Studies on the role of gut microbiota in the regulation of RegIIIβ and RegIIIγ in murine intestine

(マウス小腸の RegIIIβ および RegIIIγ の発現調節における
腸内細菌叢の役割に関する研究)

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Contents

List of abbreviations

16S rRNA	16S ribosomal ribonucleic acid
ABX	antibiotics
ANOVA	analysis of variance
ASV	amplicon sequence variants
BSA	bovine serum albumin
CDI	*Clostridium difficile* infection
cDNA	complementary deoxyribonucleic acid
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
FOS	fructooligosaccharide
FMT	fecal microbiota transplantation
GF	germ-free
GPR	G protein-coupled receptor
HDAC	histone deacetylase
HFD	high-fat diet
IL-22	interleukin-22
KES	1-kestose
mRNA	messenger ribonucleic acid
NFD	normal-fat diet
PCoA	principal coordinate analysis
PBS	phosphate-buffered saline
PCR	polymerase-chain reaction
PERMANOVA	permutational analysis of variance
REG	regenerating gene
RNA	ribonucleic acid
RT-qPCR	real-time quantitative polymerase-chain reaction
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	standard error of the mean
SCFA	short-chain fatty acid
TBS	tris-buffered saline
TSA	trichostatin A

Chapter I
Introduction

Regenerating gene family protein III (RegIII)

Regenerating gene protein (*Reg*) was firstly discovered as a gene responsible for replication, growth, and maturation of pancreatic islet β-cells and was spotted for the diabetic treatment (Terazono *et al*., 1988). Thereafter, *Reg* genes were isolated from human, rat and mouse, and were identified as a multigene family. *Reg* genes were grouped into three subclasses, *RegI*, *RegII*, and *RegIII* (Table 1), based on primary structures of proteins encoded (Okamoto, 1999)

Table I-1 The *Reg* gene family (Okamoto, 1999)

	Species				
Type	Human	Rat	Mouse	Hamster	Cow
I	*REG Iα* (*PSP*/*Lithostathine*/*PTP*) *REG Iβ*	*Reg I*	*Reg I*		
II			*Reg II*		
III	*HIP*/*PAP*	*Reg III*/*PAP II* *PAP I*/Peptide 23 *PAP III*	*Reg IIIα* *Reg IIIβ* *Reg IIIγ* *Reg IIIδ*	*INGAP*	*PTP*

Reg, Regenerating gene; *PSP*, gene for pancreatic stone protein; *HIP*, gene expressed in hepatocellular carcinoma, intestine, and pancreas; *PAP*, gene for pancreatitis-associated protein; *PTP*, gene for pancreatic thread protein; *INGAP*, gene for islet neogenesis-associated protein

The role of *Reg* gene family in human diseases, including inflammation, diabetes, and carcinogenesis, was studied, and there was a potential implication of *Reg* gene as a biomarker for therapeutic treatment (Parikh *et al*., 2012; Zhang *et al*., 2003).

RegIII is a C-type lectin family with ~16 kDa molecular mass, expressed in the intestinal epithelial cells (Miki *et al*., 2018). RegIII proteins are secreted into the intestinal lumen and exert bactericidal action by binding bacterial peptidoglycan; thus, these lectins are thought to protect against the infection with enteropathogenic

bacteria (Sassone-Corsi & Raffatellu, 2015). In addition, these lectins are secreted into the circulation and act as an anti-inflammatory cytokine (Closa *et al*., 2007).

RegIIIβ and RegIIIγ belong to the RegIII family. In mouse genome, the *Reg3b* and *Reg3g* genes are 759 bp- and 774 bp-length cDNA with 70.9% homology between mouse *Reg3b* and *Reg3g*; and both presented 5-aa insertions (aa 110–114) in the C-terminal regions (Narushima *et al*., 1997). *Reg3b* gene divided into 6 exons separated by 5 introns locates in chromosome 6, 78347868-78350449 bp; and *Reg3g* gene divided into 2 exons separated by 1 intron locates in chromosome 6, 78443252-78445855 bp in the mouse genome (Figure I-1).

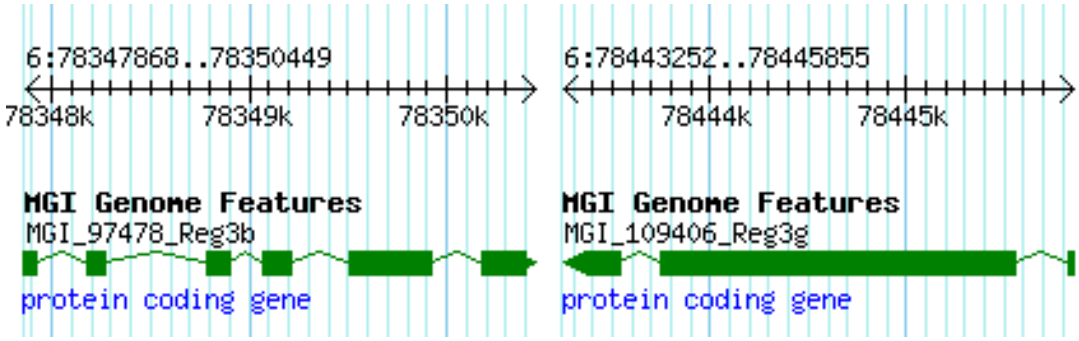


Figure I-1 Gene location of *Reg3b* (left) and *Reg3g* (right) genes in the mouse genome (Mouse Genome Database (MGD) at the Mouse Genome Informatics website, The Jackson Laboratory, Bar Harbor, Maine. World Wide Web (URL: http://www.informatics.jax.org). June, 2022).

Previous studies found that RegIIIβ provides protective function in the intestine by binding bacterial peptidoglycan and lipid A; thus, RegIIIβ kills both gram-positive and gram-negative bacteria (Miki *et al*., 2018; van Ampting *et al*., 2012). Lack of RegIIIγ reportedly increased bacterial colonization, bacterial translocation across the intestinal epithelium, and induce inflammation (Loonen *et al*., 2014; Vaishnava *et al*., 2011).

Expression of RegIIIβ and RegIIIγ in the intestinal epithelial cells is influenced by intestinal microbiota. In germ-free (GF) mice and antibiotics (ABX)-treated mice, the expression of RegIIIβ and RegIIIγ expression was reduced (Liang *et al*., 2019; Wu *et al*., 2014) and, in contrast, the expression was promoted by bacterial colonization (Atarashi *et al*., 2015; Cash *et al*., 2006). More specifically in

terms of bacterial species, intestinal RegIIIγ expression has been shown to be stimulated by *Bifidobacterium breve* in mice (Natividad *et al*., 2013).

In addition, intestinal expression of RegIIIβ and RegIIIγ is influenced by diet. Intestinal RegIIIγ expression was reportedly decreased by a high-fat diet (HFD) and increased by the supplementation of fructooligosaccharide (FOS) in drinking water (Everard *et al*., 2014). RegIIIβ was also reported to be increased by FOS supplementation (Yan *et al*., 2011). It is widely known that the consumption of HFD and indigestible saccharides such as FOS influence the composition of gut microbiota. HFD feeding decreases phylum Bacteroidetes and increases phyla Firmicutes and Proteobacteria (Bäckhed *et al*., 2005; Hildebrandt *et al.,* 2009), and FOS supplementation increases the abundance of bifidobacteria (Cummings *et al*., 2001; Goh & Klaenhammer, 2015; Mao *et al*., 2015). Therefore, it is likely that diet-induced changes in the intestinal expression of RegIIIβ and RegIIIγ are mediated by gut microbiota.

In this study, the intestinal expression of RegIIIβ and RegIIIγ was examined in the ileum part of the small intestine. Because ileum seems most susceptible to bacterial invasion due to its thinner mucosal layer that is composed of shorter and rougher villi (Cuvelier *et al*., 2001). Therefore, this study considered important to investigate the regulation of these antibacterial peptides in the ileum.

Fecal microbiota transplantation

Fecal microbiota transplantation (FMT) is defined as the administration of a donor's fecal-matter solution to the recipient's intestinal tract in order to modify gut microbiota composition and provide health benefits to recipient (Gupta *et al*., 2016). Several studies have shown that FMT from healthy donors alleviated the dysbiosis of gut microbiota in recipients, which resulted in the improvement of imbalanced health conditions including metabolic irregularity and bacterial infection (Khoruts *et al*., 2010; Silverman *et al*., 2010; Vrieze *et al*., 2012; You *et al*., 2008). In animal experiments, HFD-induced steatohepatitis was alleviated after FMT by revising altered gut microbiota composition in HFD-fed mice (Zhou *et al*., 2017). Kootte *et al.* (2012) presented the potential of FMT for the therapeutic treatment of obesity and type 2 diabetes. Gut microbiota diversity altered by *Clostridium difficile* infection (CDI) was reported to be restored by FMT, which provided the successful treatment for CDI patients (Carlucci *et al*., 2016).

In addition, FMT has been used as a tool for demonstrating the causal relationship between gut microbiota and host physiology and pathology. For instance, Turnbaugh *et al.* (2006) employed FMT to test whether gut microbiota of genetically obese mice contributes to obesity. They showed that the body fat gain was greater in GF mice receiving FMT from obese mice than those receiving FMT from lean mice, suggesting gut microbiota as a cause of obesity. Ge *et al*. (2017) showed that ABX-treated mice receiving FMT from slow-transit constipation patients showed lower fecal pellet frequency and water percentage, smaller fecal pellet size, delayed gastrointestinal transit time, and weaker spontaneous contractions of colonic smooth muscle, suggesting that gut microbiota influences gut motility.

Intestinal organoids

Organoids are derived from pluripotent stem cells or isolated organ progenitors that differentiate to form an organlike tissue exhibiting multiple cell types that self-organize to form a structure not unlike the organ *in vivo* (Lancaster & Knoblich, 2014). Intestinal organoids are pouched epithelium composed of intestinal stem cells, transit-amplifying cells, enterocytes, goblet cells, enteroendocrine cells, and Paneth cells. Since intestinal organoids were firstly cultured from rat intestine (Montgomery *et al*., 1983), the procedure of intestinal organoid culture has been developed over time. Currently, intestinal crypts including stem and Paneth cells isolated from intestine can be cultured in the three-dimensional extracellular matrix Matrigel with the culture medium that contains appropriate growth factors such as Wnt, R-spondin, and Noggin, and the resultant intestinal organoids compose of villus domain surrounded by crypt domain (Figure I-1; Sato *et al*., 2009; Sato *et al*., 2011; Miyoshi *et al*., 2013).

From the proficiency of organoid that can resemble the specific organ, human- and mice-derived intestinal organoids have been used as an *ex vivo* model for studying several issues, including infectious diseases (Castellanos-Gonzalez *et al*., 2013; Finkbeiner *et al*., 2012), tumorigenesis (Onuma *et al*., 2013), genetic disorders (Dekkers *et al*., 2013), microRNA expression (Ohsaka & Sonoyama, 2018), serotonin system (Tsuruta *et al*., 2016), and host–microbe interactions (Bartfeld *et al*., 2015; Ettayebi *et al*., 2016; Heo *et al*., 2018)

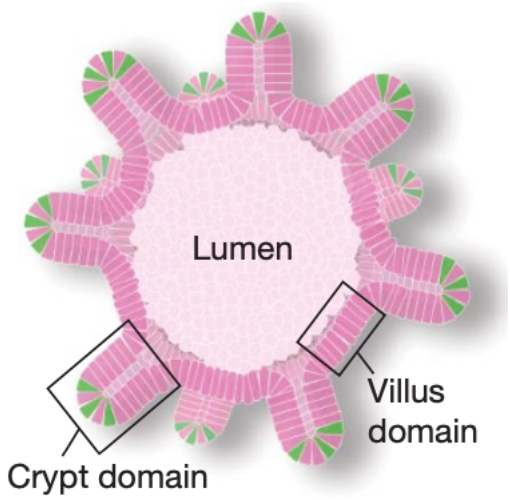


Figure I-2 Schematic representation of an intestinal organoid, consisting of a central lumen lined by villus-like epithelium and several surrounding crypt-like domains. (Sato *et al*., 2009)

Aims of the study

As described above, the intestinal expression of RegIIIβ and RegIIIγ is influenced by both gut microbiota and diet. Because HFD feeding and FOS supplementation alter both the intestinal expression of RegIIIβ and RegIIIγ and the composition of gut microbiota, this study assumed that gut microbiota may mediate diet-induced changes in the intestinal expression of RegIIIβ and RegIIIγ. Therefore, this study tested this idea and investigated underlying molecular mechanism.

The first part of this study examined to test whether changes in the intestinal expression of RegIIIβ and RegIIIγ by supplementation with 1-kestose (KES), a kind of FOS, in drinking water and by consumption of HFD are mediated by gut microbiota. To do this, this study employed FMT; thus, ABX-treated mice were received FMT from mice supplemented with and without KES and from mice fed HFD and normal-fat diet (NFD).

The second part aimed to explore the specific bacteria responsible for intestinal RegIIIβ regulation. Leptin, an adipocyte hormone discovered by Zhang *et al.* (1994), signals nutritional status to the central nervous system and peripheral organs (Pan *et al*., 2014). Leptin status is reportedly associated with the intestinal expression of RegIIIβ. Waelput *et al.* (2000) showed that leptin administration reduced the intestinal expression of RegIIIβ mRNA in leptin-deficient *ob/ob* mice. It

has been also shown that *ob/ob* mice have gut microbiota in which the composition is distinct from wild type mice (Ley *et al.* 2005). In addition to mice fed FOS and HFD, therefore, *ob/ob* mice, leptin receptor-deficient *db/db* mice, and wild type (+/+) mice were subjected to the analysis for exploring the specific bacteria associated with the intestinal expression of RegIIIβ.

