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Full Paper

Effects of the loss of maternal gut microbiota before pregnancy on gut microbiota, food allergy susceptibility, and epigenetic modification on subsequent generations

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Maternal environments affect the health of offspring in later life. Changes in epigenetic modifications may partially explain this phenomenon. The gut microbiota is a critical environmental factor that influences epigenetic modifications of host immune cells and the development of food allergies. However, whether changes in the maternal gut microbiota affect the development of food allergies and related epigenetic modifications in subsequent generations remains unclear. Here, we investigated the effects of antibiotic treatment before pregnancy on the development of the gut microbiota, food allergies, and epigenetic modifications in F1 and F2 mice. We found that pre-conception antibiotic treatment affected the gut microbiota composition in F1 but not F2 offspring. F1 mice born to antibiotic-treated mothers had a lower proportion of butyric acid-producing bacteria and, consequently, a lower butyric acid concentration in their cecal contents. The methylation level in the DNA of intestinal lamina propria lymphocytes, food allergy susceptibility, and production of antigen-specific IgE in the F1 and F2 mice were not different between those born to control and antibiotic-treated mothers. In addition, F1 mice born to antibiotic-treated mothers showed increased fecal excretion related to the stress response in a novel environment. These results suggest that the maternal gut microbiota is effectively passed onto F1 offspring but has little effect on food allergy susceptibility or DNA methylation levels in offspring.

Key words: Developmental Origins of Health and Disease, DNA methylation, gut microbiota, food allergy, antibiotics, Treg (Regulatory T cell)

INTRODUCTION

The maternal environment is linked to various disorders in offspring [1–3]. A Dutch famine study reported that subjects exposed to famine in utero showed impaired glucose tolerance compared with subjects who were not exposed to famine in utero [4]. As the Developmental Origins of Health and Disease hypothesis states, some of the mechanisms underlying this phenomenon are mediated by changes in epigenomic modifications [3, 5]. Chen *et al.* revealed that hypermethylation of the glucokinase gene was associated with impaired glucose tolerance in offspring in an animal model of maternal hyperglycemia [6]. Furthermore, Waterland *et al.* found that maternal supplementation with methyl donors increased the methylation of the intracisternal A-particle (IAP) element upstream of the agouti locus, resulting in a darker hair color in the offspring [7]. In addition to those studies, various maternal environments alter DNA methylation levels in offspring [1, 8–11]. These results indicate that disease susceptibility in offspring is related to epigenetic changes, especially DNA methylation levels that are affected by the maternal environment during the perinatal period.

The gut microbiota provides a variety of metabolites and signals that play various physiological roles in the host [12]. Some of these metabolites, including B vitamins and short-chain fatty acids (SCFAs), modulate epigenetic marks in the host cell [9, 13–15]. B vitamins, including B2, B6, B9, and

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B12, are involved in one-carbon metabolism, which produces S-adenosylmethionine as a methyl donor for DNA methylation [14]. SCFAs produced from the gut microbiota, especially butyric acid, inhibit histone deacetylases [15]. Histone deacetylase inhibitors decrease the expression of DNA methyltransferase 1, which can reduce DNA methylation levels [16]. Butyric acid facilitates extrathymic differentiation of regulatory T (Treg) cells, increasing the expression of forkhead box protein 3 (Foxp3) via epigenetic modifications [15]. Foxp3-positive Treg cells have crucial roles in coordinating immune tolerance to suppress food allergies [17]. However, whether the maternal gut microbiota affects the development of food allergies related to changes in DNA methylation status in offspring is unclear.

Food allergy is a serious health problem with an increasing prevalence in developed countries [18, 19]. Population-based estimations revealed that the prevalence of food allergy at pediatric age has exceeded 6% in more than 10 countries and regions in the world since 2018 [18]. In the US, 8–11% of children and adults suffer from food allergies [19]. A western diet, frequent use of antibiotics, and hyperclean environments, as mentioned in the hygiene hypothesis, are among the factors that increase food allergy development [20]. Several recent reports advocated that the early-life microbiota impacts the development of allergic disorders [21, 22]. As the delivery process is a crucial factor in the development of the infant gut microbiota [23], the contact between the mother and infant in early life could heavily influence the development of the infant gut microbiota [24]. Moreover, the administration of vancomycin from pregnancy to weaning increased the susceptibility to allergic asthma in mice [25]. A birth cohort study indicated that maternal antibiotic exposure during pregnancy or the first year of life is associated with eczema but not food allergies [26]. These studies suggest that maternal antibiotic exposure or changes in the maternal gut microbiota have a role in the development of allergy in subsequent generations, but the details remain unclear.

In the present study, we investigated the relationships of the maternal gut microbiota with DNA methylation levels and food allergy development in offspring. To do this, we disrupted the maternal gut microbiota by antibiotic administration in agouti viable yellow (Avy) mice, in which the methylation levels of the IAP region can be altered in response to maternal environmental factors, backcrossed into allergy-susceptible BALB/c mice.

