



Research Article

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Detection of Butyrate from a Clostridium Cluster I (Clostridium Sensu Stricto) Strain, Clostridium Tertium, Under Anaerobic Conditions

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Abstract

Lactic acid bacteria and bifidobacteria are widely used as probiotics. However, butyrate-producing bacteria remain less explored despite the critical role of butyrate in gut health. Our metagenomic analysis of the gut microbiota of obese mice given fructooligosaccharides as a prebiotic showed potential butyrate producers in Clostridium sensu stricto. In particular, the butyrate-producing ability of Clostridium tertium, which belongs to the genus Clostridium sensu stricto, had not been clarified. In this study, we examined how effective C. tertium is as a butyrate producer to evaluate its potential as a novel probiotic bacterium. Protein BLAST (BLASTP) searches for Butyrate Kinase (BUK) proteins in the genomes of C. tertium identified two proteins, and Polymerase Chain Reaction (PCR) confirmed the presence of two buk genes and Phosphate Butyryltransferase (ptb), a buk gene adjacent gene. Ion-exclusion HPLC analysis detected lactate, formate, acetate, and butyrate in cultures of C. tertium, with a butyrate concentration of 6.5 ± 1.6 mM, comparable to that produced by Clostridium butyricum under the same conditions. These results demonstrate that C. tertium is a butyrate-producing bacterium and suggests its potential as a novel probiotic species.

Keywords: Butyrate, Clostridium tertium, Clostridium butyricum, butyrate kinase (buk), Phosphate butyryltransferase (ptb)

Introduction

Butyrate-producing bacteria are emerging as important indicators of gut microbial metabolism and colonic health in humans [1]. Based on molecular phylogenetic analysis of 16S rRNA gene sequences, Clostridium was divided into 19 clusters (designated I-XIX) [2,3]. In human feces, these bacteria mainly belong to the clostridial clusters IV and XIVa [4]. A study of probiotic feeding in mice revealed no significant change in fecal microbiota, but slight increases in clostridial clusters I, XIVa and XIVb in the small intestine [5]. Further, expression levels of clostridial clusters I and XIVa

were found to be higher in healthy adults than patients with non-small-cell lung cancer [6], and clusters I and XI were greater in stool samples from autistic children than control children, but not clusters XIVa and XIVb [7]. Cluster I, the genus Clostridium sensu stricto, is the largest of the clusters, harboring 118 species including the type species, Clostridium butyricum [2,3-8]. C. butyricum efficiently ferments a wide range of carbohydrates into biofuel compounds and precursors of biomaterials, including butyrate [9,10]. C. butyricum MIYAIRI 588 strain (CBM588) has been used as a probiotic in

clinical and veterinary medicine for decades [11]. It produces butyrate that inhibits *Clostridium Difficile* (CD) infection by inhibiting CD toxin activity and inducing neutrophils and T-helper 1 (Th1) and Th17 cells to eliminate CD [11]. Oral administration of the spores of CBM588 showed that the spores of *C. butyricum* can germinate and grow in the intestine [12]. The complete genome of CBM588 has been reported [13].

Meanwhile, *Clostridium acetobutylicum* is the microorganism of choice for the industrial production of solvents (acetone, butanol and ethanol), and genetic tools for gene knockout or gene overexpression are currently available, however, tools for *C. butyricum* are not [14]. Butyrate is synthesized via pyruvate and Acetyl-Coenzyme A (CoA), mostly via the breakdown of complex polysaccharides (e.g., starch and xylan) [12]. Four pathways are known: acetyl-CoA, glutarate, succinate, and lysine [15]. The butyryl-CoA: acetate CoA transferase (BUT) and Butyrate Kinase (BUK) pathways are the two main metabolic pathways through which bacteria release butyrate [15]. Four key enzymes are critical for converting acetyl-CoA to butyrate: Thiolase (THL), β -hydroxybutyryl-CoA dehydrogenase (HBD), Phosphate Butyryltransferase (PTB), and BUK [12,15,16]. The final step from butyryl-CoA to butyrate is either catalyzed by BUT or BUK [15]. PTB converts butyryl-CoA to butyryl-phosphate, and BUK dephosphorylates butyryl-phosphate [9,15]. BUK enables both the synthesis of butyryl-phosphate and its hydrolysis back to butyrate. Butyrate is synthesized through either the ptb-buk pathway or the but pathway, but not both [9]. *C. butyricum* strains have been found to have a buk gene, while the but gene is missing [9, 15]. Recent studies on the metabolic engineering of clostridia for butyrate production have focused on *Clostridium tyrobutyricum*, *C. acetobutylicum*, and *Clostridium ljungdahlii* [17]. *C. acetobutylicum* Δ buk/ Δ ptb mutant decreased butyrate production [18,19]. A Δ buk mutant is not viable if the ptb gene is not also inactivated, probably due to the accumulation of butyryl-phosphate, which might be toxic to the cell [20]. Tanwar et al, sequenced the whole genome of three *Clostridia* species and reported that sporulation genes were most abundant in *C. butyricum* (48 genes), followed by *C. tertium* (47 genes) and *C. difficile* (32 genes) [21]. *C. tertium* was first described by Henry in 1918 as *Bacillus tertius* and was reclassified as *C. tertium* in 1923 [22,23]. It is a Gram-positive, anaerobic, aerotolerant, nontoxigenic bacterium that forms spores only under anaerobic conditions [23]. It displayed a greater sporulation efficiency than *Clostridium paraputrificum* [24]. It is an uncommon human pathogen that can spread to the blood and cause bacteremia and septicemia [25]. Most cases of *C. tertium* bacteremia occur in neutropenic patients, but it can also occur in non-neutropenic patients [26,27].

Here, we explored the butyrate production potential of *C. tertium*, with the aim to provide more comprehensive information about the organic acid production of *C. tertium* and *C. butyricum*.

Methods

Bacterial Strains and Culture Conditions

C. butyricum NBRC 3315 (*C. butyricum* Prazmowski 1880) [28]

and *C. tertium* NBRC 103192 (ATCC 14573) [22] were purchased from the National Institute of Technology and Evaluation (NITE) at <https://www.nite.go.jp/nbrc/catalogue/NBRCDispSearchServlet?lang=en> and cultured with reinforced clostridial agar (Oxoid limited/CM0151B; Kanto Chemical, Japan). The strains were cultured in thioglycollate medium enriched with vitamin K1 and hemin (TGB; Thioglycollate broth) (BD Life Sciences, USA) to measure the organic acid content. The strains were grown at 32°C for 20 h in an AnaeroPack system (Mitsubishi Gas Chemical, Japan). The AnaeroPack system included a standard-sized AnaeroPack rectangular container (135 by 197 by 95 mm; 2.5 liters) and one AnaeroPack sachet [29,30]. The anaerobic conditions in the AnaeroPack system were monitored by oxygen concentration measuring devices (Oxy-2; Ichinen Jikco, Japan) [31,32].

Detection of Bacterial Genes

Bacterial genome DNA from cultured bacteria was extracted using Isospin fecal DNA (Nippon gene, Japan). The primer sequences are listed in Table 1. PCR was performed in a 10 μ L reaction mixture containing 5 μ L, 2 $\times$ TaKaRa TaqTM HS Perfect Mix (Takara, Japan). The amplification conditions consisted of an initial denaturation step at 94°C for 3 min, followed by 25 cycles (Amp1-4) or 35 cycles (Amp5) of denaturation at 94°C for 5 s, annealing at 57.5°C for 1 s, and extension at 72°C for 5 s (Amp1-3), 16 s (Amp4) or 90 s (Amp5). A final extension was performed at 72°C for 1 min. PCR solutions were mixed with a one-sixth volume of 6 $\times$ Loading buffer orange G (Nippon gene). A molecular weight marker (50 pb DNA ladder RTU; Bio medical science, Japan) and 15% e-PAGEL (ATTO, Japan) were used for electrophoresis. Tris/glycine buffer (Fujifilm Wako Pure Chemical, Japan) was used as the running buffer. Electrophoresis was performed at a constant voltage of 50 V for 25 min (Stacking/Loading) and 300 V for 40 min (Separation). Fast BlastTM DNA stain (Bio-Rad Laboratories, USA) was used for gel staining.

