Journal of Biosciences and Medicines, 2025, 13(5), 59-74
https://www.scirp.org/journal/jbm
ISSN Online: 2327-509X
ISSN Print: 2327-5081

Effects of Long-Term Feeding of Dietary Allitol on Glucose Tolerance and Fecal Microbiota Profiles in Rats

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How to cite this paper: Matsuo, T., Mitsui, S., Takata, G., Higaki, S., Inai, R., Mochizuki, S., Yoshihara, A. and Akimitsu, K. (2025) Effects of Long-Term Feeding of Dietary Allitol on Glucose Tolerance and Fecal Microbiota Profiles in Rats. *Journal of Biosciences and Medicines*, **13**, 59-74.
https://doi.org/10.4236/jbm.2025.135006

Received: April 1, 2025
Accepted: May 16, 2025
Published: May 19, 2025

Abstract

This study aimed to investigate the effect of long-term feeding of dietary allitol on glucose tolerance and the fecal microbiota profile in rats. The basic data was obtained, and the production of butyric acid from allitol was predicted using bioinformatic techniques. Furthermore, this study examined whether the anti-diabetic effect of allitol was due to gut microbiota. Fifty male Wistar rats, aged 4 weeks, were randomly divided into two groups of 25: control (C) and allitol (A). They were fed a commercial diet containing 3% sucrose or allitol. After feeding them for 16 weeks, oral and intraperitoneal glucose tolerance tests (OGTT and IPGTT, respectively) chowed that blood glucose levels before and after glucose administration were lower in Group A than in Group C. The area under the curve (AUC) was also lower in Group A than in Group C. After 24 weeks of the feeding period, Plasma glucose, insulin, and triglyceride concentrations and HOMA-R values were significantly lower in Group A than in Group C. Taxonomic changes in the microbial communities were assessed at the genus level. Changes in the microbiota indicated a significant increase in the abundance of the genera *Blautia, Anaerostipes,* and *Acetitomaculum,* known butyric acid producers. Potential differences in the function of the microbial community were evaluated using the PICRUSt2. Regarding butyric acid metabolism-related enzymes, butyryl-CoA: acetate-CoA transferase, *trans*-2-enoyl-CoA reductase (NAD^+), butyrate kinase, and butanol dehydrogenase were significantly higher in Group A than in Group C. These results suggest that several compositional changes in the fecal microbiota and an increase in predicted butyric acid metabolism following dietary allitol supplementation. The anti-diabetic effect of allitol was confirmed; however, it was suggested

that there may be other causes of this effect besides butyric acid produced by intestinal microbiota.

Keywords

Allitol, Butyric Acid, Glucose Tolerance, Fecal Microbiota, Rat

1. Introduction

Diabetes affects approximately 10% of adults worldwide. People with diabetes have twice the risk of cardiovascular disease and approximately 20% higher incidence of cancer [1] [2]. Decreased glucose tolerance and insulin sensitivity are early signs of susceptibility to type 2 diabetes, and typically manifest as elevated fasting glucose and insulin levels [3]. Insulin is an important regulator of glucose metabolism [4], which promotes glucose uptake in peripheral tissues and inhibits its glucose production in the liver. Insufficient insulin action leads to elevated fasting blood glucose and, ultimately, type 2 diabetes [4]. In addition, insulin resistance leads to cardiometabolic complications, which occur before the onset of type 2 diabetes [5].

Since the scientific verification of the direct influence of intestinal microbiota on obesity and diabetic diseases, these microbiota and their impact on metabolic diseases have been extensively explored [6]. Recently, the relationship between glucose intolerance (type 2 diabetes mellitus) and intestinal microbiota has received considerable attention [7]-[9]. A cohort study on patients with type 2 diabetes showed that the proportion of butyric acid [a short-chain fatty acid (SCFA)]-producing bacteria in the gut microbiota was low, while that of non-butyric acid-producing bacteria was high [10]. In another cohort profile study, intestinal microbiota metagenomic sequencing analysis showed that the presence of butyric acid-producing bacteria in feces was positively correlated with the presence of pathways involved in butyrate production, and a higher butyric acid production capacity in the intestinal tract was dependent on improved insulin sensitivity after the oral glucose tolerance test [11]. Furthermore, since butyric acid could affect insulin sensitivity, increased butyrate synthesis caused by the enhancement of butyric acid-producing intestinal bacteria has the potential to prevent or treat diabetes [12].

Recently, rare sugars have gained attention owing to their potential as functional foods with anti-diabetic and anti-obesity properties. According to the International Society of Rare Sugars (2002), rare sugars are monosaccharides and their derivatives that are naturally less common than typical sugars (such as D-glucose and D-fructose) and can serve as supplements, functional food additives, and medications [13]. Allitol, a rare sugar, is a sugar alcohol formed by linking D- and L-hexoses in a process known as izumoring [14]; it is created by converting d-allulose [15]. We have previously shown that dietary allitol results in a greater increase in cecal weight and surface area of rats than maltitol [16] or fructooligosaccharide [17] (both highly fermentable carbohydrates). This suggests that allitol

is a highly fermentable sugar alcohol. Moreover, we showed that the content of cecal SCFAs, primarily butyric acid, was significantly increased by allitol [18] [19]. Therefore, we hypothesized that dietary allitol exerts its anti-diabetic effects via butyric acid production by the gut microbiota. Our previous studies [18] [19] examined the effects of several weeks of allitol intake on the gut microbiota of young, growing rats. However, to more accurately examine the relationship between glucose intolerance and dietary allitol, it would be appropriate to extend the duration of allitol intake to older age when glucose intolerance occurs more frequently. Additionally, the mechanisms responsible for the microbial activities of allitol remain unclear. Thus, this study investigates the effect of long-term feeding of dietary allitol on the glucose tolerance and fecal microbiota profiles of rats. The basic data was obtained, and the production of butyric acid from allitol was predicted using bioinformatics techniques. Furthermore, this study examined whether the anti-diabetic effect of allitol was due to the gut microbiota.

