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Aspergillus-Derived Cellulase Preparation Exhibits Prebiotic-like Effects on Gut Microbiota in Rats

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Abstract: *Aspergillus*-derived cellulase, which is involved in the production of *Aspergillus*-fermented foods, has been employed in the food and animal feed industries. However, the effect of dietary *Aspergillus* cellulase on health is poorly understood. Previously, we discovered that supplemental *Aspergillus*-derived protease and lipase preparations had substantial bifidogenic effects on rats fed a high-fat diet. Therefore, this study reports on the effects of a 0.1% dietary *Aspergillus*-derived cellulase preparation (CEL) on the gut microbiota of rats fed a high-fat diet. Gene sequencing analysis of 16S rRNA revealed that CEL treatment markedly affected the microbiota profiles of the cecal contents ($p < 0.05$). Notably, CEL markedly increased the relative abundance (RA) of typical probiotics, such as *Bifidobacterium* and *Lactobacillus*, at the genus level (28- and 5-fold, respectively, $p < 0.05$). Similarly, at the family level, CEL treatment significantly increased the RA of Bifidobacteriaceae and Lactobacillaceae ($p < 0.05$). Furthermore, CEL increased the RA of other genera, such as *Collinsella* and *Enterococcus*, but decreased the RA of *Oscillospira*, *Dorea* and *Coprobacillus* ($p < 0.05$). The effects on these genera are similar to those reported for typical prebiotic oligosaccharides. Overall, this study demonstrates the prebiotic-like effects of dietary CEL by significantly increasing *Bifidobacterium* and *Lactobacillus* abundance.

Keywords: fermentation foods; *Aspergillus*; cellulase; *Bifidobacterium*; *Lactobacillus*; prebiotic; dietary enzyme supplements; 16S rRNA gene sequencing

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1. Introduction

Aspergillus species, such as *Aspergillus oryzae* and *Aspergillus niger,* have been used in the food fermentation industry in Japan and East Asia. Extracellular hydrolysis enzymes, which are responsible for fermentation during the *Aspergillus*-associated fermentation process, are produced and released into the reaction system [1]. The extracted *Aspergillus* enzymes, such as proteases, lipases, amylases and cellulases, have been used in food processing. Previously, we found that dietary supplementation with an *A. oryzae*-derived protease preparation and purified acid protease caused a bifidogenic effect by striking an elevation in the cecal levels of *Bifidobacterium*, a typical probiotic (beneficial bacteria for host health), in rats fed a high-fat (HF) diet [2,3]. We speculated that the increase in free amino acids (available amino acids) in the gut, induced by supplemental *Aspergillus* proteases, promotes *Bifidobacterium* growth [4]. The effect of an *Aspergillus* protease preparation is similar to that of prebiotics, such as short-chain non-digestible carbohydrates, e.g., fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS), which can increase the number

of typical probiotics, *Bifidobacterium* and *Lactobacillus* [5]. Prebiotics are well known to be selectively utilized by live probiotics, and promote the growth of probiotics, resulting in health benefits for the host [5]. A subsequent study in our laboratory demonstrated the powerful bifidogenic effect of *Aspergillus* lipase in rats fed an HF diet [6].

Recently, we carried out a preliminary investigation into the bifidogenic effects of several other digestive enzyme preparations derived from *Aspergillus* in rats fed an HF diet. Using quantitative polymerase chain reaction (qPCR) analysis, we found a remarkable increase in the cecal abundance of major probiotics, including *Lactobacillus* and *Bifidobacterium* bacteria, following consumption of an *Aspergillus*-derived cellulase preparation (CEL). Generally, cellulase enzymes, such as β-1,4-endoglucanase, cellobiohydrolase and β-glucosidase, degrade cellulose to β-glucose [7]. Cellulase enzymes have been widely used in the food and animal feed industries to improve nutrient availability and promote antioxidant properties by releasing antioxidants, such as polyphenols [8,9]. Furthermore, there are many applications of cellulase in the food industry, including the tenderization of fruits, clarification of fruit juices, extraction of flavoring materials and essential oils, and improvement in the aroma and taste of food items [10]. In the production of *Aspergillus*-fermented foods, *Aspergillus*-derived cellulase is thought to play an important role in the fermentation process [1,11]. Additionally, *Aspergillus* cellulase is included as a dietary enzyme supplement for gut health [12]. However, to the best of our knowledge, limited information is available regarding how dietary exogenous cellulase modulates the gut environment. In the present study, we hypothesized that dietary supplemental CEL modulates the composition of the gut microbiota. Thus, this study used 16S rRNA gene sequencing analysis to examine the effect of CEL on the gut microbiota in rats fed an HF diet. The study was conducted with rats fed an HF diet, since HF diet-induced colon dysbiosis, inflammation and diseases have been reported to be suppressed by dietary prebiotic oligosaccharides [13]. Herein, we report the first evidence for the prebiotic-like effect of supplemental CEL on gut microbiota in rats.

