



Short Communication

Typical gut indigenous bacteria in ICR mice fed a soy protein-based normal or low-protein diet



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ABSTRACT

For patients with inflammatory bowel disease, cow's milk allergy, and lactose intolerance, soymilk is a potential alternative to cow's milk. In this study, we aimed to identify the effects of a soy protein-based low-protein diet on the body and organ weights and the gut microbiome of six-week-old mice fed a diet containing 20% (SP) or 5% (LP) soy protein for 14 days via 16S rRNA (V4) amplicon sequencing. Body weight gain (growth) and liver, spleen, and fat tissue weight were significantly suppressed by the LP diet. Operational taxonomic unit numbers and α -diversity were lower in the LP group than in the SP group. A principal coordinate analysis revealed differences in the gut microbiome compositions of SP and LP mice. The abundances of caecal *Roseburia* sp., *Alistipes* sp., and bacteria from the family *Muribaculaceae* were lower in the LP group than in the SP group. In contrast, the abundance of *Desulfovibrionaceae*, which is positively correlated with inflammation, was higher in the LP group than in the SP group. These results differed from the effects of a milk casein-based low-protein diet (reported previously). Based on these findings, we conclude that the undesirable effects of a low-protein diet and/or protein deficiency are related to changes in the gut microbiome composition and may differ depending on the kind of proteins used.

1. Introduction

Soybean is an important source of protein and has been used in traditionally processed and/or fermented foods in East Asia and other countries, such as in tofu, tempeh, natto, miso, and soy sauce. For patients with inflammatory bowel disease (IBD) and individuals with cow-milk allergy and/or lactose intolerance, soymilk is a potential alternative to cow's milk (Heyman, 2006). β -Conglycinin (BC; 7S) and glycinin (11S) are the major protein molecules in soybean that constitute ~20%–40% of the total protein content of soymilk (Krishnan and Nelson, 2011). Although BC may induce soybean allergy, the protein and its hydrolysate reportedly possess antioxidant, antibacterial, immunomodulatory, and hypocholesterolemic properties (Ferreira et al., 2010; Xiang et al., 2016; Wang et al., 2017).

The gut microbiome of mammals including human and laboratory animals have been found to contain hundreds of species and ~11 log cells/g of bacteria. These microorganisms and their metabolites are considered to have important effects on the host's health (Shanahan et al., 2021). For example, there are several reports on the correlation between typical indigenous bacterial groups and conditions such as

obesity, diabetes, ageing, immune system-related diseases, IBD, and Alzheimer's disease (Umirah et al., 2021; Ragonnaud and Biragyn, 2021). Dietary fibres and other food components such as lipids and proteins can change the composition of the gut microbiome rapidly and drastically (David et al., 2014; Wilson et al., 2020). Although most of the proteins are degraded and/or adsorbed in the stomach and small intestine, a proportion of these proteins reach the colon and caecum and are metabolised into various compounds, including amines, ammonia, phenols, H₂S, and indoles (An et al., 2014).

As protein intake is important for protein synthesis in the muscles, various protein-rich functional foods are available for different age groups (from infants to the elderly). They are used to enhance the growth of infants and children (Uauy et al., 2015) and prevent sarcopenia in the elderly (Granic et al., 2020) and are used by individuals involved in sports and exercise training (Håvard et al., 2019). Additionally, a low-protein diet and/or protein deficiency reduce immune activity (Miyazaki et al., 2018). On the other hand, there are reports about the beneficial effect of a low-protein diet on inflammation related to the liver and gut (Guo et al., 2018). It may be the case that these effects of protein concentration in the diet are related to changes in the gut microbiome (Shanahan et al., 2021).

A study on the influence of a milk casein (MC)-based low-protein diet on the gut microbiome of mice revealed a decrease in the abundance of *Bacteroides* and *Clostridium*. (Masuoka et al., 2020). However, a different

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gut microbiome composition was reported in mice fed MC or soy protein (Xia et al., 2020). Therefore, we sought to clarify the effects of a soy protein-based low-protein diet on the body and organ weights and the gut microbiome of mice via 16S rRNA (V4) amplicon sequencing.

