0.05	0.01	0.01	0.02	0.03
Tenericutes	*Mollicutes*	*RF39*	*unknown*	*unknown*	0.26	0.11	0.08	0.03	0.26	0.21	0.13	0.06	0.19	0.10	0.13	0.05
Verrucomicrobia	*Verrucomicrobiae*	*Verrucomicrobiales*	*Verrucomicrobiaceae*	*Akkermansia*	0.39	0.54	1.18	2.60	0.72	1.06	1.44	1.16	0.81	0.87	1.74	2.04

Table 7. All the data of fecal metabolites (relative area vs internal standard)

Compound name	naive		CSDS		Crp4	
	Mean	S.D.	Mean	S.D.	Mean	S.D.
1,3-Diaminopropane	0.00062	0.00017	0.00069	0.00033	0.00095	0.00040
1-Aminocyclopropane-1-carboxylic acid Homoserinelactone	N.D.	N.D.	0.00021	N.D.	N.D.	N.D.
1-Methyl-4-imidazoleacetic acid	0.01698	0.00254	0.01502	0.00297	0.01868	0.00465
1-Methyladenosine	0.00020	0.00004	0.00028	0.00006	0.00028	0.00007
1-Methylhistidine 3-Methylhistidine	0.00177	0.00045	0.00130	0.00027	0.00192	0.00064
1*H*-Imidazole-4-propionic acid	0.01659	0.00977	0.00466	0.00346	0.01926	0.01276
2'-Deoxyadenosine 5'-Deoxvadenosine	0.00178	0.00076	0.00284	0.00131	0.00290	0.00182
2'-Deoxycytidine	0.00159	0.00031	0.00193	0.00063	0.00188	0.00093
2'-Deoxyguanosine	0.00088	0.00027	0.00102	0.00042	0.00132	0.00067
2,4-Diaminobutyric acid	0.00105	0.00122	0.00055	N.D.	0.00072	0.00035
2,5-Dihydroxybenzoic acid	0.00026	0.00007	0.00041	0.00025	0.00023	0.00003
2,5-Pyrroledione	0.00047	0.00008	0.00072	0.00030	0.00071	0.00001
2,6-Diaminopimelic acid	0.00348	0.00036	0.00366	0.00104	0.00477	0.00085
2-(Creatinine-3-yl)propionic acid	0.00250	0.00039	0.00275	0.00066	0.00277	0.00040
2-Amino-2-(hydroxymethyl)-1,3-propanediol	0.00050	0.00012	0.00061	0.00015	0.00055	0.00010
2-Amino-2-methyl-1,3-propanediol	0.00042	0.00003	0.00037	0.00004	0.00047	0.00008
2-Aminoethylphosphonic acid	0.00030	0.00014	0.00023	0.00010	0.00022	0.00006
2-Aminoisobutyric acid 2-Aminobutyric acid	0.01132	0.00416	0.00758	0.00301	0.01013	0.00314
2-Deoxyglucose 6-phosphate	0.00029	0.00006	0.00017	N.D.	0.00027	0.00011
2-Deoxyribose 1-phosphate	0.00201	0.00059	0.00163	0.00087	0.00295	0.00117
2-Hydroxy-4-methylvaleric acid	0.00738	0.00340	0.00454	0.00159	0.00524	0.00337
2-Hydroxybutyric acid	0.00075	0.00035	0.00027	0.00007	0.00087	0.00072
2-Hydroxyisobutyric acid	0.00013	N.D.	N.D.	N.D.	N.D.	N.D.
2-Hydroxypyridine	N.D.	N.D.	N.D.	N.D.	0.00011	0.00001
2-Hydroxyvaleric acid	0.00307	0.00148	0.00154	0.00046	0.00216	0.00139
2-Isopropylmalic acid	0.00033	0.00011	0.00039	0.00011	0.00020	0.00009
2-Methylserine	0.00044	0.00022	0.00035	0.00003	0.00051	0.00008
2-Oxoglutaric acid	0.01308	0.00695	0.00565	0.00175	0.01401	0.00670
2-Oxoisovaleric acid	0.00415	0.00184	0.00237	0.00056	0.00352	0.00138
3'-AMP	0.00039	0.00035	0.00018	0.00005	0.00016	0.00003
3'-CMP 2'-CMP	0.00050	0.00058	0.00024	0.00014	0.00029	0.00010
3,4-Dihydroxyphenylglycol	0.00031	0.00005	0.00031	0.00002	0.00031	0.00007
3-(4-Hydroxyphenyl)propionic acid	0.01299	0.00511	0.02230	0.01493	0.00681	0.00218
3-Amino-2-piperidone	0.00039	0.00008	0.00039	0.00011	0.00038	0.00008
3-Aminobutyric acid	0.00078	0.00016	0.00041	0.00006	0.00110	0.00070
3-Aminoisobutyric acid	0.00099	0.00024	0.00112	0.00039	0.00112	0.00037
3-Aminopropane-1,2-diol	0.00029	N.D.	0.00048	0.00021	0.00034	0.00008
3-Dehydroshikimic acid	0.00019	0.00006	0.00018	0.00005	0.00014	0.00003
3-Hydroxy-3-methylglutaric acid	0.00042	0.00007	0.00047	0.00016	0.00030	0.00004
3-Hydroxybutyric acid	0.00145	0.00098	0.00048	0.00017	0.00079	0.00074
3-Hydroxypropionic acid	0.00132	0.00038	N.D.	N.D.	0.00159	0.00025
3-Methoxytyrosine	0.00029	0.00007	N.D.	N.D.	0.00026	N.D.
3-Methylguanine	0.00059	0.00009	0.00050	0.00015	0.00060	0.00012
3-Phenylpropionic acid	0.00952	0.00353	0.00610	0.00127	0.00872	0.00247
3-Phosphoglyceric acid	0.00021	N.D.	N.D.	N.D.	N.D.	N.D.
4-(β-Acetylaminoethyl)imidazole	0.00041	0.00034	0.00045	0.00025	0.00084	0.00089
4-Acetamidobutanoic acid	0.00020	0.00008	0.00014	N.D.	0.00028	0.00008
4-Guanidinobutyric acid	0.00037	0.00008	0.00038	0.00009	0.00023	N.D.
4-Methyl-2-oxovaleric acid 3-Methyl-2-oxovaleric acid	0.01813	0.00679	0.01155	0.00206	0.01713	0.00636
4-Methyl-5-thiazoleethanol	0.00018	0.00006	0.00017	0.00006	0.00022	0.00009
4-Methylthio-2-oxobutyric acid	0.00054	0.00016	0.00022	0.00006	0.00042	0.00015
4-Pyridoxic acid	0.00257	0.00024	0.00238	0.00043	0.00246	0.00043