2. Materials and Methods

2.1. Animals and Diets

Sixteen male Sprague Dawley rats (four weeks old) were purchased from Charles River, Japan. The rats were individually housed in cages in a controlled-temperature environment (23 ± 2 °C), a 12 h light–dark cycle and relative humidity of 50%–60%. After being acclimatized for 7 days, the rats were randomly divided into the following two groups based on their diet: an HF diet (control; Ctrl) [2] or an HF diet mixed with 0.1% (*w/w*) CEL (*A. niger*-derived cellulase preparation, commercial name: Cellulase A "Amano" 3, Amano Enzyme Inc. Nagoya, Japan). The optimum pH was 4.5 (stable at pH 2.0–8.0), and the cellulase activity was 30,000 U/g at pH 4.5. The optimum temperature was 55 °C. CEL had slight activities of protease and lipase equivalent to 3% of the protease activity of *Aspergillus* protease preparation (Protease A "Amano" SD, Amano Enzyme Inc. Nagoya, Japan) used in our study [6] and to less than 0.1% of the lipase activity of *Aspergillus* lipase preparation (Lipase AP12, Amano Enzyme Inc. Nagoya, Japan) used in our study [6]). The HF diet contained 30% beef tallow, 20% casein, 0.3% L-cystine, 1% vitamin mixture (AIN-93), 3.5% mineral mixture (AIN-93G), 5% cellulose, 20% sucrose and 20.2% α-corn starch. During the two-week experimental period, equal amounts of the experimental diets were given daily in food cups (9, 10, 12, 14, and 15 g on days 1, 2–4, 5–7, 8–12, and 13–14, respectively) to prevent differences in food intake. All of the given diet was consumed each day. The rats had ad libitum access to drinking water. The study protocols were approved by the Ethics Committee of Hiroshima University (protocol identity No. C15-12).

2.2. Sample Collection

At the end of the two-week treatment period, the rats were anesthetized (13:00–15:00 h) by inhaling isoflurane in a desiccator to minimize suffering, and then euthanized by decapitation. The cecum was immediately excised, and its contents were removed en-

tirely, weighed, and stored at −80 °C until subsequent analysis of cecal microbiota and organic acids.

2.3. 16S rRNA Gene-Based Microbiome Analysis

Total bacterial DNA in cecal contents was extracted using the QIAamp Stool Mini Kit, according to the manufacturer's instructions. Then, extracted bacterial DNA was quantified using NanoDrop spectrometry (NanoDrop Technology, Wilmington, DL, USA). The V1–V2 region of the 16S rRNA genes was amplified from the DNA isolated from cecal contents using the following bacterial universal primer set: 27F (5′- ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGRGTTTGATYMTGGCTCAG-3′) and 338R(5′-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTGCTGCCTCCCGTAGGAGT-3′). The following library preparation was performed as described previously [14]. Finally, all the barcoded V1–V2 PCR amplicons were sequenced using Illumina MiSeq sequencing technology at a read length of 2 × 300-bp (Illumina, San Diego, CA, USA), based on the manufacturer's instructions.

2.4. Bioinformatics Analysis

Fast Length Adjustment of SHort reads (FLASH, version 1.2.11) [15] was used to assemble the paired-end reads. Assembled reads with an average *Q*-value < 25 were filtered out using an in-house script. The same numbers of filtered reads were randomly selected from each sample and used for further analysis [6]. The selected reads were then processed using the Quantitative Insights Into Microbial Ecology pipeline (QIIME, version 1.9.1) [6]. The high-quality sequences were clustered into operational taxonomic units (OTUs) at 97% sequence similarity, and OTUs were assigned to the Greengenes database (version 13.8).

2.5. Analyses of Cecal Organic Acids and pH

The concentrations of organic acids in cecal contents were measured by gas chromatography/mass spectrometry as previously described [16]. For the analysis of pH in the cecal contents, 100 mg of freeze-dried cecal contents was mixed with 1 mL of Milli Q water. The pH value of the sample was measured by COMPACT pH Meter B-71X (Horiba Ltd., Kyoto, Japan).