MATERIALS AND METHODS

Animals

Avy and BALB/c mice were purchased from a local breeding colony (The Jackson Laboratory, Bar Harbor, ME, USA, and Charles River Laboratories Japan, Yokohama, Japan, respectively), and Avy mice were backcrossed with BALB/c mice for at least six generations. After backcrossing, 12 female mice (F0) were divided into the following two groups at 7 weeks old: the control group (Con, n=6) and antibiotic group (Ab, n=6). Mice in the Ab group were orally administered a mixture of the following four antibiotics from 8 to 10 weeks of age to disrupt broad microbiota: ampicillin (Sigma, St. Louis, MO, USA), neomycin (Tokyo Chemical Industry (TCI), Tokyo, Japan), and metronidazole (TCI), each of which were administered at 0.5 mg/100 µL/day/mouse, and vancomycin (TCI), which was administered at 0.25 mg/100 µL/day/mouse. The Con group was administered ultrapure water as a vehicle. At 2 days after finishing the administration of antibiotics or vehicle, mice in both groups were mated with male BALB/c mice. The day a vaginal plug was detected was defined as gestation day (GD) 1. Fecal samples were collected after administration of antibiotics and before delivery and stored at −80°C until analysis. Pups (F1) were co-housed with their mothers until weaning. After weaning, F1 mice were fed a standard diet (AIN-93G, Oriental Yeast, Tokyo, Japan). When the F1 mice reached 8 weeks old, fecal samples were collected and stored at −80°C. One or two F1 mice born to each F0 mouse, from a total of 11 or 12 F1 mice per group, were tested for susceptibility to food allergy at 12 weeks old. Two other mice from each litter were mated. The remaining F1 mice were euthanized to collect plasma and the liver, spleen, cecum, Peyer's patch, and large intestine, and these samples were rapidly stored at −80°C. Food allergy susceptibility was tested in F2 mice at the same age as in F1 mice. All mice were housed in cages maintained at a constant temperature (23 ± 2°C) under a 12-hr light (8:00–20:00)/12-hr dark (20:00–8:00) cycle. During the experiment, body weight was measured weekly. The University of Tokushima Animal Use Committee approved the study protocol (T28-84), and mice were maintained according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Food allergy susceptibility test

Food allergy susceptibility was investigated as reported by Kweon *et al.* [27]. First, mice were sensitized with 1 mg ovalbumin (OVA; Sigma-Aldrich) and 100 µL complete Freund's adjuvant (Nippon Becton Dickinson, Tokyo, Japan). One week after systemic priming, mice were orally administered 50 mg OVA dissolved in 100 µL PBS once every 2 days for a total of 10 times. The mice were fasted for 4 hr before oral OVA administration. At 1 hr after administration, we assessed the degree of diarrhea as an allergic reaction for stool consistency (1, normal; 2, soft; 3, viscous; 4, watery) [27]. The number of fecal pellets generated was counted for 15 min before the food allergy susceptibility test. After the last administration, the mice were euthanized, and the plasma, liver, spleen, cecum, cecal contents, Peyer's patch, and large intestine were collected and rapidly stored at −80°C.

Fecal bacteria analysis by 16S rRNA sequencing and denaturing gradient gel electrophoresis

DNA was extracted from fecal samples using a Stool DNA Isolation Mini Kit (Favorgen Biotech, Ping Tung, Taiwan), according to the manufacturer's protocol. A fragment of the 16S rRNA gene was amplified from the fecal DNA by polymerase chain reaction (PCR) using universal 16S rRNA bacterial primers for the V3 to V4 region (341F and 805R primers; Table 1). After the second PCR amplification, PCR amplicons from each sample were used for high-throughput sequencing on a MiSeq Genome Sequencer (Illumina, San Diego, CA, USA). The sequences were clustered into phylotypes using QIIME (ver. 1.8.0) with a minimum identity of 97%. The phylotype identities were analyzed by comparison of the sequences against the DNA Data Bank of Japan by BLAST analysis. Denaturing gradient gel electrophoresis was performed as reported in previous reports [28]. The concentration of the extracted DNA was adjusted to 10 ng/µL. The PCR primers used were described previously [29] (Table 1).

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Table 1. Oligonucleotide primers

Primer name	Sequence (5′-3′)	Amplicon size (bp)	Description
HAD1-GC	CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGG GGGACTCCTACGGGAGGCAGCAG	250	Eubacteria
HDA2	GTATTACCGCGGCTGCTGGCA		
341F	ACACTCTTTCCCTACACGACGCTCTTCCGATCT- NNNNN-CCTACGGGNGGCWGCAG	464	16S rRNA V3 to V4 region
805 R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-NNNNN-GACTACHVGGGTATCTAATCC		
mbbis upstream	CGGAATTCGAAAAGAGAGTAAGAAGTAAGAGAGAGAG	Size of final amplicon 290	Avy allele
mbbis downstream	GCTCTAGAAAAATTTAACACATACCTTCTAAAACCCCC		
mbbis internal	ACTCCCTCTTCTAAAACTACAAAAACTC		
-1500 F	TGTTAGGGTATTAAAGGTTGGAAGTT	230	Foxp3 enhancer allele
-1500 R	CCAATTTTCCTAAAACCAACAATAT		
Amp1 F	AGGAAGAGAAGGGGGTAGATA	466	Foxp3 CNS2 allele amplicon 1
Amp1 R	AAACTAACATTCCAAAACCAAC		
Amp2 F	ATTTGAATTGGATGTGGTTTGT	455	Foxp3 CNS2 allele amplicon 2
Amp2 R	AACCTTAAACCCCTCTAACATC		
TPH1 F	GCTCACTGCGAAGGAAGACG	145	tryptophan hydroxylase-1
TPH1 R	ACGATAGACATTGTCTTTGAAGCC		NM_001136084.2
AADC F	TCTAAAGCACAGTCACCAGGA	148	Aromatic L-amino acid decarboxylase
AADC R	GTGCTTTCGGATGTAAGCCTG		NM_001190448.1
MAO-A F	CTGGAGAACGAGCAGCTAGAG	141	Monoamine oxidase-A
MAO-A R	CGGAAGGCAGGTTCCTCTCTA		NM_173740.3
Htr3A F	CTGTGGCGATCACCGGAAG	180	5-HT receptor 3A
Htr3A R	GGCTGACTGCGTAGAATAAAGG		
Htr3B F	ATGATTCTTCTGTGGTCCTGCCTC	160	5-HT receptor 3B
Htr3B R	GTGGTGGCCTCAGCCCAGTTGT		
Htr4 F	AATTCAGCCACAACTCTAACTC	110	5-HT receptor 4
Htr4 R	CCAGCACCATGAGGAGAA		
Crhr1 F	GTACTCACGTACTCCACCGAC	147	Corticotropin- releasing hormone receptor 1
Crhr1 R	TACTCCAGGACGTTTGCCAAA		NM_007762.5
Crhr2 F	CGCAAGTGGCTTTTCCTCTTC	123	Corticotropin- releasing hormone receptor 2
Crhr2 R	CAAATCACCAGCTTCCTTGCC		NM_001288620.1
18s F	AAACGGCTACCACATCCAAG	105	18s ribosomal RNA
18s R	GGCCTCGAAAGAGTCCTGTA		NR_003278.3