The final part of this study aimed to obtain mechanistic information on the gut microbiota regulation of intestinal RegIIIβ and RegIIIγ. This study assumed that metabolites of gut microbiota are involved in the regulation of intestinal RegIIIβ and RegIIIγ. To test this idea, murine intestinal organoids were used as an *ex vivo* model; thus, the effect of supplementation of gut microbial metabolites on the expression of RegIIIβ and RegIIIγ was investigated. In addition, interleukin-22 (IL-22), a cytokine released from innate lymphoid cells, has been considered as a regulator of intestinal RegIIIβ and RegIIIγ (Miki *et al.,* 2018; Sanos *et al.,* 2011). This study therefore examined in the organoids whether IL-22 is involved in the altered expression of RegIIIβ and RegIIIγ by KES and HFD.

Chapter II

Role of gut microbiota in dietary regulation of RegIIIβ and RegIIIγ

Introduction

In the previous studies, GF mice and ABX-treated mice showed the reduced expression of intestinal RegIIIβ and RegIIIγ (Liang *et al*., 2019; Wu *et al*., 2014), leading to hypothesize that gut microbiota is essential for the intestinal expression of RegIIIβ and RegIIIγ. Expression of intestinal RegIIIβ and RegIIIγ is also influenced by diet such as FOS supplementation (Everard *et al*., 2014; Yan *et al*., 2011) and HFD consumption (Everard *et al*., 2014). FOS, a natural non-digestible oligosaccharide, selectively stimulates the growth of specific bacteria, especially bifidobacteria and provides health-promoting effect for the host (Cummings *et al*., 2001; Gibson & Roberfroid, 1995; Goh & Klaenhammer, 2015; Mao *et al*., 2015). HFD also changes gut microbiota composition by decreasing phylum Bacteroidetes and increasing phyla Firmicutes and Proteobacteria (Bäckhed *et al*., 2005; Hildebrandt *et al.,* 2009). From these findings, it is assumed that gut microbiota may mediate FOS- and HFD-induced changes in the intestinal expression of RegIIIβ and RegIIIγ. To test this idea, FMT in mice was employed in this study. Thus, ABX-treated mice were received FMT from mice supplemented with or without KES, a kind of FOS; and in separated experiment, FMT from mice fed HFD or NFD.

Materials and Methods

Animals

This study was approved by Animal Use Committee of the Hokkaido University (approval No. 14-0028 and 19-0017), and the animals were maintained in accordance with the guidelines for the care and use of laboratory animals of Hokkaido University. Five-week-old male specific pathogen free C57BL/6JJmsSlc mice, purchased from Japan SLC (Shizuoka, Japan), were housed in temperature-controlled (23 ± 2˚C) room with 12-h dark-light cycle and allowed free access to AIN-93G purified diet (Research Diet, New Brunswick, NJ, USA) and tap water.

Experimental design

After acclimatizing for 1-2 weeks, 5 experiments were performed.

In experiment (exp.) 1, 12 mice were randomly divided into 2 groups (6 mice in each group) and then fed the AIN-93G diet and supplemented either with or without ABX (neomycin [100 μg/ml, Sigma, St Louis, MO, USA], streptomycin [50 μg/ml, Wako, Osaka, Japan], penicillin [100 U/ml, Tokyo Chemical Industry, Tokyo, Japan], vancomycin [50 μg/ml, Wako], metronidazole [100 μg/ml, Wako], bacitracin [1 μg/ml, Wako], ciprofloxacin [125 μg/ml, LKT Laboratories, MN, USA], ceftazidime [100 μg/ml, LKT Laboratories], and gentamycin [170 μg/ml, Tokyo Chemical Industry])(Chevalier *et al.* 2015) in drinking water for 3 days.

In exp. 2, 12 mice were randomly divided into 2 groups (6 mice in each group) and then fed the AIN-93G diet and supplemented either with or without 4% (w/v) KES (B Food Science Co., Ltd., Chita, Japan), in drinking water for 2 weeks.

In exp. 3, 12 mice were randomly divided into 2 groups (6 mice in each group) and then fed either a NFD (D12450J, 10 kcal% fat, Research Diets) or a HFD (D12492, 60 kcal% fat, Research Diets) for 2 weeks.

On the last day of the experimental period in exp. 1, 2, and 3, mice were anesthetized with sevoflurane (Wako) inhalation and then killed by severing the carotid artery. Serum was separated from the blood samples and stored at -20˚C for western blot analysis. After a laparotomy, a 5-cm segment proximal to the ileo-cecal valve was excised from the small intestine and opened longitudinally, and the contents were collected and stored at -20˚C for DNA isolation. After washing with ice-cold phosphate-buffered saline (PBS), the mucosa of the segment was scraped off with a glass slide, snap-frozen in liquid nitrogen and stored at -80˚C for total RNA isolation.

In exp. 4 (Figure II-4a), 24 mice were randomly divided into 2 groups of donors and recipients (12 mice in each group) for FMT. Donor mice were further divided into 2 groups (6 mice in each group) and then fed the AIN-93G diet and supplemented either with or without 4% (w/v) KES in drinking water for 2 weeks. Recipient mice were fed the AIN-93G diet and tap water without KES supplementation before FMT. After FMT, recipient mice were fed the AIN-93G diet and tap water for 1 week.

In exp. 5 (Figure II-4b), 24 mice were randomly divided into 2 groups of donors and recipients (12 mice in each group) for FMT described below. Donor mice were further divided into 2 groups (6 mice in each group) and then fed either HFD or

NFD for 2 weeks. Recipient mice were fed NFD before FMT. After FMT, recipient mice were fed NFD for 1 week.

In exp. 4 and 5, serum samples, ileal contents and ileal mucosal samples in donor and recipient mice were obtained and stored as described above.

FMT

Donor mice were anesthetized with sevoflurane inhalation and then killed by severing the carotid artery. After a laparotomy, the distal half of the small intestine was excised, and the luminal contents were collected using 1-ml PBS and pooled in each group, homogenized, and then immediately subjected to FMT. Prior to receiving FMT, recipient mice were given ABX in drinking water prepared described above and intragastrically administered with omeprazole (40 mg/kg/day, Combi-Blocks, San Diego, CA, USA) once a day for 3 days in order to reduce gastric-acid secretion. On the next day of the final omeprazole administration, ABX administration was discontinued, and recipient mice were intragastrically administered with 200 μl luminal contents obtained from donor mice once a day for 2 consecutive days.

DNA isolation and real-time quantitative PCR (RT-qPCR) for bacterial enumeration

DNA was isolated from ileal contents using a QIAamp Fast DNA Stool Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. DNA samples served as a template for RT-qPCR of the 16S rRNA gene fragment to estimate the number of bacteria belonging to phylum Firmicutes, phylum Bacteroidetes and genus *Bifidobacterium* as previously described (Sasajima *et al.,* 2010). In brief, RT-qPCR was performed using a SYBR Premix Ex Taq (Takara, Otsu, Japan) with a Thermal Cycler Dice TP800 (Takara). The reaction conditions were 90°C for 10 min, followed by 45 cycles at 95°C for 30 s and 60°C for 1 min. A melting curve analysis was performed after amplification to assess the specificity of RT-qPCR. The sequences of primers used for RT-qPCR are described in Table II-1. For RT-qPCR standards, subcloned 16S rRNA gene fragments were prepared from *Blautia coccoides* (JCM 1395^{T}), *Bacteroides fragilis* (JCM11019), and *Bifidobacterium animalis* (JCM 1190^{T}) for phylum Firmicutes, phylum

Bacteroidetes, and *Bifidobacterium* spp., respectively, as previously described (Sasajima *et al.,* 2010).

RNA isolation and analysis

Total RNA was isolated from ileal mucosal samples using a ReliaPrep RNA Tissue Miniprep System (Promega Japan, Tokyo, Japan) and reverse transcribed to generate first strand cDNA using a ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan) according to the manufacturers' instructions. Murine RegIIIβ, RegIIIγ, and β-actin are encoded by *Reg3b*, *Reg3g*, and *Actb* genes, respectively. To compare the steady-state levels of these mRNAs, RT-qPCR was performed using a GeneAce SYBR qPCR Mix α No ROX (Nippon Gene, Toyama, Japan) with a Thermal Cycler Dice TP800 according to the manufacturers' instructions. Relative mRNA expression levels of each sample were computed by using cycle threshold method ($2^{-\Delta\Delta ct}$) and normalized to that of *Actb*. The sequences of primers used for RT-qPCR are described in Table II-1.

Western blot analysis

Serum samples were boiled for 3 min with a buffer composed of 30% glycerol, 0.195 M Tris-HCl pH 6.8, 9% (w/v) SDS, and 0.01% (w/v) bromophenol blue. Samples were then separated by 10% SDS-PAGE under reducing conditions. The gel was then soaked in a transfer buffer (20% methanol, 125 mM Tris, and 960 mM glycine) for 15 min before transferred electrophoretically to nitrocellulose membrane (Hybond-C, Amersham Life Science, Amersham, UK). After blocking with a blocking buffer composed of 5% (w/v) bovine serum albumin (BSA) in 20 mM Tris-HCl, pH 7.5 containing 200 mM NaCl and 0.1% (w/v) Tween-20 (Tris-buffered saline containing Tween-20, TBS-T) for 1 h at room temperature, membranes were separately incubated with primary antibody against RegIIIβ (Mouse Reg3B Antibody MAB5110, R&D systems, Minneapolis, MN, USA), RegIIIγ (REG3G Polyclonal Antibody, Thermo Fisher Scientific), and transferrin (F-8, Santa Cruz, Santa Cruz, CA) overnight at 4ºC. Primary antibodies were prepared just before using by 500-fold dilution in blocking buffer. The membranes were then washed with TBS-T for 5 min, 3 times at room temperature before being incubated with 3,000-fold dilution of horseradish peroxidase (HRP)-conjugated secondary

antibodies (Rat IgG HRP-conjugated antibody and Rabbit IgG HRP-conjugated antibody for RegIIIβ and RegIIIγ, respectively, R&D Systems) for 1 h at room temperature. The membranes were washed for 5 min, 3 times at room temperature with TBS-T. The blots were detected by ECL chemiluminescence (GE Healthcare, Menlo Park, CA, USA) according to the manufacturer's instructions and visualized with Lumi Vision PRO 400EX (AISIN, Kariya, Japan). The band intensities were quantified by ImageJ software (National Institutes of Health, Bethesda, MD, USA), and relative RegIIIβ and RegIIIγ levels for each sample were normalized to the levels for transferrin.

Statistical analysis

All results were expressed as mean ± standard error of the mean (SEM). To compare the mean values of two groups in exp. 1 and 2, an unpaired *t*-test was used. To compare the mean values of four groups in exp. 3 and 4, two-way analysis of variance (ANOVA) was used. The Tukey-Kramer *post hoc* test was applied when a significant interaction was found by two-way ANOVA. Data were analyzed using GraphPad Prism for Macintosh (version 8, GraphPad Software, San Diego, CA, USA). *P* values of <0.05 were considered to be statistically significant.

Results

ABX, KES, and HFD alter ileal expression of Reg3b and Reg3g genes (exp. 1, 2, and 3)

The mRNA levels of both *Reg3b* and *Reg3g* genes in the ileum were significantly lower in the ABX-administered mice than in the mice without ABX administration (Figure II-1a). Similarly, serum RegIIIβ and RegIIIγ levels estimated by western blot analysis were significantly lower in the ABX-administered mice than in the mice without ABX administration (Figure II-1b). The mRNA levels of both *Reg3b* and *Reg3g* genes in the ileum were significantly higher in the KES-supplemented mice than in the mice without KES supplementation (Figure II-2a) and significantly lower in the HFD-fed mice than in the NFD-fed mice (Figure II-3a). In parallel with ileal mRNA levels, serum RegIIIβ levels were significantly higher in the KES-supplemented mice than in the mice without KES supplementation (Figure

II-2b) and significantly lower in the HFD-fed mice than in the NFD-fed mice (Figure II-3b). In contrast, KES and HFD failed to alter serum RegIIIγ levels.

KES-induced increase of ileal* Reg3b *and* Reg3g *mRNA levels in donor mice was reproduced in recipient mice (exp. 4)

In this study, FMT was employed to test whether KES-induced increase of the mRNA levels of *Reg3b* and *Reg3g* genes in the ileum is mediated by gut microbiota. The abundance of *Bifidobacterium* spp. in the ileal contents tended to be influenced by both KES supplementation and FMT (Figure II-5a). When an unpaired *t*-test was applied, the abundance of *Bifidobacterium* spp. was significantly higher in the KES-supplemented donor mice than in the mice without supplementation ($p<0.01$), whereas the abundance did not differ between the mice receiving FMT from the KES-supplemented mice and the mice receiving FMT from the mice without supplementation. The mRNA levels of *Reg3b* and *Reg3g* genes in the ileum (Figure II-5b and II-5c, respectively) and serum RegIIIβ and RegIIIγ levels (Figure II-5d and II-5e, respectively) were significantly higher in the KES-supplemented donor mice than in the mice without supplementation. Similarly, the mRNA levels of *Reg3b* and *Reg3g* genes in the ileum (Figure II-5b and II-5c, respectively) and serum RegIIIβ and RegIIIγ levels (Figure II-5d and II-5e, respectively) were significantly higher in the recipient mice receiving FMT from the KES-supplemented donor mice than in the recipient mice receiving FMT from the mice without supplementation. FMT significantly decreased the ileal mRNA levels of *Reg3b* and *Reg3g* genes (Figure II-5b and II-5c, respectively) and increased the serum RegIIIγ levels (Figure II-5e).

