Title: 

Relationship of enhanced butyrate production by colonic butyrate-producing bacteria and immunomodulatory effects in normal mice fed insoluble fraction of Brassica rapa L.

Author names and affiliations:
Sachi Tanakaa, Kana Yamamotob, Kazuki Yamadab, Kanon Furuyab, and Yutaka Uyenoab

aAcademic Assembly (Institute of Agriculture), Shinshu University, Minamiminowa, Nagano, Japan, and bFaculty of Agriculture, Shinshu University, Minamiminowa, Nagano, Japan

Running title:

Microbiota change and immune regulation by B. rapa

Corresponding author:

Yutaka Uyeno
Faculty of Agriculture, Shinshu University, Minamiminowa 8304, Nagano 399-4598, Japan
Tel, Fax: +81-265-77-1650, E-mail: ytkuyeno@shinshu-u.ac.jp
Abstract

This study was performed to determine the effects of feeding a fiber-rich fraction of *Brassica* vegetables on immune response through changes in enteric bacteria and short chain fatty acid (SCFA) production in normal mice. The boiled water-insoluble fraction of *Brassica rapa* L. (nozawana), which consisted mainly of dietary fiber, was chosen as a test material. A total of 31 male C57BL/6J mice were divided into two groups and housed in a specific pathogen-free facility. The animals were fed either a control diet or the control diet plus insoluble *B. rapa* L. fraction for 2 weeks and sacrificed for determination of microbiological and SCFA profiles in lower gut samples and of immunological molecules. rRNA-based quantification indicated that the relative population of *Bacteroidetes* was markedly lower in the colon samples of the insoluble *B. rapa* L. fraction-fed group than the controls. Populations of the *Eubacterium rectale* group and *Faecalibacterium prausnitzii*, both of which are representative butyrate-producing bacteria, doubled after 2 weeks of the fraction intake accompanying a marginal increase in colonic butyrate proportion. In addition, feeding with the fraction significantly increased levels of the antiinflammatory cytokine, interleukin (IL)-10, and tended to increase splenic regulatory T cell numbers, but significantly reduced the population of cells expressing activation markers. We demonstrated that inclusion of the boiled water-insoluble fraction of *B. rapa* L. can alter the composition of the gut microbiota to decrease *Bacteroidetes* and to increase butyrate-producing bacteria, either of which may be involved in the observed shift in production of splenic IL-10.
Introduction

Animals coexist with microbial symbionts that act as an integral component of the host’s physiology in the gastrointestinal (GI) tract (1). The vast majority of GI bacteria are strict anaerobes that derive energy from fermentation, by which indigestible complex carbohydrates (cellulose, pectin, gums, beta-glucan, and lignin) are converted to short chain fatty acids (SCFA), such as acetate, propionate, and butyrate as a terminal electron acceptor (2-4). As each member of the GI microbiota has different preferences for different energy sources (5, 6), the profile of dominant species in the human gut microbiota can be potentially modified by the types of dietary carbohydrate in the diet.

Among the SCFA, butyrate acts as a primarily effective molecule on physiological regulatory systems of the host gut as well as an energy source for the colonic epithelium of the host. There has long been interest in the immunomodulatory and antiinflammatory effects of butyrate on colonic epithelial cells (7-9). The production of butyrate in the GI tract is supported by specific groups of bacteria, i.e., butyrate producers, which are considered to play important roles in maintaining gut health (10, 11). The main human colonic butyrate producers belong to two groups of gram-positive firmicutes: Faecalibacterium prausnitzii in Clostridium leptum (or Clostridial cluster IV) cluster, and Eubacterium rectale/Roseburia spp. in the Blautia cocoides (reclassified name of Clostridium cocoides, Clostridial cluster XIVa) (12-15), whereas there are other butyrate-producing groups that have been detected in humans (16-18).