Measurement of SCFAs by gas chromatography-mass spectrometry

SCFAs in cecal contents were measured using gas chromatography-mass spectrometry (GC-MS). Cecal contents were weighed, homogenized with 300 µL diethyl butyric acid (TCI) in ultrapure water (20 µg/mL) as an internal standard, and centrifuged (20,000 g, 20 min, 4°C). The supernatant was transferred to a new tube and 10 µL hydrogen chloride and 100 µL diethyl ether were added to the supernatant and mixed thoroughly. Samples were centrifuged (10,000 g, 3 min, 4°C), and the upper organic layer was collected. N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide (Sigma-Aldrich) was added to the organic layer, and the samples were derivatized at room temperature for 8 hr. Derivatized samples were analyzed using a 5977 MSD mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) equipped with a 7890B GC (Agilent Technologies). The temperature program of the oven was maintained at 40°C for 2 min, ramped up to 170°C at 15°C/min and then to 300°C at 90°C/min, and held for 5 min. Each SCFA was identified, and the concentrations of each were determined by analyzing mass spectra, m/z, retention times, and peak areas of the standards and internal control using the MassHunter Workstation (Agilent Technologies) software.

Grouping gut microbes by their contributions to SCFA production

The contributions of the identified bacterial groups to SCFA production were estimated. Butyric acid-producing bacteria were defined as those bacteria with genes for the butyric acid synthesis pathway and included over 200 bacteria, as described previously [30]. Acetic acid-producing bacteria were defined as *Bifidobacterium* spp., *Bacteroides* spp., and *Prevotella* spp., and lactic acid-producing bacteria were defined as *Enterococcus* spp., *Lactobacillus* spp., *Lactococcus* spp., and *Streptococcus* spp., which have been described previously [31–33].

Measurements of OVA-specific IgE and cortisol levels in plasma by enzyme-linked immunosorbent assay (ELISA)

The OVA-specific IgE level in plasma was measured using a DS Mouse IgE ELISA kit (DS Pharma Biomedical, Osaka, Japan) according to the manufacturer's protocol. The cortisol level in plasma was measured using a Cortisol ELISA kit

(Enzo Life Sciences, Farmingdale, NY, USA) according to the manufacturer's protocol.

Isolation of lamina propria lymphocytes (LPLs) from the large intestine

LPLs were isolated as reported previously [34]. DNA was extracted from the LPLs using a DNeasy Blood & Tissue Kit (Qiagen, Germantown, MD, USA) according to the manufacturer's protocol.

Measurement of the 5-methylcytosine (5-mC) level in LPL DNA

The 5-methylcytosine level in the DNA of LPLs was measured using a MethylFlash Global DNA Methylation (5-mC) ELISA Easy Kit (Epigentek Group Inc., Farmingdale, NY, USA) according to the manufacturer's protocol. The absorbance of each well at 450 nm was measured using a Varioskan Flash microplate reader (Thermo Fisher Scientific, Waltham, MA, USA), and the 5-mC concentration was calculated using a standard curve.

Bisulfite sequencing

DNA from LPLs was subjected to bisulfite conversion using a MethylCode Bisulfite Conversion Kit (Thermo Fisher Scientific). The IAP element was amplified using specific primers (mbbis upstream primer and mbbis downstream primer) in the first PCR and semi-nested PCR (mbbis upstream primer and mbbis internal primer; Table 1) [35]. Bisulfite DNA (2 µL) was used for the first PCR, which involved the following protocol: 94°C for 2 min followed by 35 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 30 sec. Then 1 µL of the resulting PCR product was used for the semi-nested PCR, which involved the following protocol: 94°C for 2 min followed by 35 cycles of 94°C for 30 sec, 62°C for 30 sec, and 72°C for 30 sec. The 290-bp PCR fragment was corrected for subcloning. The enhancer and CNS2 regions of the *Foxp3* gene were amplified using primers (Table 1) reported in previous reports [36, 37]. Each DNA region was amplified with EpiTaq HS DNA polymerase (Takara, Kusatsu, Japan) using the following protocol: 94°C for 2 min followed by 40 cycles of 94°C for 30 sec, 55°C for 30 sec, and finally 72°C for 30 sec. The resulting PCR fragments were subcloned using a TOPO TA cloning kit (Thermo Fisher Scientific) and then sequenced. Sequenced clones were aligned to the reference sequence in each allele. Average DNA methylation levels among at least four different clones per sample were determined.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR) analysis

Total RNA was isolated from the large intestine using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total RNA was reverse transcribed, and RT-qPCR was performed as reported previously [34] using specific primer sets (Table 1). The primer sets used to evaluate serotonin receptor genes have been reported previously [38]. The relative abundance of each target transcript was calculated by normalization to the constitutively expressed 18s mRNA level.

Statistical analysis

All results are expressed as the mean and standard deviation. Significant differences between the two groups were calculated using Student's t-test or the Mann–Whitney U-test. Principal component analysis was performed using Mass Profiler Professional (Agilent Technologies). Differences in Bray–Curtis dissimilarity were assessed by analysis of similarities (ANOSIM) in R.

