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Effect of gut microbiota on intestinal epithelial tight junction permeability in juvenile rats with food allergy

論文の区分　博士課程

著　者　名　Tulyeu Janyerkye

担当指導教員氏名　山形崇倫　教授

所　　属　自治医科大学大学院医学研究科
地域医療学系
生殖・発達医学
成育医学専攻

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EFFECT OF GUT MICROBIOTA ON INTESTINAL EPITHELIAL TIGHT JUNCTION PERMEABILITY IN JUVENILE RATS WITH FOOD ALLERGY

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Name of Author: Janyerkye Tulyeu

Name of Advising Professor: Takanori Yamagata

Affiliation: Graduate School of Medicine, Jichi Medical University

Major: Department of Pediatrics

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Summary

Background: Increased intestinal permeability is thought to underlie the pathogenesis of food allergy. The present study was conducted to investigate the relationship between gut microbiota and food allergy affecting the intestinal barrier.

Methods: Juvenile rats were divided into an OVA sensitization group and a control group, and both were subdivided into those receiving added antibiotics or probiotics (*Clostridium butyricum*, *Lactobacillus reuteri* and *Bifidobacterium breve*). The serum OVA-IgE levels were determined, and histopathological features were studied using electron microscopy and immunofluorescence staining with antibodies against tight junction (TJ)-associated proteins. Intestinal permeability was assessed using a lactulose/mannitol assay kit, and expression of mRNAs for TJ molecules was analyzed by real-time PCR. Gut microbiota in feces was analyzed on the basis of 16S rRNA metagenomics sequences.

Results: OVA-IgE levels and intestinal permeability were significantly increased in the sensitized rats relative to the controls. Although the gut mucosae were inflamed in the sensitized groups, those in rats that had received probiotics were only mildly affected. Expression of mRNAs for TJ molecules was down-regulated in rats given OVA with antibiotics, but not in those given probiotics. Immunofluorescence staining of TJ proteins was decreased in the OVA groups. TJs in rats treated with OVA and antibiotics were disrupted, but those in rats administered probiotics were not damaged. Clostridiaceae were increased in all the probiotics groups relative to the OVA-sensitized group.

Conclusion: Gut microbiota appears to play a role in regulating epithelial barrier function and probiotics may help to prevent food allergy through up-regulation of TJ proteins.

Abbreviations

Abx- antibiotics

Ag-antigen

AJ- adherent junction

BB- *Bifidobacterium breve*

CB- *Clostridium butyricum*

DC-dendritic cell

DE- desmosome

HE- hematoxylin and eosin

IBD- inflammatory bowel disease

IL-interleukin

ILCs- innate lymphoid cells

JAM- junctional adhesion molecule

LR- *Lactobacillus reuteri*

$Lgr5^{+}$- leucine rich repeat containing G protein-coupled receptor 5

OVA- ovalbumin

PDZ- postsynaptic density 95/disc-large/zonula occludens

SCFA- short chain fatty acid

SE- standard error

Th- T helper

TJ- tight junction

TSLP- thymic stromal lymphopoietin

TLR-toll like receptor

Treg- regulatory T cell

UC-ulcerative colitis

ZO-zonula occludens

Introduction

Food allergy, the pathogenesis is still unknown, has become a serious public health issue as its prevalence has increased over the last two decades, and around 10% of children are affected [1]. The rise in prevalence marked by increase of antibiotic use, vaccination, and dietary lifestyle changes, and all of them are linked to allergic and autoimmune diseases [2]. Those kind of environment changes have affected relationship between intestinal microbiota and host ecosystem which contributes to the mechanism of allergic disease [3]. Pathogenetic mechanism of food allergy involves type 2 immune response to dietary antigen (Ag)s. T helper type 2 (Th2) are differentiated from naïve T cells by food Ag stimulation, and produce interleukin (IL) -4 and IL-13 that drive B cells. B cells produce Ag-specific IgE and stimulate immune reactions in food allergy [4]. Once allergen comes to the barrier surface, epithelial cells secret inflammatory cytokines including TSLP, IL-33 and IL-25, and those cytokines stimulate dendritic cells (DCs) and antigen presenting cells which promotes immune response in the lamina propria [5]. Commensal microbiota is characterized mainly in the gastrointestinal tract and maintain intestinal homeostasis between microbe and immune system that regulate tolerance to food Ags [6]. Oral tolerance is mediated by regulatory T cells (Tregs) which are induced with the help of commensal microbiota. Microbial sensing through toll like receptor (TLRs) and IL-10 are critical role to protect against the food allergy [7]. On the other hand, some kinds of intestinal bacteria produce short chain fatty acid (SCFAs) including acetate, propionate and butyrate to demonstrate immunomodulatory properties induce the Tregs [8]. Increase of Th cells (Th1, Th2, and Th17) and their cytokines promote $Lgr5^{+}$ intestinal stem cell to promote differentiation and proliferation of intestinal epithelial cell [9]. Probiotics are live microorganisms that confer a health benefit on the host when administered in

adequate amounts [10]. Currently, the Food Allergy and Anaphylaxis Guidelines of the European Academy of Allergy and Clinical Immunology state “there is no evidence to recommend prebiotics or probiotics or other dietary supplements based on particular nutrients to prevent food allergy” [11]. The number of reports on both basic experiments and clinical trials designed to clarify the effects of probiotics or gut microbiota on allergic disorders has been increasing. *Clostridium* produces SCFAs which play an important role in the maintenance of immune tolerance and intestinal homeostasis, thus possibly preventing allergic responses [12-20]. For instance, *Clostridium butyricum* significantly ameliorates intestinal anaphylaxis symptoms in mice with food allergy [21]. *Lactobacillus* and *Bifidobacterium* strains also prevent allergic sensitization in atopic and asthmatic patients by directly interacting with the immune system [22, 23]. Pre- and postnatal *Lactobacillus reuteri* supplementation decreases allergen responsiveness in infancy [24]. Dietary, nondigestible oligosaccharides and *Bifidobacterium breve* suppress allergic inflammation in the intestine of mice [25]. Although these probiotics (*C. butyricum*, *L. reuteri*, and *B. breve*) have been widely used for their expected health benefits, evidence for their effectiveness against food allergy is still insufficient. The intestinal barrier, which acts as both a mechanical and a microbial barrier, plays an important role in the development of immune tolerance to prevent allergens passing through the intestinal epithelia from the external environment [26, 27]. Epithelial TJs consists transmembrane proteins of claudins, occludin, junctional adhesion molecule (JAM) and tricullin [28]. Their regulations contribute paracellular and transcellular transport mechanism that maintain homeostasis across the polarized cells [29]. Claudins are key components for the structure and function of TJs and approximately 27 members in human. In human, claudin 1, 2, 3, 5, 7, 8, 15, occludin and zonula occludens (ZO-1)

were highly expressed in the gastrointestinal epithelium. [30] **(Table.1)**

Table 1. Localization and expression of tight junction proteins in human intestinal disease

TJs proteins	Expression in gut	Functions in TJs	Change in expression	Related disease
Claudin-1	Jejunum	Barrier forming [31] Cation selection [35]	Increase, Decrease	UC IBD
Claudin-2	Duodenum, Jejunum	Pore forming, Cation selection [36]	Increase Increase Increase	Crohn's disease UC Celiac disease
Claudin-3	Duodenum, Jejunum, Ileum, Colon	Barrier forming [32] Cation selective [35]	Decrease Decrease Decrease	UC Crohn's disease Celiac disease
Claudin-5	Duodenum	Barrier forming [33] cation selective [35]	Decrease Decrease	Crohn's disease Celiac disease
Claudin-7	Duodenum Jejunum Ileum Colon	Barrier and pore forming [34] Differentiation of epithelial cells [40]	Decrease Decrease Decrease	UC Crohn's disease, Celiac disease
Claudin-8	Duodenum Ileum Colon	Predominantly barrier forming, Cation selection [35]	Decrease	Crohn's disease
Claudin-9	Stomach Jejunum	Barrier forming Cation selective	Decrease	*Clostridium perfingens* infection
Claudin-15	Duodenum Ileum Colon	Predominantly pore forming, Cation selection [37]	Increase	Celiac disease
ZO-1	Duodenum Jejunum, Ileum, Colon	Regulation and maintenance of TJ structure [38] [39]	Decrease Decrease Decrease	IBD, Chylous diarrhea, Celiac
Occludin	Duodenum Jejunum Ileum Colon	Paracellular permeability, maintenance and assembly of TJs [38]	Decrease Decrease	UC, Crohn's disease,

IBD: inflammatory bowel disease, UC: ulcerative colitis

On the other hand, apart from barrier functions, the claudins have the roles of affecting cellular signaling, proliferation, differentiation and receptor function which are non-barrier channel functions. [40]. However, the expression and distributions of TJ proteins (claudins and zonula occludens) are altered in many gastrointestinal disorders, and their changes are proposed to play important role for pathophysiology of food allergy disease [41]. Thus, changes paracellular permeability is thought to be associated with the pathogenesis of food allergy. Recently, it has been reported that the intestinal microbiota contributes to the organization of epithelial barrier function, as well as changing the bacterial community linked to intestinal permeability and chronic gastrointestinal disease, especially food allergy [41, 42]. However, the mechanisms underlying the relationship between intestinal epithelial barrier function and regulation of gut microbiota in the context of food allergy have not been precisely clarified. The aim of the present study was to explore the mechanism responsible for changes in the morphology and function of the intestinal barrier using a juvenile rat model of food allergy, focusing on the contribution of intestinal microbiota and the effects of probiotics (*C. butyricum*, *L. reuteri*, and *B. breve*) and antibiotics.