2. Materials and Methods

All animal procedures were approved by the Animal Care and Use Committee for Kagawa University (Approval number: 24637).

2.1. Materials

Allitol was sourced from the International Institute of Rare Sugar Research and Education (Kagawa, Japan). MF, which is a commercial rodent diet, was purchased from Oriental Yeast Co. Ltd. (Tokyo, Japan). All other reagents were provided by FUJIFILM Wako Pure Chemical Industries (Osaka, Japan) and Nacalai Tesque (Kyoto, Japan).

2.2. Animals and Diets

Fifty male Wistar rats (mean weight: 64 g, range: 50 - 77 g, and age: 4 weeks) were bought from Japan SLC (Shizuoka, Japan) and were housed separately at a temperature of 22 ± 1˚C, with lighting from 08:00 to 20:00. The rats were fed MF and water on free days. These rats were randomly divided into two groups of 25 each: control (C) and allitol (A). The experimental diets consisted of 3% sucrose or allitol added to MF. Each group was provided free access to the experimental diet and water for 40 weeks. In our previous study, the amount of allitol added was 5%, but we observed considerable cecal enlargement [18] [19]. In this long-term study, the amount of allitol added to the diet was reduced to 3% to mitigate the effects on digestive tracts and ensure the safety of rats. We have already reported on the safety of this level of allitol intake in a previous study [20]. This methodology was used in our long-term study of rare sugar d-allulose [21]-[23].

2.3. Glucose Tolerance Tests

After a feeding period of 16 weeks, oral and intraperitoneal glucose tolerance tests (OGTT and IPGTT, respectively) were performed in eight randomly selected rats

per group. Subsequently, a freshly prepared 50% glucose solution (2 g/kg body weight) was administered orally through a gavage needle (OGTT) or intraperitoneally using a syringe (5 mL, 22 G, Terumo Co., Tokyo, Japan). Blood glucose levels were measured using a glucose analyzer (Glucocard G+; Arkrey, Inc., Kyoto, Japan) at 0, 30, 60, 90, and 120 min after injection.

2.4. Plasma Biochemical Tests

After feeding them for 24 weeks, eight rats were randomly selected from each group. After overnight fasting, they were bled from the tail vein using a heparin-coated capillary tube. After blood collection, the entire sample was centrifuged at 3000 g for 15 min to obtain the plasma. Plasma concentrations of glucose, triglycerides, insulin, total cholesterol, HDL cholesterol, free fatty acids, total protein, and albumin were determined using LabAssay™ Glucose, LBIS Rat Insulin ELISA Kit, LabAssay™ Triglyceride, LabAssay™ Cholesterol, LabAssay™ HDL-Cholesterol, LabAssay™ NEFA (FFA), and A/G B-test kits [FUJIFILM Wako Chemicals, Osaka, Japan]. The homeostatic model assessment of insulin resistance (HOMA-R) was performed, and the β-cell function (HOMA-β) was calculated using the method described by Matthews *et al.* [24].

2.5. Metagenomic Analysis of the Intestinal Microbiota

After feeding them for 24 weeks, fecal samples were collected from eight rats for whom plasma biochemical tests were performed. The DNA was extracted from fecal contents using a ZymoBIOMICS DNA Mini Kit (Zymo Research, CA, USA). Microbial community profiling was performed on an Illumina MiSeq (San Diego, CA, USA) using 16S rRNA amplicon sequencing of DNA from fecal contents with primers that specifically targeted the 16S rRNA V3/V4 region. PCR amplification, purification, and quantification (according to the Illumina (CA, USA) 16S Metagenomic Sequencing Library Preparation Protocol) were outsourced and performed at Genome-Lead Co. Ltd. (Kagawa, Japan). The raw data were visualized and analyzed using QIIME2 (https://qiime2.org). The predicted functional characteristics of the microbial community based on Enzyme Commission (EC) numbers were determined using PICRUSt2 [25] and Kyoto Encyclopedia of Genes and Genomes (KEGG, https://www.genome.jp/kegg/), based on the proportion of marker gene sequences in the samples.

2.6. Data Analysis

All phenotypic data are expressed as mean and standard error (SE). Statistical analyses were performed using Excel Statistics (Social Survey Research Information Co. Ltd., Tokyo, Japan). The statistical significance of α-diversity between Groups C and A was evaluated using the Mann–Whitney U-test. The statistical significance of β-diversity between Groups C and A was evaluated using pairwise PERMANOVA. All other data from Groups C and A were analyzed using Welch's t-test. For all analyses, statistical significance was set as $p < 0.05$.

3. Results

No differences were observed in the weekly mean body weight, weight gain, or food intake between Groups C and A, whereas food efficiency was significantly lower in Group A than in Group C (Figure 1).