Organic Acid Quantification

Samples were analysed using ion-exclusion HPLC (Nexera) with an auto sampler (SIL-40C), controller (CBM-40), column oven (CTO-40C), and electrical conductivity detector (CDD-10Avp) (Shimadzu, Japan). The compounds were separated using a Shim-pack Fast-OA column (100 mm, 7.8 mm) from Shimadzu with a guard column (Shim-pack Fast-OA; 10 mm, 4.0 mm), operating at 50°C. The mobile phase was 5 mM p-toluenesulfonic acid in water, delivered at a flow rate of 0.8 mL/min. The pH buffering solution was composed of 5 mM p-toluenesulfonic acid, 20 mM Bis-Tris, and 0.1 mM ethylenediaminetetraacetic acid in water and delivered at a flow rate of 0.8 mL/min.

Metagenomic Analysis for Candidate Screening

Metagenomic analysis was conducted solely as a preliminary screening step to identify candidate butyrate-producing bacterial genera. No in vivo functional experiments were performed in this study.

Reagents

Fructooligosaccharides (FOS) and Lactose Monohydrate (Lac) were purchased from Fujifilm Wako Pure Chemical.

Mice

Six-week-old male ICR mice (n = 12) were purchased from Tokyo Laboratory Animal Science (Japan) and maintained in an isolation cabinet (Seobit, Japan). Normal diet (CE-2) and high-fat diet 32 (HFD32) were purchased from CLEA Japan. HFD32 feeding increases the body weight of ICR mice [33]. After 7 weeks on HFD32 diet, the mice (HFD-mice) were fed 2% of Lac (n = 4) or FOS (n = 4). The control mice were fed CE-2 (n = 4).

Fecal Sample Collection

After 10 more weeks, fecal samples were collected from the mice and immediately placed on dry ice and shipped to Techno-Suruga Laboratory (Japan).

DNA Extraction

DNA extraction was carried out following the procedure of Takahashi et al. [34]. Frozen fecal samples were thawed on ice; 100 mg of each sample was suspended in 4M guanidium thiocyanate, 100 mM Tris-HCl (pH9.0), and 40 mM EDTA; and the samples were then beaten with ceramic (zirconium oxide) beads using a Precellys 24 Touch (Bertin Technologies, France). DNA was extracted from the bead-treated suspensions using a GENE PREP STAR PI-480 nucleic acid isolation system (Kurabo, Japan). DNA concentrations were estimated by spectrophotometry using a NanoDrop ND-1000 instrument (Thermo Fisher Scientific, USA).

16S Rrna Gene Amplicon Sequencing

The DNA extract was sequenced using Illumina MiSeq technology (Illumina, USA) [34]. The V3-V4 region of the 16S rRNA gene was amplified by PCR in triplicate using the TaKaRa Ex Taq Hot Start Version (Takara). The primer sets were Pro341F (5'-CCTACGGGNGCASCAG-3'); Pro805R (5'-GACTACNVGGG-TATCTAATCC-3') for prokaryote and 341F (5'-CCTACGGGAGG-

CAGCA-3'); 806R (5'-GGACTACHVGGGTWTCTAAT-3') for bacteria. A 1μL sample of the combined PCR products was amplified with barcoded primers adapted for Illumina MiSeq sequencing: forward 5'-AATGATACGGCGACCACCGAGATCTACACXXXXXX-ACACTCTTTCCCTACACG ACGCTCTTCCGATCTCTG-3' and reverse 5' CAAGCAGAAGACGGCATACGAGATXXXXXXGTGACTG-GAGTTCAGACGTGTGCTCTTCCGATCTGAC-3', where X represents a barcode base. The products were purified and quantified with a QIAquick PCR Purification Kit (Qiagen, Germany) and Quant-iT™ PicoGreen™ dsDNA Assay Kit (Thermo Fisher Scientific). Equal amounts of amplicons were pooled and purified with the Gene-Read™ Size Selection Kit (Qiagen). The pooled libraries were sequenced with an Illumina MiSeq instrument and the MiSeq Reagent Kit v3 (Illumina). After removing sequences consistent with data from the Genome Reference Consortium human build 38 (GRCh38) and PhiX reads from the raw Illumina paired-end reads, the sequences were analysed using the QIIME2 software package, version 2022.2 [35]. Potential chimeric sequences were removed using DADA2 [36], then 30 and 90 bases of the 3'- regions of the forward and reverse reads were trimmed, respectively. Taxonomic classification was performed using a naive Bayes classifier trained on Greengenes 13.8, with a 99% threshold for operational taxonomic unit full-length sequences. UniFrac distances were calculated using QIIME2 software. The data collected at the end of the intervention period were statistically analysed.

Statistical Analysis

A Student's t-test was performed, and a significant difference was determined when the P-value was < 0.05.

Results

To identify the genera of gut bacteria other than Bifidobacterium from ICR mice, high fat diet induced obesity model (ICR mice) [33] were treated with FOS as a prebiotic. In humans, this induces bifidobacteria in their intestine [37], however, it does not in mice, because they have 707 to 1536 times less Bifidobacterium in their feces [38]. We designed a 10-week intervention study (Figure 1) and Figure S1-S5 & Table S1-S5).

Table S1: Number of altered bacteria obtained from Fig. S1 (domain level).

Ddomain	Lac (n = 4)	FOS (n = 4)	P value (t test)	
			One tail	Two tail
Bacteria	99.9 ± 25.0%	99.7 ± 4.7%	0.048	
Others	0.14 ± 0.14%	0.32 ± 0.14%		

Table S2: Number of significantly altered bacteria from Fig. S2 (phylum level).

Pphylum	Lac (n = 4)	FOS (n = 4)	P value (t test)	
			One tail	Two tail
Firmicutes	79.9 ± 19.9%	70.2 ± 11.9%	0.003	0.006
Bacteroidetes	14.4 ± 10.7%	23.6 ± 10.5% (1.6 fold)		

Actinobacteria	2.9 ± 3.4%	2.5 ± 2.0%		
Proteobacteria	2.3 ± 3.5%	1.5 ± 0.9% (1.5 fold)		
Candidatus Saccharibacteria	0.2 ± 0.1%	1.7 ± 1.3% (8.5 fold)		
Others	0.1 ± 0.1%	0.3 ± 0.1%	0.048	
Campilobacterota	0.03 ± 0.01%	0.09 ± 0.03% (3.0 fold)	0.018	0.036

Bas Significant difference by t test Up or Down

Table S3: Number of significantly altered bacteria from Fig. S3 (class level).

Cclass	Lac (n = 4)	FOS (n = 4)	P value (t test)	
			One tail	Two tail
Bacilli	44.7 ± 29.9%	11.6 ± 3.3% (3.9 fold)		
Clostridia	27.8 ± 24.1%	42.9 ± 7.3% (1.5 fold)		
Bacteroidia	14.4 ± 10.7%	23.5 ± 10.5% (1.6 fold)	0.003	0.006
Erysipelotrichia	7.3 ± 5.5%	15.7 ± 5.2% (2.2 fold)		
Actinobacteria	2.01 ± 3.16%	0.37 ± 0.36% (5.4 fold)		
Deltaproteobacteria	1.20 ± 1.77%	1.27 ± 0.75%		
Gammaproteobacteria	1.11 ± 1.67%	0.09 ± 0.10%		
Coriobacteriia	0.91 ± 0.22%	2.16 ± 1.80% (2.4 fold)		
Saccharibacteria_genera_incertae_sedis	0.22 ± 0.09%	1.73 ± 1.26% (7.9 fold)		
Others	0.14 ± 0.14%	0.32 ± 0.14%	0.048	
Campylobacteria	0.03 ± 0.01%	0.09 ± 0.03% (3.0 fold)	0.018	0.036
Alphaproteobacteria	0.03 ± 0.04%	0.14 ± 0.19% (4.7 fold)		
Betaproteobacteria	0.01 ± 0.01%	0.01 ± 0.01%		

Bas Significant difference by t test Up or Down

Table S4: Number of significantly altered bacteria from Fig. S4 (order level).