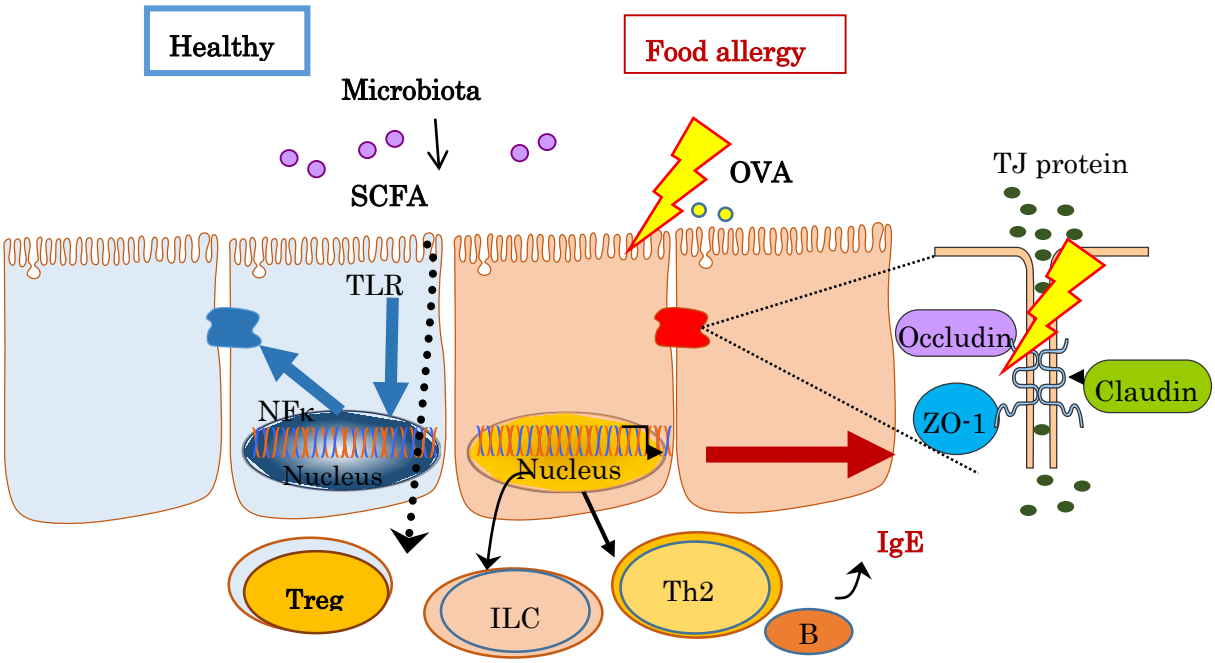


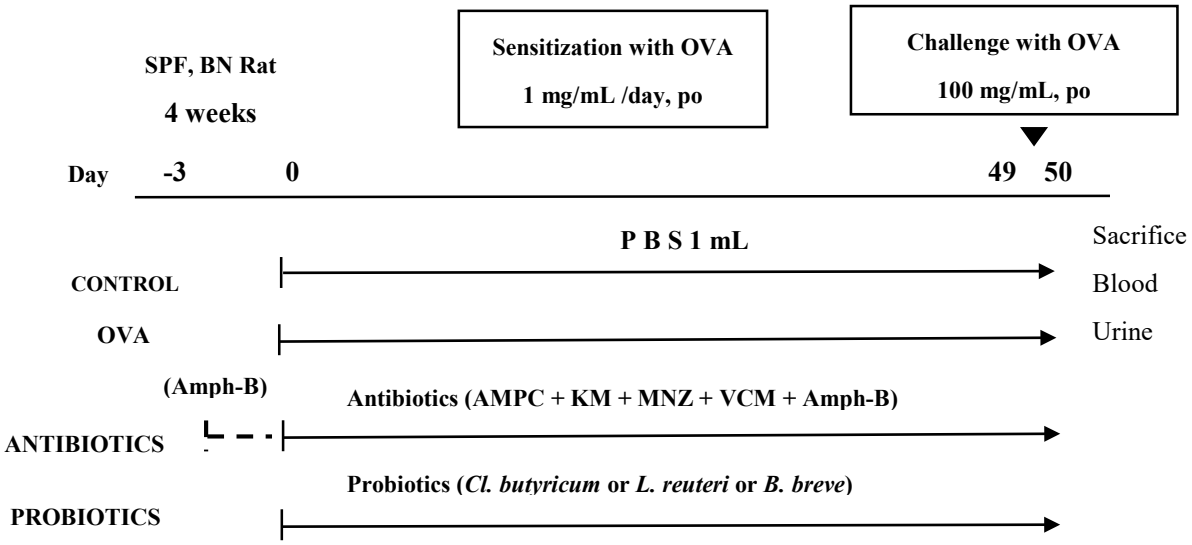
Fig. 1. Barrier regulation hypothesis in food allergy

Intestinal microbiota regulates protective and pathogenic barrier response in the intestinal epithelia. A healthy microbiota produces the SCFAs and contribute organization of epithelial barrier function through the Treg cells and maintain intestinal homeostasis. Intestinal barrier protective function prevents food Ags. Dysbiosis deteriorate protective function in epithelia and induce the intestinal permeability allow the Ags to the dysregulated epithelial barrier function and promotes allergic sensitization through the generation of Th2 pathway.

Materials and Methods

Animal handling and study design

Four-week-old male SPF rats were obtained from Charles River Laboratories (Tokyo, Japan). They were housed under specific pathogen-free conditions at 23 (±3) °C with a 12 h light/dark cycle and a relative humidity of 30–70% during experiments, and provided conventional food and water *ad libitum*. Protocols for all animal studies were approved by the Institutional Animal Ethics Committee of Jichi Medical University, in accordance with the Institutional Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology. Rats were divided into an Ag sensitized group and a control group. In the sensitized group, OVA 1 mg (Worthington Biochemical Corp, Lakewood, NJ, USA) in 1 mL PBS was administrated intragastrically daily for 48 days without the use of an adjuvant, and the control group was administered PBS 1 mL in the same way. Each group was further subdivided into one receiving antibiotics for ablation of intestinal bacteria and one receiving probiotics (*C. butyricum*, *L. reuteri*, and *B. breve*). On day 49, all OVA-sensitized rats were orally challenged with OVA (100 mg) solution in PBS 1 mL. On day 50, the intestine and blood were collected from every rat (n = 3-7 rats per group) (**Fig. 2**).



Groups

Control	Sensitization
PBS	OVA
PBS + antibiotics	OVA + antibiotics
PBS + *C. butyricum*	OVA + *C. butyricum*
PBS + *L. reuteri*	OVA + *L. reuteri*
PBS + *B. breve*	OVA + *B. breve*

Fig. 2. Protocol used for OVA sensitization, antibiotic or probiotic treatment or challenge.

Rats in the control group were sensitized with PBS (1 mL per rat, daily) and OVA-treated group, rats were sensitized with OVA (1 mg in 1 mL PBS, daily per rat) intragastrically for 48 days. Both groups were subdivided into those receiving added antibiotics (amphotericin-B, ampicillin, kanamycin, metronidazole, and vancomycin) or probiotics (*Clostridium butyricum*, *Lactobacillus reuteri* and *Bifidobacterium breve*). Amph-B;

amphotericin-B, AMPC; ampicillin, KM; kanamycin, MNZ; metronidazole, VCM; vancomycin.

Ablation of intestinal flora by antibiotic treatment

Antibiotic treatment was performed in accordance with a previous report with some modification [43]. Briefly, amphotericin-B (Fuji Pharma, Tokyo, Japan) was administered by gavage at 1 mg/kg every 12 hours before the start of the experiment. From day zero, water flasks, which are freely available to the rats, were supplemented with 1 g/L ampicillin (Astellas Pharma, Tokyo, Japan), and an antibiotic cocktail consisting of 50 mg/kg vancomycin (Shionogi Pharma, Tokyo, Japan), 100 mg/kg kanamycin (Meiji Seika Pharma, Tokyo, Japan), 100 mg/kg metronidazole (Shionogi Pharma, Tokyo, Japan). Addition to that 1 mg/kg amphotericin-B was administered by antibiotic gavage for every 12 hours. A gavage volume of 10 mL/kg body weight was delivered via a gastric tube without prior sedation. The antibiotic cocktail was prepared freshly every day, and ampicillin and water were renewed every 7th day (**Fig. 2**).

Probiotic treatment

Probiotic treatment groups comprising an Ag sensitized group and a control group were further divided into three subgroups. The probiotics in their respective dosage - C. butyricum [MIYAIRI 588®, Miyarisan Pharmaceutical Co., Ltd., Tokyo, Japan] at 1x108 CFU/mL; L. reuteri [DSM 17938, Bio Gaia Japan Co., Ltd., Stockholm, Sweden] at 1x109 CFU/mL; and B. breve [MV16, Morinaga Milk Industry Co., Ltd., Tokyo, Japan] at 5x109 CFU/mL were administered in each of the respective sensitized groups (4-7 rats per group) by daily gavage with 1 mg OVA in 1 mL PBS for 7 weeks. The control group

received the same concentrations and dosages of probiotics in PBS 1 mL in the same way (**Fig. 2**).

Measurement of serum OVA-IgE

Blood was collected from the jugular vein on days 0, 14, 28, and 50 from the start of the experiment. Each sample was allowed to clot for 1 h at room temperature and then centrifuged at 2000 × *g* (15 min, 4°C); all sera were stored at -20°C. The serum OVA-specific IgE was assayed by ELISA in accordance with the manufacturer's instructions (Cusabio Technology LLC, Houston, TX, USA). The final OD value was detected at 450 nm wavelength using a microplate reader (Benchmark Plus, Microplate Reader, Bio-Rad, USA).

Evaluation of intestinal permeability

Intestinal permeability was determined by measuring the lactulose/mannitol ratio in urine samples in each group (days 14, 28, and 50). After a 24-h fast, rats were administrated 100 mg of lactulose and 50 mg of mannitol (dissolved in 1 mL distilled water) orally. The percentage absorption of these sugars was determined from the amount of excreted lactulose and mannitol measured during the first 6 hours after ingestion using an EnzyChrom intestinal permeability assay kit (BioAssay Systems, Hayward, CA, USA) in accordance with the manufacturer's instructions. Any increase in this ratio indicated increased intestinal permeability, since lactulose is only absorbed though intercellular spaces.