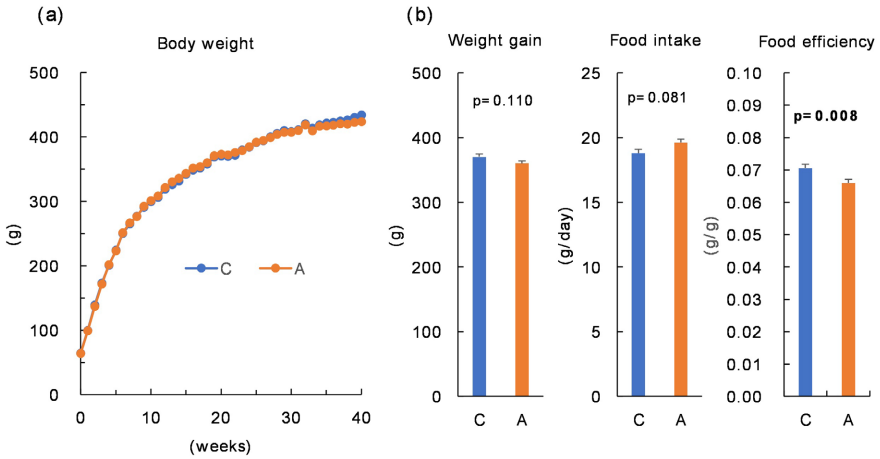

Figure 1. Weekly changes in body weight (a), weight gain, food intake, and food efficiency (b) in the control (C) and allitol (A) groups. Values are the mean ± SE for 25 rats. Significant p-values are shown in bold (Welch's t-test).

In the OGTT, the blood glucose levels before and 30 min after administration were significantly lower in Group A than in Group C (Table 1). The area under the curve (AUC) was lower in Group A; however, there was no difference in blood glucose levels at 60–120 min post-dose between the two groups (Table 1). In the IPGTT, blood glucose levels before and at 60, 90, and 120 min after administration were lower in Group A than in Group C. The AUC was significantly lower in Group A than in Group C (Table 1).

Table 1. Blood glucose concentrations (mg/dL) after glucose administration in each group of rats.

	Time after administration (min)					AUC
	0	30	60	90	120	($\times 10^4$ mg/dL × min)
(OGTT)						
C	86.8 ± 2.4	142.0 ± 5.6	139.7 ± 2.7	131.3 ± 3.8	119.0 ± 5.5	1.55 ± 0.03
A	76.0 ± 2.6	123.9 ± 3.5	135.8 ± 2.7	130.9 ± 1.8	119.5 ± 3.7	1.46 ± 0.02
p-value	**0.013**	**0.022**	0.351	0.935	0.944	0.078
(IPGTT)						
C	94.6 ± 5.5	182.9 ± 9.0	161.3 ± 5.3	155.0 ± 4.9	140.9 ± 6.0	1.85 ± 0.04
A	78.5 ± 2.6	156.4 ± 10.1	137.9 ± 5.4	120.5 ± 6.6	105.5 ± 4.2	1.52 ± 0.07
p-value	**0.043**	0.089	0.012	0.002	**<0.001**	**0.002**

Values are the mean ± SE for eight rats. C and A are abbreviations for the control and allitol groups; The significant p-values are shown in bold (Welch's t-test); OGTT: oral glucose tolerance test; IPGTT: intraperitoneal glucose tolerance test; AUC: area under the curve.

Table 2. Plasma component concentrations in each group of rats.

		C	A	*p*-value
Glucose	(mg/dL)	163.3 ± 12.6	127.6 ± 5.1	**0.028**
Insulin	(ng/mL)	2.96 ± 0.28	1.63 ± 0.19	**0.003**
Triglyceride	(mg/dL)	185.5 ± 14.0	139.8 ± 10.3	**0.027**
Total cholesterol	(mg/dL)	102.2 ± 4.3	92.3 ± 4.2	0.145
HDL-cholesterol	(mg/dL)	26.1 ± 4.1	27.4 ± 3.3	0.820
non-HDL-cholesterol	(mg/dL)	76.1 ± 3.7	64.9 ± 6.3	0.174
Free fatty acids	(mEq/L)	1.25 ± 0.09	1.31 ± 0.08	0.662
HOMA-R		29.9 ± 3.9	16.3 ± 2.9	**0.004**
HOMA-β		316.5 ± 30.6	325.1 ± 84.0	0.193
Total protein	(g/dL)	8.15 ± 0.10	8.07 ± 0.14	0.672
Albumin	(g/dL)	5.09 ± 0.11	4.95 ± 0.15	0.479
Albumin/globulin ratio		1.69 ± 0.10	1.65 ± 0.14	0.819

Values are the mean ± SE for eight rats; C and A are abbreviations for the control and allitol groups. The significant *p*-values are shown in bold (Welch's t-test).