RESULTS

Effect of administration of antibiotics before gestation on the gut microbiota of F0–F2 mice

We used a model of gut microbiota alterations after the administration of antibiotics (Fig. 1A). Body weight and food intake were not different between the Ab and Con groups during the experiment (Supplementary Fig. 1A, 1B). In F0 mice, we confirmed that the gut microbiota was considerably disrupted by antibiotic treatment and that it gradually recovered after stopping the antibiotic treatment (Supplementary Fig. 1C). A difference in the β-diversity but not α-diversity of the gut microbiota between the Con and Ab groups was apparent at both GD11 and GD18 (Fig. 1B, 1C). We found that 10 genera differed between the Ab and Con groups, with higher percentages of *Bifidobacterium* and *Lactobacillus* genera in the Ab group at GD18 (Supplementary Fig. 1D). We next investigated the effect of changes in the maternal gut microbiota induced by antibiotic treatment on the gut microbiota of F1 and F2 mice. The gut microbiota of F1 mice born to antibiotic-treated mothers was similar to the gut microbiota of F0 mice at GD18 (Fig. 1D and Supplementary Fig. 1E–1I). The F1 mice born to antibiotic-treated mothers showed differences in gut microbiota composition, including α-diversity, β-diversity, and the abundances of specific bacterial operational taxonomic units (OTUs; Figs. 1D and 2A, 2B). Sixteen bacterial genera significantly differed in abundance between the Con and Ab groups in F1 mice (Supplementary Fig. 2A, 2B). Grouping gut microbes by their contributions to SCFA production revealed substantially higher proportions of acetic acid- and lactic acid-producing bacteria and a significantly lower proportion of butyric acid-producing bacteria in the Ab group than in the Con group in F1 mice (Fig. 2C). Concomitantly, the butyric acid concentration in the cecal contents of F1 mice was significantly lower in the Ab group than in the Con group (Fig. 2D). F2 mice did not show these changes in gut microbiota diversity, the abundance of each SCFA-producing bacterium, or butyric acid concentrations in cecal contents (Fig. 2E–2H). The abundances of four bacterial OTUs were significantly different between the Con and Ab groups among the F2 mice, but the proportions of these bacteria were small (Supplementary Fig. 2C, 2D).

The DNA methylation levels in the Foxp3 gene were altered in F1 mice

We investigated DNA methylation levels in the specific enhancer and CNS2 regions, which regulate *Foxp3* gene expression [36, 37], in LPLs. The DNA methylation level in a portion of the CNS2 region was higher in the Ab group than in the Con group in the F1 but not F2 mice (Supplementary Fig. 3A). In contrast, the DNA methylation level in the IAP region of the Avy allele in F1 mice was lower in the Ab group than in the Con group (Supplementary Fig. 4A). The methylation levels in the enhancer region and whole CNS2 region of the *Foxp3* gene and whole genome in LPLs showed no differences between the Con and Ab groups (Fig. 3A and Supplementary Fig. 3B).

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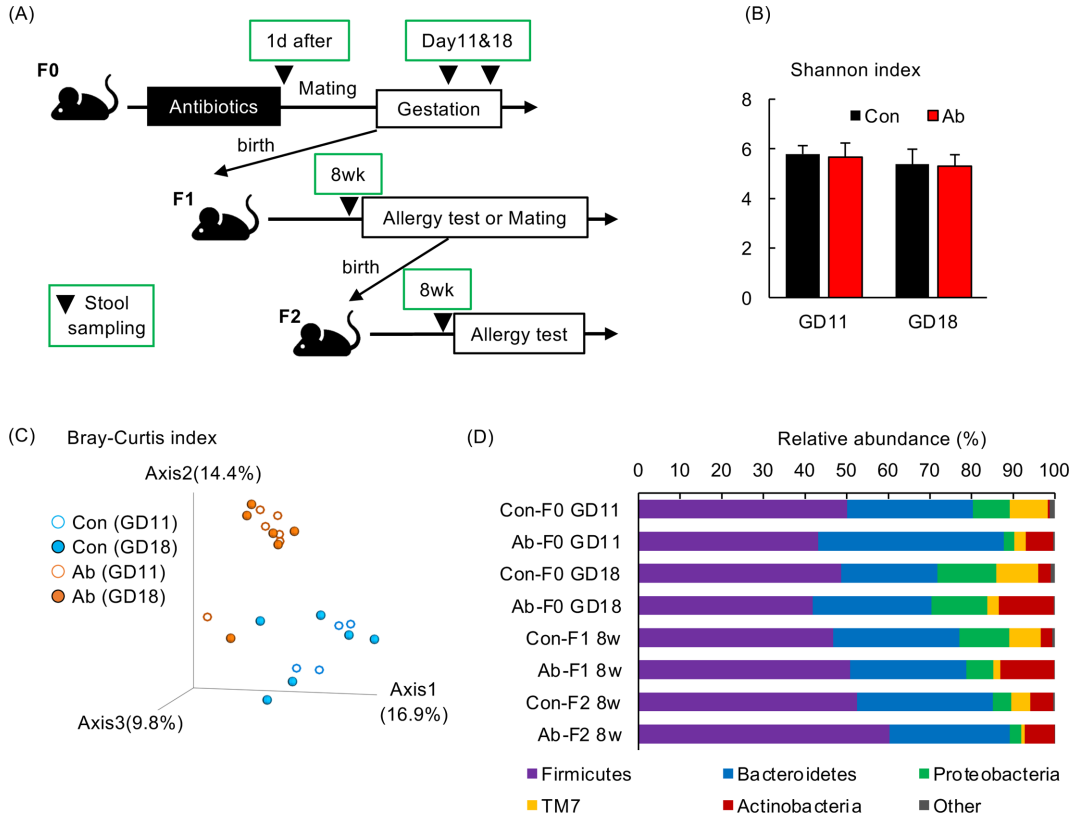

Fig. 1. Schema of the study protocol and changes in the gut microbiota in F0 mice and subsequent generations.

