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Anti-Inflammatory Effects of a Mixture of Lactic Acid Bacteria and Sodium Butyrate in Atopic Dermatitis Murine Model

Jeong A. Kim,[1,*] Sung-Hak Kim,[2,*] In Sung Kim,[1] Da Yoon Yu,[1] Sung Chan Kim,[3] Seung Ho Lee,[4] Sang Suk Lee,[5] Cheol-Heui Yun,[6] In Soon Choi,[7] and Kwang Keun Cho[1]

[1]*Department of Animal Resources Technology, Gyeongnam National University of Science and Technology, Jinju, Korea.*
[2]*Department of Animal Science, Chonnam National University, Gwangju, Korea.*
[3]*Department of Biochemistry, Institute of Cell Differentiation and Aging, College of Medicine, Hallym University, Chuncheon, Korea.*
[4]*Department of Nano-Bioengineering, Incheon National University, Incheon, Korea.*
[5]*Department of Animal Science and Technology, Sunchon National University, Sunchon, Korea.*
[6]*Department of Agricultural Biotechnology, Seoul National University, Seoul, Korea.*
[7]*Department of Life Science, Silla University, Busan, Korea.*

ABSTRACT Atopic dermatitis is a chronic and recurrent inflammatory skin disease. Recently, probiotics have been shown to suppress allergic symptoms through immunomodulatory responses. In the present study, combinatorial effects on allergic symptoms were identified in BALB/c mice fed with a mixture of four species of probiotics, *Bifidobacterium lactis*, *Lactobacillus casei*, *Lactobacillus rhamnosus*, and *Lactobacillus plantarum*, and sodium butyrate. Following sensitization with whey protein, the mice were challenged and divided into two groups: (1) mice administered with phosphate-buffered saline as a control and (2) mice administered with the probiotic mixture and sodium butyrate. Allergic symptoms were assessed by measuring ear thicknesses, serum histamine and IL-10 concentrations, and the quantities of leaked Evans blue. T cell differentiation was determined by analyzing the T cells groups in the mesenteric lymph nodes (MLNs) and spleen. To examine changes in the total gut microbiota, total fecal microflora was isolated, species identification was performed by DNA sequencing using Illumina MiSeq, and changes in intestinal beneficial bacteria were analyzed using quantitative polymerase chain reaction. Treatment with the probiotic mixture and sodium butyrate reduced ear thicknesses, the quantity of leaked Evans blue, and serum histamine values, while increasing serum IL-10 values. In the mouse model, the probiotic mixture and sodium butyrate increased Th1 and Treg cell differentiation in MLN and spleen tissues; the ratio of *Firmicutes*/*Bacteroidetes*, which is associated with reduction in allergic reactions; and microorganisms that lead to cell differentiation into Treg. These results suggest that the probiotic mixture and sodium butyrate can prevent and alleviate allergic symptoms.

KEYWORDS: • Galectin9 • *gut microbiota* • *Th1 cells/Th2 cells* • *Treg cells*

INTRODUCTION

Atopic dermatitis is accompanied with erythema, severe itching, and hemorrhage[1] and its causes include environmental changes, hyperimmune responses, stress, and genetic factors.[2] It is a type 1 hypersensitivity response mediated by mast cell degranulation and a type 2 helper T cells (Th2)-mediated allergic disease.[3] The imbalance between Th1 and Th2 cells leads to autoimmune disorder,[4] and, in particular, increases the levels of Th2 cells associated with the onset of atopy.[5] Th2 cells produce cytokines such as IL-4, IL-5, and IL-13,[6] these cytokines produce antigen-specific IgE, and the resulting IgE activates mast cells to release histamine.[7]

Probiotics are defined as live microorganisms that are beneficial to the health of the host.[8] Specifically, they have been shown to alleviate allergic reactions by modifying the structures of allergens and by inhibiting the adherence of pathogens to intestinal epithelia or mucous membranes as well as the growth of pathogens.[9] In addition, probiotics fine-tune the intestinal microflora composition to induce immune-modulatory effects.[10]

*These authors contributed equally to this study.

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Address correspondence to: Kwang Keun Cho, PhD, Department of Animal Resources Technology, Gyeongnam National University of Science and Technology, Jinju 52725, Korea, E-mail: chotwo2@gntech.ac.kr

Short chain fatty acids such as acetate, butyrate, and propionate can be produced when the gut microbiota utilizes dietary fibers as an energy source.[11,12] Especially, butyrate-producing bacteria promotes the development of tight junction leading to enhancing the function of intestine barrier, while inhibits proinflammatory signaling.[13] Mechanistically, butyrate has been known to bind to receptors such as G protein-coupled receptors GPR41, GPR43, and GPR109A to modulate cell activation, proliferation, and differentiation.[14] In terms of host immune responses in the colon, butyrate elevates the activation and proliferation of Treg cells to repress $CD4^+$ T cells, maintaining of human gut health.[15–17]

However, studies on the effects of probiotics and sodium butyrate on changes in the gut microbiota are still insufficient. In this study, we investigated the mitigation effect of probiotic mixture and sodium butyrate on atopic dermatitis in an *in vivo* mouse model.

MATERIALS AND METHODS

Animals

Three-week-old female BALB/c mice (Samtako, Osan, Korea) and 4- and 6-week-old male Sprague–Dawley rats (Samtako) were maintained at room temperature (22°C ± 1°C), with a 12-h light–dark cycle during the experimental period and were provided *ad libitum* access to food (AIN-76A; Central Lab, Seoul, Korea) and water. The probiotic mixture, which was a mixture of four species of lactic acid bacteria (*Lactobacillus casei*, *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, and *Bifidobacterium lactis* [Cell Biotech, Gimpo, Korea]), was provided at a concentration 2% wt:wt 2×10^9 CFU/g, and sodium butyrate (Sigma-Aldrich, CA, USA) was provided at a concentration of 100 mM/0.2 mL phosphate-buffered saline (PBS). After a preliminary experimental period of 1 week, the experimental animals were randomly divided into five groups: (1) control group (C: normal diet), (2) negative control group (N: normal diet+whey proteins), (3) probiotics mixture group (T1: normal diet+whey proteins+probiotics mixture), (4) sodium butyrate group (T2: normal diet+whey proteins+sodium butyrate), and (5) probiotics mixture+sodium butyrate group (T3: normal diet+whey proteins+probiotics mixture+sodium butyrate). The experimental protocol was approved by the Institutional Animal Care Board of Gyeongnam National University of Science and Technology (Approval No. 2016-7).