2.6. Data Analysis

Data are expressed as mean ± standard error. Statistical analysis was performed by Welch's t-test. Data separation in the principal coordinate analysis (PCoA) ordination of beta diversity was tested using the ANOSIM statistical test in vegan-R, and *p*-values were generated based on 999 permutations. Some bacterial taxa data were subjected to linear discriminant analysis effect size (LEfSe) analysis, which uses the two-tailed nonparametric Kruskal–Wallis test to evaluate the significance of differences between taxa. $p < 0.05$ was considered to indicate a statistically significant difference. For the relationship between organic acids and microbiota composition, Pearson's correlation coefficient (r) was calculated, and the resulting correlation matrix was visualized by using R software (version 4.0.2).

2.7. Evaluation of the Risk of Bias in the Methodology

The risk of bias of this study was assessed using the Systematic Review Centre for Laboratory Animal Experimentation (SYRCLE) risk of bias (RoB) tool [17]. Two independent authors (YY and NK) evaluated the following nine items: (1) sequence generation, (2) baseline characteristics, (3) allocation concealment, (4) random housing, (5) intervention blinding, (6) random outcome assessment, (7) outcome blinding, (8) incomplete outcome data and (9) selective outcome reporting. All items were judged as 'yes' (low risk of bias) by two authors (YY and NK) independently.

3. Results

3.1. Food Intake, Body Weight and Cecal Content Weight

The total food intake for the two weeks and the final body weight were unaffected by the dietary treatment (data not shown). The weight of the cecal contents in the CEL group was markedly greater than that in the Ctrl group (5.05 ± 0.31 g and 1.44 ± 0.06 g, respectively; $p < 0.05$).

3.2. Cecal Microbiota

For 16S rRNA gene sequencing-based microbiota analysis, a total of 417,428 high-quality reads were passed through the QIIME filter. Unweighted and weighted UniFrac PCoA and ANOSIM analyses were conducted to compare the microbial community structures (Figure 1A,B). The results of the UniFrac PCoA and ANOSIM analyses indicated that the microbial composition was distinctly separated between the Ctrl and CEL groups in both the unweighted and weighted analyses ($p < 0.05$). However, the different alpha-diversity indices indicated a lower bacterial diversity in the CEL group than in the Ctrl group (Figure 1C,D; $p < 0.05$).

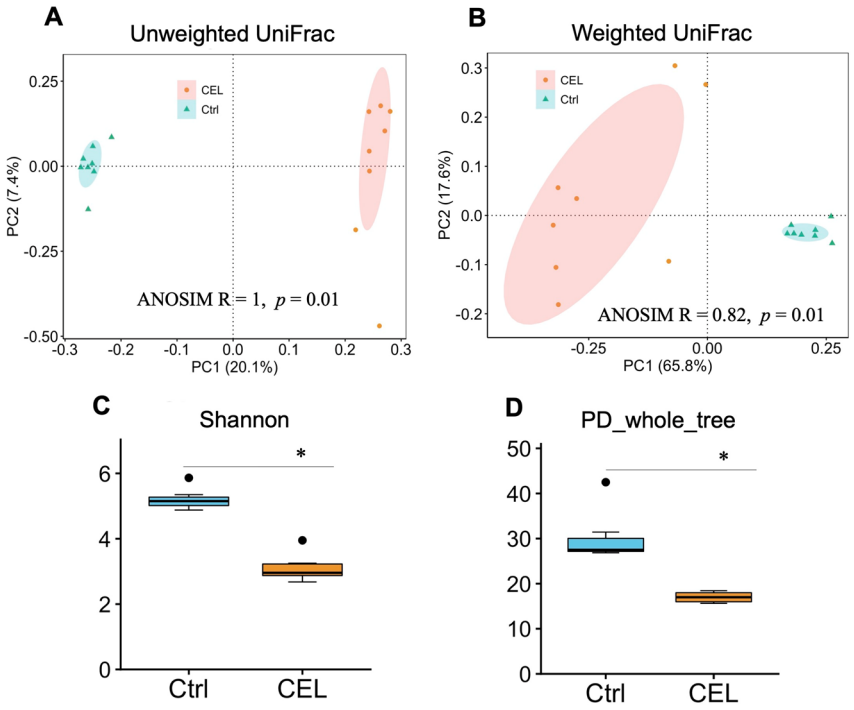

Figure 1. Effects of supplemental CEL on cecal microbiome profiles and alpha diversity. PCoA of unweighted (**A**) and weighted UniFrac (**B**) and PERMANOVA analyses were performed to compare the gut microbiome profiles of the experimental groups. The diversity of the gut microbiota within samples was measured by (**C**) Shannon index and (**D**) PD whole tree. Data are presented as a boxplot with median and min–max whiskers. The dots (•) in the boxplots are outliers. * Significantly different at $p < 0.05$ (Welch's *t*-test).