Compound name	naive		CSDS		Crp4	
	Mean	S.D.	Mean	S.D.	Mean	S.D.
5-Aminovaleric acid	0.121962	0.058019	0.065527	0.060502	0.051636	0.031686
5-Hydroxyindoleacetic acid	0.00025	9.38E-05	0.000318	8.07E-05	0.000262	0.000125
5-Hydroxylysine	0.001598	0.000181	0.001918	0.00112	0.001549	0.000404
5-Methoxyindoleacetic acid	0.00013	4.52E-05	0.00014	3.44E-05	0.000143	2.86E-05
5-Methyl-2'-deoxycytidine	0.000412	7.13E-05	0.00042	8.14E-05	0.000425	1E-04
5-Methylcytosine	0.000148	N.D.	0.000154	N.D.	N.D.	N.D.
5-Oxo-2-tetrahydrofurancarboxylic acid	N.D.	N.D.	N.D.	N.D.	0.000208	6E-05
5-Oxoproline	0.002184	0.00109	0.001006	0.000206	0.002091	0.001122
6-Aminohexanoic acid	0.003918	0.001162	0.002886	0.000233	0.003941	0.00108
6-Hydroxyhexanoic acid	0.000778	0.000153	0.000553	0.000223	0.000562	0.000328
6-Hydroxynicotinic acid	0.000321	0.000151	0.000178	8.19E-05	0.000457	0.000308
7-Methylguanine	0.00148	0.00047	0.000798	0.000201	0.001433	0.000533
8-Hydroxyoctanoic acid	0.000133	3.21E-05	0.000146	3.12E-05	0.000129	2.61E-05
Acetoacetic acid	0.000345	1.52E-06	9.82E-05	N.D.	0.000292	0.000195
Adenine	0.00791	0.00439	0.015402	0.007931	0.00817	0.003894
Adenosine	0.00131	0.000632	0.002799	0.001287	0.00234	0.001388
Adipic acid	N.D.	N.D.	N.D.	N.D.	0.000249	N.D.
Ala	0.398698	0.108875	0.340303	0.065558	0.434838	0.140068
allo-Threonine	0.000706	0.00017	0.000584	0.00013	0.000743	0.000244
Alloisoleucine	0.003497	0.002071	0.002386	0.000602	0.003021	0.001628
AMP	0.00012	2.5E-05	0.000124	1.33E-06	0.000146	N.D.
Anserine_divalent	0.000491	5.44E-05	0.000442	9.16E-05	0.000554	5.61E-05
Arg	0.011764	0.002765	0.0125	0.008677	0.013648	0.004047
Arg-Glu	0.000777	0.000206	0.000594	0.000155	0.000904	0.000279
Argininosuccinic acid	N.D.	N.D.	0.000232	5.88E-05	0.000145	3.57E-05
Ascorbate 2-glucoside	0.00062	0.000145	0.000613	0.000169	0.000726	0.000116
Ascorbic acid	0.000159	6.78E-05	0.000181	4.15E-06	0.000123	1.96E-05
Asn	0.000503	N.D.	0.000932	0.000372	0.000657	0.000135
Asp	0.164454	0.098352	0.1024	0.04329	0.140979	0.072573
Azelaic acid	0.002671	0.000855	0.002128	0.000592	0.002133	0.001003
Azetidine 2-carboxylic acid	N.D.	N.D.	0.000548	N.D.	N.D.	N.D.
Betaine	0.005847	0.000978	0.006558	0.002688	0.005662	0.00153
Betaine aldehyde_+H_2O	0.000376	0.000118	0.000417	0.000205	0.000345	4.92E-05
Betonicine	N.D.	N.D.	0.000339	0.000114	N.D.	N.D.
Cadaverine	0.00036	0.000137	0.000665	0.000179	0.000285	7.35E-05
cAMP	0.000173	4.49E-05	0.000158	3.9E-05	0.000161	3.53E-05
Carboxymethyllysine	0.001095	0.000223	0.000819	0.000145	0.001113	0.000325
Carnitine	0.001184	0.000325	0.001181	0.000276	0.001322	0.000346
Cholic acid	0.160209	0.091828	0.168993	0.071684	0.184877	0.035942
Choline	0.026136	0.004774	0.031314	0.005141	0.020295	0.006615
Cimetidine	0.000657	0.000143	0.000768	0.000269	0.00072	5.37E-05
cIMP	0.00013	4.95E-05	0.000135	3.12E-05	0.000144	1.96E-05
cis-4-Hydroxyproline	0.000713	0.000258	0.00046	7.5E-05	0.000595	0.000241
Citraconic acid	0.00037	9.66E-05	0.000168	2.47E-05	0.000359	0.000164
Citramalic acid	0.000984	0.000298	0.000464	4.94E-05	0.000786	8.94E-05
Citric acid	0.007782	0.004426	0.00706	0.002208	0.004829	0.001351
Citrulline	0.025209	0.008405	0.020051	0.008897	0.031702	0.011937
CMP	0.000136	1.34E-05	0.000156	6.32E-05	0.000224	N.D.
CMP-*N*-acetylneuraminate	N.D.	N.D.	7.51E-05	N.D.	N.D.	N.D.
Creatine	0.000279	7.02E-05	0.004302	0.007819	0.00024	7.51E-05
Creatinine	0.000428	0.000133	0.001208	0.001492	0.000556	0.000118
Crotonic acid	0.00019	1.2E-05	0.000224	N.D.	N.D.	N.D.
Cyclohexanecarboxylic acid	0.000127	1.87E-05	0.000137	5.33E-05	N.D.	N.D.