Vegetables belonging to the Brassica genus (Brassicaceae family) contain a number of nutrients with health-promoting properties, such as anticancer actions (19) and effects on cholesterol metabolism (20). These vegetables (Brassica oleracea L.
[kale, cabbage, broccoli], and B. rapa L. (turnip)] are unique in that they are rich sources of fiber, carotenoids, and foliate, as well as polyphenols and sulfur-containing compounds (21). Vegetable dietary fiber are suggested to act as effective prebiotics by inducing major shifts in gut microbial composition and affecting the mucosal immune system (22-24). However, experimental studies have yielded inconclusive results to explain how the effective components in Brassica vegetables affect the immune response through bacterial fermentation, especially whether they selectively increase butyrate production. Brassica rapa L. (nozawana), which is a traditionally and regionally planted vegetable in Japan, contains high levels of fiber (33% on a dry matter basis) (25). We chose this vegetable as an example supplement, and examined the effects of the insoluble (i.e., fiber) fraction on immunological molecules in response to the intestinal community structure in mice.

MATERIALS AND METHODS

Preparation of B. rapa L. Fresh B. rapa L. was soaked in pure water and autoclaved at 121°C for 20 minutes. The autoclaved samples were then homogenized (AM-3, Ace Homogenizer; Nissei Co. Ltd., Tokyo, Japan) and the resultant suspensions were centrifuged at 2215 × g for 10 minutes to remove large size of residue. In order to collect functional fraction of B rapa L. extract, the supernatants were also centrifuged at 20630 × g for 5 minutes and the pellets were lyophilized in a freeze-dryer (FDU-1200; Eyela, Tokyo, Japan) and used as the extracts. Samples of 1 g of fresh B. rapa L. yielded ca. 0.3 mg of residual extract, which was considered to be mainly composed of highly insoluble fiber (25, 26).
Animals and tissue sampling. Mice were cared for according to the Guide for the Care and Use of Experimental Animals of Shinshu University. Due to limitations in the capacity of the facility and animal management, the data presented in this paper were obtained over the course of three feeding trials with exactly the same design (10 animals for trial 1, 12 animals for trials 2 and 3). Five-week-old male C57BL/6 mice were housed in a specific pathogen-free facility. The standard diet (67% carbohydrate, 19% protein, 4% fat, and 4% ash) consisted mainly of casein (190 g/kg diet), corn starch (300 g/kg), sucrose (330 g/kg), cellulose (47 g/kg), soybean oil (22 g/kg), lard (18 g/kg), vitamins, and minerals. After a 1-week acclimatization period on the control diet, the animals were split into two groups. Both groups were fed the same standard diet ad libitum, and one group received oral administration of the extract of *B. rapa* L. (resuspended to 2 mg/mL water, 20 mg/kg BW/day, *B. rapa* L. group) once daily, while the other group received water in the same manner (control group). The quantity and the periods of administration of the extract were determined according to our preliminary study (unpublished data). Feed and water were supplied ad libitum. Body weight was measured once daily in this feeding period. Sonde feeding lasted 2 weeks and three animals were excluded from the study because of irregular body weight decrease (One was in the control group during trial 1 and the other two were one in the control and the other in the *B. rapa* L. during trial 2). Therefore, 16 animals in the *B. rapa* L. group and 15 animals in the control group completed the feeding trials. Mice were sacrificed by cervical dislocation, and tissues including the colon, cecum, spleen, mesenteric lymph nodes (mLN), and Peyer’s patches (PP), were collected and weighed. After measuring, colonic and cecal contents (~ 0.10 g) were subsampled in 1 mL of PBS, and mixed thoroughly to equalize the distribution in the buffer. Sampling position of colonic...
contents from each mouse was unified to the middle part of colon. Thereafter, other organs (small intestine, liver, heart, and stomach) were separated and weighed.