The LEfSe analysis results indicated that 60 bacterial taxa differed between the Ctrl and CEL groups (Figure 2; $p < 0.05$). This analysis identified that the bacterial species *Collinsella*, *Lactobacillus*, *Bifidobacterium*, *Eggerthella*, *Enterococcus*, *Akkermansia*, *Dehalobacterium*, *Adlercreutzia*, *Coprobacillus*, *Dorea*, *rc4-4*, *Oscipillospira*, *Roseburia*, *Coprococcus*, *Allobaculum*, *Ruminococcus* and *Parabacteroides* varied between the two groups.

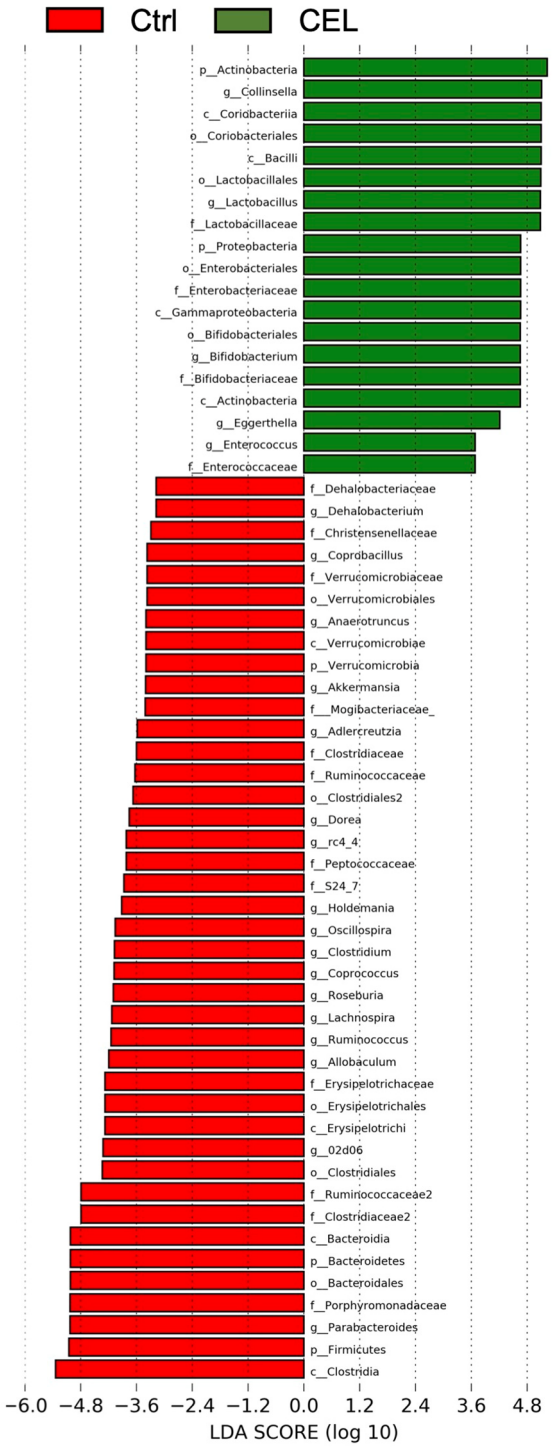

Figure 2. Different taxa between the Ctrl and CEL groups. LEfSe analysis was performed to compare the different taxa between the Ctrl and CEL groups. The two-tailed nonparametric Kruskal–Wallis test was used to evaluate the significance of differences between taxa at $p < 0.05$.