HFD-induced decrease of ileal* Reg3b *and* Reg3g *mRNA levels in donor mice was reproduced in recipient mice (exp. 5)

Similar to exp. 4, FMT was performed to test whether HFD-induced decrease of the mRNA levels of *Reg3b* and *Reg3g* genes in the ileum was mediated by gut microbiota. The Firmicutes/Bacteroidetes ratio in the ileal contents was significantly influenced by both HFD and FMT (Figure II-6a). The ratio was significantly higher in the HFD-fed mice than in the NFD-fed mice. Likewise, the ratio tended to be higher in the mice receiving FMT from the HFD-fed mice than in the mice receiving FMT from the NFD-fed mice. Also, the ratio was significantly higher in the recipient

mice than in the donor mice. The mRNA levels of *Reg3b* and *Reg3g* genes in the ileum (Figure II-6b and II-6c, respectively) and serum RegIIIβ levels (Figure II-6d) were significantly lower in the HFD-fed mice than in the NFD-fed mice. Similarly, the mRNA levels of *Reg3b* and *Reg3g* genes in the ileum and serum RegIIIβ levels were significantly lower in the mice receiving FMT from the HFD-fed mice than in the mice receiving FMT from the NFD-fed mice. No significant difference was observed in the serum RegIIIγ levels among the groups (Figure II-6e).

Discussion

The effect of gut microbiota depletion by ABX treatment (Figure II-1) was in line with previous studies (Liang *et al*., 2019; Wu *et al*., 2014) where reduced expression of the intestinal *Reg3b* and *Reg3g* genes was observed, supporting the importance of gut microbiota in the intestinal expression of *Reg3b* and *Reg3g* genes.

This study showed that the ileal mRNA expression of *Reg3g* gene was increased by KES supplementation (Figure II-2) and reduced by HFD feeding (Figure II-3). These results are in line with Everard *et al.* (2014). This study also showed that the ileal mRNA expression of *Reg3b* gene was changed in parallel with that of *Reg3g* gene in response to KES and HFD. This seems reasonable since *Reg3b* gene is usually co-expressed with *Reg3g* gene in mice (Cash *et al.* 2006). This study thus indicated that KES and HFD influenced the ileal expression of both *Reg3b* and *Reg3g* genes.

This study clearly demonstrated that mice receiving FMT reproduced the ileal mRNA expression of *Reg3b* and *Reg3g* genes in the donor mice. Ileal mRNA expression of *Reg3b* and *Reg3g* genes was higher in the mice receiving FMT from the KES-supplemented donors (Figure II-5b, c) and lower in the mice receiving FMT from the HFD-fed donors (Figure II-6b, c). In this study, the recipient mice were treated with ABX prior to receiving FMT. As described above, ABX treatment decreased the ileal mRNA expression of *Reg3b* and *Reg3g* genes. Thus, ileal mRNA expression of *Reg3b* and *Reg3g* genes in the recipient mice would reflect the recovery of gene expression by receiving FMT. Nevertheless, the present results obtained by FMT experiments suggest that KES- and HFD-induced changes in the ileal mRNA expression of *Reg3b* and *Reg3g* genes are mediated, at least in part, by gut microbiota.

This study investigated serum RegIIIβ and RegIIIγ (Figure II-5d, e and Figure II-6d, e). The serum RegIIIβ levels changed in parallel with the ileal mRNA levels, whereas inconsistent data were obtained in the serum RegIIIγ levels. These results indicate that circulating RegIIIβ would reflect the ileal expression of *Reg3b* gene. Considering that circulating RegIIIβ is thought to be associated with inflammation (Closa *et al.* 2007) and obesity (Secq *et al.* 2014), influence of diet and gut microbiota on the inflammation and obesity may be mediated, at least in part, through circulating RegIIIβ. It is true that circulating RegIIIβ and RegIIIγ might come from other organ such as skin and pancreas under specific conditions, e.g., skin lesion and bowel inflammation, whereas the most predominant tissue of RegIIIβ and RegIIIγ expression is the intestine (Shin & Seeley, 2019). Therefore, the circulating level of RegIIIβ and RegIIIγ seems reflect their expression in the intestine. However, how extent the ileum contributes to the circulating level warrants further studies.

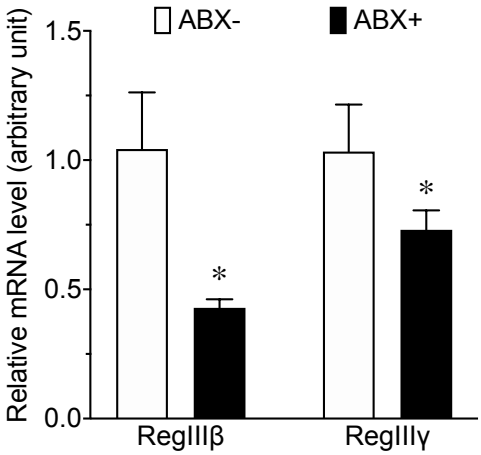
HFD feeding reportedly increases the Firmicutes/Bacteroidetes ratio (Hilderbrandt *et al.* 2009). In this study, the Firmicutes/Bacteroidetes ratio in the ileal contents was higher in the HFD-fed mice than in the NFD-mice and also higher in the mice receiving FMT from the HFD-fed donors than those receiving FMT from the NFD-fed donors (Figure II-6a). It is thus conceivable that the mice receiving FMT reproduced the gut microbiota in terms of HFD-induced increase of the Firmicutes/Bacteroidetes ratio. Because FMT itself also increased the Firmicutes/Bacteroidetes ratio, however, it should be considered that the composition of gut microbiota in the recipient mice would be not exactly identical to the composition of donors' microbiota.

The KES supplementation increased the population of *Bifidobacterium* spp. in the ileal contents (Figure II-5a), being consistent with previous studies in rats and dogs (Ide *et al.* 2020; Tochio *et al.* 2016; Watanabe *et al.* 2019). However, the recipient mice receiving FMT from the KES-supplemented mice failed to reproduce higher population of *Bifidobacterium* spp. Because Hansen *et al.* (2013) showed that dietary indigestible xylooligosaccharide increased both the population of *Bifidobacterium* spp. and the expression of *Reg3g* gene in mouse ileum, this study had initially expected that KES-induced increase of ileal expression of *Reg3b* and *Reg3g* genes would be mediated by *Bifidobacterium* spp. From the present results, however, it appears possible that other bacterial groups than *Bifidobacterium* spp.

might contribute to KES-induced increase of ileal expression of *Reg3b* and *Reg3g* genes. Further studies are required to identify the bacteria which are involved in the regulation of intestinal expression of *Reg3b* and *Reg3g* genes. Since KES could be utilized by butyrate-producing bacteria (Tanno *et al.,* 2021), the butyrate-producing bacteria might be one of the bacteria involved in the regulation of intestinal *Reg3b* and *Reg3g* gene expressions.

In conclusion, this study proposes that KES- and HFD-induced changes in the ileal expression of *Reg3b* and *Reg3g* genes and in circulating RegIIIβ levels are mediated, at least in part, by gut microbiota.

a



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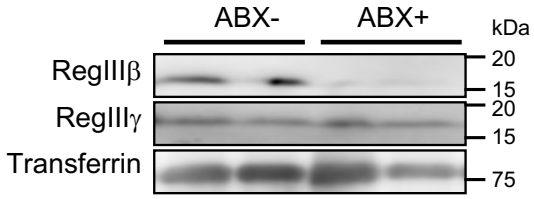


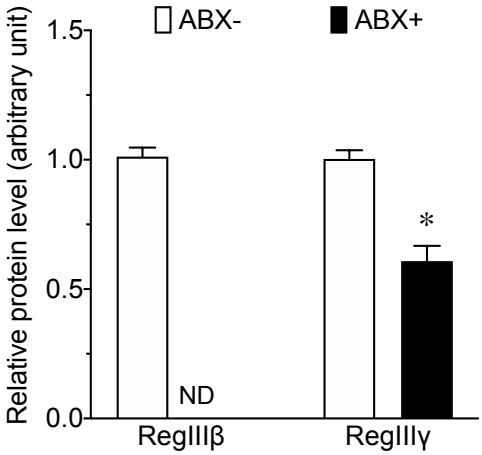
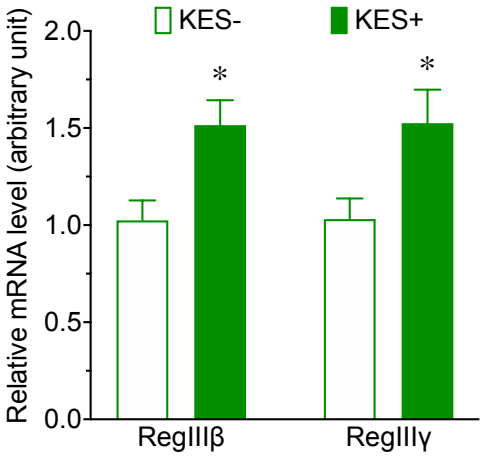


Figure II-1 Effect of ABX treatment for 3 days on the ileal RegIIIβ and RegIIIγ mRNA levels estimated by RT-qPCR (a) and serum RegIIIβ and RegIIIγ levels estimated by western blot analysis (b) in C57BL/6J mice (exp. 1). ABX-, without antibiotics supplementation in drinking water; ABX+, antibiotics supplementation in drinking water; ND, not detectable. Data are presented as mean ± SEM (n=6 in each group). *, $p < 0.05$ vs. ABX-. Insets in chart b illustrate the representative western blots.

a



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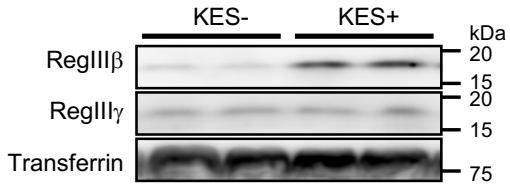


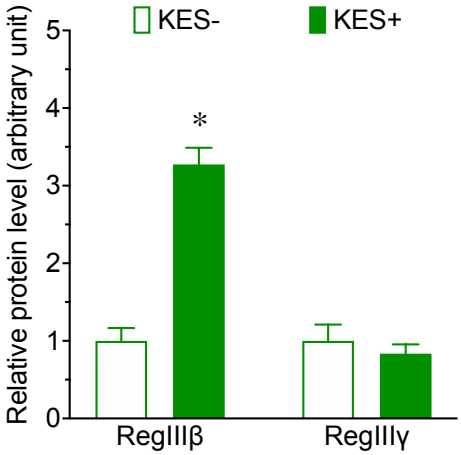
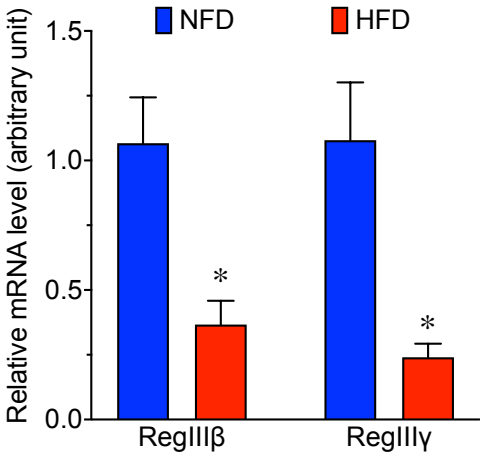
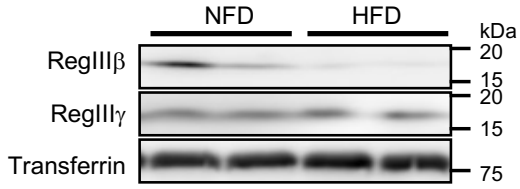


Figure II-2 Effect of KES supplementation on the ileal RegIIIβ and RegIIIγ mRNA levels estimated by RT-qPCR (a) and serum RegIIIβ and RegIIIγ levels estimated by western blot analysis (b) in C57BL/6J mice (exp. 2). KES-, without KES supplementation in drinking water; KES+, 4% (w/v) KES supplementation in drinking water. Data are expressed as means ± SEM (n=6 in each group). *, $p < 0.05$ vs. KES-. Insets in chart b illustrate the representative western blots.

a



b



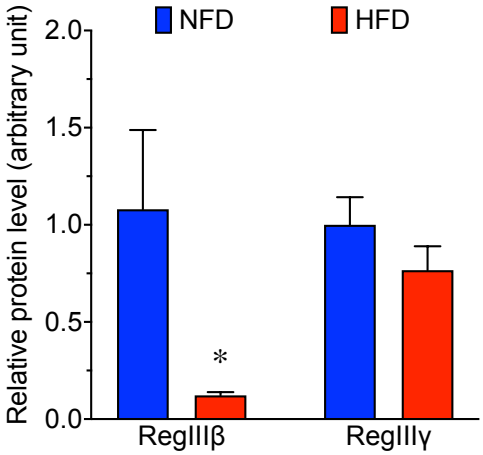
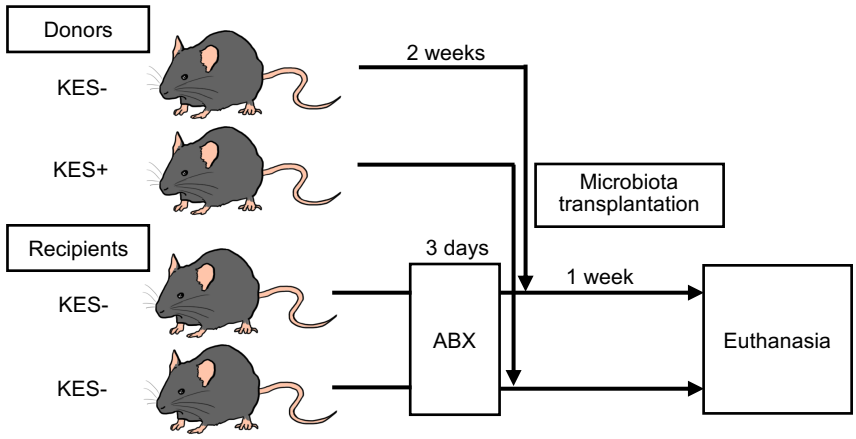


Figure II-3 Effect of HFD feeding on the ileal RegIIIβ and RegIIIγ mRNA levels estimated by RT-qPCR (a) and serum RegIIIβ and RegIIIγ levels estimated by western blot analysis (b) in C57BL/6J mice (exp. 3). NFD, normal-fat diet; HFD, high-fat diet. Data are expressed as means ± SEM (n=6 in each group). *, $p < 0.05$ vs. NFD. Insets in chart b illustrate the representative western blots.

a



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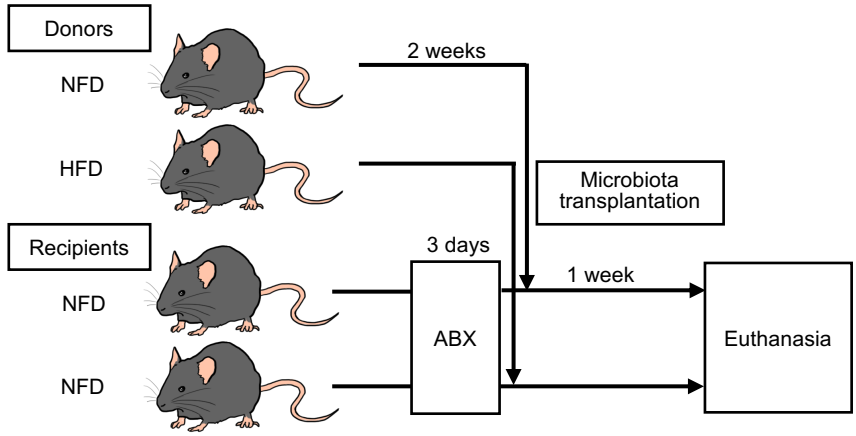


Figure II-4 Diagram of FMT experiments. Chart a shows the experiment of FMT from donor mice supplemented with KES (exp. 4), and chart b shows the experiment of FMT from donor mice fed HFD (exp. 5). KES-, without KES supplementation in drinking water; KES+, 4% (w/v) KES supplementation in drinking water.