**Microbial analyses.** Total RNAs were extracted from the prokaryotic cells in the suspensions using an RNeasy Plus Mini kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Solutions of the extracted RNA were stored at –80°C until use. An RNA-based, sequence-specific rRNA cleavage method was applied to monitor active bacterial populations in the intestinal samples (27). For detection and quantification of respective bacterial groups, the following probes were used: Bac303m (*Bacteroides* and *Prevotella*); Erec482m (*B. coccoides*-*Eubacterium rectale* group); Rfla1269 (*Ruminococcus flavefaciens*), Rbro730m (*Ruminococcus bromii*), and Fprau645 (*F. prausnitzii*); Lab158m (*Lactobacillus*-*Enterococcus* group). These probes were applied separately using the same reaction conditions as described in previous studies (28, 29). We employed two additional probes, Rrec584 (*E. rectale*) (30) and Clept866 (*C. leptum* subgroup) (31). Probe validation was conducted according to our previous report (32) using *Roseburia faecis* JCM 17581 and *Ruminococcus albus* JCM14654T as reference strains for plotting standard digestion curves. By doing so, we determined the reaction conditions (formamide percentage and digestion coefficient) as 10%, 0.90 for Rrec584 and 5%, 0.86 for Clept866. Bacterial genomic DNAs were extracted from the prokaryotic cells of the suspensions using a QIAmp DNA Stool Mini Kit (Qiagen) according to the manufacturer’s instructions. Solutions of the extracted DNA were stored at –80°C until use. Quantitative real-time PCR analysis for total bacterial DNA was performed as described previously (33). Real-time PCR primers and conditions for amplification of the butyryl-CoA:acetate CoA-transferase gene have been published previously (34, 35).
Organic acid measurements. Cooled cecal and colonic content samples (~0.05 g) were weighed and dispersed in 1 mL of sterilized water. Suspensions were centrifuged at 10000 × g at 4°C for 5 minutes. The supernatants were used to analyze the organic acids with an HPLC system equipped with an electroconductivity detector (LC-20 model; Shimadzu Corp., Kyoto, Japan) as described previously (27).

Isolation and culture of cells from spleen, mLNs, and PPs. Spleen, mLNs, and PPs were removed from each mouse in both groups, and single-cell suspensions were passed through 40-µm cell strainers (BD Falcon, Franklin Lakes, NJ). To deplete red blood cells, spleen cells were treated with 0.17 M Tris-HCl buffer (pH 7.65) containing 0.83% NH₄Cl. For culture of spleen cells, the cells were resuspended at a concentration of 1×10⁷ cells/mL in RPMI-1640 medium containing 10% FBS plus 10000 U/mL of penicillin G and 10 mg/mL of streptomycin. The cells were cultured in 96-well flat-bottomed plates in the presence or absence of 0.1 or 1 µg/mL of lipopolysaccharide (LPS) from E. coli 055:B5 (Sigma, St. Louis, MO) for 48 hours at 37°C under conditions of 5% CO₂.

Flow cytometry. For analysis of cell-surface molecules, we used FITC-conjugated anti-CD4 (GK1.5), PE-conjugated anti-CD8 (53-6.7), PE-conjugated CD11b (M1/70), APC-conjugated CD69 (H1.2F3), APC-conjugated CD11c (HL3), FITC-conjugated anti-H-2Kb (AF6-88.5), PE-conjugated anti-I-Ab (AF6-120.1), and 7-amino-actinomycin D (7-AAD). These antibodies were purchased from BioLegend (San Diego, CA). Cells from spleen, mLNs, and PPs were stained with fluorescence-labeled mAbs, and 7-AAD. The expression levels were evaluated by flow cytometry (FACSCalibur; Becton Dickinson, San Jose, CA).
**Regulatory T cells (Tregs) staining.** After staining with FITC-conjugated anti-CD4 and APC-conjugated anti-CD25 (clone:PC61; BioLegend) mAbs, cells were fixed and permeabilized with FlowX™ FoxP3 Fixation & Permeabilization Buffer Kit (R&D Systems, Minneapolis, MN). Permeabilized cells were stained with PE-conjugated anti-mouse Foxp3 mAb (clone:150D; BioLegend). Stained cells were subsequently analyzed by flow cytometry (FACSCalibur; Becton Dickinson).

**Enzyme-linked immunosorbent assay (ELISA).** The levels of interleukin (IL)-10 production in culture supernatants of the spleen cells from each mouse individually were measured using an ELISA kit (eBioscience, San Diego, CA) according to the manufacturer’s instructions. We used three-fold diluted supernatants to detect IL-10 levels by ELISA.