Among the four most abundant phyla, supplemental CEL significantly decreased the RA of Firmicutes and Bacteroidetes but enriched the RA of Actinobacteria and Proteobacteria (Figure 3A; $p < 0.05$). The top nine bacterial taxa are displayed at the family level to address the domain taxa of the microbial groups (Figure 3B); supplemental CEL significantly increased the RA of Bifidobacteriaceae (28-fold), Lactobacillaceae (5-fold), Coribacteriaceae (100-fold) and Enterobacteriaceae (13-fold) ($p < 0.05$). In contrast, it reduced the RA of bacterial taxa, including Lachnospiaceae (1.6-fold), Porphyromonadaceae

(15-fold), Ruminococcaceae (77-fold), Clostridiaceae (12-fold) and Erysipelotrichaceae (12-fold) ($p < 0.05$).

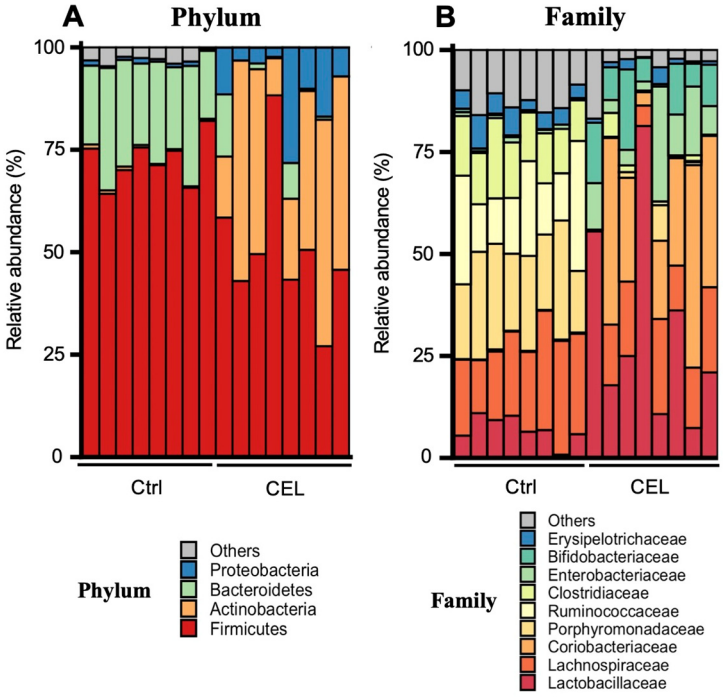

Figure 3. Effects of supplemental CEL on cecal microbiota composition at the phylum (**A**) and family (**B**) levels.

The resulting RA of the genera are shown in Table 1. Supplemental CEL significantly increased the RA of Lactobacillus (5-fold), Collinsella (526-fold), Bifidobacterium (28-fold) and Enterococcus (42-fold) ($p < 0.05$). Meanwhile, CEL significantly decreased the RA of Parabacteroides (15-fold), Allobaculum (8-fold), Oscillospira (13-fold), rc4-4, Dorea, Coprobacillus and Adlercreutzia (Table 1; $p < 0.05$). The RA of Ruminococcus and Roseburia were not significantly affected ($p > 0.05$). The genera with a mean RA less than 0.2% in all the groups were not considered for statistical analysis. The results of the effects of CEL on Lactobacillus and Bifidobacterium levels were similar to those of our preliminary study using qPCR analysis (data not shown).

Table 1. Effects of supplemental CEL on the relative abundance of genera in cecum of rats.

Phylum	Family	Genus	Ctrl	CEL
			(% of total bacteria)	
Firmicute	Lactobacillaceae	*Lactobacillus*	0.698 ± 0.108	3.186 ± 0.833 *
Actinobacteria	Coriobacteriaceae	*Collinsella*	0.005 ± 0.003	2.578 ± 0.601 **
Actinobacteria	Bifidobacteriaceae	*Bifidobacterium*	0.034 ± 0.010	0.965 ± 0.198 **
Firmicute	Enterococaceae	*Enterococcus*	0.02 ± 0.01	0.83 ± 0.15 **
Firmicute	Ruminococacceae	*Ruminococcus*	3.13 ± 1.38	0.01 ± 0.00
Firmicute	Erysipelotrichaceae	*Allobaculum*	3.03 ± 0.68	0.38 ± 0.20 **
Firmicute	Lachnospiraceae	*Roseburia*	2.60 ± 1.34	0.00 ± 0.00
Firmicute	Ruminococaceae	*Oscillospira*	2.32 ± 0.31	0.18 ± 0.08 **
Bacteroidetes	Porphyromonadaceae	*Parabacteroides*	2.19 ± 0.16	0.15 ± 0.01 **
Actinobacteria	Peptococcaceae	*rc4-4*	1.27 ± 0.16	0.00 ± 0.00 **
Firmicute	Lachnospiraceae	*Dorea*	1.12 ± 0.17	0.00 ± 0.00 **
Firmicute	Erysipelotrichaceae	*Coprobacillus*	0.35 ± 0.06	0.00 ± 0.00 **
Actinobacteria	Eggerthellaceae	*Adlercreutzia*	0.21 ± 0.00	0.00 ± 0.00 **

Mean ± SE (n = 8). * $p < 0.05$, ** $p < 0.01$ (Welch's *t*-test).