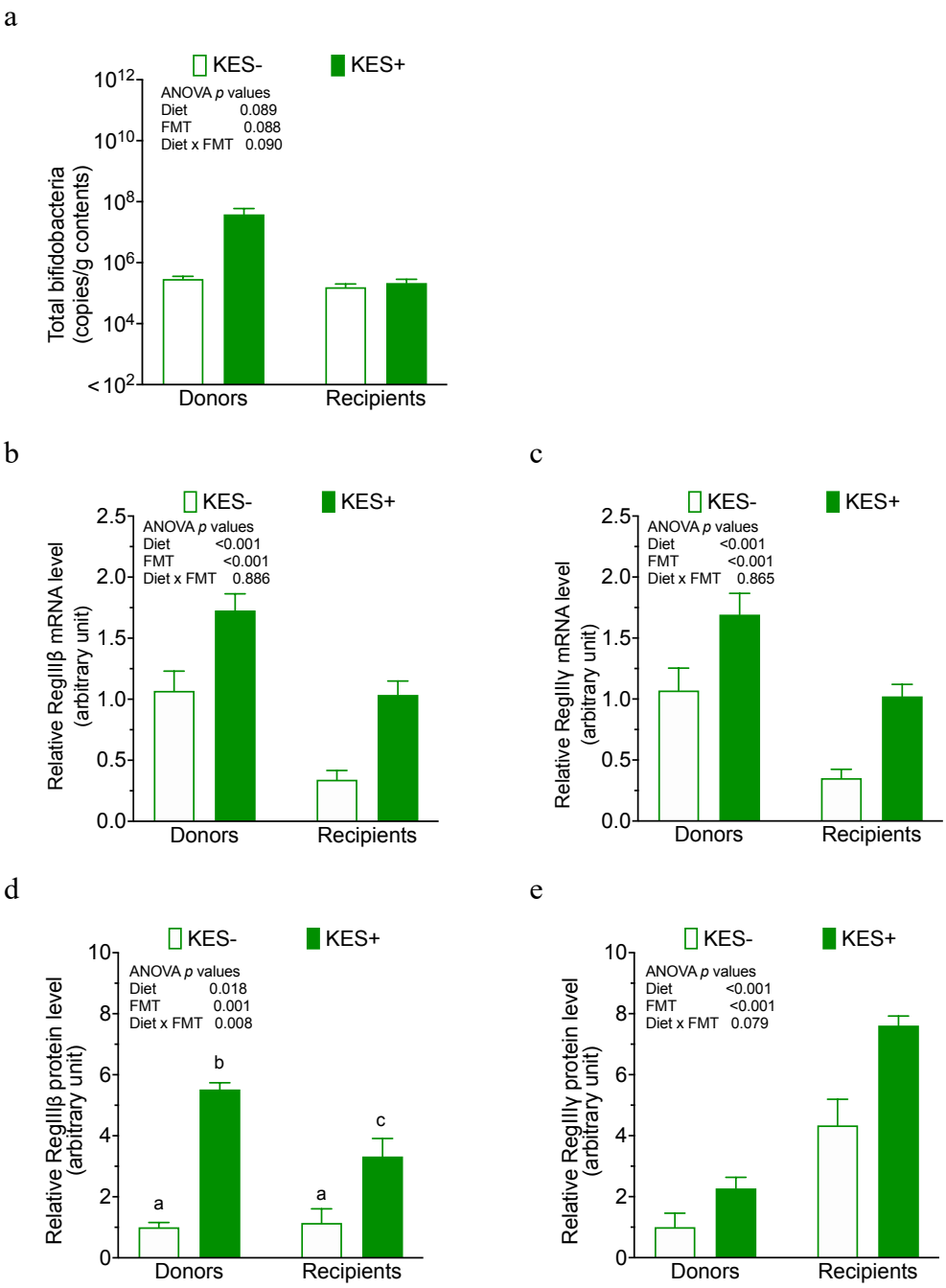


Figure II-5 Effect of KES supplementation and FMT on the population of genus *Bifidobacterium* in the ileal contents (a), ileal RegIIIβ and RegIIIγ mRNA levels (b and c, respectively), and serum RegIIIβ and RegIIIγ levels (d and e, respectively). Data are expressed as means ± SEM (n=6 in each group). KES-, without KES supplementation in the drinking water; KES+, 4% (w/v) KES supplementation in the drinking water. Two-way ANOVA was used to compare mean values between four groups, and *p* values for the effect of KES (Diet), FMT, and interaction effect of Diet x FMT are shown. In chart d, values not sharing the same letters are significantly different ($p < 0.05$).

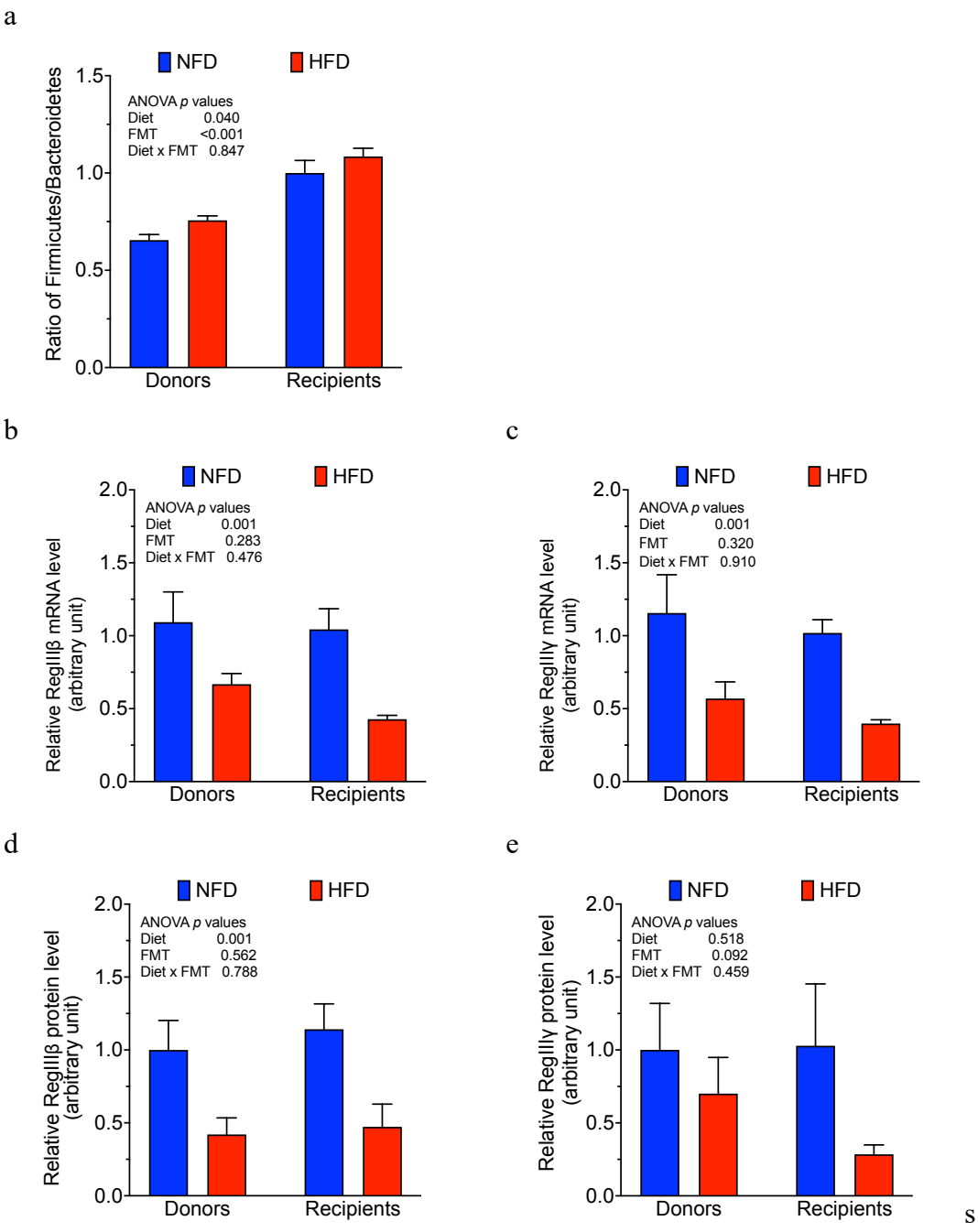


Figure II-6 Effect of HFD feeding and FMT on the Firmicutes/Bacteroidetes ratio in the ileal contents (a), ileal RegIIIβ and RegIIIγ mRNA levels (b and c, respectively), and serum RegIIIβ and RegIIIγ levels (d and e, respectively). Data are expressed as means ± SEM (n=6 in each group). Two-way ANOVA was used to compare mean values between four groups, and *p* values for the effect of HFD (Diet), FMT, and interaction effect of Diet x FMT are shown.

Table II-1 Primers used in RT-qPCR

Primer name	**Primer sequence (5′-3′)**
***Bifidobacterium* spp.**	
allbif-f	TCGCGTCYGGTGTGAAAG
allbif-r	CCACATCCAGCTCCAC
(Vermeiren *et al*., 2012)	
Phylum Firmicutes	
FirmicuteF	GTCAGCTCGTGTCGTGA
FirmicuteR	CCATTGTAKYACGTGTGT
(Bode *et al*., 2013)	
Phylum Bacteroidetes	
BacteroidF	AGCAGCCGCGGTAAT
BacteroidR	CTAHGCATTTCACCGCTA
(Edwards *et al*., 2007)	
Reg3b	
Mus-PAP/RegIIIB-F	ACAGACAAGATGCTGCCTCC
Mus-PAP/RegIIIB-R	GAGCCCTTGGGGCAACTAAT
(Udomsopagit *et al*., 2020)	
Reg3g	
Mus-RegIIIG-F	AGCCACAAGCAAGATCCCAA
Mus-RegIIIG-R	GGCCATAGTGCACACAGAGT
(Zhu *et al*., 2020)	
Actb	
Mus-b-actin-F	CTGGGACGATATGGAGAAGA
Mus-b-actin-R	AGAGGCATACAGGGACAACA
(Han *et al*., 2009)	

Chapter III
Gut microbes regulating intestinal RegIIIβ

Introduction

Study in Chapter II described the FMT experiments to show the involvement of gut microbiota in diet-induced changes in the intestinal expression of *Reg3b* and *Reg3g* genes. The findings suggested that KES- and HFD-induced changes in the ileal expression of *Reg3b* and *Reg3g* genes are mediated, at least in part, by gut microbiota. This study showed that the KES supplementation promoted the growth of *Bifidobacterium* spp. and HFD feeding increased the ratio of Firmicutes/Bacteroidetes. However, the bacteria involved in the regulation of intestinal expression of *Reg3b* and *Reg3g* genes remain to be identified. This chapter aimed to narrow down from numerous groups of intestinal bacteria to the specific bacterial group that contributes to the ileal expression of *Reg3b* and *Reg3g* genes.

Leptin is a polypeptide secreted by white adipocytes and responsible for decreasing food intake and inducing energy expenditure (Pan *et al*., 2014). A previous study showed that the expression of *Reg3b* gene was downregulated in the intestine by administration of leptin in leptin-deficient *ob/ob* mice (Waelput *et al.*, 2000). Comparing with lean wild type (+/+) mice, obese *ob/ob* mice showed lower Bacteroidetes and higher Firmicutes (Ley *et al*., 2005). Similar composition of these dominant bacterial phyla were observed in obese human (Ley *et al*., 2006; Turnbaugh *et al*., 2009). In addition to mice fed KES and HFD, therefore, *ob/ob* mice, leptin receptor-deficient *db/db* mice, and wild type (+/+) mice were subjected to the analysis for exploring the specific bacteria associated with the intestinal expression of *Reg3b* gene.

In this chapter, the structure of gut microbiota was analyzed by 16S rRNA gene sequencing. Correlation between gut microbiota data and the ileal mRNA level of *Reg3b* gene were then examined in order to explore the specific bacterial group for *Reg3b* regulation.