**Statistical analyses.** Measurements were analyzed by the unpaired Student’s *t* test with STATA 13.1 (Stata Corp, College Station, TX). In all analyses, *P* < 0.05 was taken to indicate statistical significance.

**RESULTS**

**Body weights, organ weights.** The body weights and organ weights of mice are shown in Table 1. Body weights showed no differences between the two groups at any time point during feeding period, so mice in each treatment group were considered to grow normally. There were also no differences in cecum weight between the groups. Colon weight was higher in *B. rapa* L. group, suggesting that undigested *B. rapa* L. fraction reached the colon and probably became a substrate for colonic microbial fermentation.

No difference was observed in respect to the weights of other organs (small intestine, liver, heart, and stomach; data not shown).
Analysis of colonic samples. Bacterial profiles in colonic contents of the mice are shown in Fig. 1. rRNAs of the phylum Bacteroidetes: Bacteroides and Prevotella (determined by Bac303m probe) and the phylum Firmicutes: B. coccoides-E. rectale group and C. leptum subgroup (sum of values of Erec482, Clept866, and Lact158) constituted the major fraction of the bacterial community (approximately 75% of the total 16S rRNA). At the phylum level, Bacteroidetes was lower, while Firmicutes tended to be higher in B. rapa L. (Fig. 1a). In the lower group level, the relative populations of F. prausnitzii (determined by Fprau645) and E. rectale (by Rrec584) were higher in B. rapa L. (Fig. 1b) than the control. The relative populations of R. flavefaciens and R. bromii were 1.4% ± 0.8% and 2.1% ± 0.8%, respectively, and there were no differences between groups (data not shown). The Lactobacillus-Enterococcus group was shown to constitute approximately 1% of the total rRNA of colon samples, and there were no differences between the two groups. We also determined total bacterial numbers and the butyryl-CoA:acetate CoA-transferase gene in colonic contents by quantitative PCR (Fig. 1c and 1d). While total bacterial numbers were not significantly different between treatments, the butyryl-CoA:acetate CoA-transferase gene copy numbers were higher in B. rapa L. than controls.

The total organic acids (SCFA and lactate) of the cecal and colonic contents were not different between the two groups (Fig. 2a). SCFA proportion was also determined, and the butyrate concentrations were higher in B. rapa L. than in controls (Fig. 2b and 2c). Lactate and valerate were minor constituents (< 0.5 mmol/kg sample) of the samples.

Immunological analyses. The early activation marker, CD69, was examined in spleen cells of mice orally administered the extract of B. rapa L. CD69 expression
levels on CD4^+, CD8^+, or CD11b^+ cells in B. rapa L.-administered mice were downregulated compared with controls (Fig. 3a). In addition, oral administration of B. rapa L. extract significantly decreased the expression of major histocompatibility complex (MHC) class I (H-2Kb), but not CD69 or MHC class II (I-Ab), on CD11c^+ dendritic cells (Fig. 3b).

To determine the immune regulatory effects of B. rapa L., IL-10 production by spleen cells was analyzed. Dietary supplementation of B. rapa L. extract induced IL-10 production by spleen cells stimulated with LPS (Fig. 4a). Furthermore, the proportion of Treg cells tended to increase in mice orally administered B. rapa L. extract (Fig. 4b, P = 0.06). These findings suggested that oral administration of B. rapa L. extract induces immune regulatory effects, including decreases in activation markers and increases in IL-10 production and Treg cells.