Hematoxylin and eosin (HE) staining

On day 50, intestinal samples were collected during deep anesthesia by intraperitoneal injection of Nembutal (Dainippon Sumitomo, Tokyo, Japan). For HE staining, specimens of jejunum were fixed with 4% paraformaldehyde in 50 mM phosphate buffer (Wako,

Osaka, Japan), pH 7.4, for 24 h at 4°C and stained with HE after dehydration, embedding and slicing. The structure and morphological changes were observed and analyzed using a microscope (Olympus, Tokyo, Japan). Villus length was determined by measuring the distance from the crypt base to the villus tip observed by microscopy and three to seven animals from each experimental group were evaluated, and a minimum of 15 well-oriented villi from each section were measured by ImageJ software (Version 1.50, National Institutes of Health, Bethesda, MD, USA). The degree of inflammation was evaluated using an intestinal inflammation scoring system based on the following parameters: 1) inflammatory cell infiltration, 2) damage to the surface epithelium, and 3) irregular villous and crypt loss [44]. We also counted eosinophil infiltration in the lamina propria of the jejunal mucosa.

Transmission electron microscopy

Under deep anesthesia, small pieces (about 1.5 mm×1.5 mm×2 mm) of jejunum were rapidly excised and fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 2 h at 4°C. The specimens were then post-fixed with 1% osmium tetroxide in 0.1 M phosphate buffer for 1.5 h at 4°C. The pieces of jejunum were then dehydrated in a graded ethanol series, transferred to propylene oxide, embedded in epoxy resin (Quetol 812; Nisshin EM Co., Tokyo, Japan), and polymerized for 48 h at 60°C. The specimen blocks were cut into ultrathin sections with an ultramicrotome (UCT; Leica Microsystems, Waltzer, Germany), stained with uranyl acetate and lead citrate, and then the structural and morphological changes in epithelial cells and tight junctions (TJs) were examined using a transmission electron microscope (HT7700; Hitachi, Tokyo, Japan). The apical junction length and width of the TJ and AJ of jejunum epithelial cells were measured using ImageJ software (Version 1.50, National Institutes of Health, Bethesda, MD, USA).

Representative data were obtained from 10-15 measurements per sample (n=4-7 per samples per group).

Immunofluorescence staining

The fixed jejunal samples were immersed in 30% sucrose in 50 mM PBS for 2 days at 4°C, embedded in Tissue-Tek OCT compound (Sakura FineTechnical, Tokyo, Japan) and frozen on dry ice. Cryosections (thickness: 4 μm) were obtained using a cryostat (CM3000; Leica Microsystems, Wetzlar, Germany) and blocked with 2% normal goat serum (Vector Laboratories, Burlingame, CA, USA) for 30 min at 30 °C. Tissues were incubated in antigen retrieval buffer with 0.01M citrate, pH 6.0, for 10 min at 90 °C, and then with primary antibodies against the TJ proteins claudin-1 (Cat#717800), claudin-2 (Cat#51600), claudin-7 (Cat#349100), and zonula occludens (ZO)-1 (Cat#617300) [Thermo Fisher Scientific (Waltham, MA, USA)], claudin-3 (SAB4200607) and claudin-5 (SAB4200537) [Sigma-Aldrich (St. Louis, MO, USA)] at 4°C overnight. After being washed with PBS, fixed jejunal tissues were incubated with the secondary antibody Alexa-Fluor-488-conjugated goat anti-rabbit IgG (Thermo Fisher Scientific, Waltham, MA, USA) for 30 min at 30 °C. Nuclei were visualized in Vectashield Hardset Mounting medium with DAPI (Vector Laboratories, CA, USA). Imaging was performed using a confocal laser microscope, FluoViewTM FV1000 (Olympus, Tokyo, Japan) equipped with x20 and x40 objective lenses.

Real-time PCR

After the rats had been sacrificed on day 50, 25-40 mg of jejunal tissue was collected and ground in liquid nitrogen, then transferred to a 1.5-mL EP tube. The total RNA (n = 3-7 rats per group) was extracted using TRIzol Reagent (Cat#15596018, Invitrogen, Carlsbad,

CA, USA) and reverse-transcribed to cDNA using a Superscript® VILOTM kit (Invitrogen, Carlsbad, CA, USA). Primers were designed using the Primer-Blast software package (National Center for Biotechnology Information, Rockville, Bethesda, MD, USA) based on the mRNA sequences in GenBank (National Center for Biotechnology Information, Bethesda, MD USA). These sequences are listed in **Table 2**. Real-time PCR was performed using ABI 7500 Fast Real-time PCR (ABI 7500; Applied Biosystems, Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) and samples were run in triplicate. The reaction conditions were: 50°C for 2 min, 95°C for 2 min for the holding step, followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. As an internal control, β-actin was used for standardization of transcript results, and relative gene expression levels were calculated by the (2-ΔΔCT) method.

Table 2. Primers

Primer	Sequence (5'-3')
ZO-1F	5'-ACCCACGAAGTTATGAGCA AG-3'
ZO-1R	5'-AGACTGTGGTTTCATTGC TGG-3'
OccludinF	5'-ATTCCTCTGACCTTGTC CGTG-3'
OccludinR	5'-CCTGTCGTGTAGTCG GTTTCA-3'
Claudin-2F	5'-ATTCCTCTGACCTTGTC CGTG-3'
Claudin-2R	5'-AGCCAACCGCCGTCAC AATG-3'
Claudin-8F	5'-TGTCGTGTTTGAGAA CCGCTGGG-3'
Claudin-8R	5'-ACGGACGCAG CACACATCAGTC-3'
Claudin-9F	5'-TTCCACTGGCCTTG AACTCCTCG-3'
Claudin-9R	5'-GCTGTTGCCAA TGAAGGCGGT-3'
Claudin-15F	5'-AACTGCTGGGACTT CCCGTCCAT-3'
Claudin-15R	5'-TCGATGTTGCCC ACGTTGGTGC-3'
Oncostatin-M F	5'-TAGCCCCAGTGAGTGCTTCT-3'
Oncostatin-M R	5'-CTTCCTGGTCCTCCATGTGT-3'
β-actinF	5'-GTCTCACCACTGGCA TTGTG-3'
β-actinR	5'-TCTCAGCTGTGGTGGT GAAG-3'

F: Forward; R: Reverse

Fecal DNA isolation and 16S rRNA sequencing using bioinformatic analysis

On day 50, feces were collected in a sterile tube filled with 1 mL PBS and then immediately frozen at −80 °C. For isolation of DNA, 100–300 mg of fecal material was ground with silica beads and extracted with a QIAamp DNA stool mini kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. PCR products for the V4 region of the 16S rRNA gene were amplified with region-specific primers that included the Illumina flowcell adapter (Illumina, San Diego, CA, USA) sequences and 12-base barcodes on the reverse primer. PCR production of the bacterial DNA template was quantified using Invitrogen's PicoGreen. Taxonomic classification of 16S rRNA targeting amplicon reads and the 8 most abundant bacterial sequences was performed using Illumina 16S Metagenomics workflow in the Miseq Reporter software curated by the GreenGene taxonomic database (https://basespace.illumina.com/analyses/). Alpha diversity was calculated based on the Shannon index for richness and evenness of bacterial sequences at rarefraction depth reads of the operational taxonomic unit sample.

Statistical analysis

The data were analyzed using GraphPad Prism 5.02 software and Excel 2016. Paired and unpaired Student *t* tests were used for comparisons between two groups. Differences at *p* values of <0.05, <0.01, and <0.001 were considered to be significant, and all data were expressed as the mean ± standard error (SE).

Results

Intestinal microbiota modulates OVA sensitization and gut permeability.

Food allergy was induced successfully in OVA-sensitized rats compared to controls, as confirmed by a significant (p <0.01) increase of serum-specific OVA-IgE OD values after 7 weeks of sensitization. In contrast, administration of probiotics led to a significant decrease in the OD value of IgE in the OVA sensitization groups (p <0.05) (**Fig. 3A**). An increase in the lactulose/mannitol ratio indicated that intestinal permeability was significantly increased in both the OVA sensitization (with PBS) group and the OVA sensitization with antibiotics group relative to the control groups (**Fig. 3B**). In contrast, groups subjected to OVA sensitization followed by treatment with each of the probiotics showed a significant decrease of the lactulose/mannitol ratio in comparison with the OVA sensitization alone group.