Acute hypersensitivity response

According to the method presented by de Kivit *et al.*,[18] allergic symptoms were induced in BALB/c mice using whey proteins and cholera toxin (CT; Fig. 1A). Whey protein consists of lactalbumin and lactoglobulin, which are considered to be major cow's milk allergens.[19] The probiotics mixture and sodium butyrate were dissolved in 0.2 mL PBS and orally administered once per day from 2 weeks before sensitization until the end of the experimental period. Whey proteins (20 mg) and CT (10 μg) were dissolved in 0.5 mL PBS and orally administered once per week from day 0 to 28 to sensitize the mice. At day 33, whey proteins were dissolved in PBS and were orally administered (100 mg) and intradermally injected (10 μg) to challenge the mice. Ear thickness was measured using digital calipers (Bluebird; NA500-150S) 1 h after challenging with whey proteins. The following day, the BALB/c mice were sacrificed and blood samples were collected from the vena cava; the blood samples were centrifuged for 10 min at 1300 *g* to separate the serum. Histamine levels in the separated serum were measured using the Histamine EIA kit (LDN, Germany), and IL-10 levels in the separated serum were measured using the Quantikine Human IL-10 Immunoassay kit (R&D, USA).

Histamine-induced vasodilation

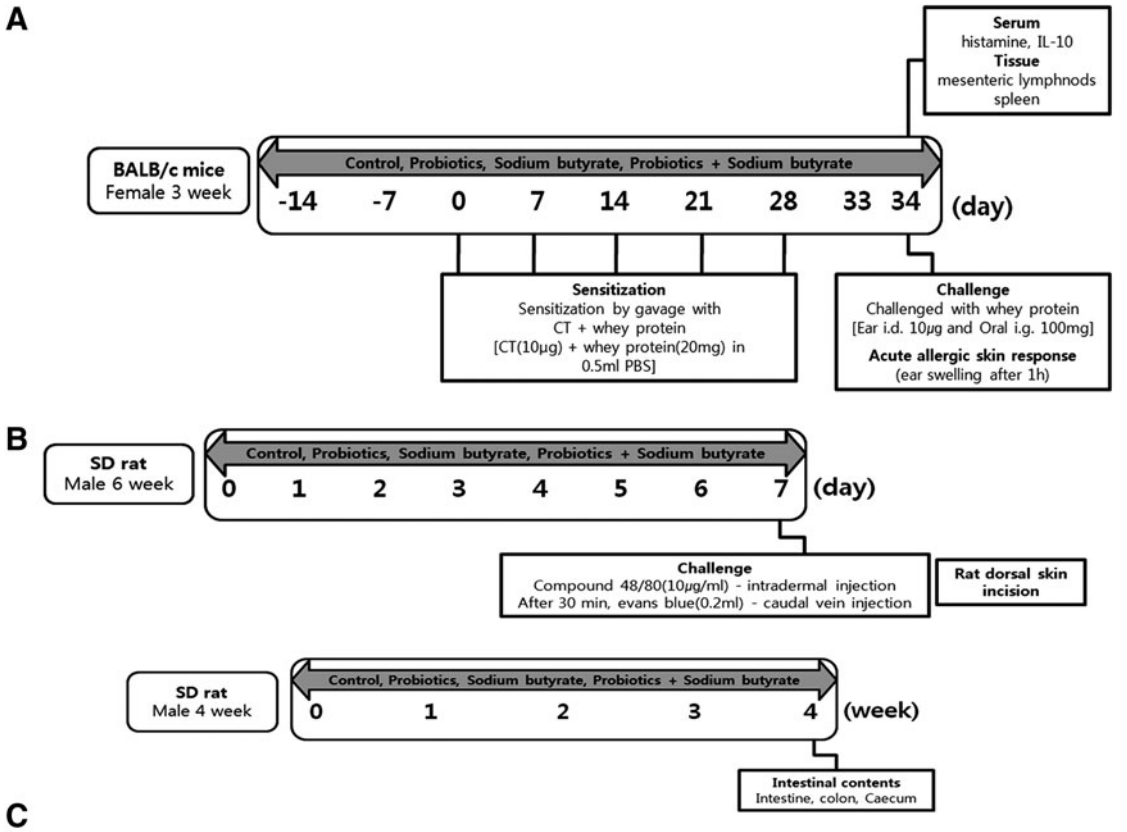
The atopic dermatitis model was established by triggering histamine release using compound 48/80 (COM; Sigma-Aldrich) (Fig. 1B).[20] In brief, 6-week-old Sprague–Dawley rats were used as atopic dermatitis models, and the probiotics mixture and sodium butyrate dissolved in 0.5 mL PBS were orally administered to the mice once per day for 7 days. Before inducing atopic dermatitis, the dorsal fur of the mice was resected using electric shavers. Next, 50 μL of COM (10 μg/mL) was intradermally injected into the dorsal dermis, and 30 min later, Evans blue (Sigma-Aldrich) was injected into the tail vein. All the animals were sacrificed 30 min there after. To measure the quantity of leaked Evans blue, the dorsal dermis of the mice was resected, maintained for 48 h in 1.0 mL of 1.0 N KOH at 37°C, followed by adding 0.6 N H_3PO_4 and acetone, and centrifuged for 10 min at 900 *g*. Next, the concentration of Evans blue was measured at 620 nm using a spectrophotometer (Biodrop Ltd., United Kingdom).