Materials and Methods

Animals

This study was approved by Animal Use Committee of the Hokkaido University (approval No. 14-0028 and 19-0017), and the animals were maintained in accordance with the guidelines for the care and use of laboratory animals of Hokkaido University. Five-week-old male C57BL/6JJmsSlc mice, 5-week-old male C57BL/6JHam-*ob/ob* (*ob/ob*) mice, 7-week-old male C57BL/6JHam +/+ (+/+) mice from Japan SLC and 5-week-old male C57BLKS/J Iar-+Lepr^{db}/+ Lepr^{db} (*db/db*) mice from IAR (Ibaraki, Japan) were housed in temperature-controlled (23 ± 2˚C) room with 12-h dark-light cycle and allowed free access to each test diet and tap water.

Experimental design

After 1-week acclimatization, +/+, *ob/ob* and *db/db* mice (n=3 in each group) were maintained with chow diet (MF, Oriental Yeast, Tokyo, Japan) and tap water *ad libitum* for 4 weeks before euthanasia. For KES administration, C57BL/6J mice were divided into 2 groups (n=3 in each group) and fed AIN-93G purified diet (Research Diet) and tap water with or without 4% (w/v) KES supplementation (B Food Science Co., Ltd.) *ad libitum* for 2 weeks. On the last day of the experimental period, mice were anesthetized with sevoflurane inhalation and then euthanatized by cervical dislocation. After a laparotomy, cecum was excised, and contents were collected and stored at -20˚C for DNA isolation followed by gut microbiota analysis. The ileal mucosa for total RNA isolation was collected as described in Chapter II.

DNA isolation and 16S rRNA gene sequencing

Bacterial genomic DNA was isolated from cecal contents using a QIAamp Fast DNA Stool Mini Kit according to the manufacturer's instructions. The V3-V4 regions of 16S rRNA gene were amplified by PCR using a universal primer set with Illumina overhang adaptor sequences (341F, 5′-TCGTCGGCAGCGTCAGATGTG

TATAAGAGACAGCCTACGGGNGGCWGCAG -3′; 805R, 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTAT CTAATCC-3′). PCR reaction was conducted with a total 50 μL that contains 2 ng/ml DNA, 0.3 pmol/ml each primer, 0.4 mM dNTPs, 0.01 U/ml KOD Fx Neo DNA polymerase (Toyobo), and 1 x KOD Fx Neo PCR buffer under the following

conditions: initial denaturation at 94°C for 2 min, followed by 25 cycles at 98°C for 10 s, 55°C for 30 s, and 68°C for 30 s, with final extension at 68°C for 7 min. The amplicons were purified with AMPure XP Beads (Beckman Coulter, Indianapolis, IN, USA), and the concentration and integrity of the purified samples were assessed using Nanodrop (Thermo Fisher Scientific, San Jose, CA, USA) and 1.5% agarose gel electrophoresis, respectively. A second PCR to attach barcode indices and adapters using the Herculase II Fusion DNA Polymerase Nextera XT Index Kit V2 (Illumina, San Diego, CA) and sequencing through 300 bp paired-end sequencing on an Illumina MiSeq platform were conducted at Macrogen Japan (Kyoto, Japan).

Analysis of gut microbiota using 16S rRNA gene sequencing data

Sequence data analyses were performed with the QIIME2 platform (version 2022.2, Boylen *et al.,* 2019). Raw sequence data were demultiplexed, followed by primers cutting. Sequences were then qualified through filtering, denoising, merging, and chimera removal using the DADA2 pipeline (Callahan *et al.,* 2016). A taxonomic classification was assigned to amplicon sequence variants (ASV) using the SILVA release 132 with taxonomic classification at >99% confidence (Quast *et al.,* 2013). α-diversity indices including the number of observed ASV, Chao1 index, and Shannon index were calculated with QIIME2 scripts. β-diversity was estimated using the un-weighted and weighted UniFrac metrics to calculate the distances betwen the samples and visualized by principal coordinate analysis (PCoA) plots using the ggplot2 package in RStudio software (version 2021.09.2+382, RStudio, Boston, MA, USA) for R (version 4.1.2), and was statistically examined using permutational analysis of variance (PERMANOVA). α- and β-diversities were analyzed by rarefying the feature table at a consistent sample depth of 10,000.

RNA isolation and analysis

Total RNA was isolated from ileal mucosa and subjected to reverse transcription followed by RT-qPCR as described in chapter II.

Statistical analysis

All results were expressed as mean ± SEM. To compare the mean values of 5 groups, Kruskal-Wallis test followed by Dunn's *post hoc* multiple comparison test

was used. Correlations between parameters were assessed by Spearman's correlation test. Data were analyzed using GraphPad Prism for Macintosh (version 8). *P* values of <0.05 were considered to be statistically significant.

Results

Ileal mRNA levels of Reg3b *gene in mice supplemented with and without KES,* +/+, ob/ob, *and* db/db *mice*

The mRNA levels of *Reg3b* gene in the ileum were significantly higher in *ob/ob* mice than in mice supplemented without KES (Figure III-1). The mRNA levels in mice supplemented with KES, +/+, and *db/db* mice did not differ from mice supplemented with KES and *ob/ob* mice. Although there was no significant difference, mice supplemented with KES tended to show higher *Reg3b* mRNA levels as compared with mice supplemented without KES, which is similar to the observation in chapter II.

Gut microbiota structure in mice supplemented with and without KES, +/+, ob/ob, *and* db/db *mice*

Microbial structure in the cecal contents was analyzed by using 16S rRNA gene sequencing. Rarefaction curve based on the number of observed ASV indicated that 10,000 sequences per sample are sufficient for capturing the α-diversity of microbiota (Figure III-2a). Chao1 and Shannon's indices (Figure III-2b and III-2c, respectively) showed significantly higher species richness and evenness, respectively, in *db/db* mice than in mice supplemented without KES. These indices in mice supplemented with KES, +/+, and *ob/ob* mice did not differ from mice supplemented with KES and *db/db* mice. Although there was no significant difference, +/+, *ob/ob*, and *db/db* mice tended to show higher Chao1 and Shannon's indices compared with mice supplemented with and without KES.

This study compared the β-diversity of microbiota among groups based on the PCoA with different statistical metrics, unweighted Unifrac distance, a phylogenetic metric considering the presence or absence of taxa, and weighted Unifrac distance, a phylogenetic metric accounting for abundance of observed taxa (Figure III-3a and III-3b, respectively). By determining the overall differences using PERMANOVA followed by pairwise test, no significant difference was observed in

both unweighted and weighted Unifrac distances (q-value = 0.122 and 0.113, respectively) among groups. At the phylum level, the relative abundance of Firmicutes and Bacteroidota was the most predominant taxa in all the mice (Figure III-3c). In mice supplemented without KES, the next two predominant taxa were Desulfobacterota and Deferribacterota, whereas other mice had smaller abundance of Desulfobacterota.

Correlation between ileal **Reg3b** *mRNA level and relative abundance of cecal bacteria*

The correlation between ileal *Reg3b* mRNA level and relative abundance of cecal bacterial taxa at the genus level was calculated manually, and top 10 genera showing positive and negative correlations are shown in Figure III-4a. The abundance of the family Ruminococcaceae_Incertae_Sedis, family Clostridia_UCG-014, and genus *Alloprevotella* showed significant positive correlation with ileal *Reg3b* mRNA level (Figure III-4b, III-4c, and III-4d, respectively).

Discussion

This study intended to explore gut bacterial taxa involved in the regulation of intestinal expression of *Reg3b* gene by comparing mice with different dietary conditions and leptin status. Significant difference in the mRNA level of *Reg3b* in the ileum was observed only between mice without KES supplementation and *ob/ob* mice (Figure III-1), which might be due to the limited replications of mice (n=3 in each group). The ileal *Reg3b* mRNA level tended to be higher in mice supplemented with KES than in mice supplemented without KES, being consistent with the observations in chapter II. Waelput *et al.* (2000) showed that leptin administration reduced the intestinal expression of *Reg3b* gene in leptin-deficient *ob/ob* mice, suggesting that leptin reduces the intestinal expression of *Reg3b*. In other words, leptin deficiency would be associated with higher expression of RegIIIβ in the intestine. Indeed, this study showed that *ob/ob* mice showed the highest expression level of *Reg3b* gene among the groups. Although no previous study showed the effect of KES supplementation on the circulating leptin, Tan *et al*. (2018) reported that dietary inulin had no influence on the circulating leptin in mice. Therefore, it could be described that findings on the *ob/ob* and *db/db* mice suggest that leptin signaling through leptin receptor is not responsible for suppressing effect of leptin on

the expression of RegIIIβ and RegIIIγ and that, indeed, the consumption of inulin, a fructan similar with KES, reportedly had no influence on the circulating leptin in mice.

Gut microbiota analysis based on the 16S rRNA gene sequences showed that Chao1 and Shannon's indices (Figure III-2) were significantly higher in *db/db* mice than in mice supplemented without KES. In addition, these two indices tended to be higher in +/+, *ob/ob*, and *db/db* mice than in mice supplemented with and without KES. The former three groups were fed the commercial chow which contains the rich and complex ingredients, such as corn, wheat bran, defatted soybean, defatted rice bran, fishmeal, defatted milk, soybean oil, and brewer's yeast. In contrast, the latter two groups were fed the AIN-93G purified diet which is formulated with refined ingredients. Therefore, diverse raw materials in the commercial chow may contribute to the higher species richness and evenness of gut microbiota (Figure III-3).

This study manually calculated the correlation coefficient between intestinal *Reg3b* mRNA level and relative abundance of cecal bacterial taxa at the genus level (Figure III-4a) and revealed that three taxa, the family Ruminococcaceae_Incertae_Sedis, family Clostridia_UCG-014, and genus *Alloprevotella*, showed significant positive correlation with the intestinal mRNA level of *Reg3b* gene (Figure III-4b, III-4c, and III-4d, respectively). Ruminococcaceae has been well known bacterial family that contains several butyrate-producing bacteria (Louis & Flint, 2009). In addition, the family Clostridia_UCG-014, previously included in the Ruminococcaceae UCG-014 genus, is linked to butyrate production (Anand *et al.* 2016). Furthermore, genus *Alloprevotella* also includes butyrate producing bacteria (Liu *et al.* 2018). From these findings, this study assumed that butyrate, a fermentation product of gut microbiota, may be a trigger of intestinal expression of *Reg3b* gene. Therefore, this possibility was examined by using intestinal organoid experiments in the next chapter.

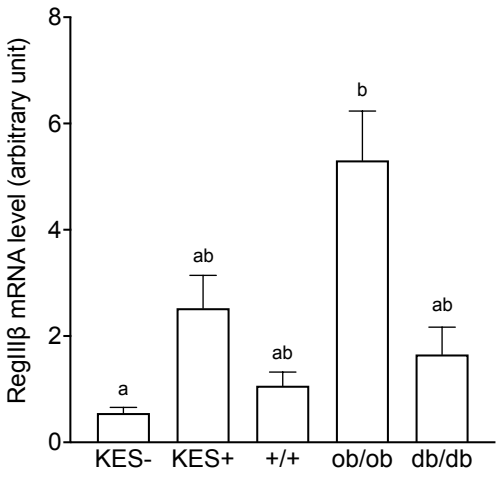


Figure III-1 Ileal mRNA levels of *Reg3* gene estimated by RT-qPCR. KES-, without KES supplementation in drinking water; KES+, 4% (w/v) KES supplementation in drinking water. Data are expressed as means ± SEM (n=3). Values not sharing common letters indicate significantly different (p<0.05).

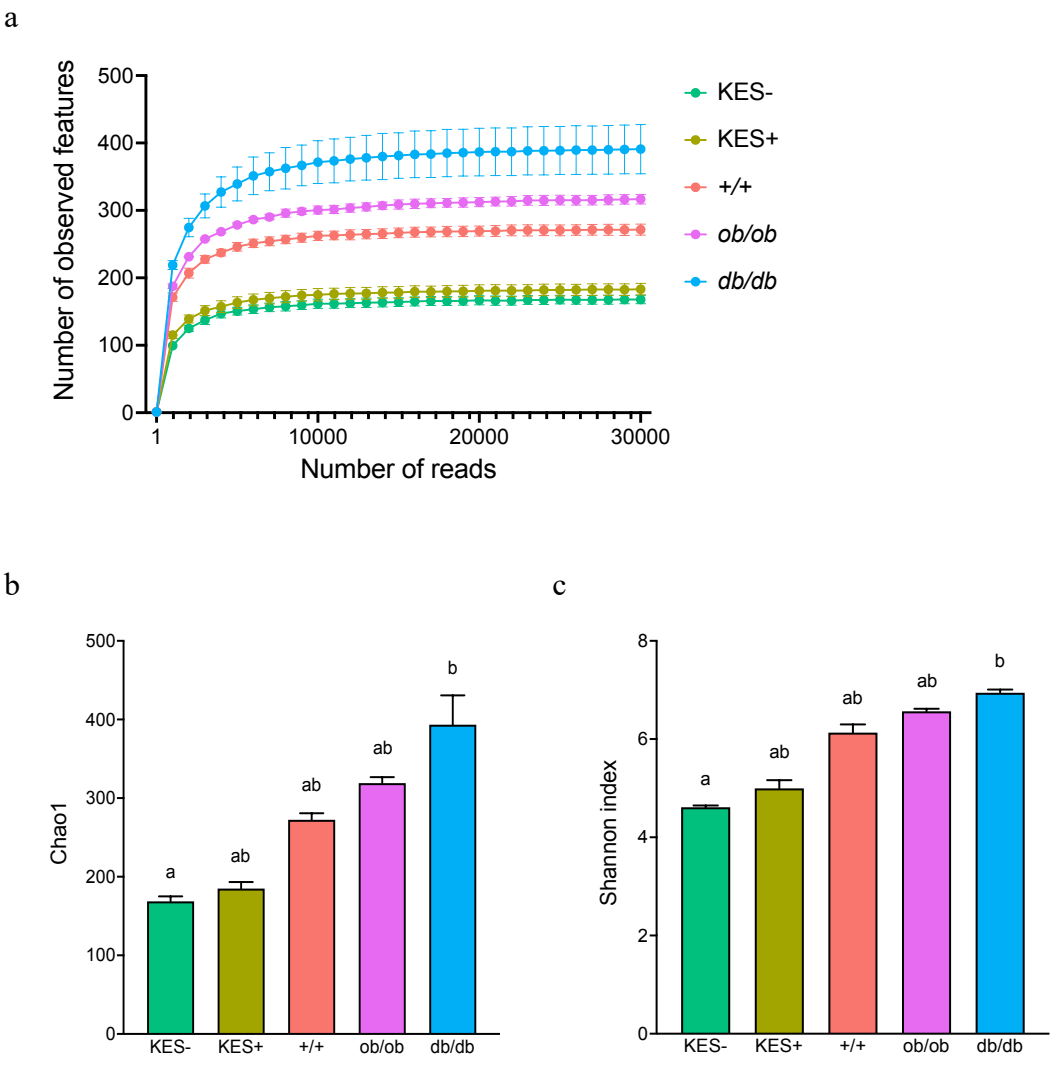


Figure III-2 Rarefaction curve and α-diversity analyses of gut microbiota in mice supplemented with and without KES (KES+ and KES-, respectively), +/+, *ob/ob*, and *db/db* mice. (a) Rarefaction curve constructed based on the number of observed ASV. (b) Chao1 richness index bar plot. (c) Shannon's evenness index bar plot. Data are presented as mean ± SEM (n=3 in each group). In charts b and c, values not sharing common letters indicate significantly different (p<0.05).