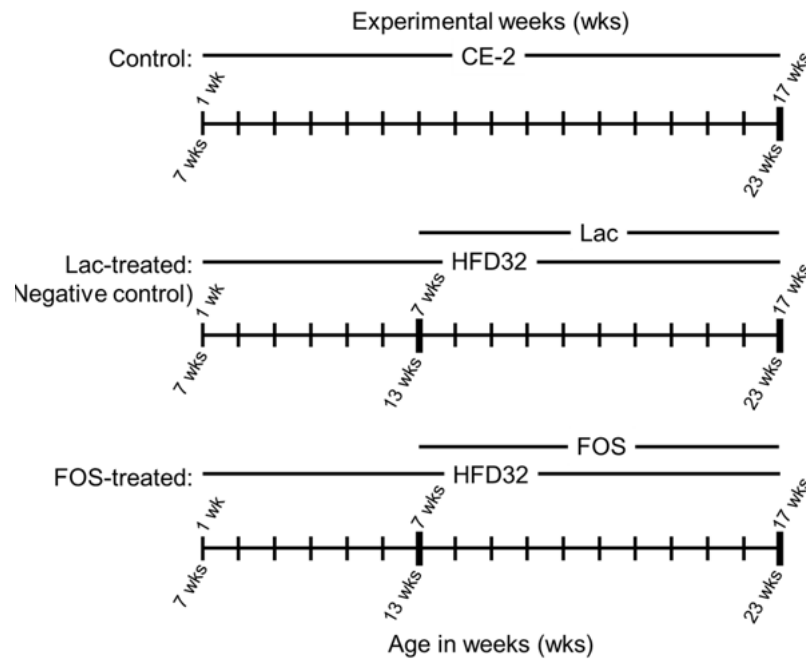
Oorder	Lac (N = 4)	FOS (N = 4)	P Value (T Test)	
			One Tail	Two Tail
Lactobacillales	44.7 ± 29.9%	11.6 ± 3.3% (3.9 fold)		
Clostridiales	27.8 ± 24.1%	42.9 ± 7.3% (1.5 fold)		
Bacteroidales	14.4 ± 10.7%	23.5 ± 10.5% (1.6 fold)	0.003	0.006
Erysipelotrichales	7.3 ± 5.5%	15.7 ± 5.2% (2.2 fold)		
Bifidobacteriales	1.9 ± 3.2%	0.4 ± 0.4% (4.8 fold)		
Desulfovibrionales	1.2 ± 1.8%	1.3 ± 0.7%		
Enterobacterales	1.11 ± 1.67%	0.09 ± 0.10%		
Eggerthellales	0.8 ± 0.1%	1.3 ± 0.6% (1.6 fold)		
Saccharibacteria_genera_incertae_sedis	0.2 ± 0.1%	1.7 ± 1.3% (8.5 fold)		
Others	0.14 ± 0.14%	0.32 ± 0.14%	0.048	
Coriobacteriales	0.08 ± 0.16%	0.85 ± 1.59% (10.6 fold)		
Mycobacteriales	0.08 ± 0.11%	0 ± 0%		
Deferribacterales	0.042 ± 0.047%	0.023 ± 0.006% (1.8 fold)		
Campylobacterales	0.028 ± 0.008%	0.092 ± 0.033% (3.3 fold)	0.018	0.036
Burkholderiales	0.007 ± 0.008%	0.007 ± 0.012%		
Kiloniellales	0.002 ± 0.004%	0.12 ± 0.19% (60 fold)		
Selenomonadales	0.004 ± 0.004%	0 ± 0%		

Bas Significant difference by t test Up or Down

Table S5: Number of significantly altered bacteria from Fig. S5 (family level).

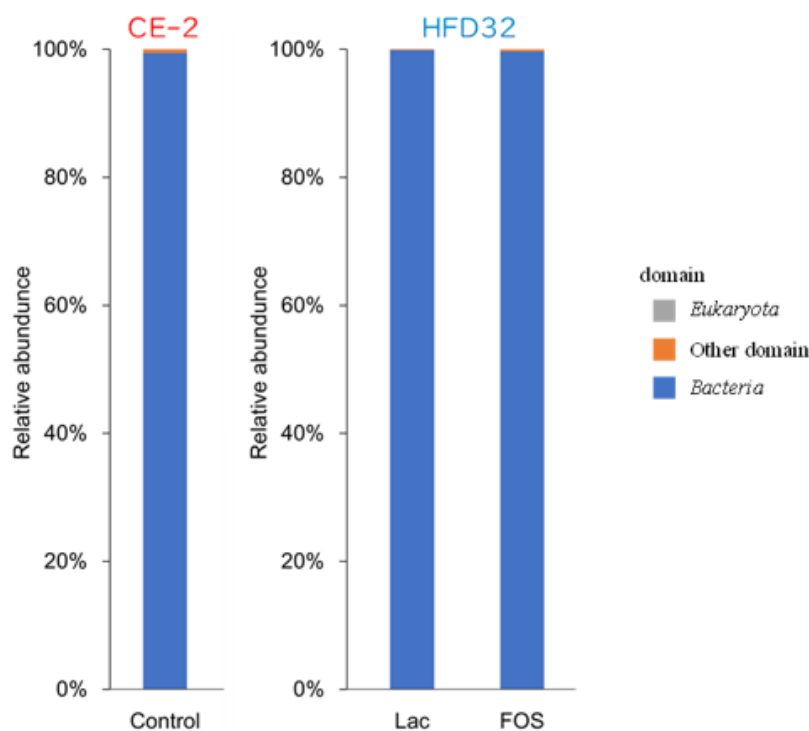
Ffamily	Lac (N = 4)	FOS (N = 4)	P Value (T Test)	
			One Tail	Two Tail
<i>Lactobacillaceae</i>	39.1 ± 31.5%	7.6 ± 1.4% (5.1 fold)		
<i>Lachnospiraceae</i>	15.7 ± 17.6%	10.2 ± 5.8% (1.5 fold)		
<i>Erysipelotrichaceae</i>	7.3 ± 5.5%	15.7 ± 5.2% (2.2 fold)		
Muribaculaceae	6.8 ± 6.3%	9.6 ± 3.6%		
<i>Clostridiaceae 1</i>	5.6 ± 5.7%	23.0 ± 8.6% (4.1 fold)	0.009	0.018
Streptococcaceae	5.5 ± 2.6%	3.9 ± 2.9%		
Peptostreptococcaceae	4.0 ± 1.9%	4.5 ± 2.0%		
<i>Bacteroidaceae</i>	2.9 ± 2.9%	6.0 ± 3.8% (2.1 fold)		0.036
Rikenellaceae	2.5 ± 1.8%	2.2 ± 0.9%		
<i>Ruminococcaceae</i>	2.2 ± 2.3%	4.5 ± 2.4% (2.0 fold)		
Bifidobacteriaceae	1.9 ± 3.2%	0.4 ± 0.4% (4.7 fold)		
<i>Porphyromonadaceae</i>	1.3 ± 1.6%	2.6 ± 1.7% (2.0 fold)		
Desulfovibrionaceae	1.2 ± 1.8%	1.3 ± 0.7%		
Enterobacteriaceae	1.1 ± 1.7%	0.09 ± 0.1% (12.2 fold)		
<i>Eggerthellaceae</i>	0.8 ± 0.09%	1.3 ± 0.6% (1.6 fold)		
<i>Odoribacteraceae</i>	0.5 ± 0.4%	2.6 ± 1.3% (5.2 fold)	0.011	0.022
Prevotellaceae	0.4 ± 0.3%	0.5 ± 0.7%		
<i>Saccharibacteria_genera_incertae_sedis</i>	0.2 ± 0.1%	1.7 ± 1.3% (8.5 fold)		
<i>Clostridiales_Incertae_Sedis XIII</i>	0.15 ± 0.24%	0.36 ± 0.37% (2.4 fold)		
Others	0.14 ± 0.14%	0.32 ± 0.14%	0.048	
Eubacteriaceae	0.13 ± 0.25%	0.04 ± 0.03% (3.3 fold)		
<i>Atopobiaceae</i>	0.08 ± 0.16%	0.85 ± 1.55% (10.6 fold)		
Corynebacteriaceae	0.08 ± 0.11%	0 ± 0%		
Enterococcaceae	0.07 ± 0.03%	0.06 ± 0.04%		
<i>Catabacteriaceae</i>	0.07 ± 0.07%	0.23 ± 0.18% (3.3 fold)		
Aerococcaceae	0.053 ± 0.096%	0.002 ± 0.002% (26.5 fold)		
Deferribacteraceae	0.04 ± 0.05%	0.02 ± 0.01% (2.0 fold)		
<i>Helicobacteraceae</i>	0.03 ± 0.01%	0.09 ± 0.03% (3.0 fold)	0.018	0.036
<i>Peptococcaceae 1</i>	0.03 ± 0.03%	0.05 ± 0.04% (1.7 fold)		
Staphylococcaceae	0.029 ± 0.030%	0.002 ± 0.001% (14.5 fold)		
Rhizobiaceae	0.02 ± 0.03%	0 ± 0%		
Carnobacteriaceae	0.0082 ± 0.0070%	0.0012 ± 0.0014% (6.8 fold)		
Sutterellaceae	0.006 ± 0.009%	0.006 ± 0.009%		
Desulfohalobiaceae	0.0054 ± 0.0069%	0.0006 ± 0.0012% (9.0 fold)		
<i>Kiloniellaceae</i>	0.002 ± 0.004%	0.122 ± 0.186% (61.0 fold)		
<i>Clostridiales_Incertae_Sedis XI</i>	0 ± 0%	0.003 ± 0.006%		