Separation and analysis of the RNA of mouse spleen and mesenteric lymph nodes

To analyze the immunomodulatory effects of the probiotics mixture and sodium butyrate, acute hypersensitivity BALB/c mice were sacrificed and mesenteric lymph node (MLN) and spleen were separated. TRIzol® was added to MLN and spleens of BALB/c mice and homogenized using Silent-Crusher M (Heidolph, Germany). According to the method presented by Chomczynski and Sacchi,[21] RNA was extracted and stored at −20°C until cDNA synthesis. cDNA was synthesized using reverse transcription-polymerase chain reaction (RT-PCR) kits (TaKaRa Co., Tokyo, Japan) according to the manufacturer's instructions. The list of the primer sequences is given in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference gene.

Analysis of gut microbiota

Four-week-old Sprague–Dawley rats were used to examine the effects of the probiotics mixture and sodium butyrate on changes in gut microbiota (Fig. 1C). Same volumes of the



C

Control	Basal diet
Probiotics	2% wt:wt $2X10^9$ CFU/g probiotics in 0.2ml PBS
Sodium butyrate	100mM/0.2ml in 0.2ml PBS
Probiotics + Sodium butyrate	2% wt:wt $2X10^9$ CFU/g probiotics + 100mM/0.2ml in 0.2ml PBS

FIG. 1. Experimental design. **(A)** Acute hypersensitivity response. **(B)** Histamine induced vasodilation. **(C)** Analysis of Intestinal microbiota. CT, cholera toxin; PBS, phosphate-buffered saline.

probiotics mixture and sodium butyrate as the acute hypersensitivity response experiment were dissolved in PBS, and 0.5 mL of the solution was orally administered once per day for 4 weeks. Four weeks later, all the animals were sacrificed and fecal samples of the Sprague–Dawley rats were collected and kept at −80°C until genomic DNA (gDNA) extraction. DNA was extracted using Fecal DNA MiniPrep Kits (Zymo Research, CA, USA), and the sequence was analyzed using Illumina MiSeq (ChunLab, Inc., Seoul, Korea).

Table 1. Reverse Transcription-Polymerase Chain Reaction Primer Sequences for Immune-Modulatory Analysis

Target gene	*Primer*	
GAPDH	Forward	5′ CCACCCAGAAGACTGTGGAT 3′
	Reverse	5′ CACATTGGGGGTAGGAACAC 3′
T-bet	Forward	5′ TCAACCAGCACCAGACAGAG 3′
	Reverse	5′ AAACATCCTGTAATGGCTTGTG 3′
Gata3	Forward	5′ CATTACCACCTATCCGCCCTATG 3′
	Reverse	5′ CACACACTCCCTGCCTTCTGT 3′
Rorγt	Forward	5′ TTCACCCCACCTCCACTG 3′
	Reverse	5′ TGCAAGGGATCACTTCAATTT 3′
Foxp3	Forward	5′ CCCATCCCCAGGAGTCTTG 3′
	Reverse	5′ CCATGACTAGGGGCACTGTA 3′
Galectin9	Forward	5′ GAGAGGAAGACACACATGCCTTTC 3′
	Reverse	5′ GACCACAGCATTCTCATCAAAACG 3′

Table 2. Quantitative Polymerase Chain Reaction Primer Sequences for Beneficial Bacteria Analysis

Target gene	*Primer*	
Universal	Forward	5′ GTGSTGCAYGGYYGTCGTCA 3′
	Reverse	5′ ACGTCRTCCMCNCCTTCCTC 3′
Faecalibacterium prausnitzii	Forward	5′ GGAGGAAGAAGGTCTTCGG 3′
	Reverse	5′ AATTCCGCCTACCTCTGCACT 3′
Ruminococcus	Forward	5′ GGCGGCYTRCTGGGCTTT 3′
	Reverse	5′ CCAGGTGGATWACTTATT GTGTTAA 3′
Bifidobacterium	Forward	5′ TCGCGTCYGGTGTGAAAG 3′
	Reverse	5′ GGTGTTCTTCCCGATATC TACA 3′
Weissella cibaria	Forward	5′ TTGATTGACATAGAACCTGAT 3′
	Reverse	5′ TTCGGTGCTAGTTCTTCAATA 3′