(A) Schematic of the study protocol. The F0 mice were administered an antibiotic mixture for 2 weeks. Stools were collected from F0 mice at 1 day after finishing the antibiotics and at 11 and 18 days of gestation (GD11 and GD18) and from F1 and F2 mice at 8 weeks old. Some F1 mice were mated, and other F1 and F2 mice were tested for susceptibility to food allergies at 12 weeks old. Control (Con) group: F0, n=6; F1, n=12; F2, n=9. Antibiotic (Ab) group: F0, n=6; F1, n=11; F2, n=10. (B, C) Changes in the gut microbiota in F0 mice. Shannon index (B) and Bray–Curtis dissimilarity index (C) in the gut microbiota of F0 mice at GD11 and GD18. Statistical differences in Bray–Curtis dissimilarity were determined by analysis of similarities (ANOSIM) ($p<0.001$). (D) The average values of the relative abundances of each bacterial phylum in the gut microbiota of each group of F0, F1, and F2 mice. "Other" represents *Cyanobacteria*, *Deferribacteres*, *Tenericutes*, and other bacterial phyla. Con group in B–D: F0, n=4 at GD11 and n=6 at GD18; F1, n=12; F2, n=9. Ab group in B–D: F0, n=6; F1, n=11; F2, n=10.

Maternal antibiotic treatment did not influence the allergic response in F1 or F2 mice

We next investigated allergy susceptibility after administering a food antigen in sensitized offspring. Both the Ab and Con groups in F1 and F2 mice showed food antigen-induced anaphylaxis responses, such as diarrhea, and the responses were enhanced by each additional dose of antigen. Allergy scores observed during the food allergy susceptibility test were not significantly different between the Ab and Con groups in either generation (Fig. 3B). According to the anaphylaxis response induced, the plasma OVA-specific IgE levels of the Con and Ab groups were similar in both generations (Fig. 3C).

F1 mice born to antibiotic-treated mothers showed significant differences in stress response

In this study, we transferred mice from their home cage to a new cage and assessed their fecal output before the allergy susceptibility test. When we counted the number of fecal pellets before each test, the fecal output was clearly higher in the Ab group than the Con group in the F1 mice at each time point (Fig. 4A). The systemic cortisol concentration, a marker of stress, was not different between the Con and Ab groups in either F1 or F2 mice (Fig. 4B). We found that the mRNA levels of serotonin metabolism-related and receptor genes were slightly downregulated, while corticotrophin receptor genes were slightly upregulated, in the colon in the Ab group in F1 but not F2 mice (Fig. 4C–4E). Although additional studies are needed, these results suggest that antibiotic-induced alterations in the maternal gut microbiota affect the gut microbiota and stress response, but not food allergy, in F1 mice.

DISCUSSION

In the present study, we investigated the effect of maternal antibiotic treatment before pregnancy on the gut microbiota, food allergy susceptibility, and DNA methylation levels in subsequent generations. Changes in butyric acid concentrations occurred in parallel with changes in the gut microbiota in F1 but not F2 mice. Although CpG site-specific and limited changes were observed, we did not detect a physiological impact of the DNA methylation level on the development of food allergies in offspring.

The first few years of life are considered a critical window in human gut microbiota development [39]. The formation of the gut microbiota can be attributed to various factors related to