Bas Significant difference by t test Up or Down



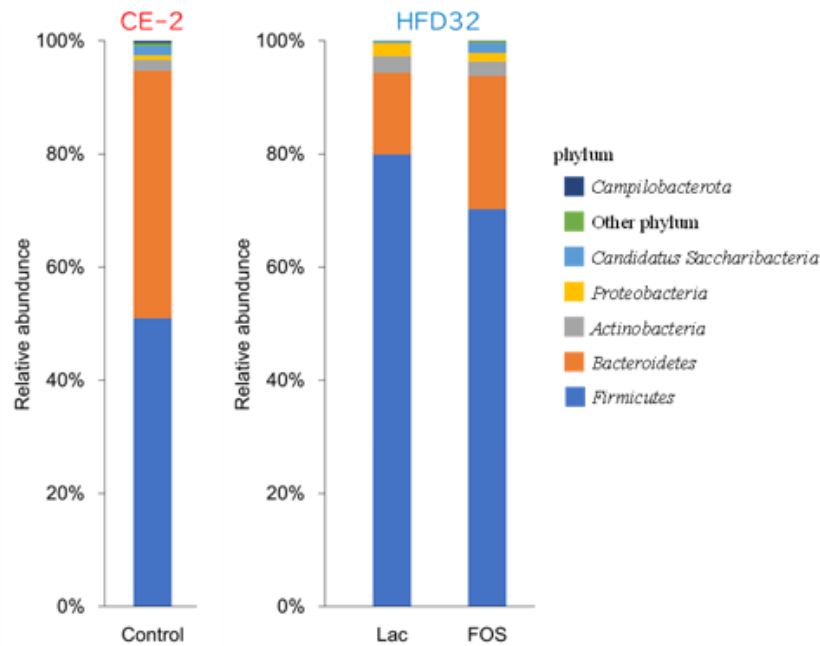
Note*: CE-2 is standard pellet chow consisting mainly of vegetable protein (soybean meal) with a proper balance of animal protein. HFD32 indicates a high-fat chow containing approximately 60% more calories per unit weight than the standard chow. Mice in the control group received tap water as drinking water from 1 to 17 weeks of age. Mice in the Lac-treated group and the FOS-treated group received drinking water supplemented with Lactic Acid (Lac) or Fructooligosaccharides (FOS), respectively, from 7 to 17 weeks of age.

Figure 1: Schematic diagram of the study design.



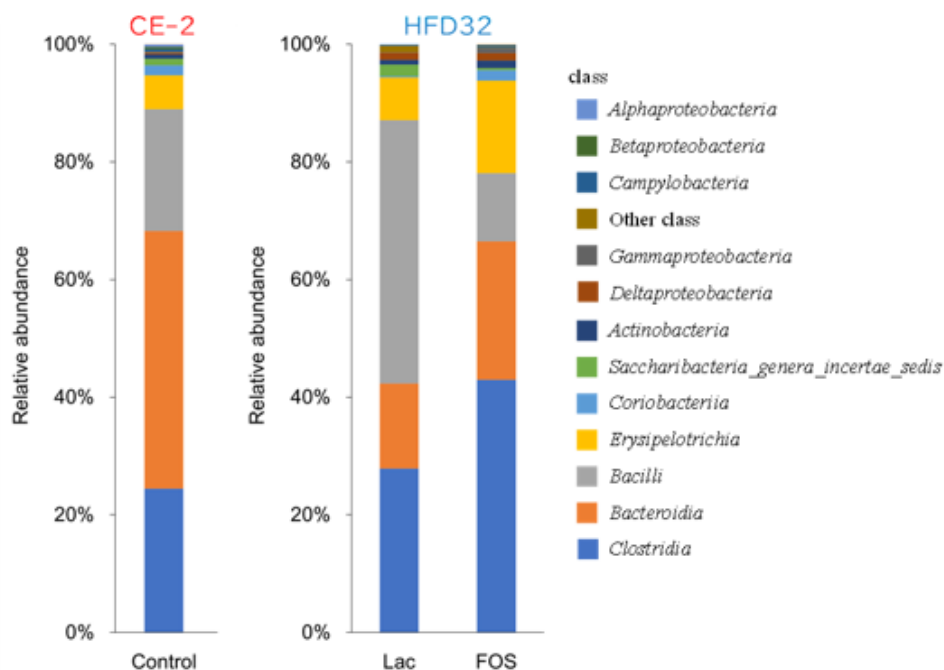
Note*: The domain levels of bacterial flora are shown. CE-2 mice (n = 4) had bacterial flora without dysbiosis (control), and Lac and FOS mice had dysbiosis caused by HFD32 [1-3]. However, there were no domain-level changes due to FOS. At the domain level, there was no significant difference in the proportion of Bacteria (■) when comparing Lac and FOS mice (see also Table S1). Others (■) was significantly increased in FOS mice (n = 4) compared to Lac mice (n = 4). Control [CE-2 mice (n = 4)] indicates normal gut microbiome. Eukaryota (■) were missing from our 16S analysis (see also Table S1).

Figure S1: Comparison of the gut microbiome relative abundance at the level of the bacterial domain.