Compound name	naive		CSDS		Crp4	
	Mean	S.D.	Mean	S.D.	Mean	S.D.
Cysteic acid	0.00182	0.000681	0.001128	0.000221	0.001973	0.000756
Cystine	0.00015	2.21E-05	0.000244	N.D.	0.000196	N.D.
Cytidine	0.003416	0.000496	0.003758	0.001215	0.003442	0.000882
Cytosine	0.001805	0.002059	0.00252	0.002674	0.000963	0.000344
Daminozide Ala-Ala	0.011289	0.004895	0.004629	0.001741	0.010773	0.005418
dAMP	0.000244	9.42E-05	0.000203	5.32E-05	0.00017	5.8E-05
dCMP	0.000348	0.000207	0.00037	0.000291	0.00037	0.00023
Diethanolamine	0.000777	0.00052	0.001787	0.001552	0.000949	0.00065
Digalacturonic acid	0.000368	0.000197	0.000801	0.00056	0.000219	0.000107
Dihydroxyacetone phosphate	0.000738	0.000204	0.000457	0.000266	0.000623	0.000215
Diphenylcarbazide	0.004342	0.000415	0.004317	0.001126	0.005229	0.000534
Dodecanedioic acid	0.002253	0.000684	0.001541	0.000596	0.001643	0.000852
dTMP	0.000287	0.000128	0.000326	0.000218	0.000375	0.000136
Dyphylline	0.004477	0.002493	0.00652	0.003419	0.005896	0.003583
Ectoine	0.000519	0.000384	0.000575	0.000208	N.D.	N.D.
Erythrose 4-phosphate	0.000124	N.D.	N.D.	N.D.	0.000132	N.D.
Ethanolamine	0.019544	0.003553	0.01593	0.00397	0.017658	0.005333
Ethyl glucuronide	0.001471	0.000178	0.001391	0.000354	0.00163	0.000212
Ferulic acid	0.002634	0.001427	0.007124	0.002859	0.002101	0.001247
FMN	0.000167	4.67E-05	N.D.	N.D.	0.000213	3.84E-05
Formiminoglutamic acid	0.001621	0.000734	0.00089	0.000233	0.001729	0.000601
Fructose 6-phosphate	0.000412	0.000268	0.000182	6.82E-05	0.000373	0.00038
Fumaric acid	0.004364	0.001782	0.004348	0.001627	0.004619	0.003404
GABA	0.005402	0.001075	0.005799	0.001368	0.008411	0.007119
Galactosamine Glucosamine	0.001852	0.001723	0.001163	0.00045	0.002623	0.002076
Gln	0.031105	0.012118	0.026783	0.016563	0.033027	0.009169
Glu	0.680785	0.265032	0.295855	0.057193	0.728424	0.321606
Glu-Glu	0.000816	0.00024	0.000737	0.000222	0.000737	0.000165
Glucaric acid	0.000135	4.05E-05	0.000302	0.000119	0.000191	7.37E-05
Gluconic acid	0.001858	0.000981	0.001834	0.000593	0.001121	0.000461
Gluconolactone	0.001318	0.000455	0.001401	7.88E-05	0.00127	0.000101
Glucosamine 6-phosphate	0.000135	N.D.	N.D.	N.D.	N.D.	N.D.
Glucose 1-phosphate	0.000681	0.00034	0.00021	6.24E-05	0.000627	0.000492
Glucose 6-phosphate	0.000929	0.001268	0.000464	0.000463	0.000541	0.00072
Glucuronic acid-1 Galacturonic acid-1	0.002598	0.000928	0.002654	0.000629	0.00186	0.000256
Glucuronic acid-2 Galacturonic acid-2	0.012901	0.005743	0.01471	0.0046	0.007941	0.001004
Glutaric acid	0.001023	0.000268	0.000943	0.000332	0.000966	0.000306
Gly	0.086458	0.026444	0.061047	0.019617	0.07631	0.024031
Gly-Asp	0.001115	0.000216	0.001203	0.000267	0.001114	0.000366
Gly-Gly	0.001176	0.00018	0.001329	0.000525	0.00108	0.000204
Gly-Leu	0.003396	0.000838	0.003511	0.001856	0.003546	0.001179
Glyceric acid	0.011923	0.003287	0.011125	0.002635	0.006561	0.001177
Glycerol	0.173539	0.030253	0.194803	0.049306	0.190731	0.035331
Glycerol 2-phosphate	0.000116	1.19E-05	N.D.	N.D.	N.D.	N.D.
Glycerol 3-phosphate	0.000877	0.000204	0.001227	0.000371	0.000835	0.000345
Glycolic acid	0.003042	0.00052	0.001861	0.000741	0.001793	0.000233
GMP	N.D.	N.D.	9.56E-05	N.D.	N.D.	N.D.
Guanine	0.002296	0.000696	0.00368	0.001307	0.003577	0.001996
Guanosine	0.000762	0.00044	0.001154	0.000594	0.001506	0.000776
Heptanoic acid	0.000234	1.55E-05	0.000166	3.04E-05	N.D.	N.D.
Hexanoic acid	0.000701	0.000277	0.000418	0.000103	0.001059	0.000782
His	0.019968	0.003811	0.017665	0.007798	0.015841	0.002864
His-Glu	0.000209	5.46E-05	0.000194	8.18E-05	0.00024	5.57E-05