**DISCUSSION**

Diet is regarded as a major contributing factor directing the bacterial population in the large intestine. Prebiotics are nondigestible food ingredients such as cellulosics, fibers, and other oligosaccharides such as resistant starch, fructooligosaccharides (FOS), and xylooligosaccharides (XOS), which beneficially affect the host by modulating the intestinal microbiota (4, 36-39). Certain dietary constituents, for example resistant starch, are known to increase the bacterial production of butyrate in the large intestine, which is generally regarded as having a beneficial effect (40). Controversially, limited ingestion of fiber in the diet was suggested to be a critical factor in disease onset linked to the gut microbiome (41).
In this experiment we profiled the changes in active populations of the microbiota instead of determining 16S rRNA gene amplicon of specific groups. Our results showed that there were significant shifts in the active populations in the colonic microbiota, whilst this method did not allow us to know the exact metabolisms that the active populations employed. For example, two remarkable responses were found regarding the composition of the colonic microbiota with the introduction of insoluble *B. rapa* L. fraction. First, the *Bacteroidetes* population was reduced in animals treated with this extract. In our previous study, long-term administration (12 weeks) of a product of kale (*Brassica oleracea*) to BL6 mice resulted in an elevated proportion of *Firmicutes* and a reduced population of *Bacteroidetes*. As a consequence, the *Bacteroidetes/Firmicutes* ratio in colon samples was lower in the kale ingestion group than controls (27). Thus, administration of *Brassica* vegetables may yield a common tendency of decreasing the population of *Bacteroidetes*, although the reasons remain unknown. Another response was a twofold increase in the *E. rectale* group and *Faecalibacterium*, both of which are representative butyrate-producing bacteria, after 2 weeks of insoluble *B. rapa* L. fraction intake, and this was probably accompanied by an increase in the molar proportion of butyrate. Although total colonic SCFA concentration remained unchanged and the increase in molar proportion of butyrate in the *B. rapa* L. group was numerically small, total colonic contents increased (Table 1), then an increase in colonic butyrate production was expected. In other reports, 5% – 10% of the total microbiota species were shown to be related to *E. rectale* and *Roseburia* and 5% – 15% were *F. prausnitzii* (13, 42, 43), in accordance with our results. Butyrate-producing strains exhibit different growth profiles on various substrates, which include starch, inulin, FOS, and XOS (10, 44, 45). In this study, we could not determine which
plant-derived components were effective as substrates for these bacteria. However, it is possible that butyrate-producing bacteria are particularly dependent on dietary fiber in the fraction to maintain the population in the colon. The nature and processing of the fiber must be determined to provide sufficient production of butyrate. We also quantified the butyryl-CoA:acetate CoA-transferase gene using degenerate primers that recognize multiple phylogenetic groups as another benchmark for determination of butyrate-producing bacteria (14, 34, 40). The results of this study suggested the increase in the copy numbers of the gene in the *B. rapa* L. group in good accordance with an increase in butyrate-producing groups, while we did not measure the expression levels of this gene. It is regarded that butyrate-CoA to butyrate pathway using extracellular acetate is involved in a major intracellular reductive pathways in the gut (46), namely, there may be metabolic relay from fiber-fermenting bacteria that produce acetate, to stimulation of butyrate producers and acetate-utilizing strains (10, 22, 47).

Butyrate is suggested to influence various aspects of gut physiology beyond simply acting as a crude caloric source (48, 49). These effects result in anticancer activity and can also be useful in the treatment of some chronic digestive diseases (50). The contributions of the gut microbiota to the development of the immune system have been extensively characterized (24, 51-55). The microbiota drives the immune system, which allows the host to tolerate the large amount of antigens present in the gut (immunological tolerance) by Treg cells (56, 57). Treg cells contribute to the homeostasis of the immune system by suppressing the immune responses of other cells via IL-10 (58). Some commensal bacteria, including fiber-fermenting species, appear to preferentially drive T-regulatory lymphocyte development (59-62). In addition, butyrate decreases intestinal expression levels of TNF-α, IL-1β, and IL-6 in patients with
Crohn’s diseases (12, 63, 64). In this context, these microbes may affect host immune function by the production of SCFAs. In the present study, the B. rapa L. fraction significantly increased production of the antiinflammatory cytokine, IL-10, and significantly reduced induction of activation markers (i.e., CD69 on CD4+, CD8+, and CD11b+ cells). Our results were generally in accordance with previous findings and support the role of the microbiota in development of the mucosal adaptive immune system. Additionally, LPS, which is released from dead cells of gram-negative bacteria such as Bacteroidetes, has been suggested to be correlated with in vivo IL-10 production (65, 66). In relation to this, in another in vitro experiment we have found induction of IL-10 production from spleen cells stimulated with the B. rapa L. extract (unpublished data), suggesting that B. rapa L. may inherently possess components which directly affect a systematic immune response unrelated to enhanced butyrate production. The reason why responses in immune molecules were not as prominent as those of the colonic bacterial community, may have been partially due to the use of healthy mice, which did not require strong immunoregulation responses.