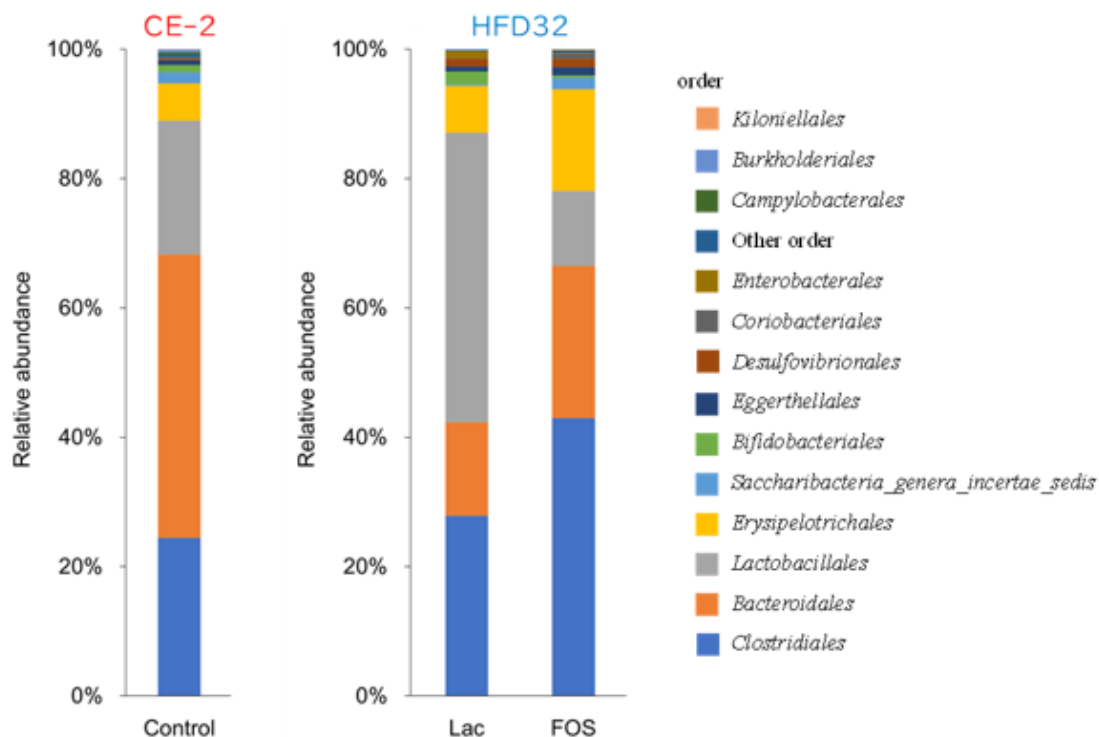
Note*: Compared to Lac mice (n = 4), FOS mice (n = 4) had an increased proportion of Bacteroidetes (■). The relative proportion of Bacteroidetes is known to be decreased in obese people compared to lean people [4]. The results show that FOS suppressed the decrease in Bacteroidetes (■), and thus may be able to improve dysbiosis. When the proportion of Bacteroidetes changes, Proteobacteria, which is usually a minority, increases [5]. Compared to Lac mice, the proportion of Bacteroidetes (■) was significantly increased in FOS mice, and the proportion of Proteobacteria (■) remained unchanged (see also Table S2). Campilobacterota (■) was significantly increased in FOS mice compared to Lac mice (see also Table S2). Control [CE-2 mice (n = 4)] indicates a normal gut microbiome.

Figure S2: Comparison of the gut microbiome relative abundance at the level of the bacterial phylum.



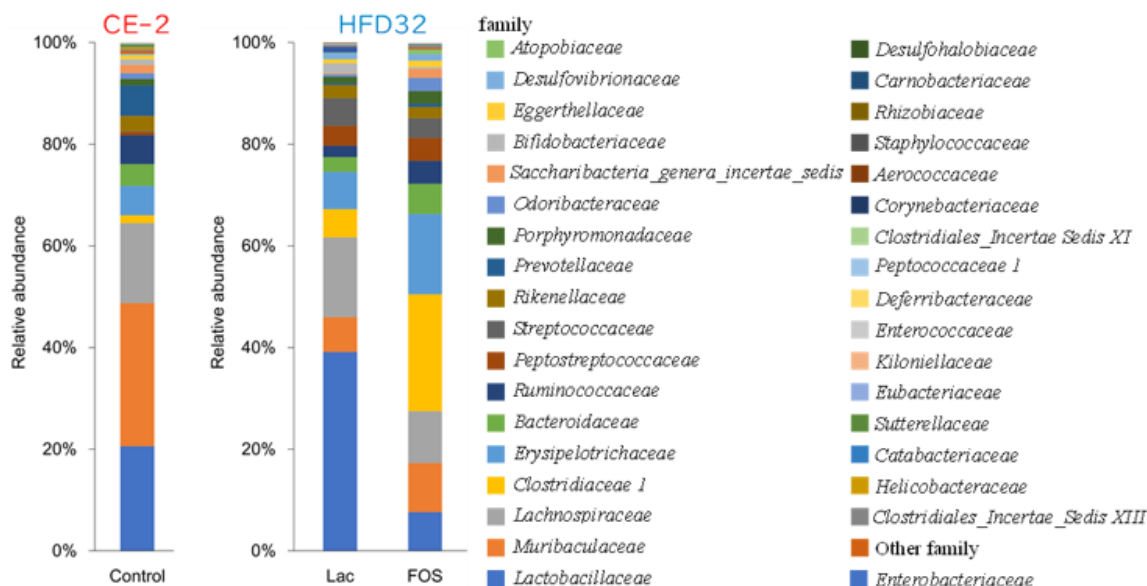
Note*: Bacteroidia (■) and Campylobacteria (■) were increased in FOS mice (n = 4) compared to Lac mice (n = 4). Details are shown in Table S3. Control [CE-2 mice (n = 4)] indicates a normal gut microbiome.

Figure S3: Comparison of the gut microbiome relative abundance at the level of the bacterial class.



Note*: Similar to the class level (Fig. S3 and Table S3), Bacteroidales (■) and Campylobacteriales (■) were significantly increased in FOS mice (n = 4) compared to Lac mice (n = 4) (see also Table S2). Control [CE-2 mice (n = 4)] indicates a normal gut microbiome.

Figure S4: Comparison of the gut microbiome relative abundance at the level of the bacterial order.