2. Materials and methods

2.1. Animal care

Animal experiments were performed in accordance with the 'Fundamental 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, Japan. The study protocol was approved by the Animal Experiment Committee of Tokyo University of Marine Science and Technology (Approval No. H31-5).

Soy protein (SP: Fujipro F) and BC products were obtained from Fuji Oil (Izumisano, Japan). The Fujipro F contained 21% β -conglycinin (7S), 41% glycinin (11S), and 38% of other lipoproteins (Kawaguchi et al., 2018). Twelve 5-week-old male ICR mice (25 ± 2 g) were purchased (Japan SLC Inc., Tokyo, Japan) and housed in metal wire cages (three mice per cage) at 22 ± 2 °C with a 12-h dark/light cycle. The mice were acclimatised to a semi-purified powder diet containing 20% (w/w) MC (Table 1) and were provided distilled water for drinking *ad libitum*. After 7 days, the mice were divided into two groups ($n = 6$, in each group)—standard protein (SP) and low protein (LP)—and were fed a diet containing either 20% (SP) or 5% (LP) soy protein for 14 days. During the feeding days 11–13, the defaecation frequency and faecal weight were measured.

2.2. Plasma lipid and glucose levels

At the experimental endpoint, the mice were exsanguinated via the abdominal vein under anaesthesia using isoflurane (Fujifilm Wako Pure Chemical Corp., Osaka, Japan), and the liver, kidneys, spleen, and epididymal fat pads were removed and weighed. After ligation with yarn, the caecum was excised and placed on ice until microbial analysis was performed. Plasma triacylglyceride (TG), total cholesterol (TC), and glucose levels were determined using commercial kits following manufacturers' instructions (Triglyceride E-Test Wako, Total Cholesterol E-Test Wako, Glucose CII-Test Wako, respectively; Fujifilm Wako Pure Chemical Corp.).

2.3. Analysis of caecal microbiota using the MiSeq system

The caecal contents were diluted using 99 vol (~5 mL) of phosphate-buffered saline (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan), and the bacterial cell count was determined via dielectrophoretic impedance measurements (Hirota et al., 2014) using a bacterial counter (PHC Ltd., Tokyo, Japan).

16S rDNA (V4) amplicon sequencing was performed by Fasmac Co., Ltd. (Atsugi, Japan) as described in a previous report (Xia et al., 2020).

Table 1
Composition of test diets (g/100 g).

	SP	LP
Soy-protein	20.0	5.0
DL-Methionine	0.3	0.3
Corn starch	15.0	30.0
Sucrose	50.0	50.0
Cellulose	5.0	5.0
Corn oil	5.0	5.0
Vitamin mix (AIN-76)	1.0	1.0
Mineral mix (AIN-76)	3.5	3.5
Choline bitartrate	0.2	0.2

Briefly, DNA was extracted from the caecal content using the MPure bacterial DNA extraction kit (MP Bio Japan, Tokyo, Japan). A DNA library was prepared using a two-step PCR (Sinclair et al., 2015). Next, the V4 region was amplified via a 23-cycle PCR using the following primers: forward 515f. And reverse 806r. Individual DNA fragments were tagged via an 8-cycle PCR using the individual mouse primers. DNA libraries were multiplexed and loaded onto an Illumina MiSeq system (Illumina, San Diego, CA, USA). Reads with a mismatched sequence at the start region were filtered using the FASTX Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/); poor-quality reads (below 20) and those shorter than 40 base pairs were omitted using Sickle (<https://github.com/ucdavis-bioinformatics/sickle>). Short-listed reads were merged using the paired-end merge script FLASH (<http://ccb.jhu.edu/software/FLASH/>), and 240–260 base pair reads were selected. Chimeras in the selected reads were identified and omitted using the QIIME2 bioinformatics pipeline (<https://qiime2.org/>). Sequences were clustered into operational taxonomic units (OTUs), with a 97% identity cut-off, using the QIIME2 workflow script and SILVA database (<https://www.arb-silva.de/>).