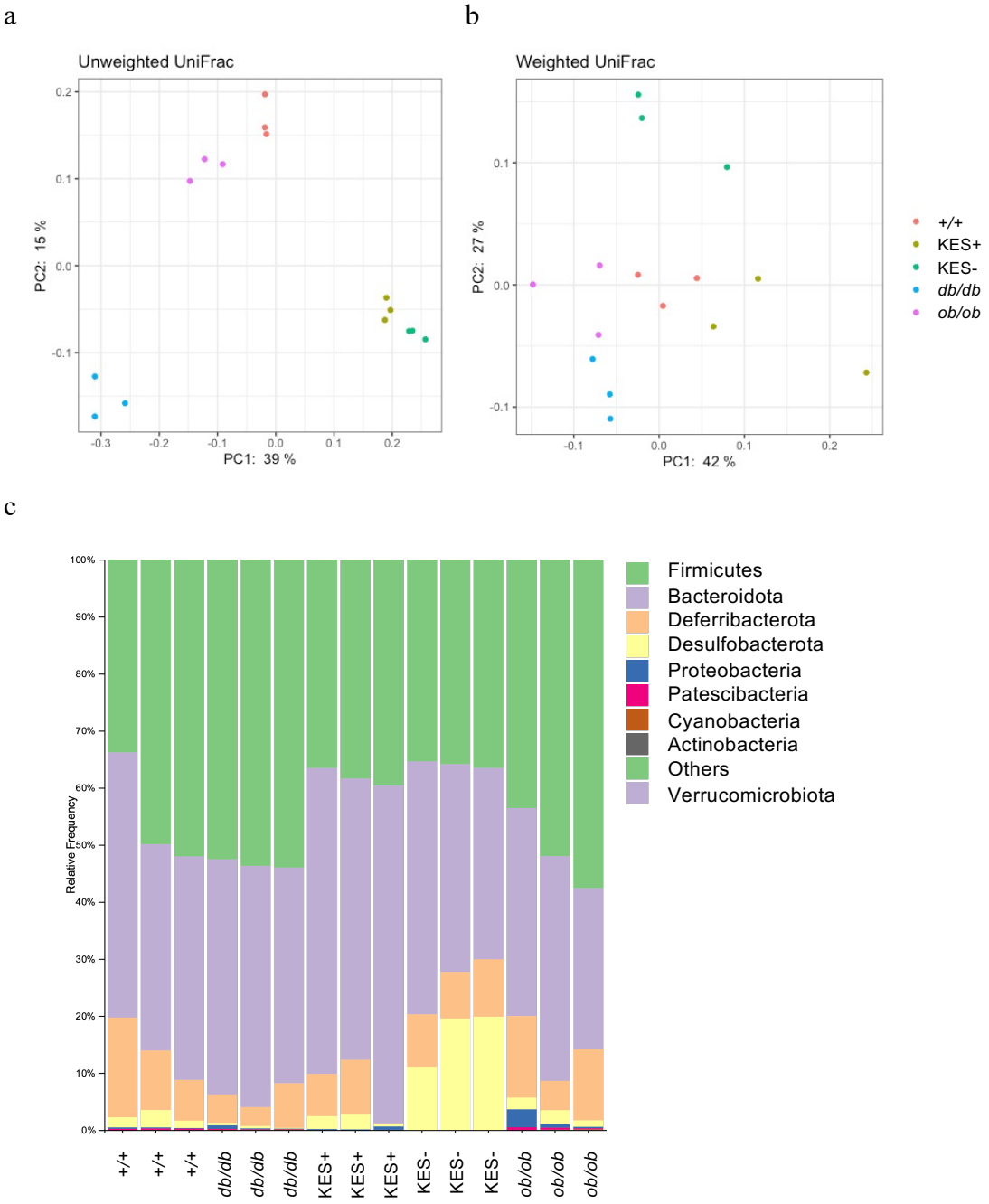


Figure III-3 β-diversity analyses of gut microbiota in mice supplemented with and without KES (KES+ and KES-, respectively), +/+, *ob/ob*, and *db/db* mice. PCoA plots of the β-diversity computed based on the (a) unweighted and (b) weighted unifrac distances. (c) Relative taxonomic abundance of cecal bacteria at the phylum level.

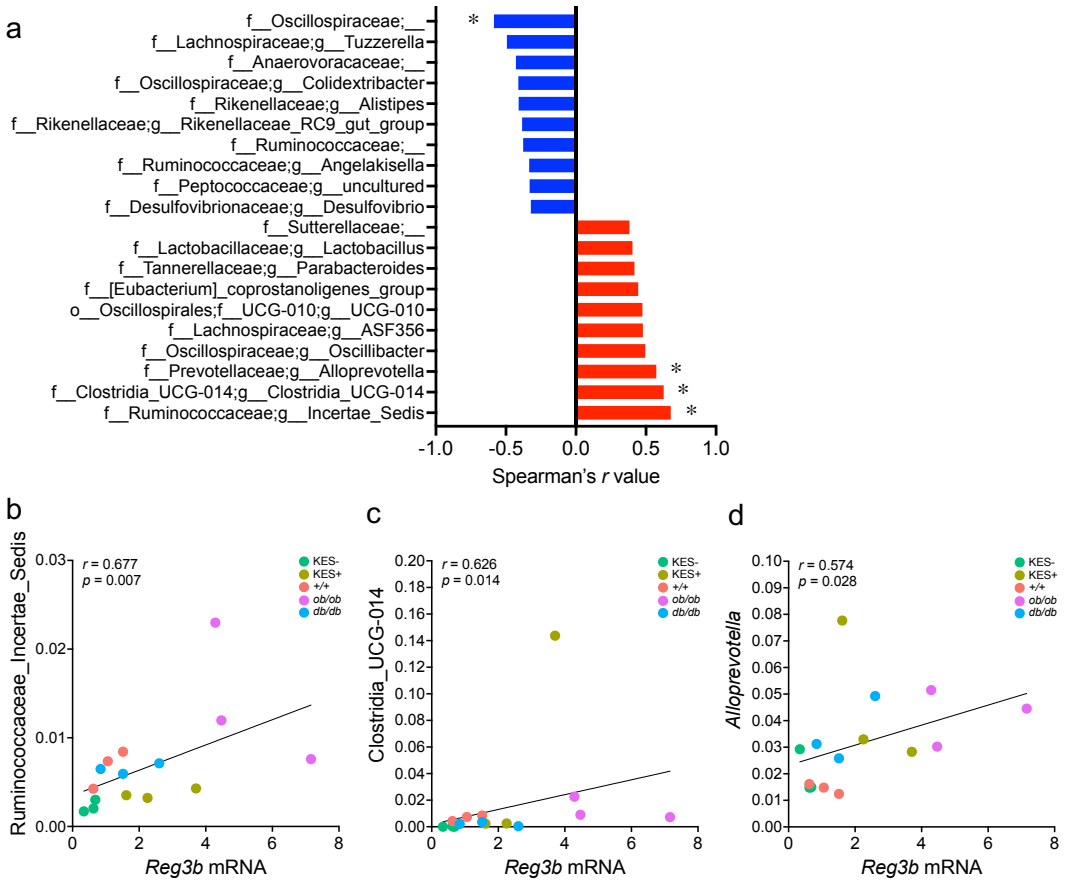


Figure III-4 Correlation between ileal *Reg3b* mRNA level and relative abundance of cecal bacterial taxa at the genus level. (a) Top 10 genera showing positive (red) and negative (blue) correlations with ileal *Reg3b* mRNA level. Asterisk shows Spearman's *p* value <0.05. (b) Correlation between ileal *Reg3b* mRNA level and relative abundance of Ruminococcaceae_Incertae_Sedis. (c) Correlation between ileal *Reg3b* mRNA level and relative abundance of Clostridia_UCG-014. (d) Correlation between ileal *Reg3b* mRNA level and relative abundance of *Alloprevotella*. KES-, without KES supplementation in drinking water; KES+, 4% (w/v) KES supplementation in drinking water.

Chapter IV

Mechanistic studies on the RegIIIβ regulation using intestinal organoids

Introduction

In the previous chapter, a significant correlation was observed between intestinal mRNA expression of *Reg3b* gene and relative abundance of the family Ruminococcaceae_Incertae_Sedis, family Clostridia_UCG-014, and genus *Alloprevotella* in the cecal contents. Because these bacterial taxa reportedly contain butyrate-producing bacteria (Anand *et al.* 2016; Liu *et al.* 2018; Louis & Flint, 2009), this study assumed that butyrate, a fermentation product of gut microbiota, may be a trigger of intestinal expression of *Reg3b* gene. In this chapter, therefore, this idea was tested by using murine intestinal organoids as an *ex vivo* model for intestinal epithelium. Concentration of SCFA in the cecal venous blood is approximately one-tenth of that in cecal contents (Nakatani *et al*., 2018). Considering the luminal concentrations of SCFA ranging from several to dozens mM, this study employed 0.1, 1 and 10 mM of SCFAs, including acetate, butyrate and propionate, in the organoid experiments.

Butyrate was previously reported as an inhibitor of histone deacetylase (HDAC) (Wu *et al.*, 2012). Epigenetic regulation by butyrate inhibition of HDAC has been thought to be one of the important molecular mechanisms by which gut microbiota influences host physiology (Fellows & Varga-Weisz, 2020; Li *et al.,* 2018). To examine whether effect of butyrate on the expression of *Reg3b* gene is due to inhibition of HDAC, this study used trichostatin A (TSA), a specific HDAC inhibitor.

IL-22, a cytokine, released from innate lymphoid cells has been considered as a regulator of intestinal RegIIIβ and RegIIIγ (Miki et al., 2018; Sanos et al., 2011). This study therefore examined in the organoids whether IL-22 is involved in the KES- and HFD-induced changes in the intestinal expression of *Reg3b* and *Reg3g* genes observed in chapter II.

Materials and Methods

Crypt isolation and culture of intestinal organoids

This study was approved by Animal Use Committee of the Hokkaido University (approval No. 14-0028 and 19-0017), and the animals were maintained in accordance with the guidelines for the care and use of laboratory animals of Hokkaido University. C57BL/6J mice were anesthetized with sevoflurane inhalation and then killed by cervical dislocation. After a laparotomy, the ileum part of small intestine was excised, and crypts were isolated and cultured to obtain the organoids as previously described (Tsuruta *et al*., 2016). In brief, the luminal contents were washed with ice-cold PBS. The tissue was opened longitudinally and washed with ice-cold PBS before cut into approximately 5-mm pieces. The tissue sections were then incubated with instant rotation in a chelating buffer (2 mM EDTA/PBS supplemented with 1 mM of DL-dithiothreitol) at 4ºC for 1 h. The tissue sections were then suspended and agitated by pipetting in a dissociation buffer (PBS supplemented with 43.4 mM sucrose and 54.9 mM of D-sorbitol). After sedimentation, supernatant was passed through a 70-μm cell strainer (BD Biosciences, San Jose, CA, USA). Crypt fraction was collected from precipitate by centrifugation at 600 rpm for 5 min and was washed by 0.1 M of gentamycin/PBS before being embedded in Matrigel (Corning, Wilmington, MA, USA) in 48-well plate (1,000 crypts/well). Culture medium composed of Advanced DMEM supplemented with 10 mM of penicillin, 10 mM of HEPES, 10 mM Glutamax (all from Invitrogen, Long Island, NY, USA), 1 mM *N*-acetylcysteine (Sigma), and B-27 Supplement (Thermo Fisher, New York, NY, USA) mixed with 50% (v/v) L-RN cell-conditioned medium containing R-spondin 3 and Noggin. L-RN cells were obtained by engineering their parental lines, L cells (ATCC CRL-2648), according to the protocol of Miyoshi & Stappenbeck (2013). For the first 3 days, culture medium was supplemented with 10 mM of human recombinant EGF (PeproTech, Rocky Hill, NJ, USA), 10 mM of CHIR 99021 (Tocris Bioscience, Bristol, UK), and 10 mM of Y-27632 (Sigma) to induce differentiation and avoid anoikis. Culture medium was changed every other day.

Organoid treatments

Differentiated organoids on day 6 were cultured in the medium supplemented with graded concentrations (0.1, 1, and 10 mM) of sodium acetate, sodium butyrate,

or sodium propionate (Wako) for 24 h. In addition, differentiated organoids on day 6 were cultured in the medium supplemented with graded concentrations of TSA (0.01, 0.05, 0.1, 0.5, and 1 μM; Sigma) for 24 h. Organoids were then incubated in cell recovery solution (Sigma) for 45 min at 4˚C followed by centrifuge at 600 g for 5 min. Collected organoids were washed with PBS and subjected to total RNA isolation.