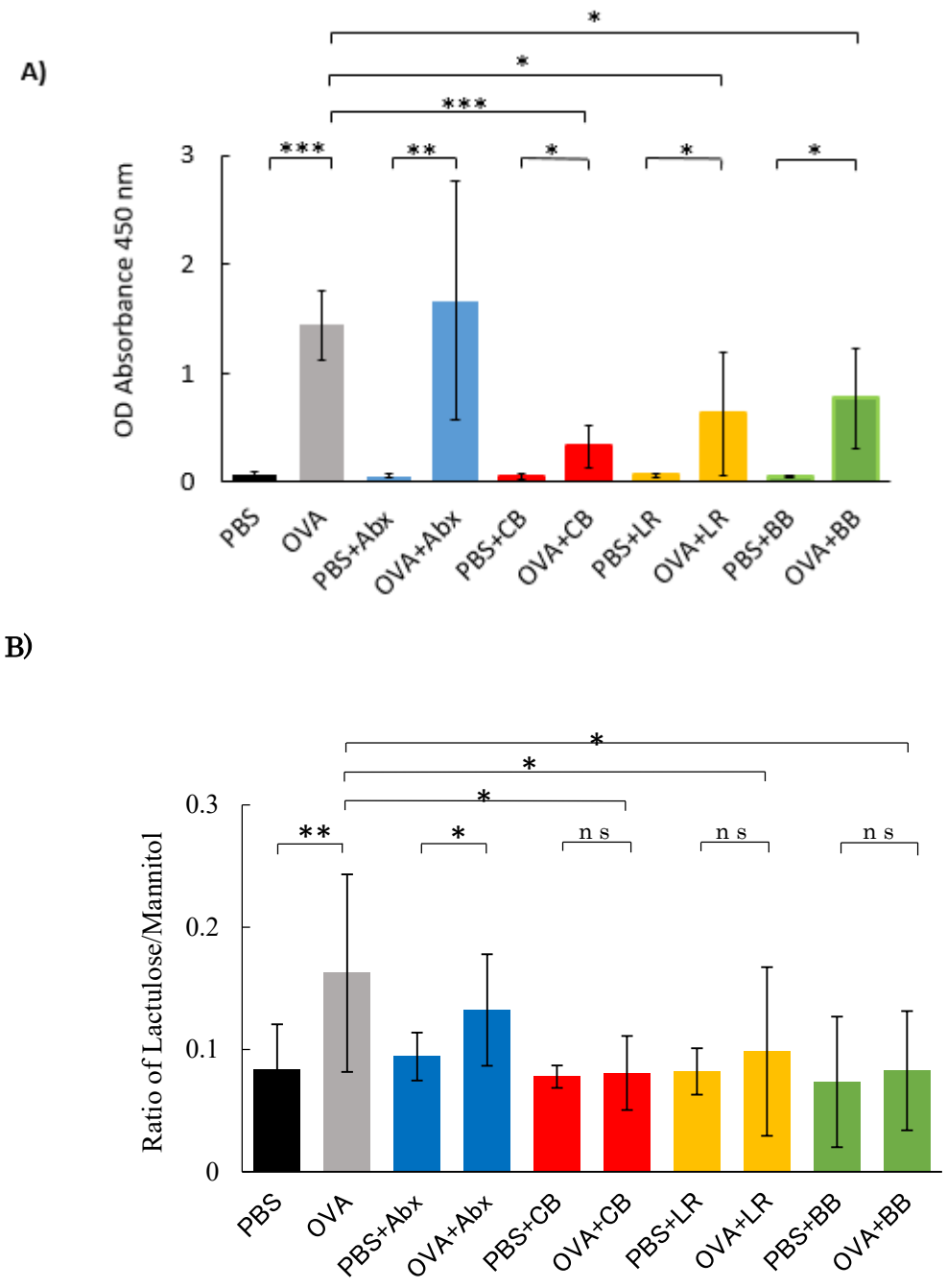


Fig. 3. A). Serum specific OVA-IgE level shows in all control and OVA-sensitized rats. **B).** Intestinal permeability assay of lactulose/mannitol metabolism and absorption. An increase in this ratio indicates increased intestinal permeability. Bar represents mean and SEM. * P <0.05, ** P <0.01, *** P <0.001 determined by Student's t test. ns; no significance, Abx; antibiotics, CB; *C. butyricum*, LR; *L. reuteri*, BB; *B. breve*.

Intestinal microbiota ameliorates OVA sensitization-induced mucosal inflammation.

HE staining showed that the jejunal mucosae were inflamed in all of the OVA sensitization groups. (**Fig. 4A**) The histological inflammation score in those groups was significantly increased (p <0.05) and eosinophil infiltration was also significantly increased (p <0.05) in all OVA sensitization groups relative to the control (**Fig. 4B and C**). In contrast, the intestinal inflammation scores and degrees of eosinophil infiltration were significantly reduced by probiotic treatment in comparison with the OVA sensitization group (**Fig. 4B and C**). Moreover, villus length in the OVA group and OVA with antibiotics treatment group was significantly reduced (p <0.05), although the probiotic treatment groups showed almost normal features similar to those in the controls (**Fig 4D**).

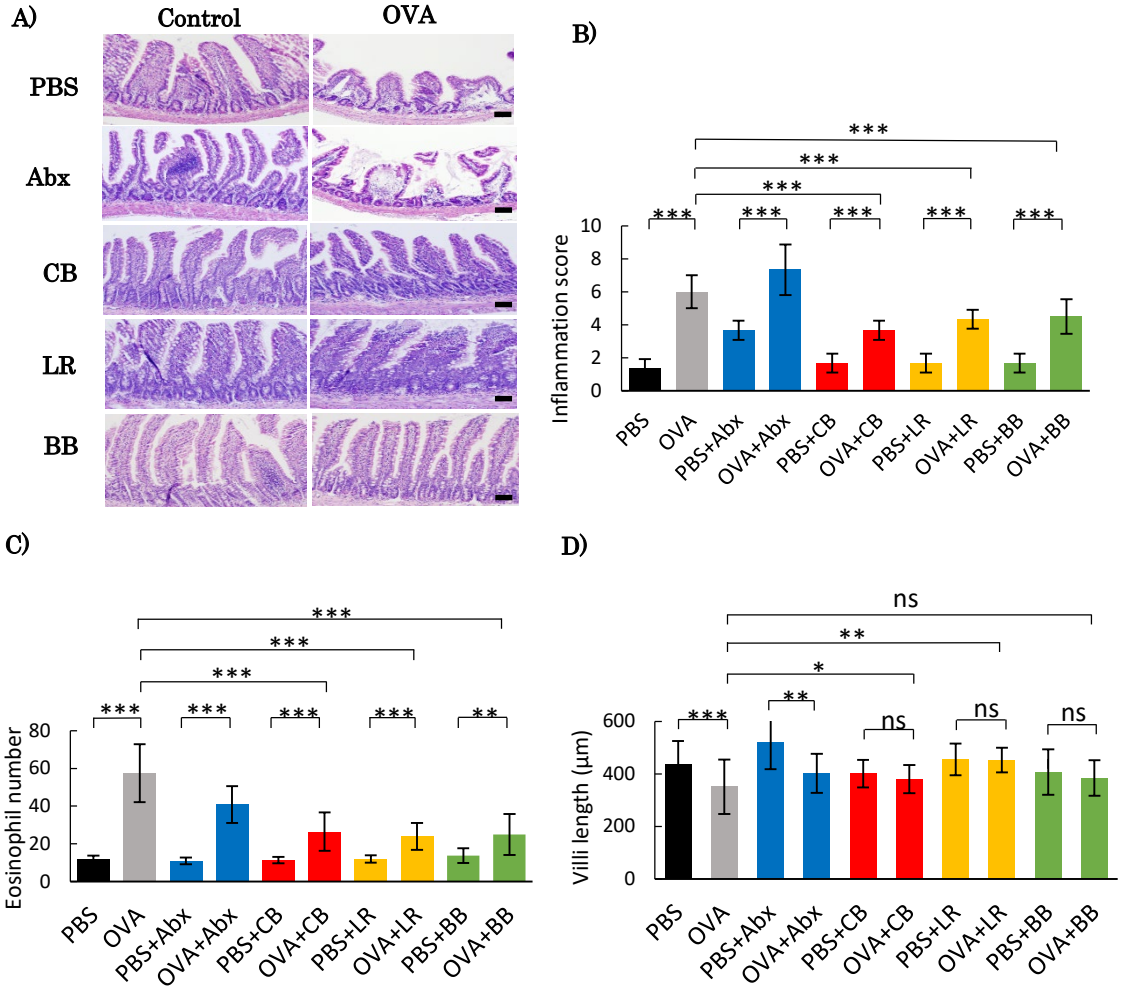
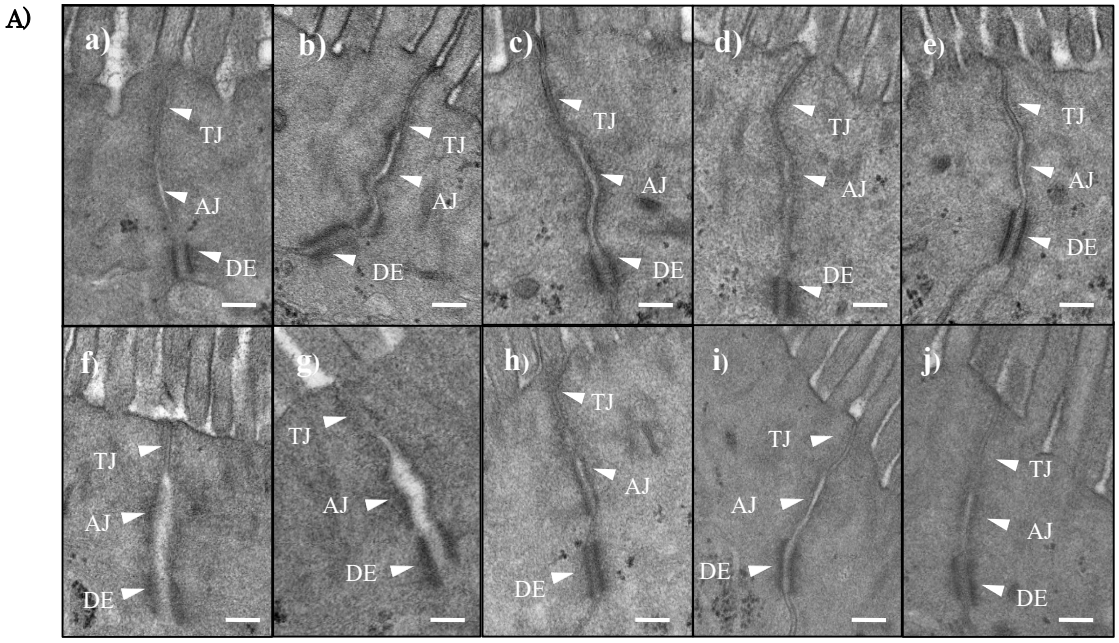


Fig. 4. A). Jejunal tissue sections were stained with Hematoxylin and Eosin for histological examination. **B)** Histological examination was performed by assigning a score for epithelial damage and leukocyte infiltration on microscopic cross-sections of the jejunum in each rat. **C).** The number of infiltrating eosinophils was counted in the lamina propria of the jejunum, and representative numbers were obtained from 10-15 measurements per sample. **D).** Villus length in jejunal epithelial cells was measured. Bar represents mean and SEM. * P <0.05, ** P <0.01, *** P <0.001 determined by Student's t test.