2.4. Statistical analysis

The results of body and organ weights and the alpha-diversity indices (diversity within a microbiome) are expressed as the mean values \pm standard error of the mean. Data were subjected to an analysis of variance and the Student's *t*-test using a statistical software package (Excel Statistic Ver. 6, Japan). A *p*-value < 0.05 was considered statistically significant. The alpha-diversity of the gut microbiome for the mice was determined using the Shannon-Wiener (*H'*) and Simpson's diversity (*D*) indices (Kim et al., 2017). The beta diversity (distance between groups based on the differences in the OTUs present in each group) was assessed using unweighted and weighted UniFrac (Lozupone and Knight, 2005) and expressed using a principal coordinate analysis (PCoA) (Bunyavanich et al., 2016).

3. Results

3.1. Body, faecal, and organ weights and plasma lipid and glucose levels

The body weight of the mice at the start (0 day) was ~33 g; the mice fed the SP diet gained 10.5 g of body weight per 14 days, whereas mice fed LP gained only 3.9 g ($p < 0.01$, Table 2). The faecal and caecal content weights tended to be high in mice that were fed the LP diet, though the difference was not significant. Compared with mice fed the SP diet, the weights of the liver and spleen were lower in mice fed the LP diet ($p < 0.01$ and 0.05, respectively). The weight of the epididymal fat pad was 40% lower in the LP group ($p < 0.05$). Plasma TG and TC levels also tended to be lower in the LP group, though the difference was not significant. Additionally, plasma glucose levels were 20% lower in mice that were fed the LP diet ($p < 0.01$).

3.2. Direct cell counts and alpha and beta diversity of the caecal microbiome

The total bacterial cell count in the caecal content of the tested mice was 11.3–11.4 log cell/g (Fig. 1A). In the 16S rDNA (V4) amplicon sequencing, the total read numbers obtained from mice fed the SP and LP diets were 99000 and 91000, respectively (Fig. 1B). Compared with the SP group, the OTU number in mice fed the LP diet was 20% lower ($p < 0.05$, Fig. 1C). The alpha-diversity Shannon-Weaver *H'* values and the Simpson's index (*D*) in mice fed LP diet were significantly lower than ones in mice fed SP diet ($p < 0.05$, Fig. 1D and E). The PCoA of the OTUs revealed that the SP and LP groups had different gut microbiome compositions (Fig. 1F and G).

Table 2

Body, organ and faecal weights, and levels of plasma lipids and glucose of tested mice.

	SP	LP
Body weight (g)		
Initial	32.9 ± 0.8	32.8 ± 0.7
14 days feeding	43.4 ± 1.7	36.5 ± 0.7**
Gain per 14 days	10.5 ± 0.9	3.8 ± 0.6**
Defecation		
Frequency (n/day/mouse)	31 ± 2	26 ± 2
Weight (g/day/mouse)	0.51 ± 0.09	0.65 ± 0.04
Organ weights (g)		
Liver	2.380 ± 0.125	1.771 ± 0.059**
Kidneys	0.650 ± 0.041	0.563 ± 0.021
Spleen	0.150 ± 0.011	0.125 ± 0.007*
Epididymal fat pads	1.916 ± 0.221	1.211 ± 0.122*
Caecum (Net)	0.280 ± 0.031	0.356 ± 0.034
Caecal content	0.206 ± 0.033	0.272 ± 0.038
Plasma lipids and glucose (mg/100 mL)		
Triacylglyceride	89.6 ± 14.7	63.0 ± 7.2
Total-cholesterol	230 ± 24	185 ± 13
Glucose	326 ± 13	262 ± 17**

Values indicate the mean and SEM ($n = 6$ for body and organ weights, $n = 4$ for plasma lipids and glucose). *and ** indicate significant differences relative to the SP group when analysed by the Student's t -test (* $p < 0.05$, ** $p < 0.01$).