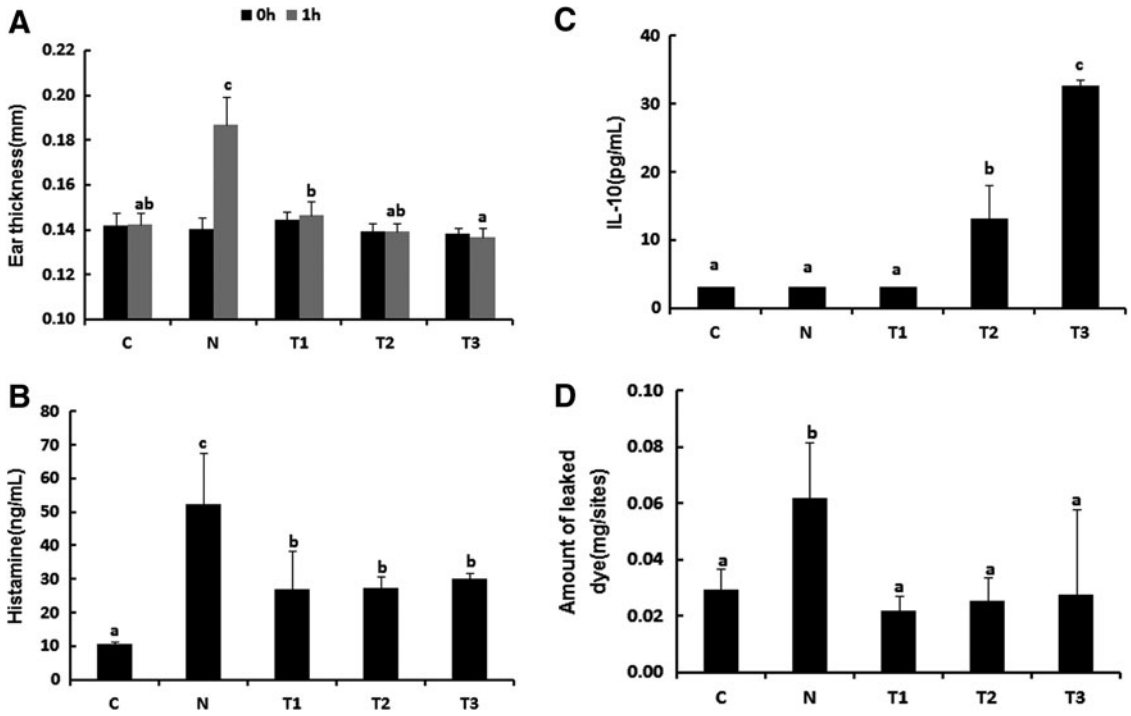


FIG. 2. Probiotic mixture and sodium butyrate suppress allergic symptoms in mice. **(A)** One hour after intradermal challenge with whey in the ear, acute hypersensitivity response was reduced in whey-sensitized mice treated with probiotics mixture and sodium butyrate compared with whey-sensitized mice ($n=6$). **(B)** Total amount of serum histamine was reduced and **(C)** serum IL-10 was increased treated with probiotics mixture and sodium butyrate at after the last challenge ($n=3$). **(D)** After an intradermal injection with compound 48/80 in the skin, amount of leaked dye was reduced in whey-sensitized mice treated with probiotics mixture and sodium butyrate compared with whey-sensitized mice ($n=5$). [a–c]Means are significantly different within the same row ($P<.05$). C, control; N, negative control group (allergy induce); T1, probiotic mixture group (2% wt:wt 2×10^9 CFU/g-*Lactobacillus casei, Lactobacillus rhamnosus, Lactobacillus plantarum, Bifidobacterium lactis*+allergy inducer); T2, sodium butyrate group (100 mM/0.2 mL-sodium butyrate+allergy inducer); T3, probiotic mixture+sodium butyrate group (probiotic mixture+sodium butyrate+allergy inducer).

Quantitative real-time PCR

As mentioned above, DNA was extracted from rat fecal samples. Quantitative PCR (qPCR) was performed using Rotor-Gene SYBR® Green PCR Kits (QIAGEN GmbH, Germany). A list of the primer sequences of intestinal beneficial bacteria is presented in Table 2. Universal was used as the reference gene.

Statistical analysis

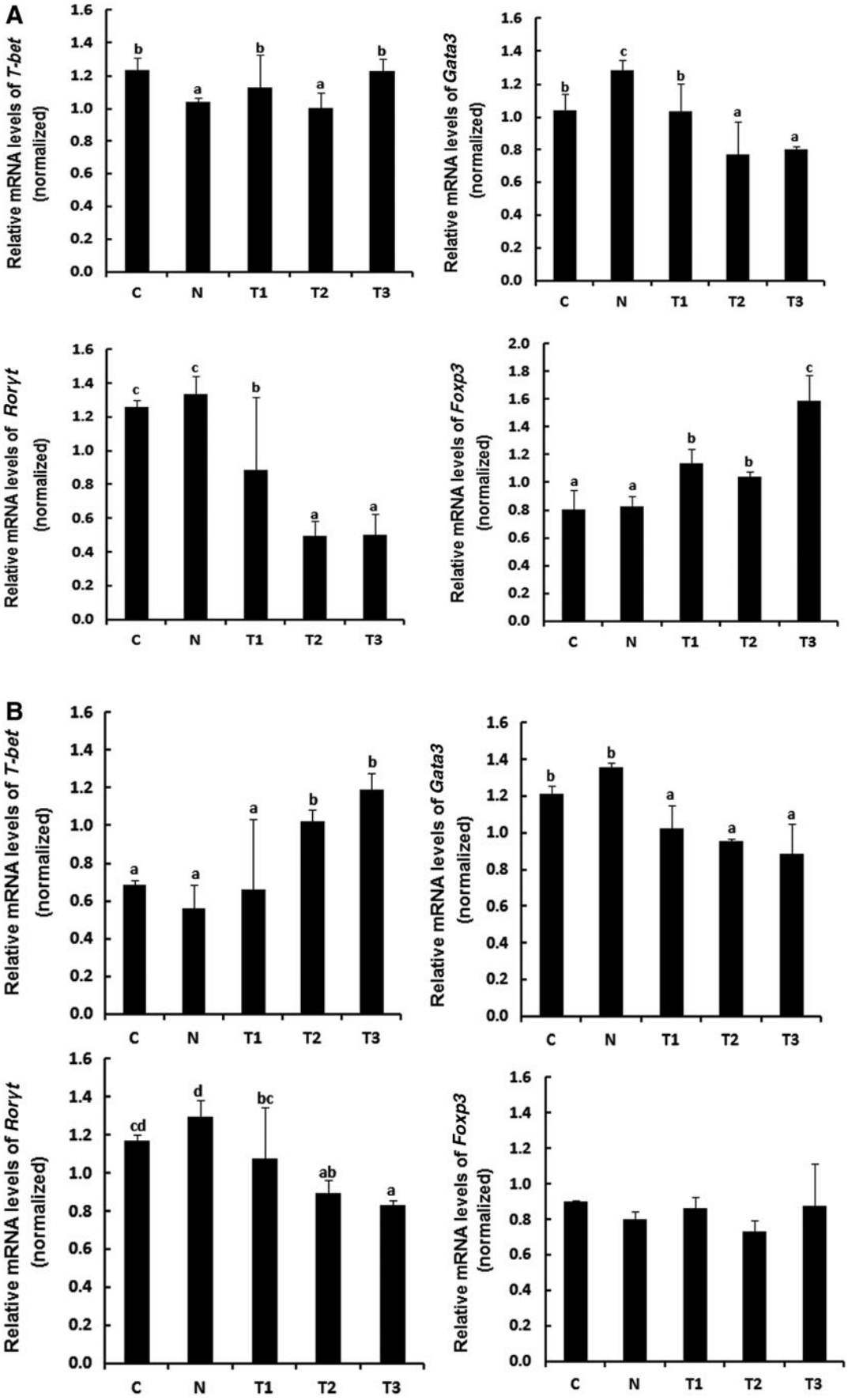
The variance of the results obtained through repeated experiments was analyzed using SPSS 12.0 (SPSS, Inc., Chicago, IL, USA). The significance of the results of analysis in the treatment groups were tested using Duncan's multiple range test at a level of $P<.05$, and intergroup significance was tested using the independent-sample *t*-test at levels of $P<.05$ and $P<.01$. All results are indicated as mean ± standard deviation.