Effects of intestinal microbiota on epithelial intercellular structures in OVA-sensitized rats.

Compared with the non-sensitized group (**5Aa-e**), damage to the TJs as well as the adherens junction was evident in intestinal villus epithelial cells in the OVA-treated group (**5Af**). Moreover, in OVA-sensitized rats given antibiotic treatment (**5Ag**), the intercellular junctions were shortened and disrupted, and intercellular spaces were widened. On the other hand, the TJs and adherens junctions in each of the OVA-sensitized and probiotic-treated groups showed almost normal features (**5Ah-j**). Quantitative analysis of the length and width of TJs revealed significant widening and shortening in the OVA-sensitized group and the OVA-sensitized and antibiotic-treated group (**5Ba and b**) relative to the control. On the other hand, the OVA sensitization group treated with probiotics did not show TJ widening, but rather TJ elongation. Observations of the adherens junction revealed results similar to those for the TJ (**5Bc and d**).


A)
a)
b)
c)
d)
e)
f)
g)
h)
i)
j)
TJ
AJ
DE

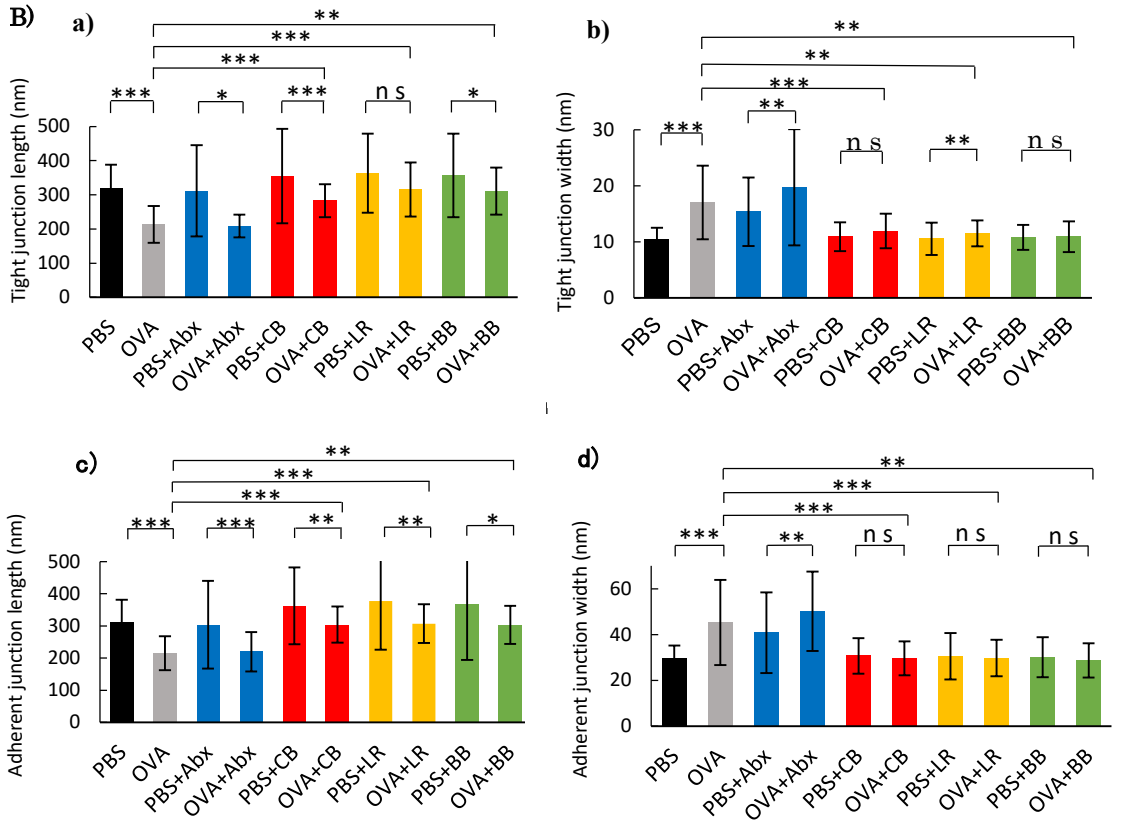

B)
a)
b)
c)
d)
Tight junction length (nm)
Tight junction width (nm)
Adherent junction length (nm)
Adherent junction width (nm)
PBS
OVA
PBS+Abx
OVA+Abx
PBS+CB
OVA+CB
PBS+LR
OVA+LR
PBS+BB
OVA+BB
n s

Fig. 5. A). Electron microscopy images of epithelial cells. **a).** PBS group. **b).** PBS + antibiotics group. **c).** PBS + *C. butyricum*-treated group. **d).** PBS + *L. reuteri*-treated group. **e).** PBS + *B. breve*-treated group. **f).** OVA sensitization group. **g).** OVA sensitization plus antibiotics group. **h).** OVA sensitization with *C. butyricum* treatment. **i).** OVA sensitization with *L. reuteri* treatment. **j).** *B. breve* treatment. Scale bar indicates 0.5 μm TJ; tight junction, AJ; adherens junction. DE; desmosome. **B). a).** Quantitative analysis of tight junction length. **b).** Quantitative analysis of tight junction width. **c).** Quantitative analysis of adherens junction length. **d).** Quantitative analysis of adherens junction width. Representative data were obtained from 10-15 measurements per sample (n=4-7 per samples per group). Bar represents mean and SEM. * P <0.05, ** P <0.01, *** P <0.001 determined by Student's *t* test. ns; no significance, Abx; antibiotics, CB; *C. butyricum*, LR; *L. reuteri*, BB; *B. breve*.

Intestinal microbiota is related to expression of TJ proteins and their localization.

Expression and localization of key TJ proteins in each group indicated that they were modulated by not only OVA sensitization but also the microbiota composition in epithelial cells. As shown in **Fig. 6**, TJ-barrier regulating proteins such as ZO-1 (p <0 .05), occludin (p <0.05), claudin-8 (p <0.05), claudin-9 (p <0.05), and claudin-15 (p <0.05) were significantly down-regulated in OVA-sensitized rats relative to the PBS controls. However, expression of claudin-2 was not significantly increased.

In the OVA-sensitized and antibiotic-treated rats, ZO-1 (p <0.05), occludin (p <0.01), claudin-8 (p <0.01), claudin-9 (p <0.01) and claudin-15 (p <0.01) were also down-regulated relative to the PBS with antibiotics group. On the other hand, in each of the groups treated with probiotics (*C. butyricum*, *L. reuteri*, and *B. breve*), the expression levels of TJ proteins (ZO-1, occludin, claudin-2, -8, -9, -15) were not down-regulated relative to the OVA-sensitized PBS group. Furthermore, administration of *C. butyricum* and *B. breve* to OVA-sensitized rats resulted in up-regulation of occludin and claudin-15 expression, respectively. On the other hand, the expression of claudin-9 was down-regulated by *B. breve* treatment in OVA-sensitized rats relative to OVA-sensitized PBS rats.

As shown in **Fig. 7**, immunofluorescence staining for intestinal TJ proteins such as ZO-1, claudin-1, -2, -3, -5, and -7 in each group indicated that not only OVA sensitization but also microbiota composition appeared to be related to the localization of those proteins in epithelial cells. In the control group, ZO-1 was expressed in the apical membrane of epithelial cells. In the OVA-sensitized group and OVA-sensitized groups treated with antibiotics, ZO-1 expression was lost in the apical membrane. Claudin-1, -3, -5 and -7 were stained in the apicolateral area in the control groups. In the OVA-sensitized group

and OVA-sensitized groups treated with antibiotics, the expression of these proteins was weaker and located mainly in the basolateral membrane of the villi. In contrast, in each of the probiotic-treated groups, the expression of claudin proteins was mild and their localizations were similar to those in the controls.