3.3. Microbiome composition

3.3.1. Phylum level

The predominant bacterial phylum in the tested mice was Firmicutes, and its abundance was higher in mice fed the LP diet (84%) than in those fed the SP diet (70%) ($p < 0.01$, Fig. 2A). In contrast, the abundance of the second-most dominant phylum, Bacteroidota, was higher in the SP (26%) than in the LP (9.5%) group ($p < 0.01$). The ratio of the Firmicutes to Bacteroidota (F/B) was three times higher in mice fed the LP diet ($p < 0.01$, Fig. 2B). The phylum Desulfobacterota was also dominant in mice fed the LP (4.9%) rather than the SP (1.7%) diet ($p < 0.05$). The abundance of Actinobacteria tended to be higher in the SP group (1.7%) than

in the LP group (0.8%), though the difference was not significant.

3.3.2. Family level

As shown in Fig. 2C, in the case of families that belonged to the phylum Firmicutes, *Lachnospiraceae* (14%–24%), *Erysipelotrichaceae* (16%–21%), *Clostridiaceae* (10%–23%), *Lactibacillaceae* (9.6%–20%), and *Oscillospiraceae* (3.1%–5.3%) were dominant. Among these dominant families, bacteria from the family *Clostridiaceae* in mice fed the LP diet were significantly higher than in mice fed the SP diet ($p < 0.05$). Bacteria from other dominant families besides *Lachnospiraceae* tended to be high in mice fed the LP diet. Certain Gram-positive cocci, such as *Streptococcaceae* (0.08%–0.46%) and *Peptococcaceae* (0.13%–0.30%) were higher in mice fed the SP diet than in those fed the LP diet ($p < 0.05$), although their abundance was not high. With regard to the phylum Bacteroidota, the dominant family was *Muribaculaceae* (7.5%–20%), followed by *Bacteroidaceae* (1.2%–4.4%). Both the families in the SP group were 2.6 and 3.6 times higher than in the LP group ($p < 0.05$ and 0.01), respectively. Almost all of the Desulfobacteria OTUs, which were high in the LP group, were defined as *Desulfovibrionaceae*.

3.3.3. Genus level

In the case of genera that belonged to the family *Lachnospiraceae*, *Roseburia* (7.1%) and two *Lachnospiraceae* OTUs (4.4%–7.3%) were dominant in mice fed the SP diet (Fig. 2D). Among these genera, the abundance of *Roseburia* was low in mice fed the LP diet (0.1%, $p < 0.05$). The abundance of the genus *Alistipes* (family *Rikenellaceae*, phylum *Bacteroidota*) was higher in the SP group than in the LP group ($p < 0.05$), though it was not the dominant genus (1.0%). *Enterorhabdus*, which belong to the family *Eggerthellaceae*, and Actinobacteria were higher in mice fed SP (0.62%) rather than the LP diet (0.21%, $p < 0.01$).

3.4. Dominant OTUs

The heat map of dominant OTUs with an abundance of 0.5% or more is shown in Fig. 3. Species names of the predominant and typical OTUs defined using BLASTn by the National Center for Biotechnology Information (NCBI, <https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and DDBJ that displayed 95% or more similarity are also shown. In Firmicutes, the top