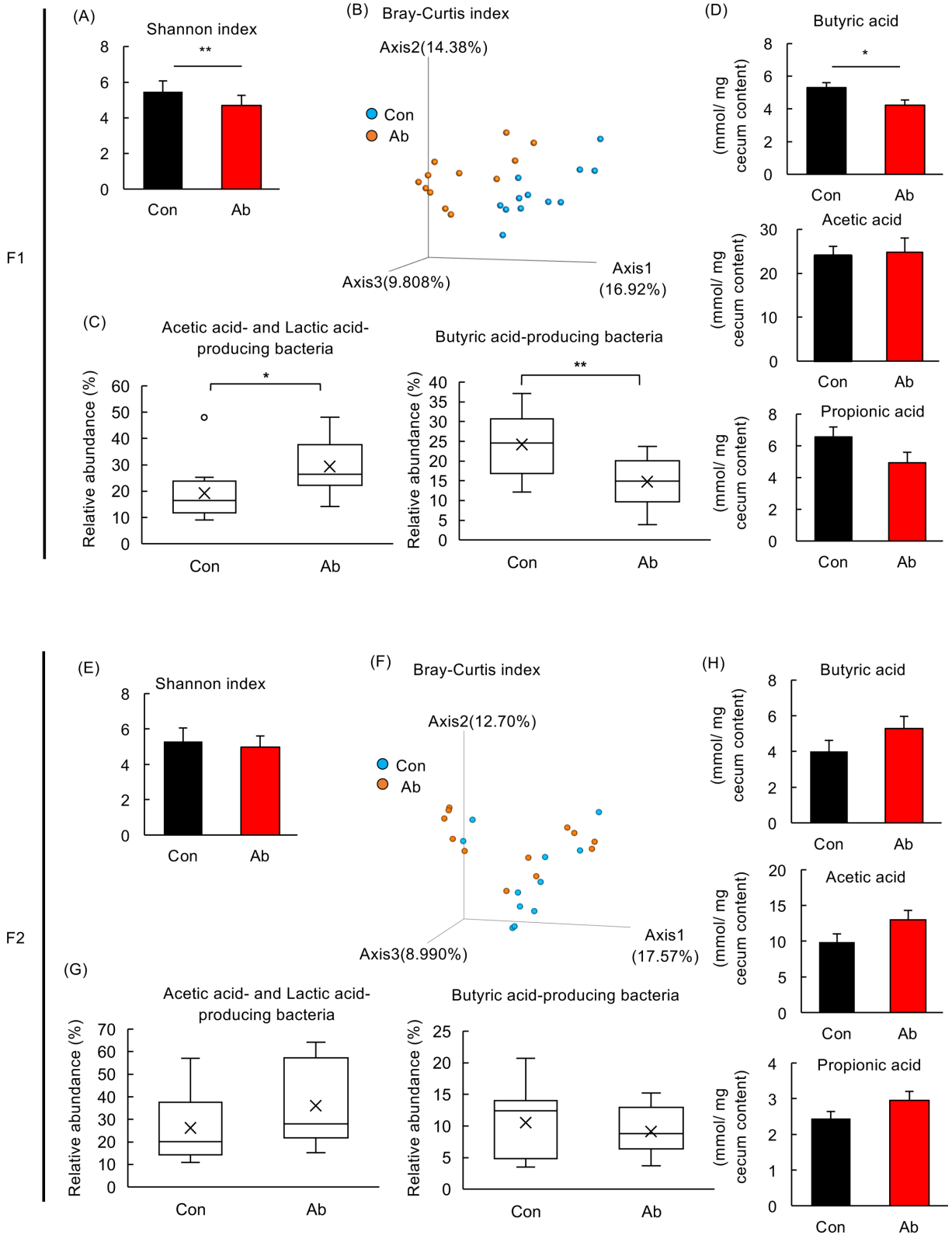

Fig. 2. Changes in the gut microbiota and short-chain-fatty acid (SCFA) concentration in the F1 and F2 mice.

(A, B, E, F) Shannon index (A, E) and Bray–Curtis dissimilarity index (B, F) in the F1 (A, B) and F2 (E, F) mouse gut microbiota at 8 weeks. Statistical differences in Bray–Curtis dissimilarity were determined by analysis of similarities (ANOSIM) using R ($p<0.001$). (C, G) The relative abundances of the gut microbiota according to SCFA production in F1 mice (C) and F2 mice (G). (D, H) The SCFA concentrations in the cecal contents of the F1 (D) and F2 (H) mice. Control (Con) group: F1, n=12; F2, n=9. Antibiotic (Ab) group: F1, n=11; F2, n=10. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

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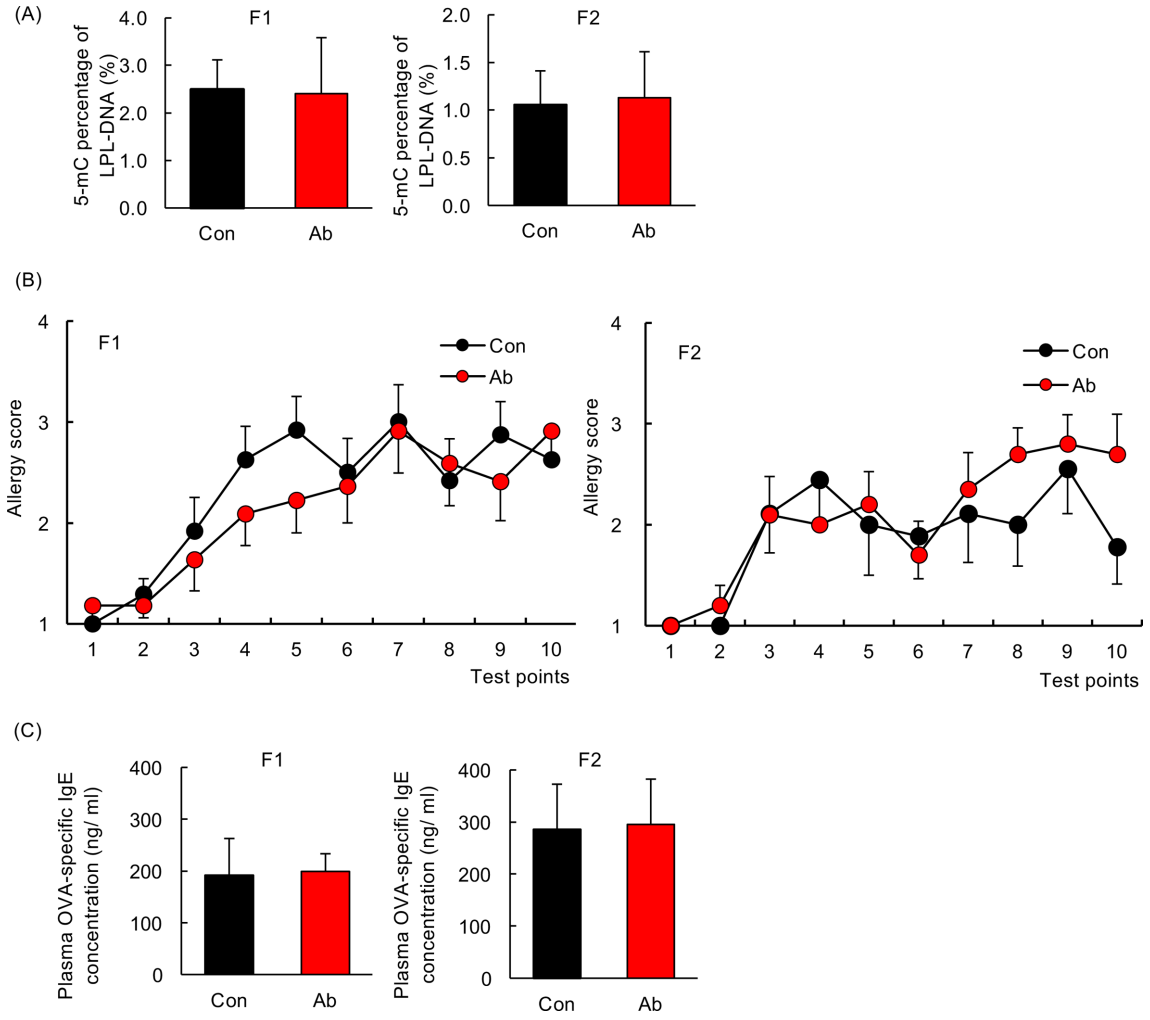

Fig. 3. DNA methylation levels, food allergy susceptibility, and plasma ovalbumin (OVA)-specific IgE levels in the F1 and F2 mice.