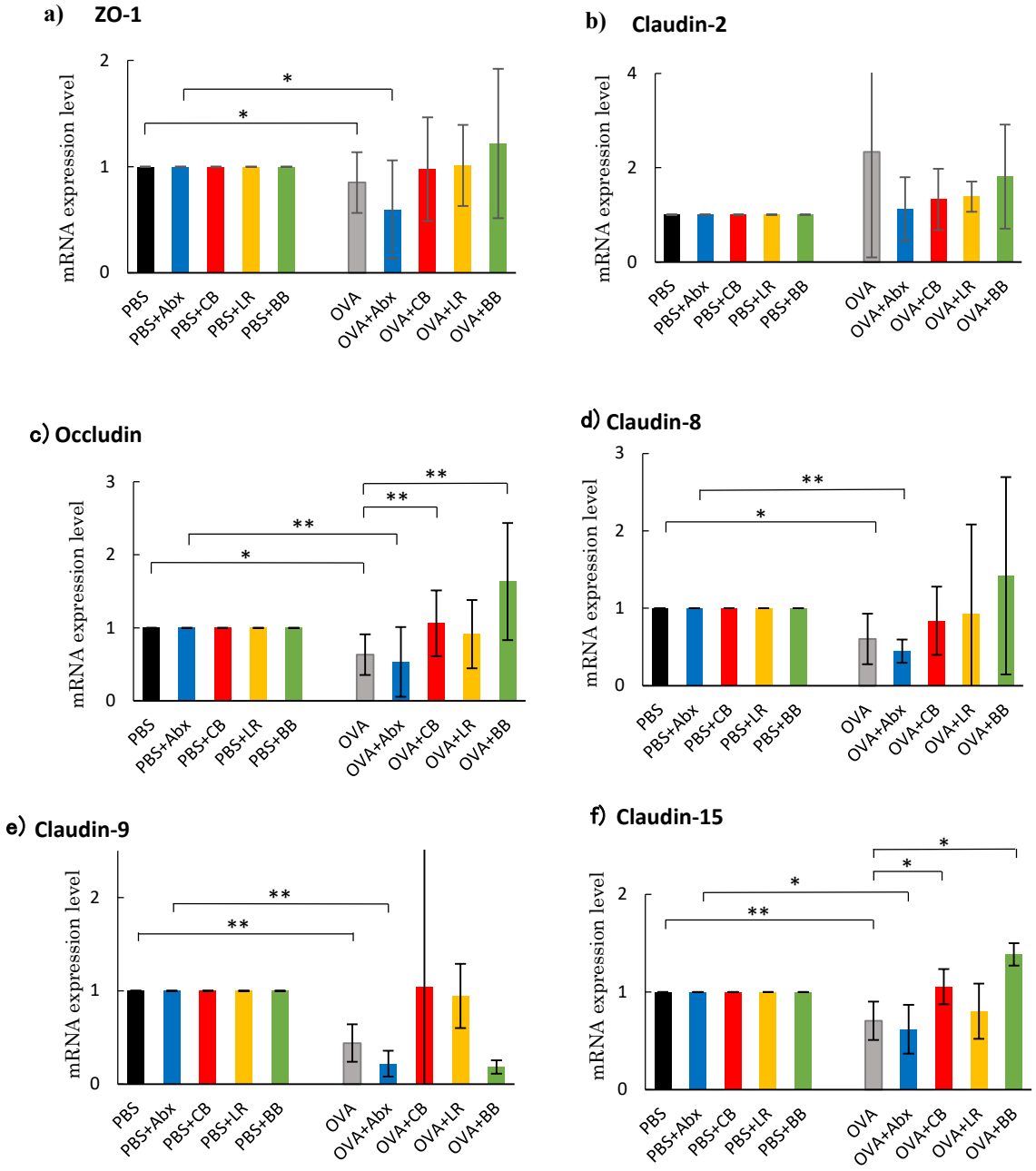


Fig. 6. Total RNA was extracted from jejunal tissue of rats, and the mRNA expression of tight junction-related molecules was examined by real time RT-PCR. Data are presented as the fold change in gene expression relative to the control (PBS). **a).** ZO-1, **b).** occludin, **c).** claudin-2, **d).** claudin-8, **e).** claudin-9, **f).** claudin-15. Each value is represented as the mean ±SE. Bar indicates significant difference between the control and treatment groups

at * P <0.05, ** P <0.01 determined by Student's t test. ns; no significance, Abx; antibiotics, CB; *C. butyricum*, LR; *L. reuteri*, BB; *B. breve*.

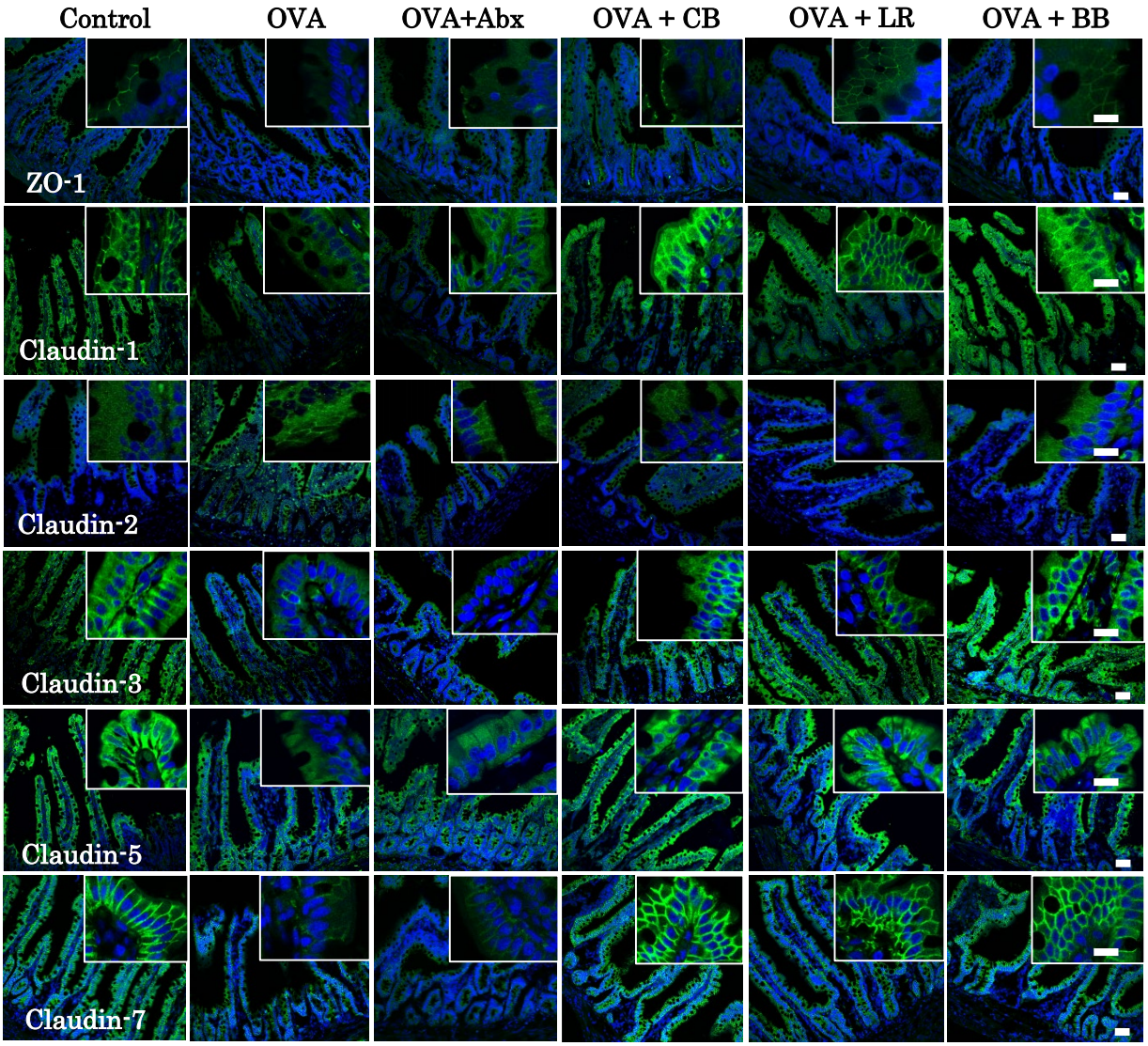
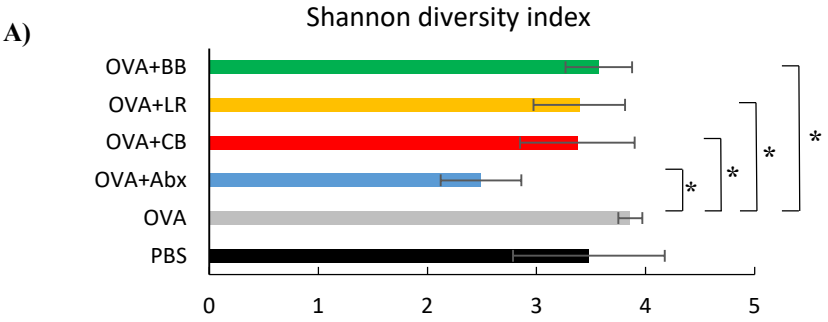


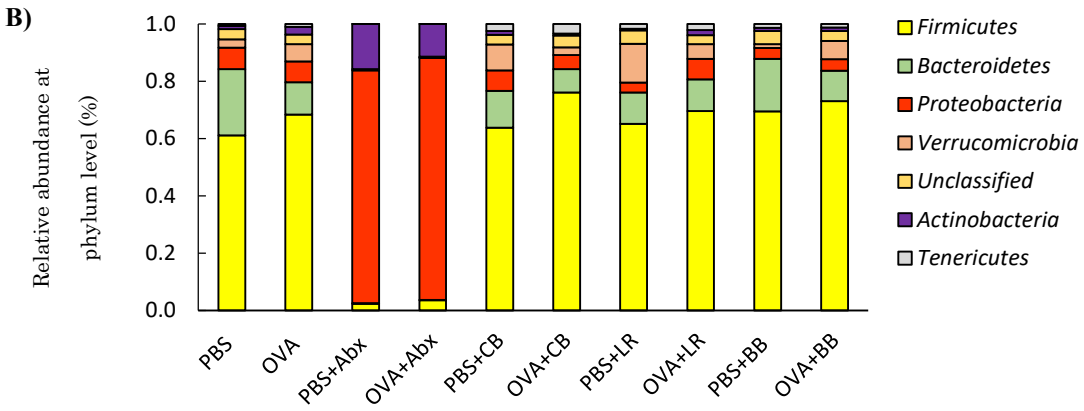
Fig. 7. Representative confocal images of tight junction-related proteins. Cryosections of jejunal tissue were immunolabeled for ZO-1, and claudin-1, -2, -3, -5, -7 (in green). Nuclei were visualized with DAPI (in blue). Size bars, 50 μm (small) and 20 μm (large). Abx; antibiotics, CB; *C. butyricum*, LR; *L. reuteri*, BB; *B. breve*.