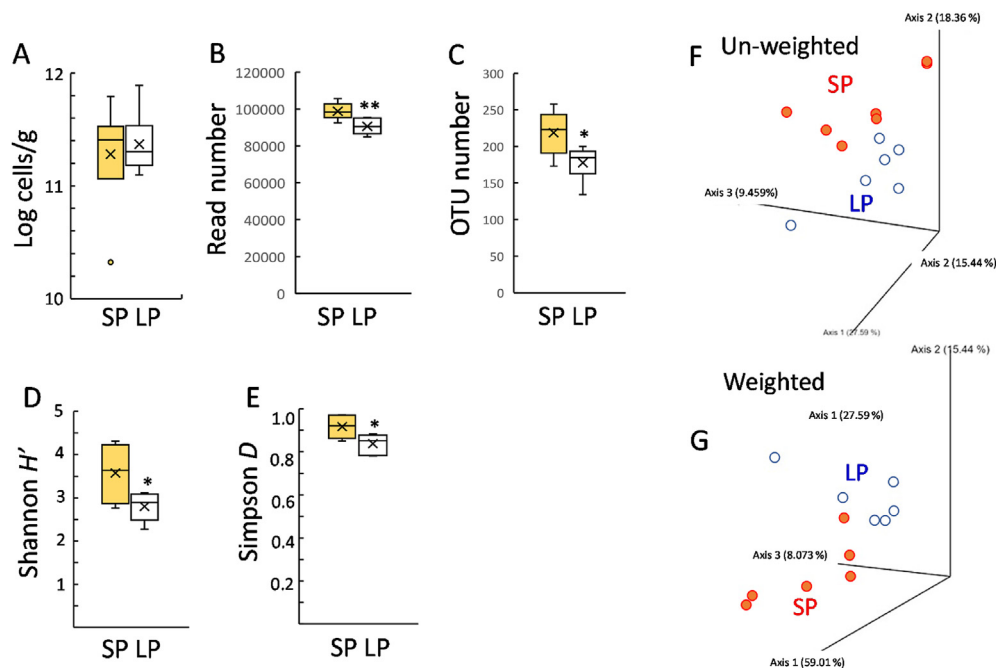


Fig. 1. Total bacterial count (A), total read number (B), operational taxonomic units (OTU) number (C), Shannon index (D), Simpson's index (E) and principal coordinate analysis (PCoA; F and G) of the OTUs in the caecal microbiome of the mice fed a diet containing 20% (SP) or 5% soy protein (LP).

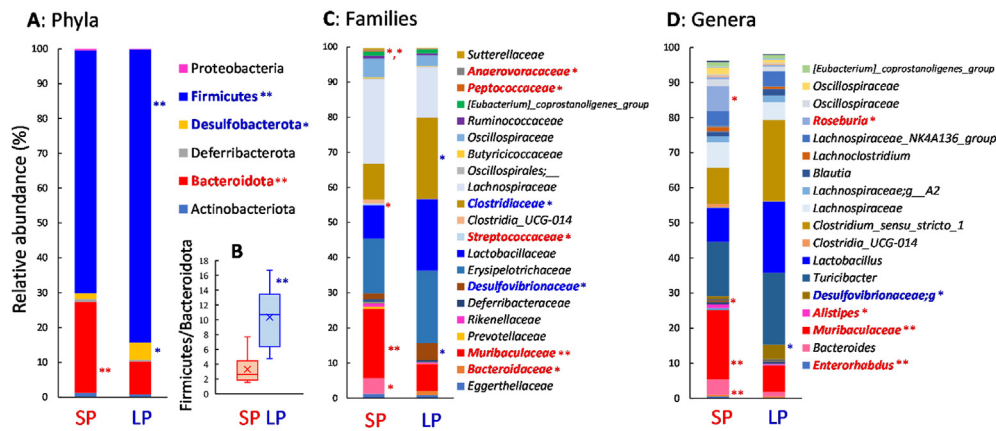


Fig. 2. Composition of the caecal microbiome at the phylum (A), family (C), and genus (D) levels in the mice fed a diet containing 20% (SP) or 5% (LP) soy protein. (B) The ratio of Firmicutes to Bacteroidota. * and ** indicate significant differences between the groups determined by the Student's *t*-test (**p* < 0.05, ***p* < 0.01).

four dominant OTUs, *Turicibacter*, *Clostridium sensu stricto*, *Lactobacillus*, and Lachnospiraceae NK4A136 group were defined as *T. sanguinis*-, *C. disporicum*-, *L. murinus*-, and *Kineothrix alysoides*-like bacteria, respectively. There was no significant difference for the dominant OTUs between the two diet groups, though *C. disporicum*-like bacteria tended to be high in mice fed an LP diet. The *Roseburia* OTU that showed high abundance in the SP group could not be defined, and the most similar bacteria that were identified using BLASTn was *R. intestinalis* (similarity = 91%).