Oral administration of B. rapa L. extract significantly decreased the expression of MHC class I, but not CD69 or MHC class II on dendritic cells. MHC class I is generally used in the presentation of endogenous antigens to CD8+ T cells. In some cases, however, exogenous antigen can enter the MHC class I presentation pathway of dendritic cells (cross-presentation) (67). In addition, IL-10 inhibits MHC class I expression on dendritic cells and converts immature into tolerogenic type (68). In this study, the expression levels of CD69 and MHC class II on dendritic cells were not changed by the oral administration of B. rapa L, but MHC class I expression decreased. Also, IL-10 production from spleen cells stimulated with LPS was higher in mice orally
administered with *B. rapa* L. extract. So, dietary *B. rapa* L might induce the functional
or population changes in dendritic cells by the enhancement of IL-10 production.

Taken together, our findings not only indicate synchronized relationships
between changes in the GI bacterial community structure in normal mice fed insoluble
*B. rapa* L. fraction and colonic induction of splenic expression of IL-10. These changes
probably occurred concurrently due to the induction of Treg cells. This study also
implies increased generation of butyrate derived from a food component rich in dietary
fiber may have a suppressive effect on gut immune functions mediated by changes in
the microbiota. SCFAs have been reported to act through cell-surface signaling
receptors, such as G protein-coupled receptors (GPR), to achieve some of their
functions, including immunological responses (7, 69, 70). Determination of the
responses of GPRs will be necessary to evaluate the increased butyrate production by *B.
rapa* L. administration acting jointly with the immune responses. Further studies are
also required to determine which components in the insoluble fraction of *B. rapa* L.
affect the butyrate producers and other members of the microbiota.

**ACKNOWLEDGMENTS**

This work was financially supported by Integration Research for Agriculture and
Interdisciplinary Fields from the Ministry of Agriculture, Forestry, and Fisheries of
Japan. The authors thank S. Yonekura and H. Fujii for their suggestions regarding this
work and T. Shimosato for his critical peer review of the manuscript.
REFERENCES


commercial vegetable juice, the intake of fresh fruit and komatsuna (Brassica rapa
L. var. perviridis) juice mixture reduces serum cholesterol in middle-aged men: a

and human cancer risk: epidemiologic evidence and mechanistic basis. Pharmacol

22. Louis P, Flint HJ. 2009. Diversity, metabolism and microbial ecology of
butyrate-producing bacteria from the human large intestine. FEMS Microbiol Lett

23. Hooper LV, Macpherson AJ. 2010. Immune adaptations that maintain homeostasis
with the intestinal microbiota. Nat Rev Immunol 10:159-169.


Nozawana (Leaves, raw; Item No, 06229), Standard Tables of Food
Composition in Japan (Fifth Edition), Tokyo.


microbial ecology with ingestion of kale. Benef Microbes 5:345-349.

An rRNA-based analysis for evaluating the effect of heat stress on the rumen


**Figure legends**

FIG. 1. Effects of oral administration of the extract of *B. rapa* L. on the colonic bacterial community and the level of a gene involved in butyrate generation. Relative bacteria population at the phylum level (*Bacteroidetes*: *Bacteroides*, and *Prevotella*, and *Firmicutes*: *B. coccoides-E. rectale* group and *C. leptum* subgroup) (a). Relative bacterial populations in the representative butyrate-producing bacteria (*E. rectale* and *F. prausnitzii*) (b). Data shown in Fig. 1a and 1b were obtained using the sequence-specific rRNA cleavage method. Total bacterial 16S rRNA copies in colonic samples (c). Data are expressed as relative gene copy numbers per colonic contents assuming the average of the control group as 100. The relative amount of butyryl-CoA CoA transferase gene per 16S rRNA gene (d). Error bars indicate standard deviation (SD) for all mice used in three independent experiments performed together (Control group, *n* = 15; *B. rapa* L. group, *n* = 16). The significance of differences between control (blank bar) and *B. rapa* L. (filled bar) groups were determined by Student’s *t* test (*, *P* < 0.05).