Compound name	naive		CSDS		Crp4	
	Mean	S.D.	Mean	S.D.	Mean	S.D.
Histamine	0.000193	N.D.	0.000243	N.D.	0.000528	0.000205
Histidinol	N.D.	N.D.	N.D.	N.D.	7.8E-05	N.D.
Homocarnosine	0.000135	3.47E-05	0.000147	2.19E-05	0.000117	2.48E-06
Homocitrulline	0.000822	0.000128	0.000722	0.000199	0.000762	0.00022
Homocysteic acid	0.000105	1.18E-05	0.00011	1.74E-05	0.000116	2.34E-05
Homoserine	0.006423	0.002163	0.003212	0.000698	0.006893	0.003136
Homovanillic acid	0.00078	0.000273	0.000405	6.02E-05	0.000785	0.000315
Hydroxyindole	0.001887	0.000222	0.001856	0.000297	0.00215	0.000481
Hydroxyproline	0.010616	0.004134	0.008793	0.002994	0.007968	0.00395
Hypotaurine	0.000435	0.000151	0.000408	3.02E-05	0.000454	7.16E-05
Hypoxanthine	0.159686	0.059208	0.076001	0.030919	0.147222	0.048871
Ile	0.152056	0.033504	0.110904	0.04063	0.139863	0.044727
Imidazole-4-acetic acid	N.D.	N.D.	0.00033	N.D.	0.000336	0.000308
Imidazole-4-methanol	0.000481	0.000213	0.000527	0.000125	0.000479	0.000256
Imidazolelactic acid	0.000275	0.000113	0.000471	0.00017	0.000362	0.000176
Indole-3-acetic acid	0.000274	7.34E-05	0.000315	8.43E-05	0.000231	8.23E-05
Inosine	0.008574	0.003988	0.005917	0.005153	0.016578	0.014507
Isethionic acid	0.00122	0.002072	0.000381	0.00022	0.000369	0.000171
Isoamylamine	N.D.	N.D.	N.D.	N.D.	0.005593	N.D.
Isobutyric acid Butyric acid	0.105586	0.049927	0.064962	0.044534	0.113579	0.090151
Isocitric acid	0.000433	N.D.	N.D.	N.D.	N.D.	N.D.
Isoglutamic acid	0.016566	0.010423	0.004554	0.001918	0.019063	0.014994
Isopropanolamine	0.001486	0.000193	0.002205	0.000765	0.001486	0.000477
Isovaleric acid Valeric acid	0.029833	0.010352	0.018039	0.002843	0.025816	0.011199
Isovalerylalanine *N*-Acetylleucine	0.000214	5.13E-05	0.000159	1.3E-05	0.00019	4.64E-05
Kojic acid	0.001075	0.000215	0.001008	0.000262	0.001022	0.000124
Kynurenic acid	0.000109	2.1E-05	0.000114	3.39E-05	0.000141	9.53E-06
Lactic acid	0.195511	0.083869	0.153629	0.059531	0.111622	0.067228
Lauric acid	0.000195	9.84E-06	0.000152	2.99E-05	0.000151	N.D.
Leu	0.213415	0.040676	0.156594	0.061918	0.198728	0.064978
Loperamide	N.D.	N.D.	N.D.	N.D.	0.000326	4.54E-06
Lys	0.209441	0.053193	0.132497	0.025826	0.226391	0.073281
Malic acid	0.063788	0.031826	0.055595	0.020562	0.066251	0.034661
Melatonin	0.001397	0.000107	0.001377	0.000279	0.001533	0.000107
Met	0.072054	0.018464	0.046387	0.013598	0.070259	0.02717
Methionine sulfoxide	0.006163	0.001484	0.004171	0.000474	0.006236	0.001709
Mevalolactone	0.002477	N.D.	0.002103	0.00012	N.D.	N.D.
Mevalonic acid	0.000279	5E-05	0.00049	0.000195	0.000289	4.81E-05
Mucic acid	0.000218	7.87E-06	0.000282	3.01E-05	0.00019	N.D.
myo-Inositol 1-phosphate *myo*-Inositol 3-phosphate	0.000556	0.000234	0.000319	0.000126	0.000413	9.3E-05
myo-Inositol 2-phosphate	0.000354	5.89E-05	0.000699	0.000281	0.000395	0.000128
N,*N*-Dimethylglycine	0.002287	0.001859	0.002308	0.00207	0.002134	0.00101
N,*N*-Dimethylhistidine	0.00028	1.47E-05	0.000297	0.000127	0.00033	6.54E-05
N-Acetyl-β-alanine	0.00064	0.00011	0.000603	0.000191	0.000422	0.000184
N-Acetylalanine	0.000257	7.75E-05	0.000201	5.51E-05	0.000204	5.37E-05
N-Acetylasparagine	0.00015	2.71E-05	0.000165	8.62E-05	0.000164	7.57E-05
N-Acetylaspartic acid	0.000435	9.18E-05	0.000488	0.000184	0.00044	0.00023
N-Acetylgalactosamine *N*-Acetylmannosamine *N*-Acetylglucosamine	0.036627	0.009249	0.042755	0.014919	0.04456	0.018249
N-Acetylglucosamine 1-phosphate	0.000114	1.32E-07	0.000175	N.D.	N.D.	N.D.
N-Acetylglucosamine 6-phosphate	N.D.	N.D.	N.D.	N.D.	6.41E-05	N.D.
N-Acetylglucosylamine	N.D.	N.D.	0.000376	6.77E-05	0.000433	0.000173