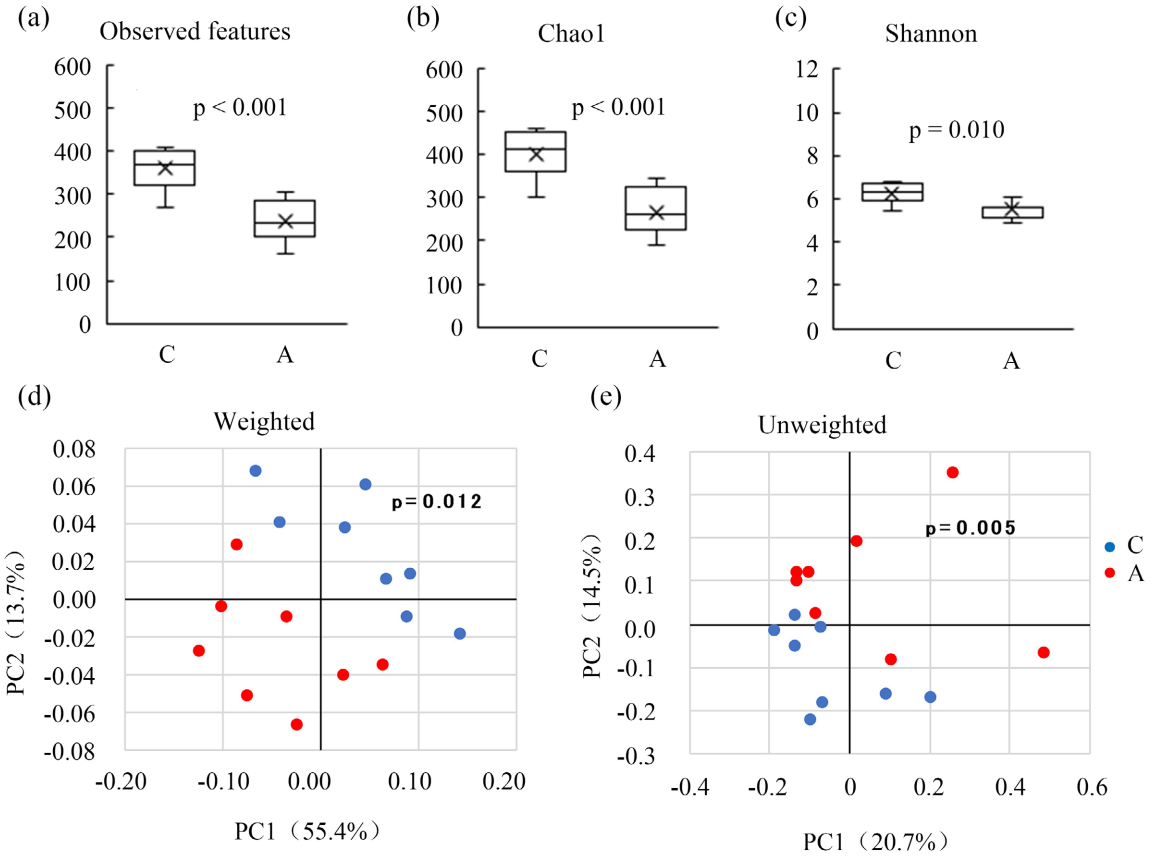

Figure 2. *α*-Diversity indices and principal coordinate analysis plots of the control (C) and allitol (A) groups. *α*-Diversity indices, the observed features (a) and Chao 1 (b) index (OTU richness estimation), and the Shannon (c) indices (OTU evenness estimation) were compared using the Mann–Whitney *U*-test. *β*-Diversity was calculated using weighted (d) and unweighted (e) UniFrac distances, and significance was analyzed using the pairwise PERMANOVA test. Significant P-values are shown in bold. OTU: operational taxonomic unit.

Plasma glucose, insulin, and triglyceride concentrations and HOMA-R values were significantly lower in Group A than in Group C (Table 2). There were no significant differences in the other plasma parameters between the two groups (Table 2).

α-Diversity between the two groups was compared using three different indices: the observed features (Figure 2(a)) and Chao 1 (Figure 2(b)) (operational taxonomy unit (OUT) richness estimation) and Shannon (Figure 2(c)) indices (OTU evenness estimation). These *α*-diversities were significantly lower in Group A than in Group C.

The overall structure of the fecal microbiota between Groups C and A was evaluated using the *β*-diversity indices calculated for weighted (Figure 2(d)) and unweighted (Figure 2(e)) UniFrac distances. Principal coordinate analysis (PCoA) revealed the microbial structural differences between Groups C and A in terms of weighted (p = 0.012) and unweighted (p = 0.015) UniFrac distances.