(A) Percentage of methylated cytosines in the whole genome of LPLs in F1 mice (left panel) and F2 mice (right panel) determined by ELISA. (B) Food allergy susceptibility determined according to the incidence of diarrhea. OVA (50 mg) was administered at each test point, and the diarrhea incidence over 1 hr was measured in F1 mice (upper panel) and F2 mice (lower panel). (C) Plasma OVA-specific IgE levels in F1 mice (left panel) and F2 mice (right panel). Control (Con) group: F1, n=12; F2, n=9. Antibiotic (Ab) group: F1, n=11; F2, n=10.

contact with the mother, including mode of delivery, diet, and the gut and skin microbiotas. In the present study, we found that administration of antibiotics before pregnancy in the F0 mother affected the gut microbiota of the F1 but not F2 mice. These generational changes in the gut microbiota suggest several possibilities. First, as suggested in some reports, changes in the skin or vaginal microbiota may have a greater impact than those in the gut microbiota on gut microbiota development in offspring [24]. However, generally, there are significant differences among the gut, skin, and vaginal microbiotas, but the effect of changes in the skin and vaginal microbiotas on the development of the gut microbiota in offspring has remained unclear [40]. In addition, the maternal gut microbiota during pregnancy has been shown to undergo dramatic changes and remodeling [41]. Unfortunately, we did not examine whether remodeling of the F1 gut microbiota during gestation reduces the differences between the Ab and Con groups. As a second possibility, genetic factors may be of greater importance than the maternal gut microbiota. Korach–Rechtman *et al.* reported that the gut microbiota of pups is more strongly affected by genetic background than by the maternal gut microbiota [42]. In their study, the authors hypothesized that host genetic variations are associated with differences in the mucosal gut structure, bile acid metabolism, olfactory receptor activity, and the immune system. Additional factors, such as the direct effect of antibiotic treatment on oocytes, may have contributed to the generational differences. In our F2 mice, the Ab and Con groups showed few differences in their gut microbiotas. These results are consistent with the DNA methylation levels and suggest that antibiotic treatment has little effect on genetic factors, including epigenomic changes, involved in gut microbiota development in the second generation.

Disruption of the gut microbiota induced by antibiotic treatment enhances OVA-induced food allergy responses [25, 43–47]. Some studies have shown that early-life antibiotic treatment significantly increased allergy susceptibility [43, 44]. In contrast, maternal antibiotic treatment has little to no effect on increased allergy susceptibility [45, 46]. These results suggest that the administration of antibiotics during a critical window of

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gut microbiota development accelerates the allergy response to stimuli. In other words, specific changes in the gut microbiota critically affect the susceptibility to developing food allergies. For example, Atarashi *et al.* showed that *Clostridium* species activated Treg cells and improved allergy susceptibility [47]. Furthermore, the abundance of *Lachnospiraceae* was reportedly higher in food allergy patients [48]. In this study, the abundance of *Clostridium* species was not changed in either group, but *Lachnospiraceae* was more abundant in the Con group in F1 mice (Supplementary Fig. 2). Moreover, lactate-producing bacteria, such as *Bifidobacterium* and *Lactococcus* species, which were more abundant in the Ab group than the Con group in F1 mice