Compound name	naive		CSDS		Crp4	
	Mean	S.D.	Mean	S.D.	Mean	S.D.
N-Acetylglutamic acid	0.003557	0.001297	0.001981	0.000789	0.00356	0.001163
N-Acetylglutamine	0.000436	4.73E-05	0.000433	6.07E-05	0.000432	8.77E-05
N-Acetylglycine	0.000189	0.000151	0.000364	0.000315	0.000125	N.D.
N-Acetylhistidine	0.001543	0.000777	0.001162	0.00037	0.000861	0.000167
N-Acetyllysine	0.007959	0.001934	0.006389	0.000992	0.01063	0.004778
N-Acetylmethionine	0.00044	0.000117	0.000333	9.34E-05	0.000368	0.000112
N-Acetylmuramic acid	0.00355	0.001157	0.003086	0.001082	0.005008	0.001853
N-Acetylneuraminic acid	0.019562	0.004925	0.022195	0.004558	0.017563	0.00176
N-Acetylornithine	0.004638	0.001137	0.003273	0.00052	0.004652	0.001858
N-Acetylputrescine	0.002605	0.001086	0.003179	0.001718	0.001485	0.000906
N-Acetylserine	0.001775	0.001107	0.003345	0.002088	0.001072	0.000339
N-Acetyltryptophan	0.000442	1.52E-05	0.000432	7.46E-05	0.000482	5.03E-05
N-Carbamoylaspartic acid	0.000174	6.5E-05	0.000237	N.D.	0.000214	3.46E-06
N-Formylmethionine	0.000202	2.44E-05	0.000207	2.55E-05	0.000185	2.54E-05
N-Methylalanine	0.005981	0.003004	0.002038	0.000966	0.006244	0.005136
N-Methylglutamic acid	0.000708	0.000502	0.000321	6.93E-05	0.000594	0.00038
N-Methylnorsalsolinol	0.000501	0.000103	0.000456	8.75E-05	0.000512	0.000167
N-Methylproline	0.005333	0.003299	0.003691	0.002838	0.002802	0.003034
N^2-Acetylaminoadipic acid	0.000545	0.000251	0.000263	2.77E-05	0.000466	0.000184
N^2-Phenylacetylglutamine	0.000686	0.00012	0.000646	0.000144	0.000769	8.72E-05
N^2-Succinylornithine	0.000985	0.000546	0.001245	0.00043	0.001576	0.001286
N^5-Ethylglutamine	0.0023	0.001104	0.001233	0.000478	0.002178	0.001002
N^6,*N*^{6},*N*^{6}-Trimethyllysine	0.001008	0.000396	0.00058	0.000111	0.000821	0.000357
N^6-Acetyllysine	0.001799	0.000506	0.00094	0.000167	0.001796	0.0006
N^6-Methyladenine	0.000416	9.4E-05	0.000369	9.02E-05	0.000431	0.000143
N^6-Methyllysine	0.000935	0.000319	0.00066	0.000151	0.000911	0.000346
N^8-Acetylspermidine	0.001291	0.000648	0.000777	0.000353	0.001257	0.000528
Nicotinic acid	0.074068	0.024796	0.042703	0.013337	0.075163	0.027114
N_ω-Methylarginine	0.00013	N.D.	N.D.	N.D.	N.D.	N.D.
O-Acetylhomoserine 2-Aminoadipic acid	0.000886	0.000189	0.000987	0.000259	0.000807	6.9E-05
O-Acetylserine	0.000596	0.000265	0.00066	0.000506	0.000465	0.000187
o-Coumaric acid *p*-Coumaric acid	0.000217	4.94E-05	0.000284	0.000105	0.000146	2.35E-05
o-Hydroxybenzoic acid	0.000249	9.28E-05	0.000309	0.000216	0.000192	4.32E-05
Octanoic acid	0.000209	N.D.	N.D.	N.D.	0.000338	N.D.
Ornithine	0.082143	0.019759	0.057638	0.021198	0.079585	0.034459
Orotic acid	N.D.	N.D.	N.D.	N.D.	0.001144	0.000187
Oxypurinol	N.D.	N.D.	N.D.	N.D.	0.003444	N.D.
p-Aminobenzoic acid	0.001149	0.00035	0.000647	0.000196	0.001146	0.000596
p-Aminophenol *m*-Aminophenol	N.D.	N.D.	0.000345	7.56E-05	0.000249	N.D.
p-Hydroxybenzoic acid	0.000256	5.99E-05	0.000528	0.000448	N.D.	N.D.
p-Hydroxymandelic acid	0.000368	7.24E-05	0.000325	7.4E-05	0.000305	5.49E-05
p-Hydroxyphenylacetic acid	0.008522	0.003999	0.006748	0.003459	0.005116	0.002353
p-Hydroxyphenylpyruvic acid	0.000859	0.000188	0.00058	0.000128	0.000651	0.00025
p-Toluic acid *m*-Toluic acid *o*-Toluic acid	0.001382	0.00034	0.001051	0.000532	0.001224	0.000274
Pantothenic acid	0.010582	0.003282	0.00746	0.0025	0.01344	0.004075
Penicillamine	0.000829	0.000175	0.000564	6.46E-05	0.000812	0.000152
Phe	0.108583	0.021767	0.082683	0.0395	0.1009	0.035863
Phenylpyruvic acid	0.001124	0.000489	0.000575	0.000141	0.000928	0.000392
Phosphorylcholine	0.000613	0.000446	0.0009	0.000266	N.D.	N.D.
Phthalic acid	0.000243	4.23E-05	0.000258	5.51E-05	0.000252	3.44E-05
Picolinic acid	0.000372	7.68E-05	0.000267	N.D.	N.D.	N.D.
Pimelic acid	0.000367	6.01E-05	0.000332	8.91E-05	0.000347	0.000106