In a separate experiment, differentiated organoids on day 6 were cultured in the medium supplemented with rIL-22 (5 ng/ ml, Biolegend, San Diego, CA, USA) for 24 h. Total RNA was then isolated as described above.

RNA isolation and analysis

Total RNA was isolated from organoids and subjected to reverse transcription followed by RT-qPCR as described in chapter II. The sequences of primers for *Il22* are as follows: IL-22F, CGATCTCTGATGGCTGTCCT; IL-22R, ACGCAAGCATTTCTCAGAGA. Total RNA samples isolated from ileum of mice supplemented with and without KES and mice fed NFD and HFD in chapter II were also subjected to *Il22* mRNA analysis.

Statistical analysis

All results were expressed as mean ± SEM. One-way ANOVA followed by Dunnett's *post hoc* test were used to compare mean values between multiple groups. Data were analyzed using GraphPad Prism for Macintosh (version 8). *P* values of <0.05 were considered to be statistically significant.

Results

Propionate and butyrate promote the expression of* Reg3b *and* Reg3g *genes in the intestinal organoids

This study successfully cultured murine intestinal organoids. Figure IV-1 shows the organoids on day 6 of culture, showing the typical structure of mature intestinal organoids, consisting of a central cyst structure and surrounding crypt-like budding structures. Graded concentrations of acetate, propionate, and butyrate were added to the culture medium of the intestinal organoids (Figure IV-2). *Reg3b* and *Reg3g* mRNA levels were not significantly changed by any concentration of acetate.

In contrast, *Reg3b* and *Reg3g* mRNA levels were significantly higher in the organoids treated with 10 mM propionate as well as 1 and 10 mM butyrate.

HDAC inhibitor TSA promotes the expression of Reg3b *and* Reg3g *genes in the intestinal organoids*

TSA was used as the HDAC inhibitor in the intestinal organoids. *Reg3b* and *Reg3g* mRNA levels were increased by TSA supplementation in a dose dependent manner, reached the peak at 0.1 μM TSA, and decreased at 0.5 and 1 μM of TSA (Figure IV-3).

IL-22 may not be responsible for HFD- and KES-induced alteration of ileal Reg3b *mRNA levels*

The supplementation of rIL-22 for 24 h increased approximately thousandfold the mRNA levels of *Reg3b* gene in mouse intestinal organoids (Figure IV-4a). However, the mRNA levels of *Il22* gene in the ileum tended to be lower (p = 0.057) in the KES-supplemented mice than in the mice without supplementation (Figure IV-4b) and were the same between the NFD-fed mice and HFD-fed mice (Figure IV-4c).

Discussion

As expected, the mRNA levels of *Reg3b* and *Reg3g* genes were increased by supplementation of butyrate in the intestinal organoids (Figure IV-2). In addition, propionate but not acetate increased the mRNA levels. Recently, Pearce *et al.* (2020) reported that 24-h culture of murine intestinal organoids with 5 mM propionate and butyrate, but not acetate, promote the expression of *Reg3b* and *Reg3g* genes, which is in line with this study.

Similar to butyrate (Wu *et al.*, 2012), propionate has also been reported to be an HDAC inhibitor (Thangaraju *et al.,* 2006). This study therefore assumed that butyrate and propionate may promote the intestinal expression of *Reg3b* and *Reg3g* genes through HDAC inhibition. To test this idea, an HDAC inhibitor TSA was added to the culture medium of the organoids. As expected, TSA supplementation promoted the expression of *Reg3b* and *Reg3g* genes (Figure IV-3), supporting the hypothesis described above. Therefore, it is likely that propionate and butyrate

promote the expression of *Reg3b* and *Reg3g* genes, at least in part, *via* HDAC inhibition in the intestinal organoids.

Other molecular mechanisms for propionate and butyrate stimulation of *Reg3b* and *Reg3g* genes should be considered. Short-chain fatty acids (SCFAs) including propionate and butyrate are known to serve as signaling molecules between the gut microbiota and host through G protein-coupled receptor (GPR) 41, GPR43, and GPR109a that belong to the free-fatty acid receptor family (Brown *et al.,* 2003; Thangaraju *et al.,* 2009). In addition, these receptors are reportedly expressed in the intestine (Dass *et al*., 2007; Duca *et al*., 2012). Therefore, one may speculate that propionate and butyrate could promote the intestinal expression of *Reg3b* and *Reg3g* genes *via* these receptors. Indeed, Zhao *et al.* (2018) showed that genetic deletion of GPR43 decreased the intestinal *Reg3g* expression in mice. They also found that butyrate supplementation induced *Reg3g* expression in mouse intestine and intestinal organoids in a GPR43-dependent manner. Bajic *et al.* (2020) observed that both GPR43 gene knockout mice and GPR109a gene knockout mice showed reduced expression of *Reg3b* gene in the cecum. They also showed that 1 mM of propionate supplementation increased the expression of *Reg3b* and *Reg3g* genes in the intestinal organoids cultured from wild type mice, but not GPR43 gene knockout mice. Furthermore, they showed that GF mice supplemented with 150 mM of propionate in drinking water showed the increased expression of *Reg3b* and *Reg3g* genes in the intestine. From these findings, it was suggested that butyrate- and propionate-induced expression of *Reg3b* and *Reg3g* depends on the signaling through GPR43 and GPR109a.

IL-22 released from innate lymphoid cells has been considered as a regulator of intestinal *Reg3b* and *Reg3g* (Miki *et al.* 2018; Sanos *et al.* 2011). Indeed, this study showed a marked increase of *Reg3b* gene expression by rIL-22 supplementation in the murine intestinal organoids. Expression of *Il22* gene in the colon was reportedly reduced by HFD feeding (Gulhane *et al.* 2016; Zou *et al.* 2018) and increased by the supplementation with an indigestible fructan inulin (Zou *et al.* 2018); thus, these raise the possibility that the KES-induced increase and HFD-induced reduction of the mRNA expression of *Reg3b* and *Reg3g* genes would be mediated by IL-22. In this study, however, neither KES supplementation nor HFD feeding influenced the expression of *Il22* gene in the ileum. Thus, it is unlikely that IL-22 is involved in the KES- and HFD-induced changes in the ileal expression of

Reg3b and *Reg3g* genes. In addition, other lymphoid organs, such as lymph nodes and spleen, are also the source of IL-22 (Sanos *et al*., 2011). It is also possible that IL-22 secreted from other organs might affect the *Reg3b* and *Reg3g* genes *in vivo*.

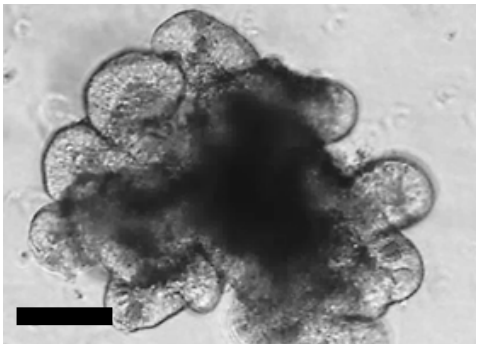


Figure IV-1 A representative light-microscopic visualization of murine intestinal organoids. Mature organoids were obtained after incubating intestinal crypts for 6 days. Scale bars indicate 100 μm.

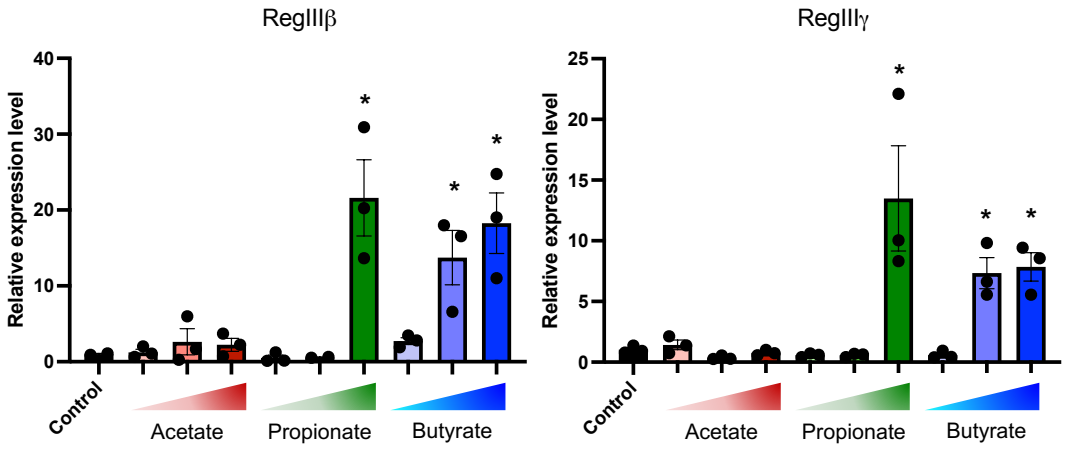


Figure IV-2 Effect of graded concentrations of acetate, propionate, and butyrate (0.1, 1, and 10 mM) on *Reg3b* (left) and *Reg3g* (right) mRNA levels in murine intestinal organoids. Data are expressed as means ± SEM. *, $p < 0.05$ vs. control by one-way ANOVA followed by Dunnett's *post hoc* test.

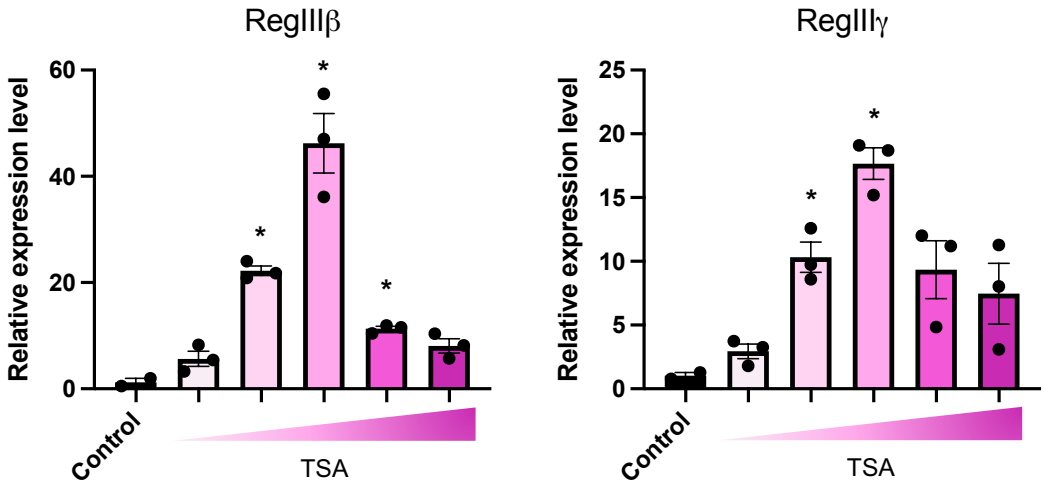


Figure IV-3 Effect of graded concentrations of TSA (0.01, 0.05, 0.1, 0.5, and 1 μM) on *Reg3b* (left) and *Reg3g* (right) mRNA levels in murine intestinal organoids. Data are expressed as means ± SEM. *, $p < 0.05$ vs. control by one-way ANOVA followed by Dunnett's *post hoc* test.

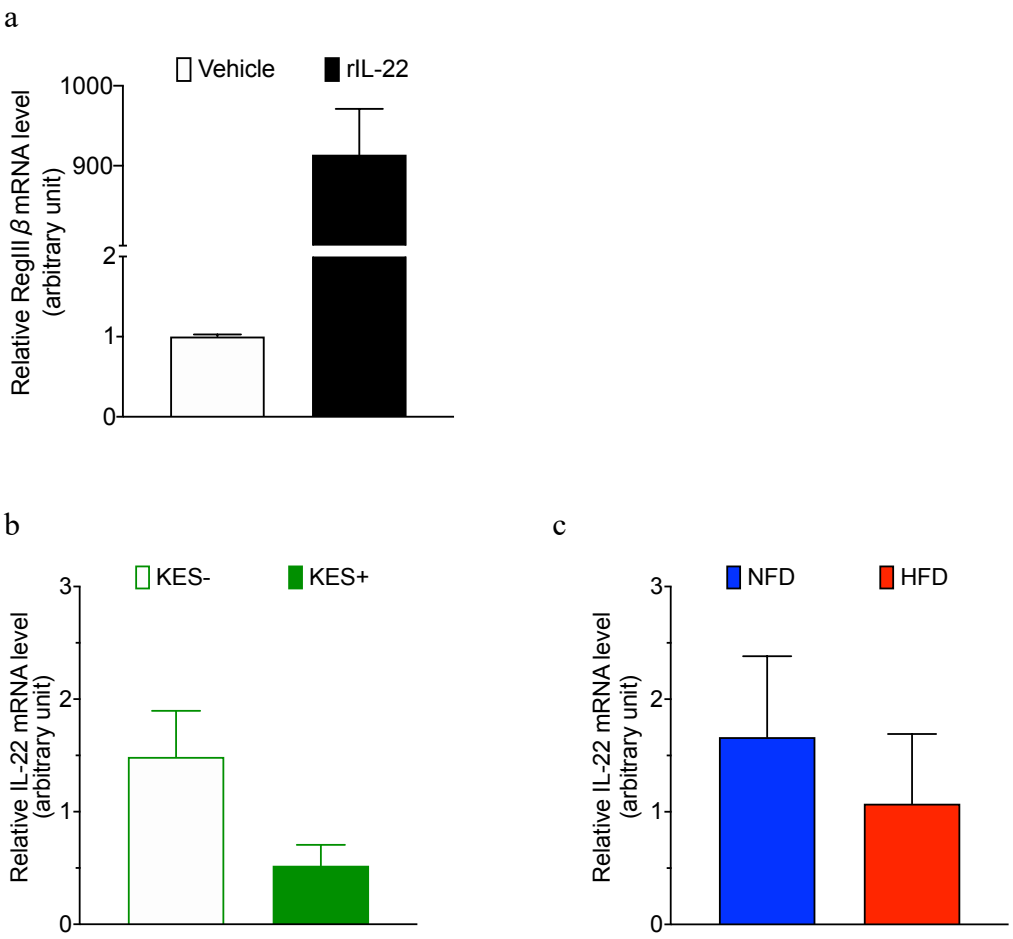


Figure IV-4 Relationship between IL-22 and *Reg3b* gene. In chart a, the effect of rIL-22 supplementation on *Reg3b* mRNA expression in murine intestinal organoids is shown. The effect of KES supplementation and HFD on the ileal mRNA expression of *Il22* gene in C57BL/6J mice are shown in charts b and c, respectively. Data are expressed as means ± SEM (n=6 in each group). KES-, without KES supplementation in drinking water; KES+, 4% (w/v) KES supplementation in drinking water.