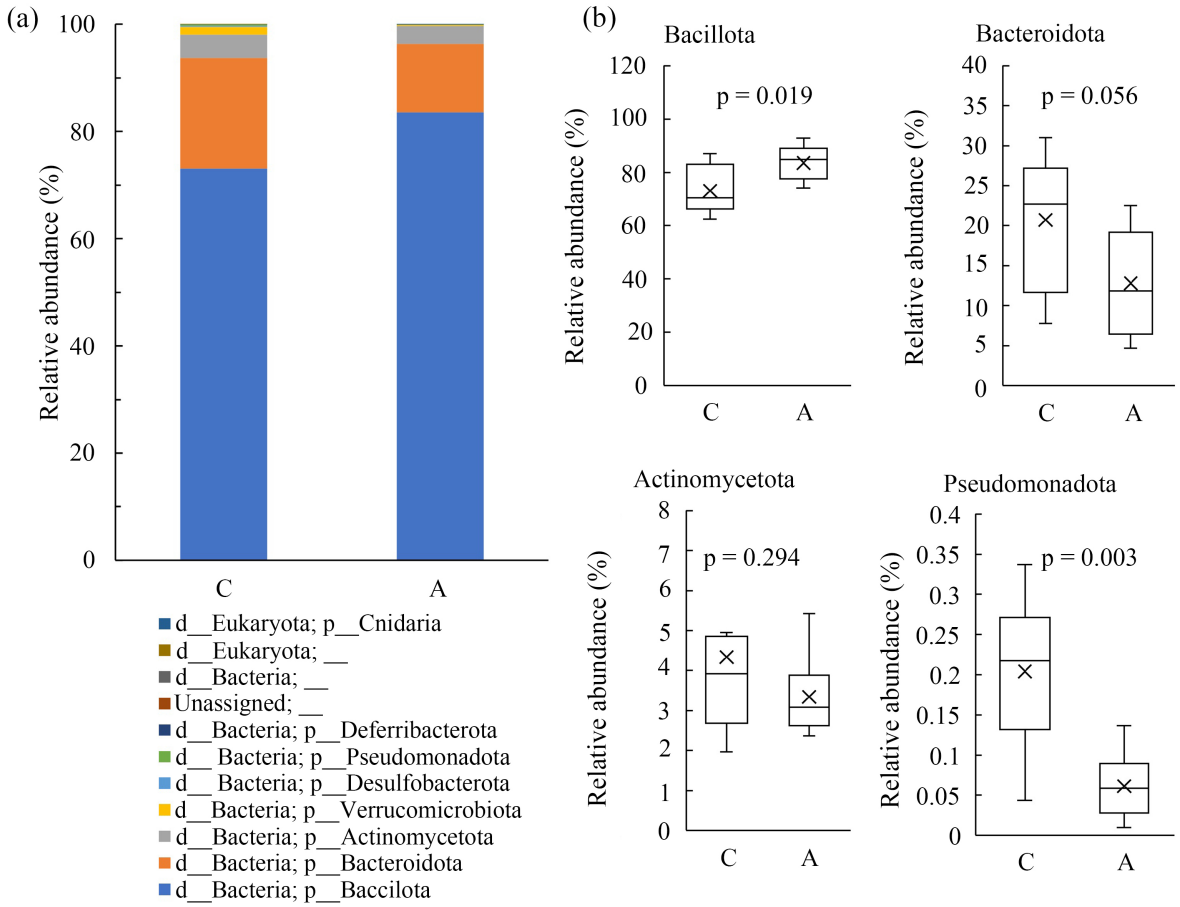

Figure 3. Relative abundance of the taxonomic composition of the fecal microbial community at the phylum level in the control (C) and allitol (A) groups. Each component of the cumulative bar graph on the left indicates a phylum. The right side of the image shows the representative phyla evaluated using Welch's *t*-test. The significant p-values are shown in bold.

Differences in fecal microbial structure were taxonomically assessed at the phy-

lum level (**Figure 3(a)**) Consistent with a previous study [26], the microbiota composition of both groups was dominated by four main phyla: Bacillota, Bacteroidetes, Actinomycetota, and Pseudomonadota (**Figure 3(b)**). Group A had a significantly higher abundance of the phylum Bacteroidetes but also demonstrated a significantly lower abundance of Bacillota and Pseudomonadota than Group C.

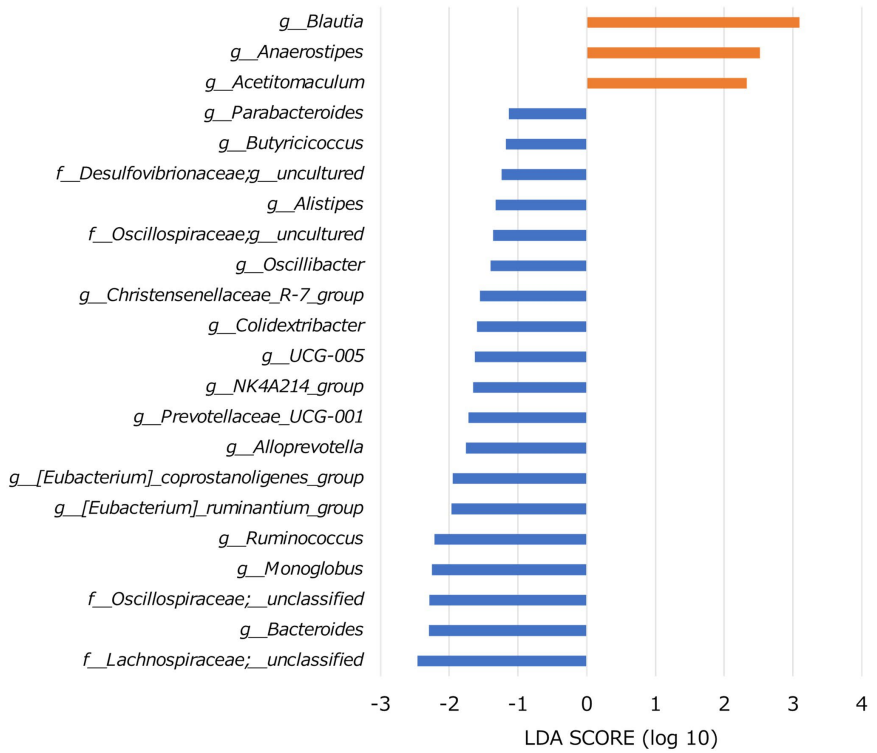

Figure 4. Comparative analyses of the taxonomic composition of the microbial community at the genus level. The significant differences ($p < 0.05$) in genera between the control and allitol groups were presented. Genera that increased with the allitol diet are indicated by red bars, and genera that decreased are indicated by blue bars. LDA: linear discriminant analysis.

Taxonomic changes in the microbial communities were assessed at the genus level. As shown in **Figure 4** and **Table 3**, changes in the microbiota indicated a significant increase in the abundance of three genera and a significant decrease in the abundance of nineteen genera in Group A compared to Group C. These were characterized by an increase in the abundance of the genera *Blautia, Anaerostipes,* and *Acetitomaculum* and by a decrease in the abundance of the genera *Parabacteroides, Butyricicoccus,* uncultured *Desulfovibrionacea*, Alistipe, uncultured *Oscillospiraceae, Oscillibacter, Christensenellaceae_R-7_group, Colidextribacter, UCG-005, NK4A214_group, Prevotellaceae_UCG-001, Alloprevotella, [Eubacterium]_coprostanoligenes_group, [Eubacterium]_ruminantium_group, Ruminococcus, Monoglobus,* unclassified *Oscillospiraceae*, *Bacteroides,* and unclassified *Lachnospiraceae* (**Table 3**).