3.3. Cecal Organic Acids and pH

Table 2 indicates the concentrations of cecal organic acids. Treatment with CEL significantly increased the concentrations of lactate (219-fold) and total organic acids (3-fold), while it significantly decreased those of acetate (4-fold), propionate (10-fold) and n-butyrate (5-fold) ($p < 0.05$). There was a significant inverse association of lactate with propionate, n-butyrate and acetate levels (r = −0.91, r = −0.82 and r = −0.74, respectively; $p < 0.01$). Figure 4 further indicates the relationship between the levels of organic acids and various bacteria. There was a strong correlation between lactate levels and the RA of the lactate-producing bacteria *Lactobacillus*, *Bifidobacterium* and *Enterococcus*. In general, the RA of genera such as *Oscillospira*, *Dorea* and *Coprobacillus* had a strong positive association with propionate levels, but a strong negative association with lactate levels (Figure 4). The pH in the cecal contents of the CEL group was significantly lower than that in the Ctrl group (5.40 ± 0.10 and 8.29 ± 0.20, respectively; $p < 0.01$). There was a strong inverse association between the pH values and the levels of total organic acids (r = −0.97; $p < 0.001$).

Table 2. Effects of supplemental CEL on the levels of organic acids in cecum of rats.

Organic Acids	Ctrl	CEL
	(μmol/g dry wt of cecal contents)	
Acetate	40.3 ± 5.9	10.8 ± 4.0 *
Propionate	11.1 ± 0.0	1.1 ± 0.2 *
n-Butyrate	7.8 ± 0.9	1.5 ± 1.2 *
Lactate	0.9 ± 0.1	188.7 ± 13.1 *
Succinate	13.9 ± 3.1	12.5 ± 3.3
Total organic acids	75.2 ± 8.8	213.9 ± 10.0 *

Mean ± SE (n = 8). * $p < 0.05$ (Welch's *t*-test).

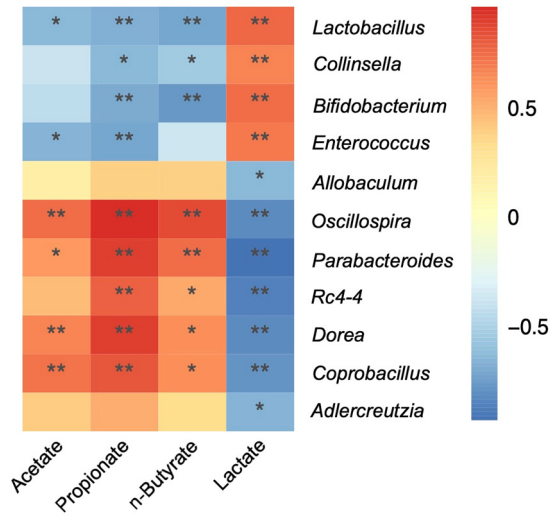

Figure 4. Correlation matrix (correlation coefficient) between the levels of organic acids and the relative abundances of genera. * $p < 0.05$, ** $p < 0.01$.

4. Discussion

4.1. Bifidobacterium and Lactobacillus

Our previous study revealed that the dietary consumption of 0.1% *Aspergillus* lipase and protease preparations for two weeks markedly increased the RA of *Bifidobacterium* in the cecum, but not *Lactobacillus* [3,6]. Moreover, this study discovered that the consumption of 0.1% *Aspergillus* cellulase preparation significantly increased the RA of both probiotics, such as *Bifidobacterium* and *Lactobacillus*. Hence, our findings suggest a potential role of *Aspergillus* cellulase as a prebiotic-like ingredient for enhancing typical probiotic levels,

i.e., bacteria of the genera *Bifidobacterium* and *Lactobacillus*. Because a wide range of plant-derived foods contain cellulose, cellulase preparations may be effective prebiotics for use in the food and animal feed industries. Currently, various prebiotics are well known for providing health benefits by enhancing the abundance of probiotics [18]. To the best of our knowledge, this study, along with previous studies [3,6] on *Aspergillus* protease and lipase, implies that *Aspergillus*-derived cellulase may be a new type of "prebiotic digestive enzyme", as proposed by our recent study [6].