Muribaculaceae OTUs that showed higher abundance in the SP group were defined as *Muribaculum* sp. like bacteria. The other *Muribaculaceae* OTU, which was also abundant in the SP group, was defined as *Duncanella muris*. Although *Bacteroidaceae* abundance was significantly higher in mice fed an SP diet, the abundance of the dominant *Bacteroidaceae* OTUs identified as *Phocaeicola sartorii*, *Bacteroides rodentium*, and *Bacteroides acidifaciens*-like bacteria tended to be higher in mice fed an SP diet than in those fed an LP diet, but the difference was not significant.

In the case of OTUs from Bacteroidota, the three dominant

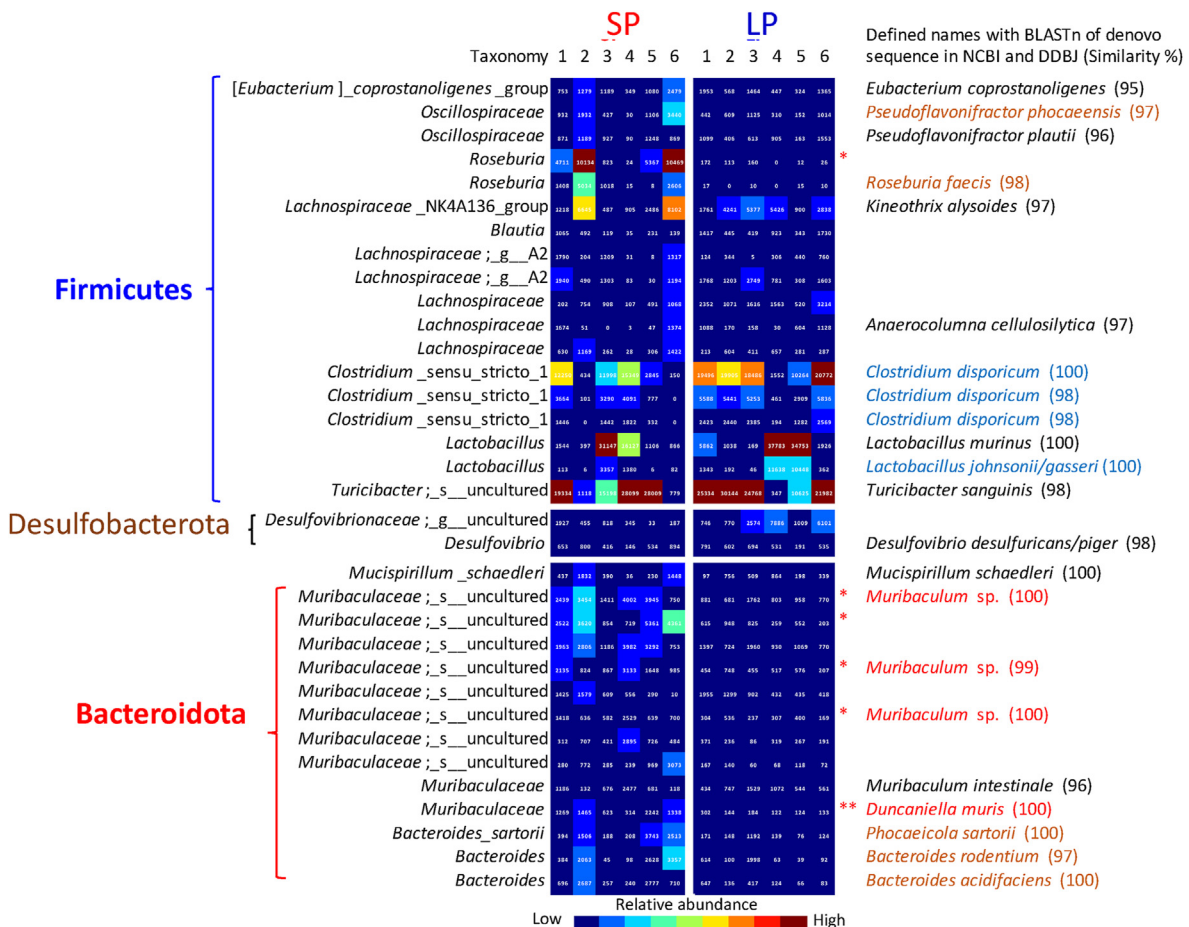


Fig. 3. Heat map showing the relative abundance of identified OTUs filtered by 0.5% counts of all identified OTUs in the caecal microbiome in mice fed a diet containing 20% (SP) or 5% (LP) soy protein. * and ** indicate significant differences between the groups as determined by the Student's *t*-test (**p* < 0.05, ***p* < 0.01).

4. Discussion

There are several reports about the suppression of weight gain in young mice and rats fed on MC- and soy protein-based low-protein diets (Chaumontet et al., 2019). Liver weight loss in mice fed a low-protein diet has also been reported (Pérez-Martí et al., 2017). Lower spleen weight in mice fed an LP diet can be regarded as a result of the effect of low protein intake on the immune system (Lewicki et al., 2014). The suppressive effects of a low-protein diet on fat tissue weight and the level of plasma lipids and glucose has been shown in rat models of type 2 diabetes (T2D) (Kitada et al., 2018).

Sequencing of the 16S rRNA amplicon revealed that among the typical bacterial families in the gut of mice fed an LP diet, *Clostridiaceae* and *Desulfovibrionaceae* showed high abundance, and *Muribaculaceae* showed low abundance (Fig. 2C). Additionally, the abundance of the genus *Roseburia* was significantly lower in the LP group than in the SP group (Fig. 2D). These results are not in agreement with those from a previous study on mice maintained on MC (Masuoka et al., 2020). The variation may be due to the different effects exerted by soybean and MC on the gut microbiome. For example, the abundance of the families *Bacteroidaceae* and *Muribaculaceae* was lower and higher, respectively, in mice fed 20% soy protein than in mice fed 20% MC (Xia et al., 2020).