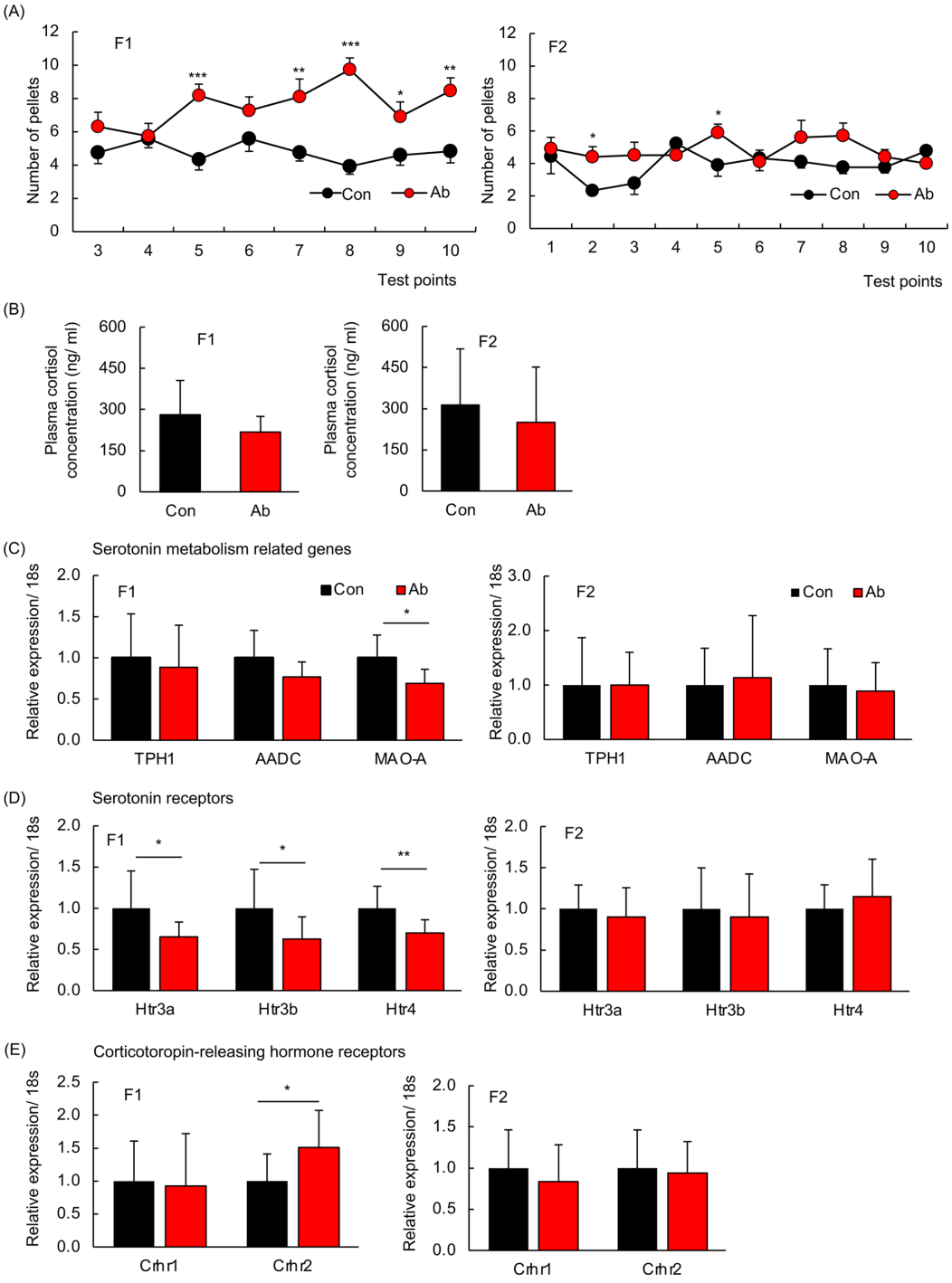

Fig. 4. Changes in stress-related parameters in F1 and F2 mice.

(A) Number of fecal pellets produced during the 15 min period before the food allergy susceptibility test at each time point. Mice were moved to a new cage before each ovalbumin (OVA) administration, and pellet output was measured before OVA administration. (B) Plasma cortisol concentration. (C–E) Changes in the relative mRNA expression of serotonin metabolism-related genes (C), serotonin receptor-related genes (D), and corticotropin-releasing hormone receptor genes (E) in the colon. F1 mice (left panel) and F2 mice (right panel) are shown. Control (Con) group: F1, n=12; F2, n=9. Antibiotic (Ab) group: F1, n=11; F2, n=10. *p<0.05, **p<0.01, ***p<0.001.

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(Supplementary Fig. 2), also have anti-allergic effects [49, 50]. We also focused on SCFA-producing bacteria and found a lower proportion of butyric acid-producing bacteria in the Ab group than in the Con group in F1 mice. The counterbalance between butyric acid-producing and lactate-producing bacteria may explain why antibiotic-treated F1 mice showed no difference in food allergy susceptibility. Another possibility is that the method used in our study for comparing food allergy susceptibility might not be sensitive enough to determine the difference in allergy tolerance. Another method might be more sensitive and reveal the effects of the maternal gut microbiota impact on F1 and F2 mice [51].

Mice feel stress after transfer to a new cage, and this stress is manifested as an increased output of fecal pellets under stressful conditions [52, 53]. Furthermore, dysbiosis induced by maternal administration of antibiotics or a high-fat diet during pregnancy alters fetal neurodevelopment or social deficits in offspring, respectively [54, 55]. The gut microbiota and its metabolites, SCFAs, serotonin, and gut peptides, which stimulate the enteric mucosa, may affect the physiological stress response in the central nervous system [56]. Enterochromaffin cells produce serotonin in the colon, and some bacteria promote serotonin biosynthesis in these cells [57]. In our study, F1 mice born to antibiotic-treated mothers showed slight alterations in stress responses, concomitant with significant reductions in the mRNA expression of serotonin and corticotropin metabolism-related genes in the colon. The hypothalamus–pituitary–adrenal axis controls the corticotropin-releasing hormone (CRH) receptor and regulates stress-induced responses [58]. Under prolonged stress, the brain releases CRH, which is sensed by CRH receptors in the gut to regulate intestinal motility [59]. Although additional studies are needed to clarify the mechanism of stress response changes, the findings of this study suggest that antibiotic-induced alterations in the maternal gut microbiota may have a stronger impact on stress responses compared with food allergies.

As a limitation of this study, we aimed to investigate the DNA methylation level of the IAP element to investigate the relationships among the gut microbiota, metastable epialleles, and the host immune system. Our study was based on the hypothesis that the methylation level of the IAP region is metastable over the entire lifespan [7, 35]. The DNA methylation level in the IAP region was lower in the Ab group than in the Con group in F1 mice (Supplementary Fig. 4). However, we could not properly amplify the IAP region from bisulfite DNA by PCR in the F2 mice. It is possible that a mutation occurred in the long terminal repeat region upstream of the IAP element or that a retrotransposon insertion occurred in abnormal transfer [60]. Therefore, in this experiment, we evaluated whole genome methylation to compare epigenetic modifications instead of methylation of the IAP region.

In conclusion, our results indicate that changes in the maternal gut microbiota induced by antibiotic administration alter the gut microbiota and butyric acid-concentrations in F1 mice but have minimal impact on food allergy susceptibility or DNA methylation levels. We also found that changes in the gut microbiota of the F0 generation diminished in subsequent generations. Our findings may provide valuable information on the safe use of antimicrobials in women of childbearing age.

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