RESULTS

Allergic symptoms in mice

To examine the effects of administration of the probiotics mixture and sodium butyrate on the allergic symptoms, we first measured the ear thickness, which is a pathological phenotype in mice orally sensitized and challenged with whey protein. As shown in Figure 2A, ear thickness decreased significantly in the T1, T2, and T3 groups compared with that in the N group (Fig. 2A; $P<.05$). Next, we examined the histamine and IL-10 levels because allergic

FIG. 3. Whey-allergic mice fed probiotics mixture and sodium butyrate show enhanced Th1- and Treg-cell development in MLNs and spleen. T cell polarization in **(A)** MLN and **(B)** spleen was evaluated by analyzing the expression of T-bet (Th1), GATA3 (Th2), RORγT (Th17), and Foxp3 (Treg). The probiotics mixture and sodium butyrate increased the differentiation of Th1 and Treg cells and decreased the differentiation of Th2 and Th17 cells. **(C)** *Galectin9* is expressed by intestinal epithelial cells and the probiotics mixture and sodium butyrate increased the *Galectin9* in MLN and spleen. [a–d]Means are significantly different within the same row ($P<.05$). C, control; N, negative control group (allergy inducer); T1, probiotic mixture group (2% wt:wt 2×10^9 CFU/g-*L. casei, L. rhamnosus, L. plantarum, B. lactis*+allergy inducer); T2, sodium butyrate group (100 mM/0.2 mL-sodium butyrate+allergy inducer); T3, probiotic mixture+sodium butyrate group (probiotic mixture+sodium butyrate+allergy inducer). MLN, mesenteric lymph node.


A
Relative mRNA levels of T-bet (normalized)
Relative mRNA levels of Gata3 (normalized)
Relative mRNA levels of Rorγt (normalized)
Relative mRNA levels of Foxp3 (normalized)
C
N
T1
T2
T3
B
Relative mRNA levels of T-bet (normalized)
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Relative mRNA levels of Rorγt (normalized)
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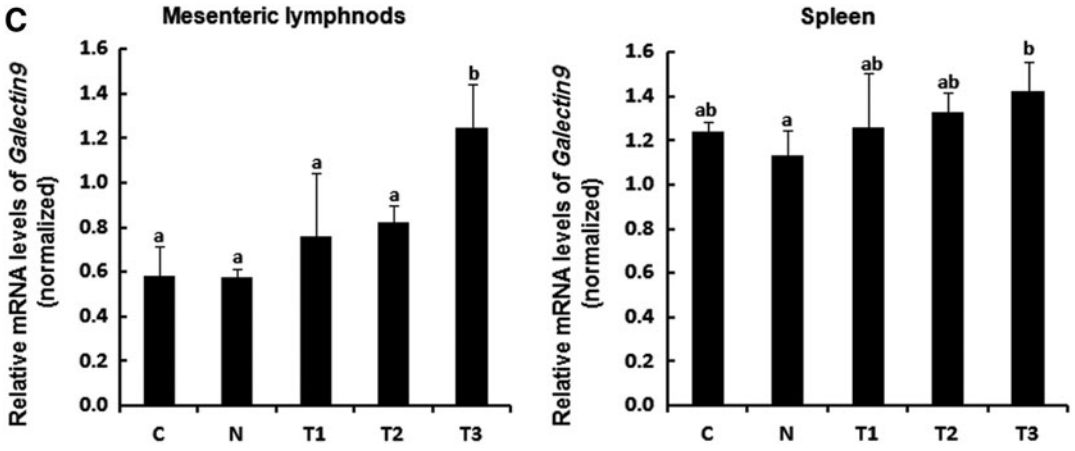


FIG. 3. (*Continued*).

symptoms are accompanied with increases in serum histamine levels and decreases in serum IL-10 levels due to increases in the Th2 cells immune response.[5] Serum histamine levels decreased significantly in all treatment groups, whereas IL-10 concentration increased in the T2 and T3 groups compared with those in the N group (Fig. 2B, C; $P < .05$). Similar results were also observed in mice intradermally injected with COM, an allergic reaction inducer. Reduced Evans blue staining was observed in the dorsal dermis of mice in the T1, T2, and T3 groups due to vasodilation (Fig. 2D). Therefore, these results suggest that the probiotics mixture and sodium butyrate inhibit allergic symptoms.