FIG. 2. Effects of oral administration of the extract of *B. rapa* L. on total SCFA concentrations in cecum and colon samples (a), and relative molar proportions of acetate (C2), propionate (C3), and butyrate (C4) in the cecum sample (b) and colon sample (c). Data are represented in the same manner as described in Fig. 1.

FIG. 3. Oral administration of the extract of *B. rapa* L. down-regulates the expression of CD69 in spleen cells. The extract of *B. rapa* L. (20 mg/kg BW/day) or water was administered orally to mice for 14 days, and then spleen cells were collected. Spleen cells were stained with anti-CD4, anti-CD8α, anti-CD11c, and anti-CD69 mAbs and
expression levels of the early activation marker, CD69, on CD4^+ T cells, CD8^+ T cells, and CD11b^+ cells were evaluated by flow cytometry (a). Flow cytometry was performed to determine the expression of CD69, H-2Kb, or I-Ab on CD11c^+ cells using anti-CD69, anti-H-2Kb, anti-I-Ab, and anti-CD11c mAbs (b). Data are presented in the same manner as in Fig. 1.

FIG. 4. Enhancement of IL-10 production and Treg cells in mice orally administered the extract of *B. rapa* L. Sampling of spleen cells was conducted in the same manner as described in Fig. 3. Spleen cells (5×10^5 cells/well) from mice were stimulated with LPS (0.1, 1 µg/mL) for 48 hours. IL-10 production levels in the supernatants were measured by ELISA (a). Spleen cells from mice orally administered the extract of *B. rapa* L. (20 mg/kg BW/day) or water were stained with anti-CD4, anti-CD25, and anti-Foxp3 mAbs, and the percentages of Treg cells were evaluated by flow cytometry (b). Data are presented in the same manner as in Fig. 1.
TABLE 1 Body weight and organ weights of cecum and colon in mice orally administered water (control) or *B. rapa* L. extract

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th><em>B. rapa</em> L.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>20.5 ± 0.9</td>
<td>20.2 ± 0.9</td>
</tr>
<tr>
<td>Cecum (mg)</td>
<td>537.9 ± 131.1</td>
<td>492.5 ± 86.4</td>
</tr>
<tr>
<td>Colon (mg)</td>
<td>218.6 ± 39.6</td>
<td>251.5 ± 47.0*</td>
</tr>
</tbody>
</table>

* Data was expressed mean± SD.

* Significant difference between control and *B. rapa* L. group (*P* < 0.05).
(Fig. 1)

(a) Relative population of Bacteroidetes and Firmicutes.

(b) CoA-transferase genes per 16S rRNA gene.

Control B. rapa L.

(c) 16S rRNA genes (relative %).

Control B. rapa L.

(d) CoA-transferase genes per 16S rRNA gene.

Control B. rapa L.
Concentration (mmol/L)

Proportion (mol%)

(a) (b) (c)

(Fig. 2)
Fig. 3

(a) Proportion (%) of CD4+CD69+, CD8+CD69+, and CD11b+CD69+. The graph shows a comparison between control and B. rapa L. treatments, with asterisks indicating significant differences.

(b) Proportion (%) of CD11c+CD69+, CD11c+H-2Kb+, and CD11c+I-Ab+. Similar comparisons are made between control and B. rapa L. treatments, with asterisks indicating significant differences.
(Fig. 4)

(a) IL-10 (ng/mL)

<table>
<thead>
<tr>
<th>Condition</th>
<th>IL-10 (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1</td>
</tr>
<tr>
<td>LPS (0.1 μg/ml)</td>
<td>2</td>
</tr>
<tr>
<td>LPS (1 μg/ml)</td>
<td>5</td>
</tr>
</tbody>
</table>

(b) Treg cells (%)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Treg cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.5</td>
</tr>
<tr>
<td>B. rapa L.</td>
<td>8.0</td>
</tr>
</tbody>
</table>