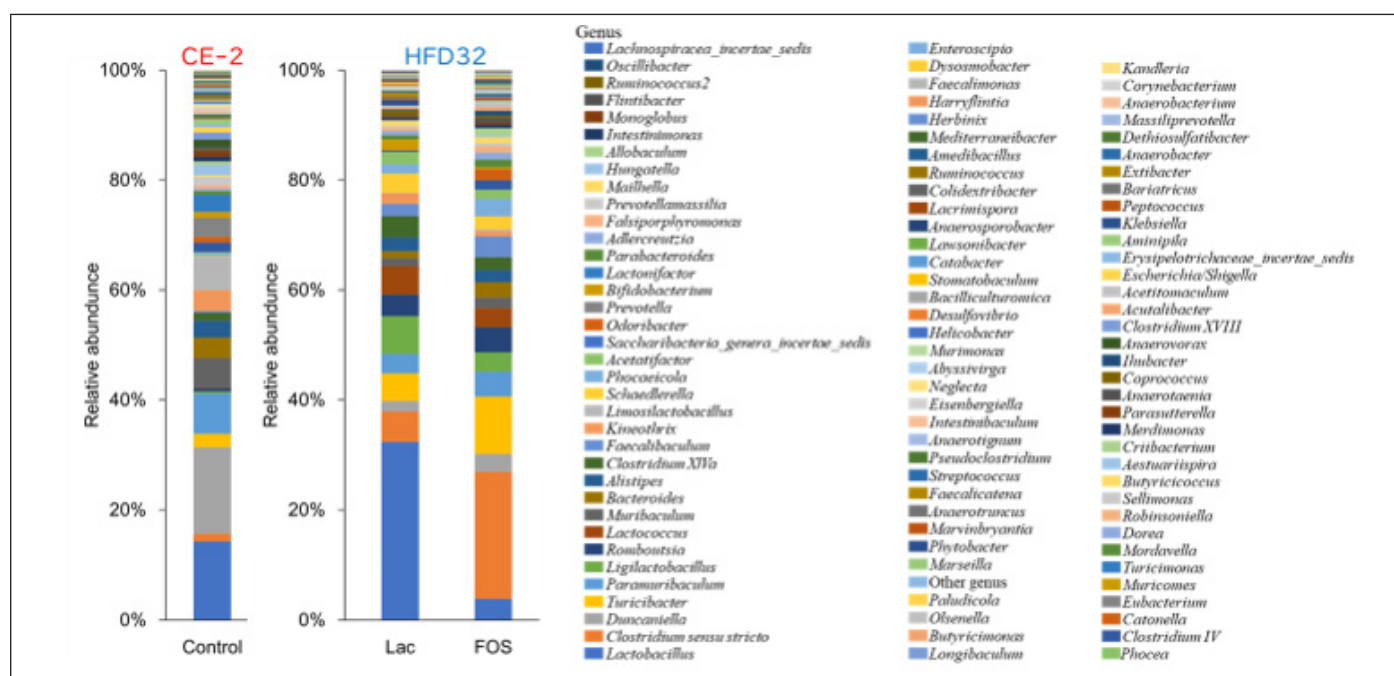


Note*: Clostridiales order bacteria are classified into 19 families [6]. Among the 19 classified bacterial families, there are Clostridiaceae 1, Clostridiaceae 2, Clostridiaceae 3, and Clostridiaceae 4. Clostridiaceae 1 includes Sarcina, Anaerobacter (■ in Fig. 2), Clostridium [Clostridium sensu stricto (■), Clostridium XIVa (■), Clostridium IV (■), and Clostridium XVIII (■) in Fig. 2], Caloramator, Thermobrachium, and Oxobacter. Clostridiaceae 1 (■) was significantly increased in FOS mice (n = 4) compared to Lac mice (n = 4). Bacteroidaceae (■) was significantly increased in FOS mice compared to Lac mice (see also Table S5). In mice, Odoribacteraceae (■) was significantly increased (see also Table S5). Helicobacteraceae (■) was significantly increased in FOS mice compared to Lac mice (see also Table S5). Control [CE-2 mice (n = 4)] indicates a normal gut microbiome.

Figure S5: Comparison of the gut microbiome relative abundance at the level of the bacterial family.

At domain level (Fig. S1 and Table S1), there was no significant change of Bacteria between Lac and FOS. At phylum (Fig. S2 and Table S2), class (Fig. S3 and Table S3), and order (Fig. S4 and Table S4) levels, two lineages, Bacteroidetes and Campylobacterota, significantly increased [comparison between Lac and FOS treatment; phylum Bacteroidetes increased 1.6-fold, Campylobacterota increased 3.0-fold; class Bacteroidia increased 1.6-fold, class Campylobacterota increased 3.0-fold; order Bacteroidales increased 1.6-fold, and order Campylobacterales increased 3.3-fold]. At family level (Fig. S5 and Table S5), one family, containing Clostridiaceae 1 (4.1-fold), Bacteroidaceae (2.1-fold), Odoribacteraceae (5.2-fold), and Helicobacteraceae (3.0-fold) significantly increased. At genus level (Fig. 2 and Table S6), 13 genera increased and two genera decreased. No increase in Bifidobacteria was detected following FOS treatment (Table S6). In contrast, the abundance of the genera *Clostridium sensu stricto* [39,40], *Butyrivibrio* [41], *Odoribacter*

[42], and *Bacteroides* [43], which produce short-chain fatty acids, was significantly increased, strongly suggesting that FOS alters the balance of the gut microbiota in mice. Initially, we focused on *Clostridium sensu stricto* (Clostridium Cluster I) and examined the gut microbiota in high-fat diet 32 (HFD32)-fed ICR mice with or without the complex polysaccharide FOS (Figs. S1–S5 and Fig 2; Tables S1–S7). FOS increased *Clostridium sensu stricto* in mice feces (Fig. 2; Table S6–S7). Four Genera (*Clostridium sensu stricto*, *Bacteroides*, *Butyrivibrio*, and *Odoribacter*) were identified as butyrate producers in HFD-fed ICR mice after FOS administration (Table S7). Prebiotics [particularly FOS and Galactooligosaccharides (GOS)] appear to promote an increase in the abundance of bifidobacteria in the gut microbiota [44]. Predictably, *Bifidobacterium* did not increase in FOS mice (n = 4) compared to Lac mice (n = 4) (Figure 2) and (Table S6 and S7).



Note*: The relative abundance of the 16S rRNA genus detected in feces from Control (n = 4), Lac (n = 4) and FOS (n = 4). Each color represents a distinct bacterial genus.

Figure 2: Bacterial composition of the studied samples at the genus level.

Table S6: Number of significantly altered bacteria from Fig. 2 (genus level).

Ggenus	Lac (n = 4)	FOS (n = 4)	P value (t test)	
			One tail	Two tail
<i>Lactobacillus</i>	32.2 ± 33.9%	3.8 ± 2.9% (8.5 fold)		
<i>Ligilactobacillus</i>	6.8 ± 5.1%	3.5 ± 2.7% (1.9 fold)		
<i>Clostridium sensu stricto</i>	5.6 ± 5.7%	23.0 ± 8.6% (4.1 fold)	0.009	0.018
<i>Lactococcus</i>	5.2 ± 2.5%	3.5 ± 2.8% (1.5 fold)		
<i>Turicibacter</i>	5.0 ± 6.1%	10.4 ± 5.9% (2.1 fold)		
<i>Romboutsia</i>	3.9 ± 1.9%	4.5 ± 2.0%		

<i>Clostridium XIva</i>	3.8 ± 4.0%	2.3 ± 1.4% (1.7 fold)	
<i>Schaedlerella</i>	3.5 ± 3.3%	2.3 ± 2.1% (1.5 fold)	
<i>Paramuribaculum</i>	3.5 ± 3.3%	4.5 ± 1.9%	
<i>Alistipes</i>	2.5 ± 1.8%	2.2 ± 0.9%	
<i>Faecalibaculum</i>	2.2 ± 3.0%	3.8 ± 3.6% (1.7 fold)	
<i>Acetatifactor</i>	2.2 ± 3.6%	1.6 ± 1.0%	
<i>Kineothrix</i>	2.0 ± 3.1%	0.9 ± 0.7% (2.2 fold)	
<i>Duncaniella</i>	1.9 ± 1.9%	3.2 ± 1.2% (1.9 fold)	
<i>Bifidobacterium</i>	1.9 ± 3.2%	0.4 ± 0.4% (4.8 fold)	
<i>Phocaeicola</i>	1.7 ± 1.9%	3.2 ± 2.2% (1.9 fold)	0.035
<i>Muribaculum</i>	1.4 ± 1.2%	1.9 ± 0.9%	
<i>Bacteroides</i>	1.3 ± 1.1%	2.7 ± 1.6% (2.1 fold)	0.036
<i>Mailhella</i>	0.9 ± 1.3%	1.1 ± 0.6%	
<i>Ruminococcus2</i>	0.9 ± 1.8%	0.4 ± 0.4% (2.3 fold)	
<i>Phytobacter</i>	0.96 ± 1.50%	0.02 ± 0.03% (48 fold)	
<i>Adlercreutzia</i>	0.8 ± 0.1%	1.3 ± 0.6% (1.6 fold)	
<i>Faecalicatena</i>	0.8 ± 1.2%	0.1 ± 0.0% (8.0 fold)	
<i>Parabacteroides</i>	0.7 ± 1.0%	1.4 ± 1.0% (2.0 fold)	
<i>Falsiporphyromonas</i>	0.5 ± 0.6%	1.2 ± 0.8% (2.4 fold)	0.014 0.029
<i>Prevotellamassilia</i>	0.4 ± 0.3%	0.5 ± 0.7%	
<i>Intestinimonas</i>	0.4 ± 0.3%	0.6 ± 0.2% (1.5 fold)	
<i>Butyricimonas</i>	0.4 ± 0.5%	0.7 ± 0.4% (1.8 fold)	0.037
<i>Anaerotruncus</i>	0.4 ± 0.7%	0.2 ± 0.2% (2 fold)	
<i>Flintibacter</i>	0.3 ± 0.2%	0.8 ± 0.5% (2.7 fold)	
<i>Streptococcus</i>	0.3 ± 0.2%	0.4 ± 0.2%	
<i>Desulfovibrio</i>	0.3 ± 0.5%	0.2 ± 0.2% (1.5 fold)	
<i>Murimonas</i>	0.3 ± 0.7%	0.2 ± 0.2% (1.5 fold)	
<i>Lacrimispora</i>	0.295 ± 0.339%	0.028 ± 0.026% (10.5 fold)	
<i>Saccharibacteria_genera_incertae_sedis</i>	0.22 ± 0.09%	1.73 ± 1.26% (7.9 fold)	
<i>Monoglobus</i>	0.2 ± 0.2%	0.4 ± 0.4% (2 fold)	
<i>Marvinbryantia</i>	0.2 ± 0.1%	0.4 ± 0.5% (2 fold)	
<i>Stomatobaculum</i>	0.2 ± 0.2%	0.1 ± 0.1% (2 fold)	
<i>Butyricicoccus</i>	0.1595 ± 0.2974%	0.0073 ± 0.0028% (21.8 fold)	
<i>Abyssivirga</i>	0.148 ± 0.046%	0.130 ± 0.032%	
<i>Odoribacter</i>	0.14 ± 0.18%	1.96 ± 1.11% (14 fold)	0.022 0.044
<i>Dorea</i>	0.140 ± 0.091%	0.028 ± 0.028% (5.0 fold)	0.036
<i>Neglecta</i>	0.137 ± 0.243%	0.044 ± 0.030% (3.1 fold)	
<i>Mediterraneibacter</i>	0.138 ± 0.151%	0.029 ± 0.020% (4.8 fold)	
<i>Bacilliculturomica</i>	0.13 ± 0.24%	0.30 ± 0.40% (2.3 fold)	
<i>Klebsiella</i>	0.13 ± 0.15%	0.0 ± 0.0%	
<i>Eubacterium</i>	0.12 ± 0.25%	0.0 ± 0.0%	
<i>Bariatricus</i>	0.12 ± 0.19%	0.0 ± 0.0%	
<i>Hungatella</i>	0.1 ± 0.1%	0.3 ± 0.3%	
<i>Others</i>	0.1 ± 0.1%	0.3 ± 0.1%	0.048
<i>Oscillibacter</i>	0.1 ± 0.1%	0.8 ± 0.6% (8.0 fold)	0.047