Compound name	naive		CSDS		Crp4	
	Mean	S.D.	Mean	S.D.	Mean	S.D.
Pipecolic acid	0.00492	0.001541	0.004539	0.001558	0.003969	0.001398
Piperidine	0.000912	0.000359	0.000783	0.00019	0.000733	0.000267
Pro	0.134677	0.018226	0.121447	0.023889	0.117042	0.027328
Propionic acid	0.071227	0.020905	0.04873	0.016245	0.045379	0.009561
Prostaglandin E_2	0.003168	0.000407	0.003026	0.000603	0.003444	0.000164
Purine	0.000418	8.24E-05	N.D.	N.D.	0.000413	0.000102
Putrescine	0.005162	0.001861	0.008505	0.004716	0.005416	0.002695
Pyridoxal	0.002003	0.000145	0.001602	0.000336	0.001731	0.000195
Pyridoxamine	0.00161	0.000636	0.000862	0.0005	0.002271	0.00066
Pyridoxamine 5'-phosphate	0.000531	0.00029	0.000268	5.39E-06	0.000703	0.000488
Pyridoxine	0.001876	0.001199	0.002139	0.000934	0.001117	0.00041
Pyrophosphate	0.012274	0.001499	0.009672	0.001973	0.012245	0.002482
Pyruvic acid	0.003029	0.000861	0.003779	0.001547	0.003196	0.000814
Quinic acid	0.001182	0.000118	0.001181	0.000234	0.001265	0.000158
Quinolinic acid	0.00041	0.000136	0.000361	9.51E-05	0.000498	0.000175
Riboflavin	0.0009	8.24E-05	0.000738	0.000165	0.001052	0.000265
Ribose 5-phosphate	0.00079	0.000561	0.000471	0.000378	0.000744	0.000647
Ribulose 5-phosphate	0.002644	0.001373	0.002505	0.001651	0.004632	0.003063
S-Adenosylmethionine	0.000573	N.D.	0.000372	0.000233	0.001918	0.001415
S-Sulfocysteine	0.000234	6.19E-05	0.000376	0.000172	0.000239	6.57E-05
Saccharopine	0.000847	0.000251	0.000654	0.000115	0.000927	0.000258
Sarcosine	0.007153	0.002514	0.008578	0.007167	0.005303	0.001168
SDMA	0.000655	0.000128	0.000529	0.000148	0.000549	0.000143
Sebacic acid	0.001223	0.000244	0.001	0.000288	0.000941	0.000407
Sedoheptulose 7-phosphate	0.001796	0.001594	0.000545	0.00038	0.001159	0.001578
Ser	0.059905	0.007109	0.053825	0.016166	0.053348	0.011878
Ser-Glu	0.001668	0.000287	0.001351	0.000336	0.001853	0.000891
Serotonin	0.000323	9.14E-05	0.000401	0.000122	0.000437	8.22E-05
Sinapic acid	0.0008	0.000113	0.000905	0.000181	0.000816	9.01E-05
Spermidine	0.020068	0.009368	0.011338	0.007048	0.024574	0.010878
Stachydrine	0.00055	0.000166	0.000711	0.000326	0.00044	0.00014
Suberic acid	0.001265	0.000177	0.001201	0.000328	0.00121	0.000344
Succinic acid	0.110001	0.064798	0.062137	0.036094	0.0913	0.043954
Syringic acid	0.000217	6.23E-05	0.000276	5.48E-05	0.00021	5.86E-05
Taurine	0.04152	0.050556	0.047825	0.049037	0.037053	0.025805
Taurocholic acid	0.001032	0.00088	0.002503	0.002473	0.0007	0.000218
Terephthalic acid	0.000707	0.00011	0.000755	3.64E-05	0.000734	4.62E-05
Thiamine	0.001734	0.000342	0.000996	0.000423	0.001912	0.000822
Thiamine diphosphate	0.000194	0.000103	0.000108	N.D.	0.000237	9.81E-05
Thiamine phosphate	0.000604	0.00013	0.000451	0.000153	0.000707	0.000205
Thr	0.089341	0.020491	0.061793	0.015146	0.088605	0.026827
Thr-Asp	0.000938	0.000118	0.000806	0.000207	0.001001	0.000433
threo-β-Methylaspartic acid	0.000735	0.000225	0.000457	4.33E-05	0.00072	0.000265
Threonic acid	N.D.	N.D.	0.00063	7.93E-05	0.000687	0.000268
Thymidine	0.002798	0.000324	0.002798	0.000668	0.003666	0.001029
Thymine	0.014199	0.003962	0.014153	0.00665	0.017247	0.006135
trans-Glutaconic acid	0.005726	0.002964	0.001807	0.00075	0.004943	0.003579
Trehalose 6-phosphate	0.000196	8.02E-06	N.D.	N.D.	0.000147	N.D.
Trigonelline	N.D.	N.D.	0.000771	N.D.	N.D.	N.D.
Trimethylamine	0.035522	0.011909	0.022105	0.006717	0.033877	0.008928
Trimethylamine *N*-oxide	0.000442	7.48E-05	0.000274	4.33E-05	0.000282	5.28E-05
Tropic acid 3-Phenyllactic acid 3-(2-Hydroxyphenyl)propionic acid *m*-Ethoxybenzoic acid *p*-Methoxyphenylacetic acid Atrolactic acid	0.00156	0.000797	0.000908	0.000171	0.001056	0.000625

Compound name	naive		CSDS		Crp4	
	Mean	S.D.	Mean	S.D.	Mean	S.D.
Trp	0.01009	0.00071	0.012529	0.011027	0.0091	0.00296
Tryptamine	N.D.	N.D.	N.D.	N.D.	0.000183	1.09E-05
Tyr	0.058287	0.01331	0.041661	0.020115	0.057451	0.023289
Tyr-Glu	0.000351	2.94E-05	0.000317	6.85E-05	0.000382	0.000101
Tyramine	0.000356	9.39E-05	0.000385	0.000104	0.000591	0.000251
UMP	0.000213	N.D.	0.000126	N.D.	0.000153	N.D.
Undecanoic acid	0.000138	1.6E-05	0.000125	3.13E-05	0.000162	2.35E-05
Uracil	0.034032	0.008771	0.019551	0.006578	0.035936	0.010285
Uridine	0.007276	0.002367	0.004355	0.001334	0.008383	0.00421
Urocanic acid	0.00431	0.000463	0.003566	0.000476	0.00565	0.002149
Val	0.174883	0.038409	0.127777	0.05845	0.155071	0.047241
Xanthine	0.067344	0.021117	0.035805	0.01071	0.061896	0.020195
Xanthosine	0.000593	0.000248	0.000292	0.000185	0.000947	0.001211
Xanthurenic acid	N.D.	N.D.	0.000129	N.D.	0.000225	N.D.
β-Ala	0.012548	0.0033	0.00758	0.00194	0.016364	0.008494
β-Ala-Lys	0.000344	0.00019	0.000287	0.000115	0.000676	0.000673
β-Tyr	0.000232	N.D.	0.000185	6.56E-05	0.000189	N.D.
γ-Butyrobetaine	0.015485	0.006745	0.008516	0.001836	0.015222	0.008677
γ-Glu-2-aminobutyric acid	0.000791	0.00033	0.000578	0.000141	0.000843	0.000264
γ-Glu-Val-Gly	0.001324	0.000331	0.001407	0.000273	0.001539	0.000538

N.D.: not detected.

For peaks that were not identified as a single metabolite, multiple candidates are described.

Chapter 4. Discussion

This study showed that psychological stress decreases α-defensin secretion from Paneth cells, which induces disruption of homeostasis in the intestinal metabolites via imbalance of the intestinal microbiota, dysbiosis in CSDS model. Furthermore, the oral α-defensin administration rescues dysbiosis and recovers homeostasis in the metabolites, suggesting that psychological stress-induced dysbiosis is related to Paneth cell dysfunction.