Previously, we discovered that dietary *Aspergillus*-derived acid protease had a strong bifidogenic effect [3], but dietary *Aspergillus*-derived alkaline protease had no effect (Yang et al., unpublished results). In the study, we speculated that the acid protease might be stable under acidic conditions, such as the stomach, and relatively resistant to gut digestive proteases. This might enable the intact acid protease to be delivered to the colon lumen, increasing the number of colonic free amino acids, which are essential for *Bifidobacterium* growth. In this context, we were interested to see that the CEL remains stable under acidic conditions (pH 2.0). We believe that this acid-resistant property might partially contribute to the substantial increase in *Bifidobacterium* and *Lactobacillus*.

Cellulase is responsible for the hydrolytic conversion of cellulose to metabolites, including shorter cello-polysaccharides, cello-oligosaccharides (COS), cellobiose and beta-glucose [7,8]. According to research, dietary supplemental COS significantly enhances the abundance of *Lactobacillus* bacteria in pig jejunal contents [19]. Furthermore, a recent in vitro study reported that COS treatment significantly enhanced the growth of *Lactobacillus* bacteria [20]. Thus, the enrichment of *Lactobacillus* by CEL may be, at least partially, mediated through mechanisms involving COS. However, neither of these studies indicated any effect of COS on the abundance of *Bifidobacterium*. In this study, CEL treatment markedly increased the RA of *Bifidobacterium* (a 28-fold increase). Accordingly, COS cannot account for the strong bifidogenic effect of CEL. Therefore, further studies are necessary to elucidate the mechanisms underlying the bifidogenic effect of supplemental *Aspergillus* cellulase.

4.2. Other Genera

This study further indicated that CEL markedly increased the RA of *Collinsella* and *Enterococcus*, but decreased the RA of seven genera, including *Parabacteroides*, *Allobaculum*, *Oscillospira*, *rc4-4*, *Dorea*, *Coprobacillus* and *Adlercreutzia*. There is very limited information about the effects of prebiotic oligosaccharides on *Parabacteroides*, *Allobaculum*, *rc4-4* and *Adlercreutzia*, as well as their roles in gut health. Therefore, the implications of modulating *Collinsella*, *Enterococcus*, *Oscillospira*, *Dorea* and *Coprobacillus* by CEL are discussed below.

Accumulating evidence indicates that the treatment of rats with inulin and oligosaccharides enhances *Collinsella* and *Bifidobacterium* abundance in the guts [21]. A recent study also revealed that *Aspergillus* protease and lipase preparations significantly increased the RA of *Collinsella* [6]. These findings are similar to our current results, indicating a marked increase in the level of *Collinsella,* as induced by CEL. *Collinsella* species might be beneficial to health; their enhanced abundance following dietary supplementation with oligofructose-enriched inulin in obese women is associated with an improved profile of hippurate, a microbial co-metabolite, indicating a healthier phenotype [22]. Furthermore, *Collinsella* exists at lower abundance in patients with inflammatory bowel disease or chronic pancreatitis than in healthy controls [23]. A study by Saalman et al. [24] suggested the potential use of this genus in treating inflammatory bowel disease. Overall, this study suggests that the significant increase in the abundance of *Collinsella* by CEL is beneficial to health; however, further studies are necessary to validate this position.

Additionally, the current study indicates a higher abundance of *Enterococcus* species in rats fed CEL. *Enterococcus faecalis* improves host health [25,26] and is clinically relevant for the treatment of chronic recurrent bronchitis [27]. Some *Enterococcus* species are employed as probiotics and in the production of feed additives to prevent diarrhea in animals [24]. Interestingly, several *Enterococcus* species isolated from food possess antioxidant activities [28]. Studies have shown that prebiotic oligosaccharides enhance the abundance of

Enterococcus species in mice and in perioperative colorectal cancer patients [29,30]. In addition, our previous study showed a significant increase in the RA of *Enterococcus* in rats fed *Aspergillus* protease and lipase preparations [6]. The higher abundance of commensal *Enterococcus* in rats fed CEL might be beneficial to the rats' health. However, *Enterococcus* species are a leading cause of hospital-associated bacteremia, endocarditis and urinary tract infections [31]. Therefore, further studies are necessary to determine the implications of increased *Enterococcus* in rats administered CEL.