T cell differentiation in MLN and spleen

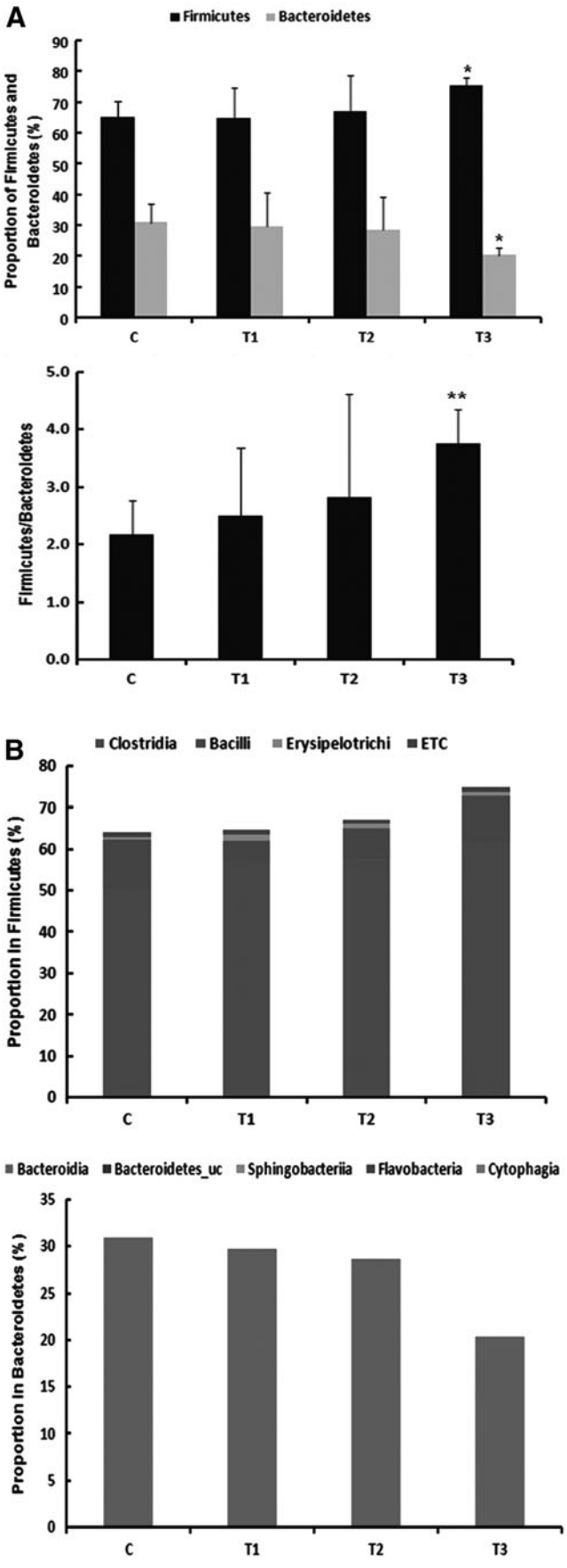
To understand the effects of the probiotics mixture and sodium butyrate on changes in T cells population, mRNA levels of *T-bet*, *Gata3*, *Rorγt*, and *Foxp3*, which are transcription factors specific to Th1, Th2, Th17, and Treg cells, respectively, were analyzed by RT-PCR in MLN and spleen tissues from whey-allergic mice. mRNA expression of *T-bet* and *Foxp3* increased in the T1, T2, and T3-treated MLN, but the difference between T2-treated MLN and the N group was not significant (Fig. 3A). However, the treatment with T1, T2, and T3 caused a marked decrease in GATA3 and *Rorγt* mRNA expression. In particular, the T3 group exhibited a significant increase in the mRNA expression of *Foxp3* by 1.9-fold compared with the N group, indicating that the treatment with probiotics mixture and sodium butyrate may induce immunomodulatory responses by changing the balance of Th1, Th2, and Treg cells, resulting in alleviating atopic dermatitis occurring due to immune imbalance states. mRNA expression of *T-bet* increased in the T2 and T3-treated spleen, but there is no statistical significant in the *Foxp3* mRNA expression of experimental groups in spleen (Fig. 3B). To further determine the effect of the probiotics mixture and sodium butyrate on *Galectin9* expression, *Galectin9* mRNA expression in MLN and spleen was determined by RT-PCR. *Galectin9* modulates mast cell degranulation and T cell differentiation.[18] As shown in Figure 3C, *Galectin9* expression increased significantly in the MLN of the T3 group compared with that in the MLN of the N group ($P < .05$). However, in the spleen, *Galectin9* expression increased slightly in the T1 and T2 groups and increased significantly in the T3 group ($P < .05$). It is known that *Gata3* gene is a specific marker during Th2 cell differentiation.[22] In the Figure 3, due to the low expression level of *Gata3* in T2 groups, we believe probiotic mixture and sodium butyrate group reduces Th2 cell differentiation. Our results suggest that treatment with the probiotics mixture+sodium butyrate reduces Th2 cell differentiation while increasing Th1 and Treg cell differentiation. Therefore, we propose that probiotics mixtures and sodium butyrate can alleviate atopic dermatitis through immunomodulation.

Effects of probiotics mixture and sodium butyrate on gut microbiota

Gastrointestinal microbiota influence Th1, Th2, and Treg maturation.[23] As shown in Figure 4A, the ratio of *Firmicutes* to *Bacteroides* increased significantly in the T3 group compared with that in the C, T1, and T2 groups. In addition, the results of the analysis of gut microbiota indicated that *Firmicutes* (64.85–75.31%) existed in the highest ratio, which was more than half of the total population of gut microbiota. When the results were analyzed at the class level, *Clostridia* was dominant at 50.52–62.17% in the *Firmicutes* (Fig. 4B).

Effects of probiotics mixture and sodium butyrate on increases in intestinal beneficial bacteria

We could not observe synergistic effects of combinatorial treatment of probiotics mixtures and sodium butyrate to regulate intestinal beneficial bacteria such as *Faecalibacterium prausnitzii* spp. and *Weissella cibaria*. In Figure 5, it seems to be more effective of sodium butyrate to increase their populations. It is known that *F. prausnitzii* spp. promotes Treg cell differentiation by producing butyrate



as a butyrate-producing bacteria in the gut.[24] In addition, *W. cibaria* has been shown to block Th2 cell differentiation as well as IgE.[25] Taken together, we concluded T2 group containing sodium butyrate repress the symptoms of atopy dermatitis.

Increase in immunomodulatory effects by probiotics mixture and sodium butyrate

Among the gut microbiota, *Clostridia* has been implicated in preventing diseases such as food allergies.[26] As shown in Table 3, *Clostridia* population increased significantly to 62.17% in the T3 group compared with the C group with a ratio of 50.52%. In particular, the IV and XIVa clusters of *Clostridia* alleviate allergy and inflammation by inducing Treg cells.[27] Therefore, we verified the immunomodulatory effects of *Clostridia* clusters IV and XIVa in gut microbiota. According to our results, at the genus level, *Eubacterium*, which belongs to *Clostridia* cluster XIVa, increased significantly in the T3 group to 10.54% compared with that in the C group, where its proportion was 5.55% ($P < .01$). In addition, *Clostridium*, which belongs to *Clostridia* clusters XIVa and IV, increased significantly in the T1 (7.00%) and T3 groups (8.49%) compared with that in the C group (5.09%; $P < .05$). *Clostridium* was also shown to exist in the highest ratio in the T3 group. *Eubacterium* (7.37%) and *Clostridium* (7.18%) increased in the T2 group; however, the differences were not significant. Therefore, the present study indicates that the probiotics mixture and sodium butyrate can induce Treg cells through increases in *Clostridia* clusters IV and XIVa and increase IL-10 concentrations in the blood, there by alleviating allergic reactions.