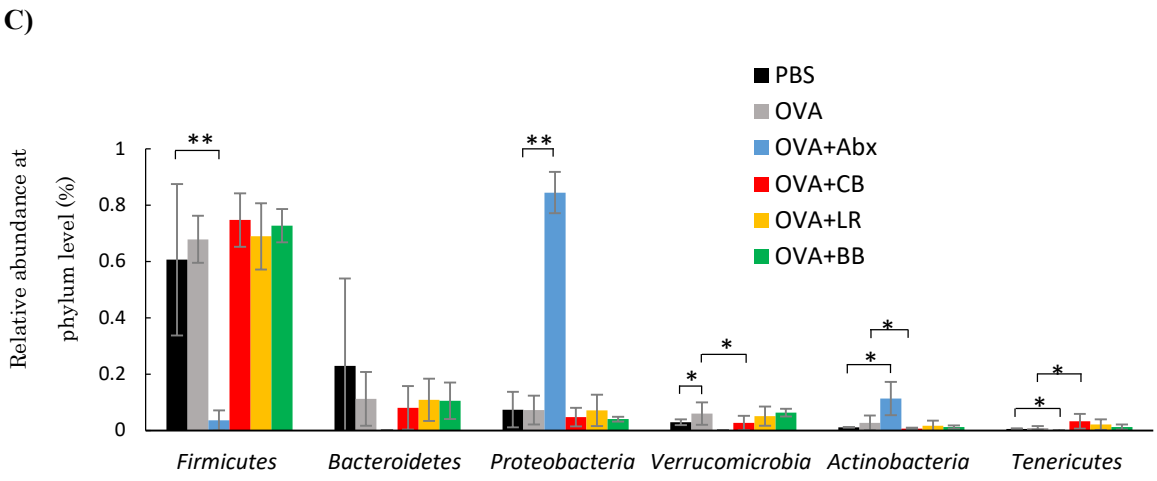
Influence of OVA sensitization on composition of the fecal microbiota.

Analysis of the overall bacterial community structure demonstrated that rats treated with probiotics had a significantly less diverse community than OVA-sensitized rats without probiotic treatment (Shannon's index, OVA = 3.9±0.1vs OVA + *C. butyricum* =3.4 ±0.5; OVA + *L. reuteri* =3.4±0.4; OVA+*B. breve* =3.5±0.3: **Fig. 8A**).

Comparison of relative abundance at the phylum level between the control and sensitization groups demonstrated enrichment of Firmicutes and Verrucomicrobio in the OVA sensitization group. In the antibiotic-treated groups, sequences specific for Proteobacteria and Actinobacteria were evident (**Fig. 8B**). The family Ruminococcaceae were significantly enriched in the OVA group and reduced in the groups treated with *C. butyricum* and *L. reuteri*. Clostridiaceae were significantly increased in all of the groups administered probiotics (*C. butyricum*, *L. reuteri*, and *B. breve*) relative to the OVA-sensitization group (**Fig. 8D**). *Blautia* and *Bacteroides* were reduced in all of the OVA sensitization groups. On the other hand, *Clostridium* was enriched in OVA-sensitized *C. butyricum*-treated rats in comparison with the OVA group. *Oscillospira* was significantly enriched in the OVA groups and reduced by administration of *C. butyricum* and *L. reuteri* (**Fig. 8E**). *Alkaliphilus* was significantly enriched in all of the OVA groups treated with probiotics (*C. butyricum*, *L. reuteri*, and *B. breve*).


A)
Shannon diversity index
OVA+BB
OVA+LR
OVA+CB
OVA+Abx
OVA
PBS
0
1
2
3
4
5
*
*
*
*


B)
Relative abundance at phylum level (%)
1.0
0.8
0.6
0.4
0.2
0.0
PBS
OVA
PBS+Abx
OVA+Abx
PBS+CB
OVA+CB
PBS+LR
OVA+LR
PBS+BB
OVA+BB
Firmicutes
Bacteroidetes
Proteobacteria
Verrucomicrobia
Unclassified
Actinobacteria
Tenericutes


C)
Relative abundance at phylum level (%)
1
0.8
0.6
0.4
0.2
0
PBS
OVA
OVA+Abx
OVA+CB
OVA+LR
OVA+BB
**
**
*
*
*
*
*
*
Firmicutes
Bacteroidetes
Proteobacteria
Verrucomicrobia
Actinobacteria
Tenericutes

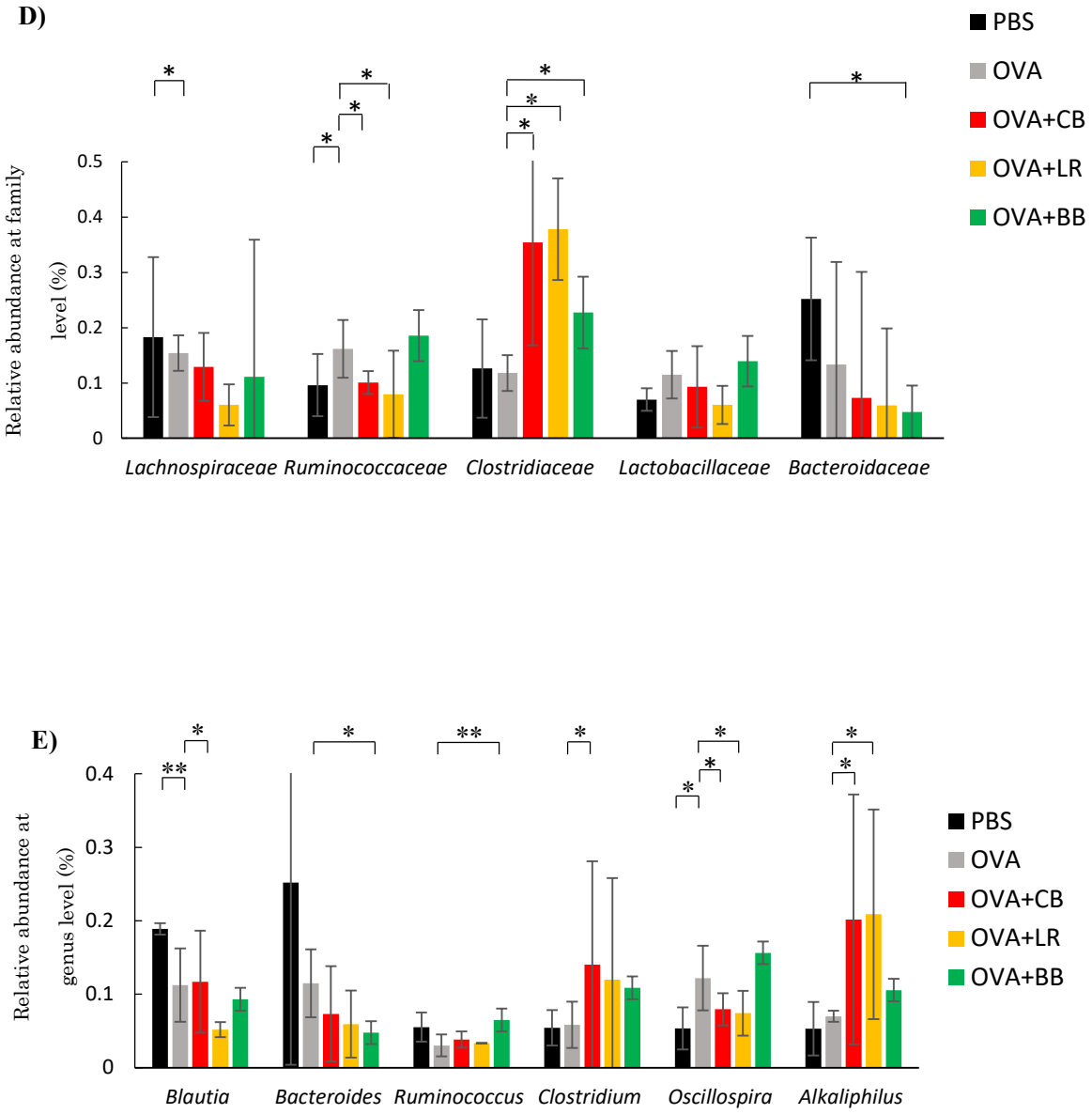


Fig. 8. A). Diversity profiles of the gut microbiota. **B and C).** Phylum-level taxonomic distributions of the microbial communities in fecal contents. **D).** Composition of the gut microbiota at the family level. **E).** Composition of the gut microbiota at the genus level. Bar represents mean and SEM. * P <0.05, ** P <0.01 determined by Student's t test. ns; no significance, Abx; antibiotics, CB; *C. butyricum*, LR; *L. reuteri*, BB; *B. breve*.

Summary of the results by group:

Fig. 9 illustrated summary of the results.

OVA sensitization

Gut permeability, the serum-specific OVA-IgE level, intestinal mucosal inflammation score and mucosal eosinophilic infiltration were increased. TJ structures were disrupted, and expression of TJ proteins was mislocalized (ZO-1, claudin-1, -3, -5, and 7) and down-regulated (ZO-1, occludin, and claudin- 8, -9, -15). Ruminococcaceae and the genus *Oscillospira* were enriched in the microbiota.

OVA sensitization with antibiotics

Gut permeability was increased, and allergic reactions were more enhanced by antibiotic treatment in the OVA sensitization group, which showed a higher level of serum-specific OVA-IgE and severe inflammation of the mucosae, as well as TJ structural damage. Expression of TJ proteins (ZO-1, claudin- 8, -9, and 15) was markedly down-regulated, and low expression of the proteins (ZO-1, claudin-1, -3, -5, -and 7) was detected. The composition of gut microbiota was quite different from that of the commensal bacterial flora.

OVA sensitization with probiotics

Neither gut permeability nor the serum-specific OVA-IgE level was increased, and allergic mucosal inflammation was mild. TJ proteins were not down-regulated and showed a similar intensity of immunofluorescence staining to that in controls. The gut microbiota demonstrated less diversity in comparison with rats subjected to OVA sensitization, and enrichment of specific bacterial sequences (Clostridiaceae and *Clostridium*).