It is worth noting that the CEL treatment significantly decreased the abundance of bacteria from the following genera: *Oscillospira*, *Dorea* and *Coprobacillus*. These findings agree with previous research on typical prebiotic oligosaccharides [32–36], and *Aspergillus* protease and lipase preparations (6). The current information on the roles of *Oscillospira*, *Dorea* and *Coprobacillus* in gut health is limited. Therefore, the implications of their modulation remain unclear.

4.3. Bacterial Diversity

In this study, contrary to expectations, CEL treatment significantly lowered bacterial diversity compared to the control. Microbial diversity is considered beneficial for community stability and host health [37,38]. However, this may not always be the case, and assumptions of increased diversity could be oversimplified for complicated interactive mechanisms in health and disease [39]. We believe that the reduced bacterial diversity in the CEL group might be associated with the depletion of several bacterial species, including *Parabacteroides*, *Allobaculum*, *Oscillospira*, *Dorea* and *Coprobacillus* (Figure 2, Table 1).

4.4. Organic Acids

Furthermore, in this study, it is interesting that CEL markedly increased cecal lactate levels, which were significantly associated with the modulation of the RA of lactate-producing bacteria, such as *Lactobacillus*, *Bifidobacterium* and *Enterococcus*. Meanwhile, CEL decreased the levels of other organic acids, such as acetate, propionate and butyrate. Notably, there was a significant inverse relationship between lactate and propionate levels. Propionate is produced microbially from lactate in the human colon [40]. Thus, CEL may reduce the metabolic conversion of lactate into propionate. Lactate has previously been studied in vitro for its free radical scavenging and antioxidant properties [41]. According to recent studies, lactate exhibits an inflammatory or anti-inflammatory role depending on its effects on immune cells and disease types [42]. Therefore, the implications of lactate accumulation in the CEL group remain unexplored. Additionally, CEL increased the total organic acids and lowered the pH. Interestingly, there was a strong inverse association between total organic acids and pH. The increased organic acids by CEL may cause the lower pH.

4.5. Limitations of This Study

One limitation of this study was that the cellulase preparation was crude and unpurified, despite having high cellulase activity. Therefore, factors related to cellulase preparation, besides the cellulase itself, may be responsible for modulating the gut microbiota. Since the activities of protease and lipase in CEL were slight (Section 2.1: Animals and Diets), the possibility that the protease and lipase in CEL modulate the gut microbiota was neglected. Further research is necessary to determine the effects of purified *Aspergillus*-derived cellulase on intestinal microbiota, as well as the relationship between cellulase activity and gut microbiota modulation. Although the cellulase preparation used here is crude, the preparation is actually used for the improvement of food digestion and food production. Therefore, the finding of the prebiotic-like effect of CEL is of great significance in terms of application.

5. Conclusions

Our results indicate that CEL treatment increased the RA of typical probiotics, such as *Bifidobacterium* and *Lactobacillus*. CEL also modulated the RA of other genera, including *Collinsella*, *Enterococcus*, *Oscillospira*, *Dorea* and *Coprobacillus*, as reported for typical prebiotic oligosaccharides. These findings suggest a potential role for *Aspergillus* cellulase as a prebiotic digestive enzyme in the food and animal feed industries, in addition to the established benefits to food digestion. This study may also help to elucidate the health benefits of *Aspergillus*-fermented foods and dietary enzyme supplements containing *Aspergillus* cellulase. Interestingly, the modulations of the genera *Bifidobacterium*, *Collinsella*, *Enterococcus*, *Oscillospira*, *Dorea* and *Coprobacillus* are similar to those reported for *Aspergillus* protease and lipase preparations [6]. Thus, the colonic digestion of carbohydrates, proteins and lipids may have a similar impact on these genera. Currently, our group is conducting metabolomics studies to elucidate the mechanisms through which *Aspergillus* cellulase modulates microbiota, as well as the effects of *Aspergillus* cellulase on gut health and diseases.

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Data Availability Statement: The data of the 16S rRNA gene sequences of gut microbiota presented in this study are available from the DDBJ database (http://getentry.ddbj.nig.ac.jp/) (accessed on 8 October 2021) under accession number DRA012837. Further inquiries can be directed to the corresponding authors.

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