Table 3. Relative abundance (%) of cecal microbiota at the genus level in each group of rats.

Domein	Phylum	Class	Ordaer	Family	Genus	C	A	*p*-value
Bacteria	Bacillota	Clostridia	Lachnospirales	*Lachnospiraceae*	*Blautia*	1.43 ± 0.39	13.8 ± 1.44	<0.001
Bacteria	Bacillota	Clostridia	Lachnospirales	*Lachnospiraceae*	*Anaerostipes*	0.00 ± 0.00	3.28 ± 0.00	0.006
Bacteria	Bacillota	Clostridia	Lachnospirales	*Lachnospiraceae*	*Acetitomaculum*	0.80 ± 0.29	2.92 ± 0.59	0.034
Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	*Tannerellaceae*	*Parabacteroides*	0.21 ± 0.03	0.07 ± 0.02	0.009
Bacteria	Bacillota	Clostridia	Oscillospirales	*Butyricicoccaceae*	*Butyricicoccus*	0.19 ± 0.06	0.04 ± 0.02	0.042
Bacteria	Desulfobacterota	Desulfovibrionia	Desulfovibrionale	*Desulfovibrionacea*	*uncultured*	0.18 ± 0.04	0.01 ± 0.00	0.007
Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	*Rikenellaceae*	*Alistipes*	0.31 ± 0.07	0.10 ± 0.03	0.031
Bacteria	Bacillota	Clostridia	Oscillospirales	*Oscillospiraceae*	*uncultured*	0.37 ± 0.06	0.13 ± 0.03	0.015
Bacteria	Bacillota	Clostridia	Oscillospirales	*Oscillospiraceae*	*Oscillibacter*	0.35 ± 0.10	0.10 ± 0.04	0.037
Bacteria	Bacillota	Clostridia	Christensenellales	*Christensenellaceae*	*Christensenel-laceae_R-7_group*	0.59 ± 0.06	0.24 ± 0.02	<0.001
Bacteria	Bacillota	Clostridia	Oscillospirales	*Oscillospiraceae*	*Colidextribacter*	0.55 ± 0.15	0.16 ± 0.08	0.032
Bacteria	Bacillota	Clostridia	Oscillospirales	*Oscillospiraceae*	*UCG-005*	0.52 ± 0.07	0.09 ± 0.03	0.003
Bacteria	Bacillota	Clostridia	Oscillospirales	*Oscillospiraceae*	*NK4A214_group*	0.69 ± 0.11	0.13 ± 0.03	0.015
Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	*Prevotellaceae*	*Prevotel-laceae_UCG-001*	0.66 ± 0.10	0.13 ± 0.05	0.003
Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	*Prevotellaceae*	*Alloprevotella*	0.72 ± 0.01	0.14 ± 0.07	0.043
Bacteria	Bacillota	Clostridia	Oscillospirales	*[Eubacterium]_coprostanoligenes_group*	*[Eubacterium]_coprostanoligenes_group*	1.34 ± 0.18	0.45 ± 0.10	0.003
Bacteria	Bacillota	Clostridia	Lachnospirales	*Lachnospiraceae*	*[Eubacterium]_ruminantium_group*	1.02 ± 0.26	0.09 ± 0.06	0.016
Bacteria	Bacillota	Clostridia	Oscillospirales	*Ruminococcaceae*	*Ruminococcus*	2.44 ± 0.47	0.79 ± 0.21	0.032
Bacteria	Bacillota	Clostridia	Monoglobales	*Monoglobaceae*	*Monoglobus*	3.53 ± 0.50	1.54 ± 0.35	0.010
Bacteria	Bacillota	Clostridia	Oscillospirales	*Oscillospiraceae*	*unclassified*	2.87 ± 0.56	0.92 ± 0.30	0.013
Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	*Bacteroidaceae*	*Bacteroides*	3.53 ± 0.50	1.55 ± 0.35	0.010
Bacteria	Bacillota	Clostridia	Lachnospirales	*Lachnospiraceae*	*unclassified*	10.5 ± 0.47	7.56± 0.67	0.034

Values are the mean ± SE for eight rats. C and A are abbreviations for the control and allitol groups; The p-values were obtained using Welch's t-test.

Using multiple regression analysis, three genera of fecal bacteria were identified as predictor variables for plasma glucose levels, and six genera were identified as predictor variables for plasma insulin, triglyceride, and HOMA-R values. The correlations between these bacteria and plasma glucose, insulin, triglyceride, and HOMA-R levels are shown in **Table 4**. The abundance of *Anaerostipes* had a negative correlation with the plasma glucose levels, while the abundance of *Ruminococcus* and *Prevotellaceae_UCG-001* had a positive correlation. The abundance of *Ruminococcus, Alloprevotella*, and *Parabacteroides* had a positive correlation with plasma insulin levels. The abundance of *Anaerostipes* had a negative correlation with plasma triglyceride levels, while the abundances of *[Eubacterium]_ru-*

minantium_group, *Parabacteroides*, and *Oscillibacter* had a positive correlation. The abundance of *Bacteroides*, *Ruminococcus*, and *Alloprevotella* had a positive correlation with the HOMA-R value.