DISCUSSION

Atopic dermatitis accompanies serum histamine release[6] and IL-10 decrease by mast cell degranulation.[28] In this study, we demonstrate the effects of the treatment with probiotic mixture and sodium butyrate to alleviate atopic dermatitis *in vivo*. Because COM induces mast cell degranulation and histamine releases in systemic anaphylaxis, it has been used for establishing vasodilation in atopic dermatitis model.[29] Several studies have shown that a probiotic, *Lactococcus chungangensis*, could improve atopic dermatitis symptoms in a COM-induced mouse model.[30] Using this model, we observed that a composite mixture of four species

FIG. 4. Pyrosequencing analysis of the fecal microbiota composition from mice fed the probiotics mixture and sodium butyrate. **(A)** Relative abundance ratio of *Firmicutes* and *Bacteroidetes* in fecal microbiota. **(B)** Population of Firmicutes and Bacteroidet at class level. *Means are significantly different within the same row ($P < .05$), **Means are significantly different within the same row ($P < .01$). C, control; T1, probiotic mixture group (2% wt:wt 2×10^9 CFU/g-*L. casei, L. rhamnosus, L. plantarum, B. lactis*+allergy inducer); T2, sodium butyrate group (100 mM/0.2 mL-sodium butyrate+allergy inducer); T3, probiotic mixture+sodium butyrate group (probiotic mixture+sodium butyrate+allergy inducer).

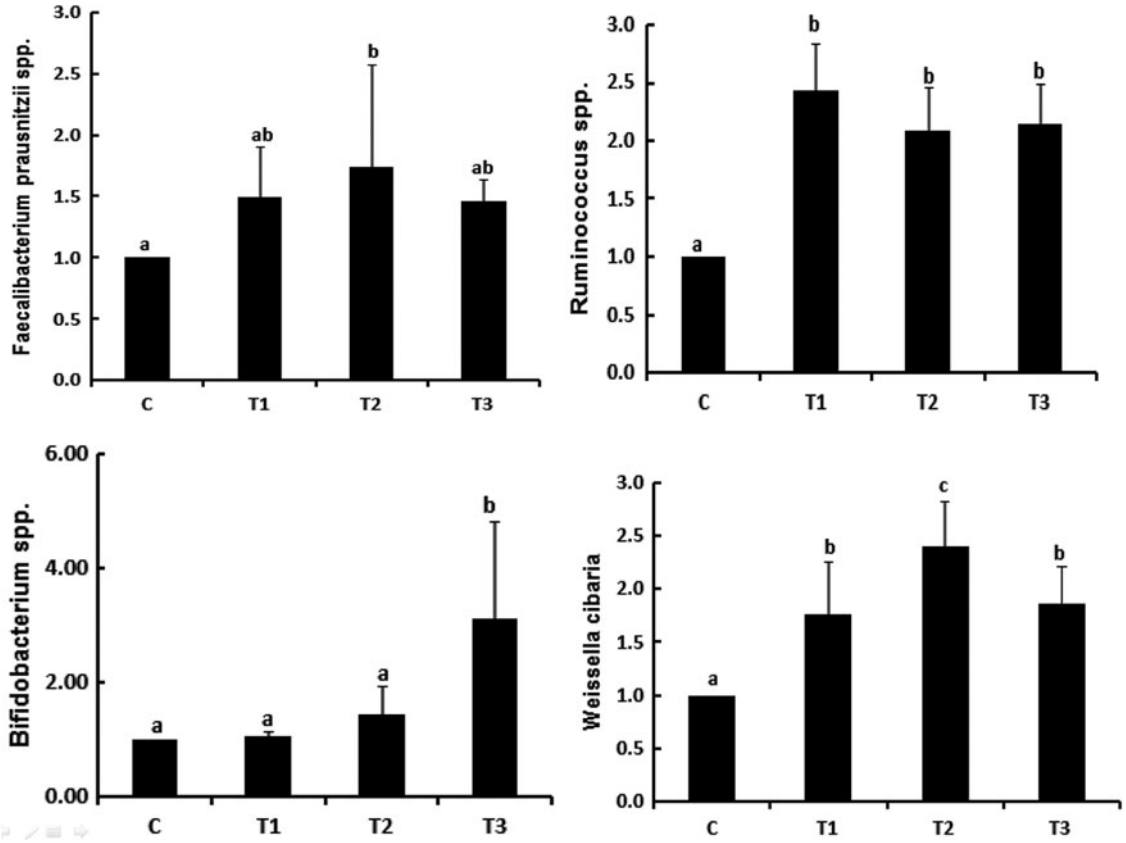


FIG. 5. The abundance of functional bacteria in the feces of probiotics mixture and sodium butyrate by quantitative PCR. [a–c]Means are significantly different within the same row ($P < .05$). C, control; T1, probiotic mixture group (2% wt:wt 2×10^9 CFU/g-*L. casei, L. rhamnosus, L. plantarum, B. lactis*+allergy inducer); T2, sodium butyrate group (100 mM/0.2 mL-sodium butyrate+allergy inducer); T3, probiotic mixture+sodium butyrate group (probiotic mixture+sodium butyrate+allergy inducer). PCR, polymerase chain reaction.