<i>Pseudoclostridium</i>	0.10 ± 0.15%	0.13 ± 0.13%	
<i>Anaerotignum</i>	0.1 ± 0.1%	0.3 ± 0.2% (3.0 fold)	
<i>Limosilactobacillus</i>	0.086 ± 0.096%	0.31 ± 0.26% (3.6 fold)	
<i>Olsenella</i>	0.082 ± 0.156%	0.850 ± 1.554% (10.4 fold)	
<i>Corynebacterium</i>	0.08 ± 0.11%	0.0 ± 0.0%	
<i>Lachnospiraceae_incertae_sedis</i>	0.078 ± 0.149%	0.031 ± 0.031% (2.5 fold)	
<i>Phoceae</i>	0.076 ± 0.131%	0.129 ± 0.093% (1.7 fold)	
<i>Catabacter</i>	0.074 ± 0.074%	0.229 ± 0.177% (3.1 fold)	
<i>Merdimonas</i>	0.073 ± 0.090%	0.065 ± 0.049%	
<i>Criibacterium</i>	0.0680 ± 0.0734%	0.0055 ± 0.0042% (12.4 fold)	0.05
<i>Paludicola</i>	0.064 ± 0.093%	0.084 ± 0.037%	
<i>Eisenbergiella</i>	0.062 ± 0.066%	0.117 ± 0.073% (1.9 fold)	
<i>Coproccoccus</i>	0.040 ± 0.051%	0.043 ± 0.028%	
<i>Acetitomaculum</i>	0.030 ± 0.060%	0.023 ± 0.016%	
<i>Dysosmobacter</i>	0.034 ± 0.045%	0.193 ± 0.154% (5.7 fold)	0.047
<i>Clostridium IV</i>	0.033 ± 0.043%	0.051 ± 0.021% (1.5 fold)	
<i>Peptococcus</i>	0.032 ± 0.026%	0.051 ± 0.035% (1.6 fold)	
<i>Lawsonibacter</i>	0.029 ± 0.022%	0.251 ± 0.294% (8.7 fold)	
<i>Sellimonas</i>	0.029 ± 0.035%	0.133 ± 0.187% (4.6 fold)	
<i>Clostridium XVIII</i>	0.029 ± 0.058%	0.100 ± 0.149% (3.4 fold)	
<i>Acutalibacter</i>	0.029 ± 0.031%	0.086 ± 0.053% (3.0 fold)	0.033
<i>Helicobacter</i>	0.028 ± 0.008%	0.92 ± 0.033% (32.9 fold)	0.018
<i>Amedibacillus</i>	0.027 ± 0.033%	0.089 ± 0.060% (3.3 fold)	0.014
<i>Colidextribacter</i>	0.025 ± 0.020%	0.254 ± 0.184% (10.2 fold)	0.042
<i>Robinsoniella</i>	0.025 ± 0.024%	0.073 ± 0.095% (3.0 fold)	
<i>Faecalimonas</i>	0.2 ± 0.4%	0.1 ± 0.1% (2.0 fold)	
<i>Harryflintia</i>	0.020 ± 0.021%	0.212 ± 0.344% (10.6 fold)	
<i>Enteroscipio</i>	0.018 ± 0.011%	0.029 ± 0.022% (1.6 fold)	
<i>Muricomes</i>	0.0172 ± 0.0274%	0.0067 ± 0.0070% (2.6 fold)	
<i>Aminipila</i>	0.0154 ± 0.0127%	0.0361 ± 0.0238% (2.3 fold)	
<i>Extibacter</i>	0.014 ± 0.011%	0.018 ± 0.018%	
<i>Escherichia/Shigella</i>	0.013 ± 0.017%	0.068 ± 0.103% (5.2 fold)	
<i>Anaerosporobacter</i>	0.012 ± 0.014%	0.322 ± 0.509% (26.8 fold)	
<i>Anaerobacter</i>	0.012 ± 0.012%	0.014 ± 0.0065	
<i>Parasutterella</i>	0.0091 ± 0.0018%	0.0012 ± 0.0014% (4.3 fold)	
<i>Anaerotaenia</i>	0.0054 ± 0.0063%	0.0122 ± 0.0045% (2.3 fold)	0.044
<i>Aestuariaispira</i>	0.0054 ± 0.0399%	0.1217 ± 0.18615 (22.5 fold)	
<i>Turicimonas</i>	0.0054 ± 0.0069%	0.0043 ± 0.0086%	
<i>Mordavella</i>	0.0036 ± 0.0051%	0.0201 ± 0.0280% (5.6 fold)	
<i>Longibaculum</i>	0.0036 ± 0.0031%	0.0031 ± 0.0046%	
<i>Anaerobacterium</i>	0.0027 ± 0.0054%	0.0061 ± 0.0076% (2.3 fold)	
<i>Ihubacter</i>	0.0027 ± 0.0054%	0.0239 ± 0.0200% (8.9 fold)	
<i>Anaerovorax</i>	0.0018 ± 0.0021%	0.0037 ± 0.0032% (2.1 fold)	
<i>Erysipelotrichaceae_incertae_sedis</i>	0.00091 ± 0.00181%	0.00428 ± 0.00542% (4.7 fold)	
<i>Lactonifactor</i>	0.00091 ± 0.00181%	0.00061 ± 0.0012% (1.5 fold)	

Allobaculum	0.0 ± 0.0%	1.3 ± 2.6%	
Prevotella	0.0 ± 0.0%	0.0031 ± 0.0031%	
Dethiosulfatibacter	0.0 ± 0.0%	0.003 ± 0.006%	
Massiliprevotella	0.0 ± 0.0%	0.0 ± 0.0%	
Catonella	0.0 ± 0.0%	0.0 ± 0.0%	
Ruminococcus	0.0 ± 0.0%	0.0 ± 0.0%	
Herbinix	0.0 ± 0.0%	0.0 ± 0.0%	
Marseilla	0.0 ± 0.0%	0.0 ± 0.0%	
Intestinibaculum	0.0 ± 0.0%	0.0 ± 0.0%	
Kandleria	0.0 ± 0.0%	0.0 ± 0.0%	

Bas Significant difference by t test Up or Down

Table S7: Number of significantly altered bacteria from Figure S2-S5 and Figure 2.