Table 4. Correlation between cecal microbiota and plasma biochemical components.

	Glucose		Insulin		Triglyceride		HOMA-R	
Genus	*R*	*p*-value	*R*	*p*-value	*R*	*p*-value	*R*	*p*-value
Acetitomaculum			−0.46	0.071				
Anaerostipes	−0.56	**0.023**			−0.63	**0.008**		
Bacteroides							0.43	**0.025**
[Eubacterium]_ruminantium_group					0.57	**0.049**	−0.05	0.072
Ruminococcus	0.26	**0.004**	0.23	**0.011**			0.25	**0.003**
Alloprevotella			0.83	**<0.001**	0.47	0.149	0.86	**<0.001**
Prevotellaceae_UCG-001	0.94	**<0.001**						
NK4A214_group			−0.29	0.081				
UCG-005							−0.03	0.090
Parabacteroides			0.39	**0.043**	0.60	**0.036**		
Colidextribacter							0.04	0.172
Oscillibacter			0.03	0.077	0.65	**<0.001**		
Butyricicoccus					0.50	0.052		

R: correlation coefficient. The significant p-values are shown in bold.

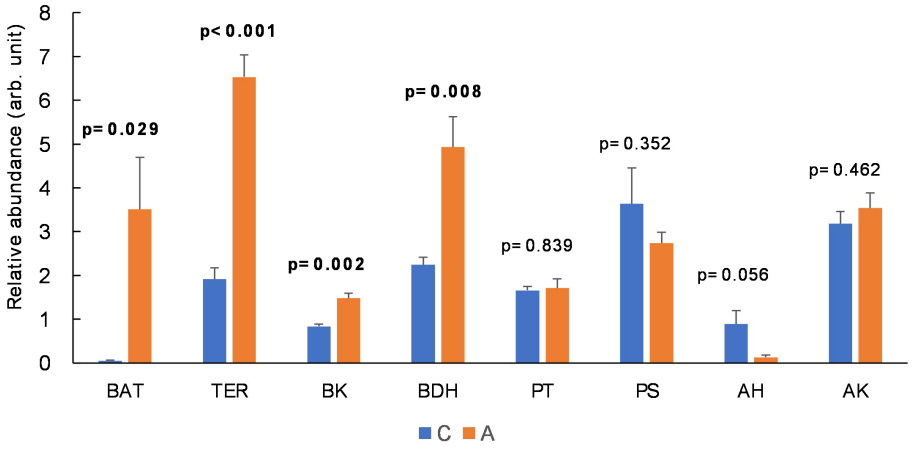

Figure 5. Relative abundance of predicted enzymes in the fecal microbial community related to SCFA metabolism in the control (C) and allitol (A) groups. Values are the mean and SE for eight rats. The p-values were obtained using Welch's *t*-test. The significant p-values are shown in bold. BAT: butyryl-CoA: acetate-CoA transferase; TER: *trans*-2-enoyl-CoA reductase (NAD^+); BK: butyrate kinase; BDH: butanol dehydrogenase; PT: propionyl-CoA transferase; PS: propionyl-CoA synthetase; AH: acetyl-CoA hydrolase; AK: acetate kinase.

Potential differences in the function of the microbial community were evalu-

ated using PICRUSt2 software. The relative abundance of the predicted enzymes in the fecal microbiota associated with SCFA metabolism in Groups C and A is shown in **Figure 5**. Regarding butyric acid metabolism-related enzymes, butyryl-CoA enzymes [acetate-CoA transferase (BAT), *trans*-2-enoyl-CoA reductase (NAD^+) (TER), butyrate kinase (BK), and butanol dehydrogenase (BHD)] were significantly higher in Group A than in Group C. However, regarding propionic and acetic acid metabolism-related enzymes, no differences in propionyl-CoA transferase (PT), propionyl-CoA synthetase (PS), acetyl-CoA hydrolase (AH), and acetate kinase (AK) were observed between the two groups.

4. Discussion

At 16 weeks of this study, for both OGTT and IPGTT, fasting blood glucose and the increase in blood glucose levels after glucose administration were lower in Group A than in Group C (**Table 1**). Furthermore, the AUC in IPGTT was significantly lower in group A, and the AUC in OGTT tended to be lower ($p = 0.078$) in group A. After 24 weeks, the fasting plasma glucose and insulin levels and HOMA-R values were lower in Group A than in Group C (**Table 2**). These results indicate the anti-diabetic effects of a long-term allitol diet. These results indicate the anti-diabetic effects of a long-term allitol diet. To the best of our knowledge, these findings are the first of their kind. Since we previously reported the anti-obesity effects of allitol [17] [18] [27] [28], it is possible that the results of this experiment are secondary to a reduction in body fat. However, as there was no difference in body weight between the two groups (**Figure 1**), it is unclear whether the anti-diabetic effect of allitol was due to a reduction in body fat. In previous studies, 5% allitol was added to the diets [17] [18] [27] [28]. In contrast, in this study, the dose was reduced to 3% to confirm the long-term effects of dietary allitol. This could have resulted in the negligible weight difference between the two groups in this study; however, this remains unclear since the body mass was not examined.