TABLE 3. PYROSEQUENCING ANALYSIS OF THE FECAL MICROBIOTA COMPOSITION FROM MICE FED THE PROBIOTICS MIXTURE AND SODIUM BUTYRATE

	Treatments			
	C n = *4* *Mean% (SD)*	*T1* n = *4* *Mean% (SD)*	*T2* n = *4* *Mean% (SD)*	*T3* n = *4* *Mean% (SD)*
Firmicutes	65.01 (5.15)	64.85 (9.92)	67.11 (11.64)	75.31 (2.49)*
Clostridia	50.52 (6.87)	56.99 (11.81)	57.87 (8.23)	62.17 (4.02)*
Lachnospiraceae	33.69 (3.74)	37.57 (9.15)	38.93 (7.52)	41.40 (2.65)*
Ruminococcaceae	13.56 (1.33)	13.52 (3.79)	14.99 (4.34)	17.50 (1.82)*
Eubacterium	5.55 (1.71)	6.26 (3.61)	7.37 (2.77)	10.54 (1.96)**
Clostridium	5.09 (0.74)	7.00 (1.28)*	7.18 (3.09)	8.49 (2.48)*
Bacilli	11.87 (4.38)	5.18 (3.69)	7.08 (2.60)	10.65 (3.80)
Lactobacillaceae	11.76 (4.36)	5.12 (3.69)	7.00 (2.56)	10.55 (3.78)
Lactobacillales_uc	0.03 (0.01)	0.02 (0.01)	0.02 (0.01)	0.03 (0.01)
Streptococcaceae	0.04 (0.02)	0.03 (0.01)	0.03 (0.01)	0.04 (0.01)
Bacteroidetes	30.99 (6.15)	29.78 (10.81)	28.65 (10.52)	20.33 (2.53)*
Bacteroidia	30.98 (6.16)	29.77 (10.81)	28.65 (10.51)	22.13 (5.74)*
Prevotellaceae	18.60 (12.19)	14.84 (11.32)	13.75 (11.66)	2.91 (1.64)*
S24-7_f	10.09 (6.51)	10.88 (1.47)	9.13 (1.87)	9.86 (1.60)

*Means are significantly different within the same row ($P < .05$).

**Means are significantly different within the same row ($P < .01$).

C, control; SD, standard deviation; T1, probiotics mixture group (2% wt:wt 2×10^9 CFU/g-*Lactobacillus casei, Lactobacillus rhamnosus, Lactobacillus plantarum, Bifidobacterium lactis*+allergy induce); T2, sodium butyrate group (100 mM/0.2 mL-sodium butyrate+allergy induce); T3, probiotics mixture+sodium butyrate group (probiotics mixture+sodium butyrate+allergy induce).

of probiotic bacteria and sodium butyrate mitigated atopic dermatitis symptoms due to histamine secretion.

Atopic dermatitis is characterized by Th1/Th2 cells imbalance and dominant Th2 cells-mediated responses.[31,32–34] Recent studies have reported that Treg cells could trigger inflammatory responses in atopic dermatitis through Th2 cells regulation.[25,35] In addition, *Galectin9* is associated with the alleviation of allergy-related diseases as it induces Th1 and Treg cells immune responses and directly inhibits mast cell degranulation.[18] In MLN and spleen tissues of atopic dermatitis mouse models, we demonstrated that the probiotics mixture and sodium butyrate increased the populations of Th1 and Treg cells and *Galectin9* expression, while reducing the population of Th2 and Th17 cells. These data imply that probiotics mixture and sodium butyrate can induce positive effects by alleviating symptoms of atopic dermatitis.

Symbiotic microorganisms in the large intestine have significant effects on metabolism and immunity[36] and cause allergic airway inflammation in response to allergens.[37] Therefore, we hypothesized that administration of a probiotic mixture supplemented with sodium butyrate may be able to modulate atopic dermatitis by immunoregulation of the changes in intestinal symbiotic microorganisms. Most of the intestinal microorganisms (90–99%) are composed of *Firmicutes* and *Bacteroidetes*, and 50–80% of *Firmicutes* are primarily Clostridium clusters.[38] In this study, we have shown that treatment with probiotics mixture and sodium butyrate increased *Firmicutes* while decreasing *Bacteroidetes* in the gut microbiota of rats. In addition, consistent with our findings, it has been shown that *Firmicutes* (phylum) and *Clostridia* (class) are present in high numbers in infants after completely treating milk allergies, whereas *Bacteroidetes* (phylum) are present in high numbers in infants suffering from milk allergies.[39] Microorganisms belonging to the increasing *Firmicutes* population and the decreasing *Bacteroidetes* population were analyzed. *Bacteroidia* (class) and *Prevotellaceae* (family) were found to be the dominant species in *Bacteroidetes*. The population of intestinal *Prevotellaceae* (family), a pathogenic bacterium that causes chronic intestinal inflammation,[40] decreased following administration of the probiotics mixture and sodium butyrate. When the families belonging to *Firmicutes* were analyzed, *Lachnospiraceae* and *Ruminococcaceae* were shown to be the dominant intestinal species, and their numbers increased after administration of the probiotics mixture and sodium butyrate. *Lachnospiraceae* and *Ruminococcaceae* are butyrate-producing bacteria.[41] Gut microbiota utilize dietary fiber as an energy source to produce lactate and acetate, which are converted to butyrate by butyrate-producing bacteria.[17] *Bacteroidia* probiotics exhibit a butyrogenic effect through interaction with bacteria that produce butyrate,[12] and butyrate binds to receptors such as G protein-coupled receptors GPR43 and GPR109a to stimulate the differentiation of Treg cells.[42] In a recent study, it was found that butyrate stimulates Treg cells to increase the production of IL-10.[16] In addition, changes in the gut microbiota reduce the Th2 cell population increase caused by allergic reactions and promote Treg cell development,[43] indicating that there is a correlation between gut microbiota and Treg cells during an allergic response.[44] It is plausible that probiotics exert immunomodulatory effects by increasing butyrate that activates GPR43- and GPR109a-mediated signaling pathways. *Clostridia* is classified into 19 clusters, of which clusters IV, XIVa, and XVIII are known as Treg-inducing strains.[45] In this study, *Eubacterium* belonging to *Clostridia* cluster XIVa and *Clostridium* belonging to *Clostridia* clusters XIVa and IV increased significantly following administration of the probiotics mixture and sodium butyrate. However, the increase in *Ruminococcus,* belonging to the *Clostridia* cluster XIVa, was not remarkable.

In conclusion, the intake of probiotics mixture promotes butyrate production by increasing the population of butyrate-producing bacteria. Butyrate enhances Treg cells, thereby acting as an immunomodulatory agent, to mitigate and prevent atopic dermatitis. In future studies, the pathways through which probiotics play the role of an immunomodulator between the gut microbiota and T cell differentiation should be investigated in detail.

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AUTHOR DISCLOSURE STATEMENT

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