Various animal models of depression such as CSDS model, forced swimming model and fear conditioning model have been constructed. However, acute stress models including forced swimming model have limitations in characterizing depression which is a long-term progressive disease. On the other hand, CSDS model, chronic and mild stress model, has been considered a suitable model to characterize depression, which is supported by the evidence that CSDS model is sensitive to chronic antidepressant administration [35]. It has been known that the disruption of intestinal microbiota and metabolites is induced by psychological stress in the CSDS model [16] and that antibiotic treatment improves behavioral abnormalities [36]. Thus, CSDS model is considered to be a suitable model for analyzing the relationship between depression and the intestinal microbiota and metabolites.

It is revealed that CSDS loading decreases the amount of secreted α-defensin into the intestinal lumen (Fig. 4). Since the number of Paneth cells and Crp1 positive granule area were reduced in the small intestine in CSDS group (Fig. 5a-c), the reduction in fecal Crp1 may be due to Paneth cell dysfunction. Psychological stress by mother-infant separation at birth has been reported to decrease the number of rat Paneth cells in the

small intestine via activation of corticotropin-releasing factor (CRF), and Paneth cell number has remained reduced even after weaning [37]. CRF activation has been also known in CSDS model [38]. The results in this study that α-defensin decreased due to CSDS loading and the reduction continued for 20 days after the end of CSDS loading are consistent with these previous findings, and importantly, abnormalities of α-defensin, the effector of innate enteric immunity, secreted by Paneth cells were verified for the first time.

In addition, gene expression analysis of small intestinal tissue revealed that expression of *CHOP*, a marker of endoplasmic reticulum stress (ER stress), increased in CSDS group. In Crohn's disease, mutations and defects in genes related to ER stress and autophagy induce morphological abnormalities of Paneth cell [24], thus ER stress is a factor leading to Paneth cell damage. Since social defeat stress load and subsequent elevated corticotropin have been reported to increase ER stress [39,40], the decrease in Paneth cell number and α-defensin secretion due to CSDS in this study may be mediated by ER stress in Paneth cell. However, further observation over time is necessary to understand the details of the mechanism.

α-Defensins regulate the composition of intestinal microbiota, and α-defensin deficiency diminishes diversity and affects the intestinal microbiota composition including *Firmicutes* and *Bacteroidetes*, leading to dysbiosis [28,40,41]. Previously, reduction in *Firmicutes* and increase in *Bacteroidetes* have been reported in depression patients [43]. This study revealed that α-defensin decrease due to CSDS induces reduction of *Firmicutes* and increase of *Bacteroidetes* in the intestinal microbiota and further indicated a positive correlation between the amount of α-defensin and the intestinal microbiota including *Akkermansia*. It has been known that *Akkermansia* increases by α-defensin administration [44,45], and correlates strongly with stress

tolerance in CSDS model [46], suggesting α-defensin regulates the intestinal microbiota in depression. Although the status of dysbiosis varies depending on certain pathophysiology and models, since β-diversity approached to naïve group by α-defensin administration, α-defensin may have a function to maintain homeostasis of the intestinal microbiota in response to the imbalance of the intestinal ecological system.

Dysbiosis in depression has been reported to be diverse between studies due to individual differences in diet, region, race, etc[47]. On the other hand, metabolic processes of microbiota are relatively conserved compared to high variation of the intestinal microbiota among individuals [48], indicating that individuals may have taxonomically different but functionally similar microbiota. Therefore, analyzing intestinal microbial metabolites is important in this study. Five metabolites including pyridoxamine, pyridoxamine 5'-phosphate, β-alanine, 3-aminobutyric acid, and 1H-imidazole-4-propionic acid are significantly reduced with α-defensin reduction due to CSDS and fully recovered by administration of α-defensin. Pyridoxamine, one form of vitamin B6, is important for synthesis of many neurotransmitters including serotonin, dopamine, noradrenaline, GABA, histamine, glycine, and d-serine, and currently used as therapeutics for autism [49]. Vitamin B6 administration has been also reported to attenuate depression-like behaviors in depression model mice induced by dexamethasone [50]. In addition, administration of β-alanine has been known to improve depression-like behaviors in post-traumatic stress disorder (PTSD) model mice by increasing carnosine levels in the brain and maintaining hippocampal brain-derived neurotrophic factor (BDNF) expression, an important target of antidepressants [51,52]. In this study, those metabolites were recovered to normal by administration of α-defensin, suggesting that α-defensin affects brain function through microbial metabolites by maintaining

homeostasis in the intestinal microbiota.

Other metabolites that reduced with α-defensin decrease due to CSDS and recovered to the same levels as naïve group by administration of α-defensin include lysine, pantothenic acid, thiamine phosphate, glutamic acid, and uracil. Although these metabolites correlated with α-defensin, no direct correlation was observed with the intestinal microbiota at genus level. The reason why no metabolite correlates with microbiota may be because taxonomically similar bacteria often involve in the same function [48], and further the metabolism of the intestinal microbiota can be affected by crosstalk among bacteria in addition to phenotypic changes of the bacteria themselves [53,54]. In addition, it has been reported that long-term deficiency of lysine increases anxiety and psychological stress, and chronic anxiety is attenuated by interventions of lysine-enriched diet [55]. Pantothenic acid has been known to stimulate cortisol secretion by increasing adrenal sensitivities to adrenocorticotropic hormone (ACTH) [56]. Thiamine-phosphate has been known as a potential biomarker for depression since people with higher concentration of thiamine-phosphate in erythrocyte have lower symptoms of depression [57]. Glutamic acid has been reported as a major excitatory neurotransmitter that regulates higher functions including learning and memory in the central nervous system of mammals [58] and serves as an important material for GABA synthesis in the brain. Furthermore, it has been known that SNPs localized in uracil-processing genes potentially regulate the onset and development of depression [59]. Collectively, the reduction of multiple intestinal metabolites which are reported to function in resolving or defending against depression, anxiety, and psychological stress links to the decline of α-defensin, and these metabolites are recovered to the normal extent by α-defensin administration in this study, providing the important new

findings to improve understanding of depression in relation to the gut-brain axis. There are several metabolites that changed along with α-defensin decrease while no correlation with the amount of α-defensin was observed (Fig. 13). Since psychological stress has been well known to affect many biological events in the host, it remains controversial whether biological disruptions are causes or effects. Especially, the gastrointestinal function is affected by psychological stress as represented in irritable bowel syndrome [60], and the metabolism of the intestinal microbiota and the host closely interacts each other to create a complicated metabolic system [53]. Thus, it is speculated that the change of these metabolites was a secondary effect of improving dysbiosis by administration of α-defensin to affect the host intestinal function, and since responses to psychological stress largely vary depending on host, i.e., animal model [61], direct correlation with α-defensin could not be observed. However, detailed underlying mechanisms of these findings remain unclear and future study is necessary to further understand the gut-brain axis in depression.