The important finding of this study was that dietary allitol significantly increased the phylum Bacillota and decreased the phyla Bacteroidetes, Actinomycetota, and Pseudomonadota [**Figure 3(b)**]. At the genus level, in Group A, the abundance of *Blautia, Anaerostipes,* and *Acetitomaculum* (known butyric acid and SCFA producers) [29]-[31] belonging to the phylum Bacillota was significantly increased by dietary allitol (**Figure 4**, **Table 3**). Moreover, the plasma glucose and triglyceride levels had a significant negative correlation with the abundance of *Anaerostipes*, while the plasma insulin levels had a negative correlation with the abundance of *Acetitomaculum* ($P = 0.071$) (**Table 4**). PICRUSt2 gut microbiota analysis revealed that the levels of butyric acid-metabolizing enzymes were significantly increased by allitol intake. BAT and TER are key enzymes involved in butyric acid production, and their predicted enzyme levels increased 65- and 3.5-fold, respectively, with long-term allitol intake (**Figure 5**). In contrast, there were no differences in the levels of enzymes related to the production of

propionic acid (PT and PS) and acetic acid (AH and AK) between the two groups. We previously reported that the amount of short-chain fatty acids (especially butyrate) in the cecum was greatly increased by allitol intake [18]. The results of this study support our previous findings. SCFAs, such as butyric acid, control a wide range of cellular and physiological functions in the body by serving as ligands for G protein-coupled receptors (GPCRs) [32]. GPR41 and GPR43, the most significant SCFA receptors in the GPCR family [33], are highly expressed in sympathetic ganglia and fatty tissues, respectively [33] [34]. SCFAs can increase energy expenditure and lipolysis via these receptors [33] [34]. Acetic, propionic, and butyric acids are the most common SCFAs found in mammalian bodies, particularly in the intestinal tract [35]. Butyric acid has the most significant systemic effect among SCFAs. A significant association between butyric acid and diabetes has been reported [36]-[38]. Research on microbiota composition has shown that individuals with diabetes have decreased levels of bacteria that produce butyric acid [39]. Based on these findings, the anti-diabetic effect of allitol could be due to butyric acid produced by certain intestinal microbiota.

In contrast, changes in the microbiota indicated a significant decrease in the abundance of many genera in Group A compared to Group C (**Figure 4**, **Table 3**). In particular, the abundance of *Bacteroides, Ruminococcus,* and *Alloprevotella* in Group A was significantly decreased by the dietary allitol (**Figure 4**, **Table 3**), and HOMA-R showed significant correlation to the abundance of these genera (**Table 4**). The relative abundance of *Ruminococcus* present is positively correlated with type 2 diabetes [40]. Moreover, Wang *et al.* [41] reported that the *Bacteroides* enterotype is an independent risk factor for type 2 diabetes because it decreases insulin sensitivity. Factors other than butyric acid, such as the action of certain intestinal microbiota, could also have an influence on the anti-diabetic effects of allitol, but the details are unknown. Functional analysis using PICRUSt2 revealed that more than 300 metabolic pathways were altered by the allitol diet (data not shown). Therefore, it is necessary to examine the various functions of allitol as a functional carbohydrate.

The changes in the relative abundance of the fecal microbiota due to dietary allitol were explained. First, the overall differences in microbial structure between Groups C and A were analyzed using both weighted and unweighted UniFrac distances. As shown in **Figure 2(d)** and **Figure 2(e)**, the weighted PCoA indicated significant structural differences between the two groups. Thus, a shift from Group C to Group A was observed in the composition of fecal microbiota. α-Diversity indices, the observed features, Chao 1, and Shannon indices were significantly lower in Group A than in Group C (**Figures 2(a)-(c)**). In our previous study, there was no significant difference in the α-diversity index between the control and allitol groups. This could be due to the fact that in the previous study the rats were fed a high-fat diet for 8 weeks, whereas in the current study they were fed a high-carbohydrate diet for an even longer period. The decrease in intestinal bacterial diversity with the allitol diet was unexpected and will be explored in the future.

This study has some limitations. First, the amount of body fat and butyric acid in

the blood and the cecum of rats was not measured. This was due to the fact that only a small amount of blood was collected and that the rats were not dissected after the end of the experiment. Second, using multiple regression analysis, correlations between some genera and diabetes indicators were identified; however, these causal relationships are unknown. Third, this study solely examined allitol without comparing it to other uncommon sugars, such as d-allulose or non-digestible sugars. Allitol could be more efficient in producing butyric acid compared to other sugars; this needs further investigation. Fourth, we examined the fecal microbiota in detail and did not analyze other tissue biomarkers. Alterations in the fecal microbiota may affect liver, adipose tissue, and muscle biomarkers. Fifth, the relative abundance of the taxonomic composition of feces significantly varied for each rat. The suitability of the strain of rats (Wistar) and the number of animals (n = 8) used in the fecal metagenome analyses needs further exploration. However, there is scope for reconsidering these limitations.

In conclusion, this study demonstrated several compositional changes in the fecal microbiota and an increase in predicted butyric acid metabolism because of the dietary allitol supplementation. The anti-diabetic effect of allitol was confirmed; however, it was suggested that there may be other causes of this effect besides the butyric acid produced by the intestinal microbiota. However, since allitol intake increases the abundance of bacterial genera such as *Blautia* and *Anaerostipese,* which are associated with butyric acid-producing effects, the fecal microbiota profile is probably involved in the anti-diabetic effects of dietary allitol. Although several beneficial effects of butyric acid have been reported, the unknown effects of dietary allitol (a butyrate precursor) need further investigation.

Acknowledgements

We would like to thank Editage (http://www.editage.jp/) for editing the English language.

Conflicts of Interest

The authors declare no conflicts of interest associated with this manuscript.

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