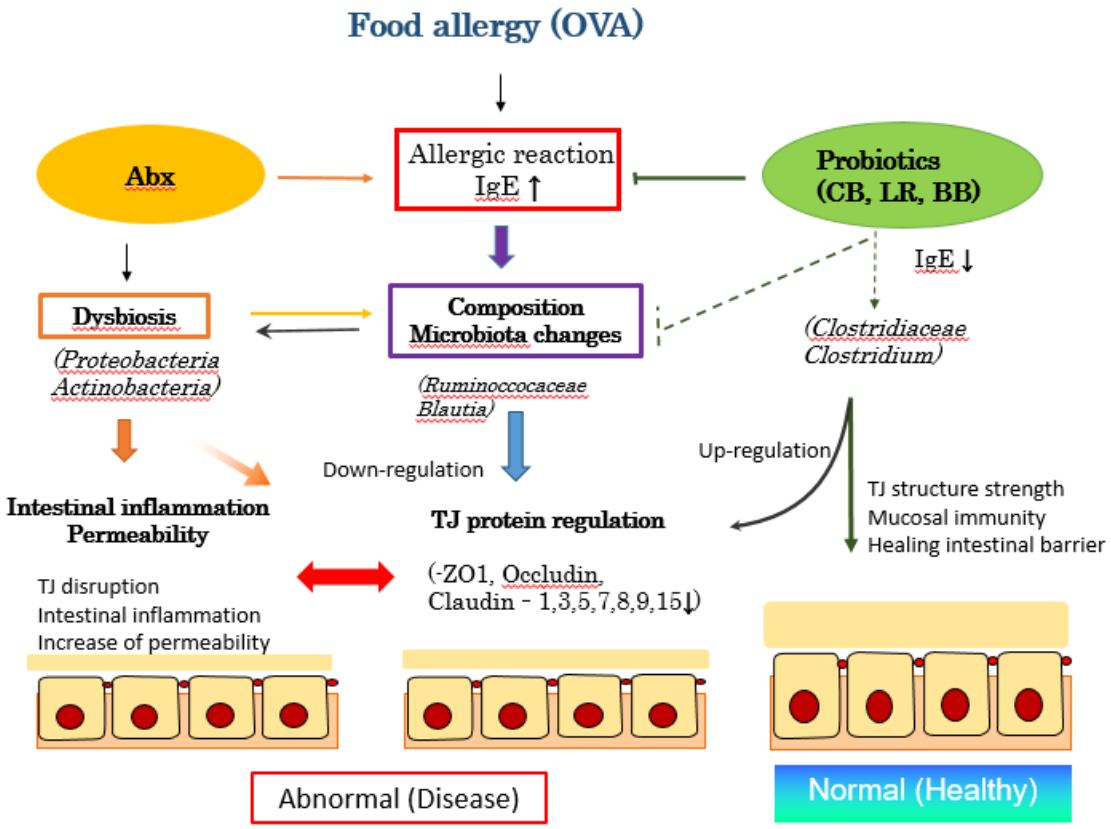


Fig. 9. Mechanism of intestinal microbiota in maintain barrier function in food allergy. Probiotics leads to increase the Clostridaceae bacterial community and upregulate TJ proteins in intestinal epithelia protect against food allergy. Additionally, Antibiotics increase the inflammation and permeability in intestine through the downregulation of TJ proteins.

Discussion

In the present study using juvenile rats with food allergy as a model, we found that not only Ag (OVA) sensitization but also antibiotic treatment induced leaky gut, causing allergen absorption and food allergy, presumably through significant expression of key TJ proteins and mucosal infiltration. Moreover, administration of probiotics prevented this increase in intestinal permeability, presumably through an increase in Clostridiaceae and also a significant influence on the expression of TJ proteins and mucosal infiltration.

One of the functions of the epithelial barrier is to prevent macromolecular Ags and other harmful substances from being absorbed [45, 46]. It is reported that intestinal permeability may increase when the intestinal mucosal barrier function is damaged during allergy, and that such epithelial barrier dysfunction leads to excessive transport of macromolecular substances, which are absorbed into the deep tissues and have the potential to induce Ag-related intestinal inflammation or hypersensitivity [47-50]. The results of our lactulose/mannitol assay showed that intestinal permeability was increased after OVA sensitization, and this was further confirmed by electron microscopy (shortened and widened intercellular TJs of intestinal villus epithelial cells). These changes in OVA-sensitized rats were not prevented by administration of antibiotics but were prevented by treatment with probiotics, as further confirmed by electron microscopy. These findings are in line with a previous report indicating that TJ and adherens junction microstructure is markedly disrupted and widened after OVA sensitization and treatment with antibiotics [23].

TJs play an important role in the maintenance of intestinal permeability, which is considered to determine selective cellular absorption [51, 52]. TJs comprise multiple proteins forming a functional complex, and the major function of most TJ proteins is to

maintain the integrity of the epithelial barrier. Transmembrane proteins, such as occludin, claudins, and junctional adhesion molecules are structural proteins arranged in a linear manner, whereas the cytoplasmic adhesion proteins, ZO-1, -2, and -3, form a supporting cytoskeletal structure [53]. These proteins play a pivotal role in the regulation of TJ permeability and intestinal barrier function [28, 31, 40, 54-56]. Our real-time PCR study showed that OVA down-regulated the expression of ZO-1, occludin, and claudin-8, -9, and -15. These findings are with a previous similar study of OVA-sensitized rats, which demonstrated significant down-regulation of the TJ-mediating proteins ZO-1, and claudin -8 and -15 [57]. However, localization and distribution of TJ proteins, ZO-1, claudin 1, 2, 3, 5, and 7 were described firstly in our experiment. In OVA induced and antibiotics administration rats TJ proteins of ZO-1, claudin1, 3, 5 and 7 were mislocalized and low expression level determined compared to control and probiotics treated group. These results suggest that OVA induces damage to the intestinal barrier, and that TJ permeability is related to the expression and regulation of these proteins. Although a previous report has indicated that elevated expression of claudin-2 in epithelial cells plays an important role in epithelial barrier dysfunction as well as the pathogenesis of intestinal antigen-specific hypersensitivity [58], we found no significant increase of claudin-2 expression in the present study. This may have been partly due to the experimental design, as another previous report that adopted a similar experimental design also demonstrated no significant differences in the claudin-2 level between an Ag sensitization group and a control group [43]. On the other hand, treatment with *C. butyricum* and *B. breve* up-regulated the expression of both occludin and claudin-15. Similar beneficial effects of probiotics on the association between intestinal permeability and TJ protein expression have been reported. In Bet v1 pollen-sensitized mice, a mixture of *Lactobacillus* strains

up-regulated the expression of occludin and the TJ molecule ZO-1, and improved the function of the gut epithelial barrier [23]. In rats treated with dextran sulfate sodium, which has been widely used as a model for inducing acute and chronic colitis, a probiotic mixture of strain VSL#3 was shown to protect against increased intestinal permeability by up-regulating of expression of occludin, ZO-1 and claudins 1-5 [59].

The microbiota of OVA-sensitized rats was significantly more diverse than that of each of the probiotic treatment groups. This is compatible with previous clinical reports that demonstrated that the microbiota of children with food allergy was significantly more diverse than that of healthy controls [60-62]. In our study, the bacterial phylum characteristics were similar to those in previous reports showing that Firmicutes were greater in children with food allergy than in healthy controls [61-63]. Bacterial families characteristic of OVA-sensitized rats included Ruminococcaceae, which is a representative member of the Firmicutes. Clostridiaceae were also significantly enriched by treatment with *C. butyricum*, *L. reuteri*, and *B. breve*. *C. butyricum* can produce butyrate, which is one of the SCFAs and the main energy source for enterocyte regeneration [64]. It is reported that a combination of specific immunotherapy with *C. butyricum* significantly enforces the therapeutic effect against food allergen-related inflammation in mouse intestine [20]. Stefka et al. reported that *Clostridium* regulates innate lymphoid cell function to alter gut epithelial permeability and reduce allergen uptake into the systemic circulation [18]. Furthermore, Berni Canani et al. reported that extensively hydrolyzed casein formula supplemented with *Lactobacillus rhamnosus* GG promoted tolerance in infants with cow's milk allergy by influencing the butyrate-producing bacterial strains [62]. Bacteria-produced SCFAs critically regulate both the proportions and functional capabilities of colonic Tregs [8, 65]. In our present study,

Alkaliphilus, which is a member of the Clostridiaceae, was significantly enriched in the probiotic (*C. butyricum*, *L. reuteri*, and *B. breve*)-treated OVA groups. It has been reported that a strain of *Alkaliphilus* had the potential to correlate with gut SCFA production [66]. In our study, we detected a beneficial effect of *Alkaliphilus* on intestinal barrier function in OVA-sensitized rats, probably by inducing production of SCFA.

We found that OVA sensitization alone changed the composition of gut microbiota in comparison to controls, in terms of the relative abundance of bacterial phyla (**Fig. 8B and C**). Similarly, Andreassen et al. recently reported that allergen immunization in a food allergy model induced profound changes in the composition of the gut microbiome [67]. This is considered to indicate that the gut mucosal immune system can affect the composition of gut commensal bacteria. In other words, there is a bidirectional interaction between gut microbiota formation and the mucosal immune system which leads to allergic reaction or inflammation. Those kind of inflammation also could have damaged the intestinal barrier function through complex gut ecosystem.

From this viewpoint, it may not be surprising that Firmicutes and Actinobacteria were not enriched in OVA-sensitized rats, despite administration of *C. butyricum* and *L. reuteri*, both of which belong to the Firmicutes, and *B. breve*, which belongs to the Actinobacteria. The gut microbiota signatures could have been affected by OVA sensitization through complex ecosystem effects.

Because the jejunum, where most food absorption occurs in the digestive tract, plays an important role in epithelial barrier function in food allergy, we focused on the jejunum in the present study. However, microbiota composition and immune cell composition varies throughout the intestine, and these regional variations will influence the interactions between them. Therefore, further investigation of different segments of the

gastrointestinal tract (ileum, colon, etc.) is needed. We acknowledge that the sample size used in this study was small. Despite the use of inbred rodents, standardized maintenance, handling, and exposure to the animals in the same groups, there was considerable inter-individual variation in the immune responses. Some of the immunized animals even appeared to be non- or low responders, [67] which means that our results might have been affected by non-immune response bias.

Conclusion

Eradication of commensal bacteria is able to promote OVA sensitization through an increase of intestinal permeability by influencing TJ protein regulation and mucosal inflammation. On the other hand, administration of probiotics is able to suppress the level of sensitization by maintaining intestinal permeability, regulating the expression of TJ proteins and suppression of the mucosal inflammation. Our findings support the use of probiotic supplementation to prevent or treat food allergy.

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