The dominant genus in the family *Clostridiaceae* in the LP diet-fed group was identified as *C. disporicum* (Fig. 3). Though an increase in the abundance of *C. disporicum* in murine caecum fed fermented loofah and some spices (cumin and coriander) has been reported (Shikano et al., 2019; Xia et al., 2019), the abundance decreased on a diet containing egg white, some edible algae, and algal polysaccharides (Takei et al., 2019; Takei et al., 2020; Xia et al., 2020). Although functional reports of *C. disporicum* are limited, the epimerase activity of this species, which produces ursodeoxycholic acid (UDCA) from chenodeoxycholic acid (CDCA) was recently reported (Tawtep et al., 2017). This might be correlated with low liver and fat tissue weights observed with the LP diet (Table 2).

Recently, bacteria from the family *Desulfovibrionaceae* have been identified as harmful. For example, there are reports about a positive relationship between endotoxin, which induces inflammation, and the abundance of bacteria from the family *Desulfovibrionaceae* (Zhai et al., 2019; Chen et al., 2020). An increase in gut *Desulfovibrionaceae* in rats fed a diet containing beef and sucrose stimulated oxidative stress (Hecke et al., 2019). In contrast, the prevalence of the bacteria from this family was decreased by the use of some dietary fibres such as inulin and fucoidan, antioxidants such as rutin, quercetin, and resveratrol, and food materials rich in dietary fibres and antioxidants such as edible brown algae and wasabi (Zhao et al., 2017; Guo et al., 2018; Li et al., 2019; Takei et al., 2019; Thomaz et al., 2020). The relationship between dietary protein deficiency, liver injury, oxidative stresses, and amelioration of that with the use of antioxidants has been reported (Maiti and Chatterjee, 2000; Nkosi et al., 2006).