Chapter V
General discussion

This study aimed to elucidate the role of gut microbiota in the regulation of intestinal expression of antimicrobial peptides RegIIIβ and RegIIIγ. Initially, this study showed that ABX-treatment reduced the intestinal expression of *Reg3b* and *Reg3g* genes. These data are consistent with previous studies (Liang *et al.*, 2019; Wu *et al*., 2014) and suggest that gut microbiota is required for the intestinal expression of *Reg3b* and *Reg3g* genes. Next, this study confirmed the previous findings that the intestinal expression of *Reg3g* genes was reduced by consumption of HFD and increased by supplementation of indigestible saccharides (Everard *et al.,* 2014). FMT experiments in this study showed that the intestinal expression of *Reg3b* and *Reg3g* genes was lower in mice receiving FMT from HFD-fed mice than in those receiving FMT from NFD-fed mice and higher in mice receiving FMT from KES-supplemented mice than in those receiving FMT from mice without KES supplementation, suggesting that HFD- and KES-induced changes in the intestinal expression of *Reg3b* and *Reg3g* genes are mediated, at least in part, by gut microbiota. Comprehensive analysis of gut microbiota by using 16S rRNA gene sequencing indicated that butyrate-producing bacteria, i.e., the family Ruminococcaceae_Incertae_Sedis, family Clostridia_UCG-014, and genus *Alloprevotella* are possibly related to the regulation of intestinal expression of *Reg3b* gene. Furthermore, organoid experiments in this study suggested that butyrate and propionate upregulate the expression of *Reg3b* and *Reg3g* genes, at least in part, *via* HDAC inhibition. Together, this study described the crucial role of gut microbiota in diet-induced changes in the intestinal expression of *Reg3b* and *Reg3g* genes.

Discussions of the limitations of this study is warranted. First, this study observed a significant positive correlation between the abundance of the family Ruminococcaceae_Incertae_Sedis, family Clostridia_UCG-014, and genus *Alloprevotella* and the intestinal mRNA level of *Reg3b* gene. From this finding, this study proposed that these bacterial groups are involved in the regulation of intestinal expression of *Reg3b* gene. However, the causal relationship of these bacterial groups and the intestinal expression of *Reg3b* gene remains to be shown. To do this, the family Ruminococcaceae_Incertae_Sedis, family Clostridia_UCG-014, and genus

Alloprevotella should be cultured in the intestinal-mimic conditioned medium in order to observe the production of butyrate and propionate by these bacteria. Furthermore, it should be required to show the effect of oral administration of these bacteria on the intestinal expression of *Reg3b* gene. Recently, Ahn *et al.* (2022) demonstrated that oral administration of *Ruminococcus gnavus* ameliorated the ovalbumin-induced allergic skin inflammation in ABX-treated mice. The authors described that these effects are mediated by the enhancement of SCFA production. It is possible that this mechanism is related to the function of RegIIIβ.

Second, this study employed the intestinal organoids to show that HDAC inhibition upregulates the expression of *Reg3b* and *Reg3g* genes, whereas this should be demonstrated *in vivo*. In fact, previous studies have shown that the administration of TSA in murine models attenuated asthma (Choi *et al.,* 2005), colitis (Glauben *et al.,* 2006), and multiple sclerosis (Jayaraman *et al.,* 2017). Therefore, it could be possible to test whether TSA administration increases the intestinal expression of *Reg3b* and *Reg3g* genes.

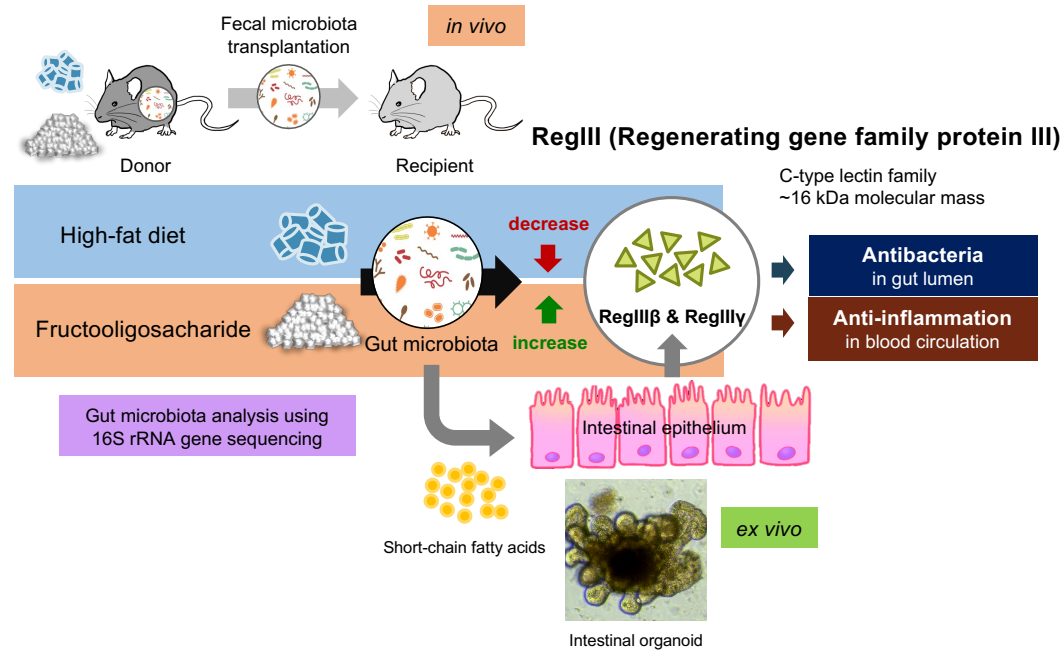
Third, gut microbiota-independent regulation of *Reg3b* and *Reg3g* genes should be considered. Although the FMT experiments in this study suggested the gut microbiota-dependent regulation of *Reg3b* and *Reg3g* genes, this does not necessarily deny the possibility of gut microbiota-independent regulation. To test this possibility, it would be useful to examine the effect of KES supplementation and HFD feeding on the intestinal expression of *Reg3b* and *Reg3g* genes in GF mice or ABX-treated mice. Alternatively, the intestinal organoids may be incubated with KES and free-fatty acids.

Fourth, SCFA-independent regulation of *Reg3b* and *Reg3g* genes should also be considered. Natividad *et al.* (2013) reported that monocolonization with the probiotic *Bifidobacterium breve*, but not with the nonprobiotic commensal *Escherichia coli* JM83, upregulated *Reg3g* expression in GF mice. They showed that *B. breve*-induced *Reg3g* expression was abrogated in mice lacking MyD88/Ticam1 signaling. In addition, both live and heat-inactivated *B. breve* but not spent culture medium induced the expression of *Reg3g* gene. Kobayashi *et al.* (2022) demonstrated that membrane vesicles released from *Clostridium* spp. promoted the expression of *Reg3b* and *Reg3g* genes *via* MyD88/TRIF signaling in primary cultured mouse small intestinal epithelial cells. These findings suggest that SCFA-

independent mechanisms through bacterial cellular components and released membrane vesicles are involved in the regulation of *Reg3b* and *Reg3g* genes.

As described above, a number of issues remain to be solved for elucidating the role of gut microbiota and also diet in the regulation of intestinal expression of antimicrobial peptides RegIIIβ and RegIIIγ. Nevertheless, this study proposes a novel mechanism that butyrate and propionate, fermentation products of gut microbiota, regulate the expression of *Reg3b* and *Reg3g* genes *via* HDAC inhibition. Considering that RegIIIβ and RegIIIγ exert barrier function against pathogenic bacteria in the intestine, the present findings shed light on the novel strategy to control the intestinal infectious diseases.

a



b

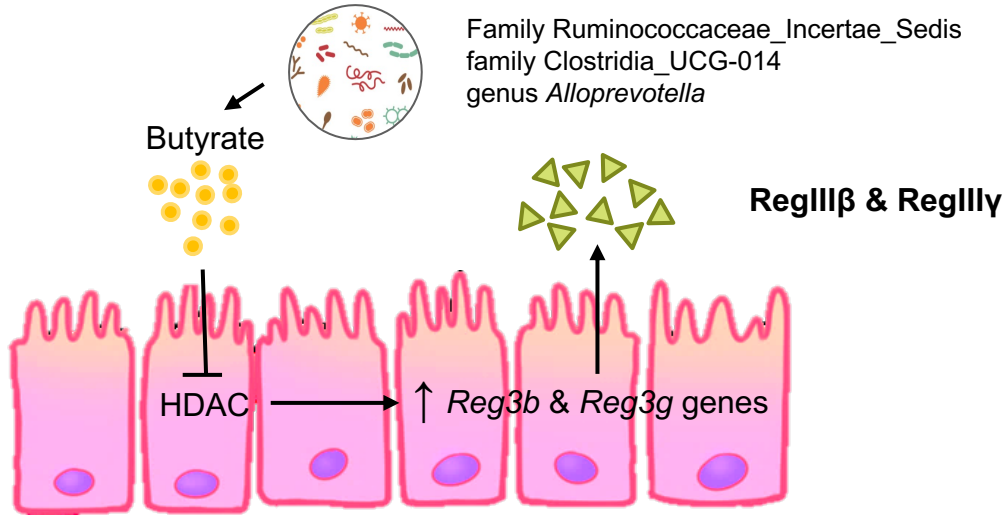


Figure V-1 The overview of this study (a) and the regulation mechanism of intestinal RegIIIβ and RegIIIγ proposed in this study (b).

Chapter VI

Summary

RegIIIβ and RegIIIγ are C-type lectins expressed in the intestinal epithelial cells. These lectins are secreted into the intestinal lumen and exert bactericidal action whereby intestinal infection is prevented. The intestinal expression of RegIIIβ and RegIIIγ is influenced by both gut microbiota and diet. Because consumption of high-fat diet (HFD) and indigestible saccharides including fructooligosaccharide (FOS) alter both the intestinal expression of RegIIIβ and RegIIIγ and the composition of gut microbiota, this study assumed that gut microbiota may mediate diet-induced changes in the intestinal expression of RegIIIβ and RegIIIγ. Therefore, this study tested this idea and investigated underlying molecular mechanism.

1. Role of gut microbiota in dietary regulation of RegIIIβ and RegIIIγ

This study tested whether changes in the intestinal expression of RegIIIβ and RegIIIγ by supplementation with 1-kestose (KES), a kind of FOS, and by consumption of HFD are mediated by gut microbiota. To do this, this study employed fecal microbiota transplantation (FMT); thus, antibiotics-treated mice were received FMT from mice supplemented with and without KES and from mice fed HFD and normal-fat diet (NFD). Intestinal mRNA levels of *Reg3b* and *Reg3g* genes were lower in mice receiving FMT from HFD-fed mice than in those receiving FMT from NFD-fed mice and higher in mice receiving FMT from KES-supplemented mice than in those receiving FMT from mice without KES supplementation. These results suggest that HFD- and KES-induced changes in the intestinal expression of *Reg3b* and *Reg3g* genes are mediated, at least in part, by gut microbiota.

2. Gut microbes regulating intestinal RegIIIβ

This study explored the specific bacteria responsible for intestinal RegIIIβ regulation. Leptin, an adipocyte hormone, signals nutritional status to the central nervous system and peripheral organs. Because leptin status is reportedly associated with the intestinal expression of *Reg3b*, leptin deficient *ob/ob* mice, leptin receptor-deficient *db/db* mice, and wild type (+/+) mice were included in analysis. The

composition of gut microbiota in mice supplemented with and without KES, *ob/ob* mice, *db/db* mice, and +/+ mice was analyzed by 16S rRNA gene sequencing. The data showed that the abundance of the family Ruminococcaceae_Incertae_Sedis, family Clostridia_UCG-014, and genus *Alloprevotella* is positively correlated with the intestinal mRNA level of *Reg3b*. Because these bacterial groups contain several butyrate-producing bacteria, this study assumed that butyrate, a fermentation product of gut microbiota, may be a trigger of intestinal expression of *Reg3b* gene.

3. Mechanistic studies on the RegIIIβ regulation using intestinal organoids

Murine intestinal organoids were used as an *ex vivo* model for intestinal epithelium. The mRNA levels of *Reg3b* and *Reg3g* genes were increased by supplementation of butyrate and another fermentation product propionate in the intestinal organoids. Butyrate and propionate regulate the expression of several genes by inhibiting histone deacetylase (HDAC). Supplementation of trichostatin A, an HDAC inhibitor, increased the expression of *Reg3b* and *Reg3g* genes in the organoids, suggesting that butyrate and propionate upregulate the intestinal expression of *Reg3b* and *Reg3g* genes through HDAC inhibition.

Overall, this study presented the crucial role of gut microbiota in diet-induced changes in the intestinal expression of antibacterial peptides, RegIIIβ and RegIIIγ. Mechanistically, this study proposes that butyrate and propionate, fermentation products of gut microbiota, regulate the expression of *Reg3b* and *Reg3g* genes through HDAC inhibition. The present findings therefore shed light on the novel strategy to control the intestinal infectious diseases.

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