In this study, Crp4 was used for administration to CSDS group because Crp4 is reported to have the most potent bactericidal activities among Crps [21] and the amount of administered Crp4 can be monitored since C57BL/6 mice do not express Crp4 genetically [62]. It has been known that Crp1 family consisted from Crp1-3 and 6, is the most abundant α-defensins in mice [63-66]. Surprisingly, administration of Crp4 increased the amount of Crp1 in the CSDS mice (Fig. 8). Considering the results that administration of Crp4 rescued the dysbiosis and the impaired intestinal metabolites due to CSDS loading, it is suggested that administration of Crp4 improved host microenvironment for Paneth cells, resulting in the increase of Crp1 secretion. This speculation is supported by evidence on the intestinal ecological system, including that the intestinal

commensal microbiota positively affects Paneth cell development and function, i.e., Crp secretions by comparing the germ-free and the conventional mice [20,67,68].

This study provides a novel insight that is associated with the pathogenesis and pathophysiology of depression. The results in this study suggest a mechanism leading to depression (Fig. 14). Psychological stress immediately decreases α-defensin secretion from Paneth cells in the small intestine at early stage, leading to dysbiosis and further disrupting homeostasis of intestinal metabolites. In the gut-brain axis, the disrupted intestinal ecological system may affect brain function through some unrevealed pathways to develop or worsen depression. Dysbiosis reported in depression patients and depression model animals largely varies probably due to individual differences relating such as diet and race [47]. This study clarified a previously unknown important link between intestinal microbial metabolic profiles and upstream host-derived regulator, α-defensin, and further contributes to understanding mechanisms of depression. Although long-term observation is required to clarify systemic effects including behavior in future studies to understand whole picture of the gut-brain axis in depression, the new relationship between α-defensin and depression shown in this study may contribute to development for prevention and therapeutics of depression.

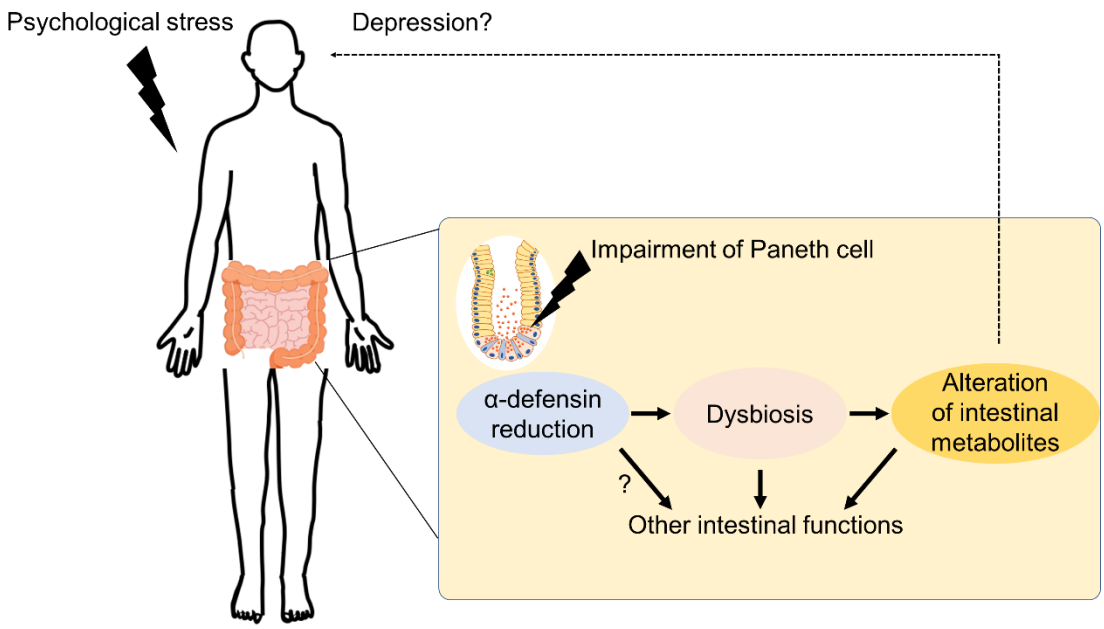


Figure 14. A novel mechanism based on α-defensin in the CSDS model.

Psychological stress decreases α-defensin secreted by Paneth cells in the intestine and disrupts the homeostasis of the composition of intestinal metabolites via dysbiosis. α-Defensin administration improves these abnormalities. Thus, disruption of homeostasis in microbial metabolites may affect brain function and result in the onset or progression of depression. However, the relationship between microbial metabolites and brain function is shown as dotted lines since the direct effects on the brain have not been verified.

Chapter 5. Summary

1. CSDS decreases α-defensin secretion from Paneth cells.
2. α-Defensin decrease due to CSDS induces dysbiosis and the dysbiosis is improved by α-defensin administration.
3. α-Defensin decrease due to psychological stress induces disruption of metabolites via dysbiosis, which is improved by α-defensin administration

It is known that psychological stress-induced dysbiosis and subsequent abnormalities in metabolite composition are associated with the onset and exacerbation of depression. α-Defensin secreted by Paneth cells, an effector of intestinal innate immunity, is an important regulator of the intestinal microbiota and contributes to the maintenance of homeostasis of intestinal ecological system. In this study, dysbiosis and subsequent disruption of metabolites under psychological stress are induced by α-defensin decrease in a mouse CSDS model, a model of psychological stress-induced depression, and α-defensin administration improved these abnormalities. Although long-term observation of systemic effects including behavior is necessary to reveal the whole picture of depression, the novel insight into gut-brain axis provided in this study may contribute to the development of prevention and treatment of depression.

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