Muribaculum, which had a low abundance in mice fed an LP diet, is a dominant genus in the murine gut (Lagkouvardos et al., 2016). Its abundance increases when mice are fed dietary fibre- and protein-rich foods such as rice bran and cyanobacteria, and this increase exerts an ameliorative effect on obesity and T2D in model mice (Shibayama et al., 2019; Yuan et al., 2020; Taniguchi et al., 2021). Furthermore, studies have suggested a correlation of the predominantly commensal *Muribaculum* sp. with the host immune system and metabolism, particularly carbohydrate metabolism (Lagkouvardos et al., 2016; Graham et al., 2018; Chun et al., 2020).

Multiple reports on the butyrate-producing species of *Roseburia*, which also showed low abundance in the LP group, have indicated their role as a marker of host health that can be correlated with its beneficial properties (Tamanai-Shacoori et al., 2017). For example, the relationship between *Roseburia* abundance and amelioration of signs of colitis, IBD, obesity, T2D, and neuronal system conditions, as well as immunomodulation, have been reported (Imharan et al., 2017; Gurung et al., 2019;

Luo et al., 2019; Zheng et al., 2019; Nishiwaki et al., 2020). Although the ameliorative effect of a low-protein diet on gut inflammation has been described earlier (Guo et al., 2018), soy protein and its hydrolysate have antioxidant, antibacterial, immunomodulatory, and hypocholesterolemic properties (Ferreira et al., 2010; Xiang et al., 2016; Wang et al., 2017).

The results of our study regarding the effect of a low-protein diet on the gut microbiome have important differences relative to previous reports about mice maintained using MC (Masuoka et al., 2020). In our study, we observed that a low-protein diet was undesirable for growth, the gut microbiome, and its environment when mice were maintained using soy protein. The bacteria that showed an increase or decrease in prevalence in the gut of mice kept on a low-protein diet in this study can be regarded as soy protein susceptible gut indigenous bacteria (SIB). It has been shown that certain food materials and their SIBs have a synergistic effect on the host (Wilck et al., 2017). Isolation of the SIBs using a proper culturing method may help clarify the relationship between proteins and the gut microbiome.

5. Conclusion

In this study, we characterised the effects of a low-protein diet in mice that were administered soy protein, and measured the body and organ weights, and analysed the gut microbiome of six-week-old mice fed a diet containing 20% (SP) or 5% (LP) soy protein for 14 days via 16S rRNA (V4) amplicon sequencing. The body weight gain (growth), and the liver, spleen, and fat tissue weights were significantly suppressed in mice on the LP diet. Further, the OTU number and α -diversity were lower in the LP group. A PCoA revealed that SP and LP fed mice had different gut microbiome compositions. The caecal *Roseburia* sp., *Alistipes* sp., and bacteria from the family *Muribaculaceae* in the LP group were lower than that in the SP group. In contrast, the abundance of bacteria from the family *Desulfovibrionaceae* was high in the LP group. These results differ from the effects of a low-protein diet maintained using MC. We concluded that the undesirable effects of a low-protein diet and/or protein deficiency are related to changes in the gut microbiome composition and that these effects may differ depending on the kinds of proteins used.

CRedit authorship contribution statement

Saori Nakamura: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization. **Takashi Kuda:** Conceptualization, Methodology, Validation, Formal analysis, Resources, Data curation, Writing – review & editing, Visualization, Supervision, Project administration. **Yuko Midorikawa:** Investigation. **Hajime Takahashi:** Conceptualization, Methodology, Supervision. **Bon Kimura:** Conceptualization, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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