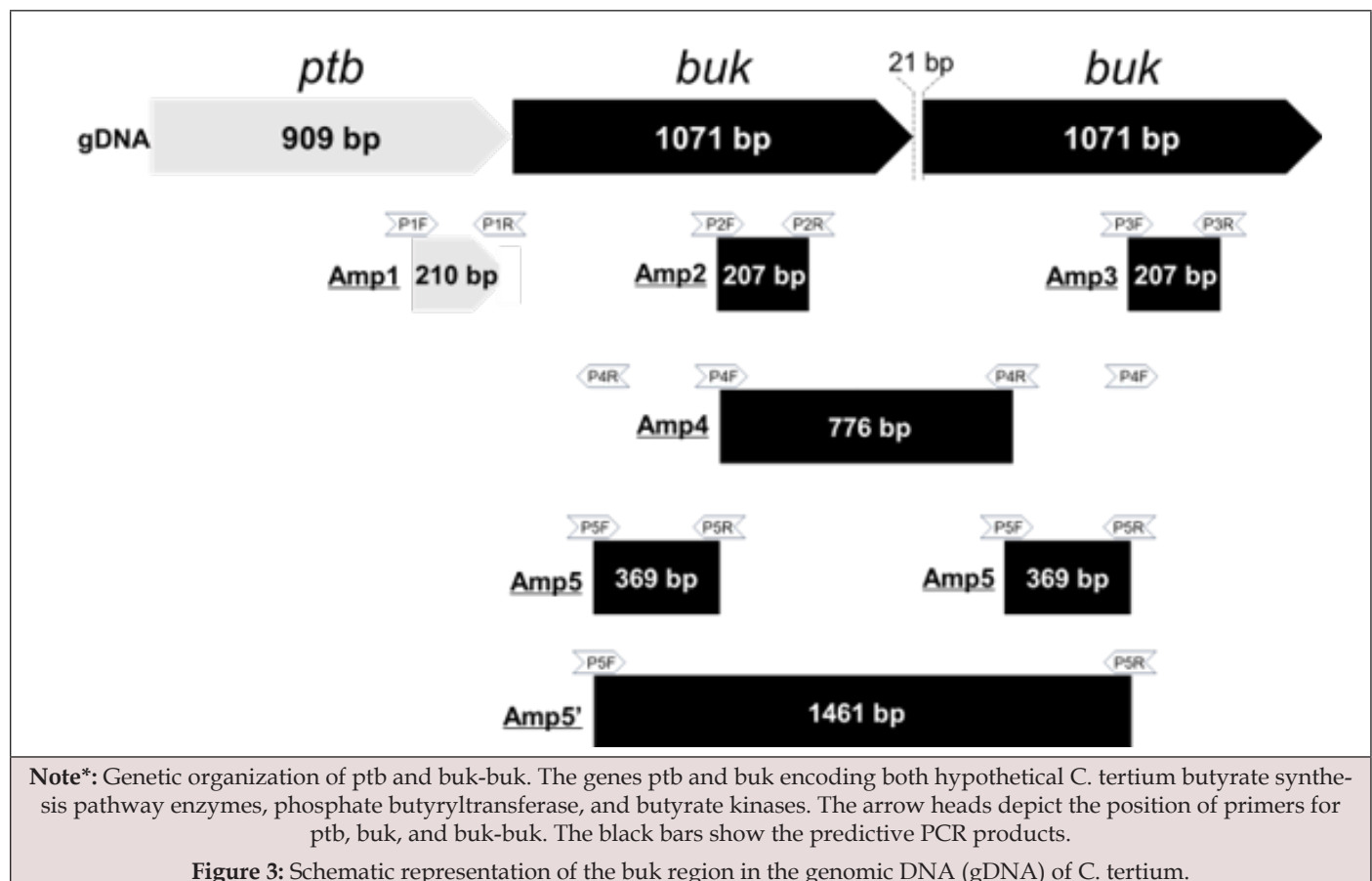
Biological classification	Lac (n = 4)	FOS (n = 4)	P value (t test)	
			One tail	Two tail
Pphylum				
<i>Bacteroidetes</i>	14.4 ± 10.7%	23.6 ± 10.5% (1.6 fold)	0.003	0.006
<i>Campilobacterota</i>	0.03 ± 0.01%	0.09 ± 0.03% (3.0 fold)	0.018	0.036
Cclass				
<i>Bacteroidia</i>	14.4 ± 10.7%	23.5 ± 10.5% (1.6 fold)	0.003	0.006
<i>Campylobacteria</i>	0.03 ± 0.01%	0.09 ± 0.03% (3.0 fold)	0.018	0.036
Oorder				
<i>Bacteroidales</i>	14.4 ± 10.7%	23.5 ± 10.5% (1.6 fold)	0.003	0.006
<i>Campylobacterales</i>	0.028 ± 0.008%	0.092 ± 0.033% (3.3 fold)	0.018	0.036
Ffamily				
<i>Clostridiaceae 1</i>	5.6 ± 5.7%	23.0 ± 8.6% (4.1 fold)	0.009	0.018
<i>Bacteroidaceae</i>	2.9 ± 2.9%	6.0 ± 3.8% (2.1 fold)	0.036	
<i>Odoribacteraceae</i>	0.5 ± 0.4%	2.6 ± 1.3% (5.2 fold)	0.011	0.022
<i>Helicobacteraceae</i>	0.03 ± 0.01%	0.09 ± 0.03% (3.0 fold)	0.018	0.036
Ggenus				
<i>Clostridium sensu stricto</i>	5.6 ± 5.7%	23.0 ± 8.6% (4.1 fold)	0.009	0.018
<i>Phocaeicola</i>	1.7 ± 1.9%	3.2 ± 2.2% (1.9 fold)	0.035	
<i>Bacteroides</i>	1.3 ± 1.1%	2.7 ± 1.6% (2.1 fold)	0.036	
<i>Falsiporphyrmonas</i>	0.5 ± 0.6%	1.2 ± 0.8% (2.4 fold)	0.014	0.029
<i>Butyricimonas</i>	0.4 ± 0.5%	0.7 ± 0.4% (1.8 fold)	0.037	
<i>Odoribacter</i>	0.14 ± 0.18%	1.96 ± 1.11% (14 fold)	0.022	
<i>Dorea</i>	0.140 ± 0.091%	0.028 ± 0.028% (5.0 fold)	0.036	0.044
<i>Oscillibacter</i>	0.1 ± 0.1%	0.8 ± 0.6% (8.0 fold)	0.047	
<i>Criibacterium</i>	0.0680 ± 0.0734%	0.0055 ± 0.0042% (12.4 fold)	0.05	
<i>Dysosmobacter</i>	0.034 ± 0.045%	0.193 ± 0.154% (5.7 fold)	0.047	
<i>Acutalibacter</i>	0.029 ± 0.031%	0.086 ± 0.053% (3.0 fold)	0.033	
<i>Helicobacter</i>	0.028 ± 0.008%	0.92 ± 0.033% (32.9 fold)	0.018	0.036
<i>Amedibacillus</i>	0.027 ± 0.033%	0.089 ± 0.060% (3.3 fold)	0.014	0.028
<i>Colidextribacter</i>	0.025 ± 0.020%	0.254 ± 0.184% (10.2 fold)	0.042	
<i>Anaerotaenia</i>	0.0054 ± 0.0063%	0.0122 ± 0.0045% (2.3 fold)	0.044	

Up or Down

As shown in Fig. 2, *Clostridium sensu stricto*, which decomposes glucose into acetate, butyrate, and valerate through fermentation [39,40], *Phocaeicola*, of which genus only *Phocaeicola abscessus* species is known [45], *Bacteroides*, which are characterized by being Gram-negative rods, non-spore forming, non-motile obligate anaerobes and in whose carbohydrate fermentation the main end products are acetate and succinate and also feature SCFAs such as butyrate [43], *Falsiporphyromonas*, which is an obligate anaerobic, Gram-negative, non-sporulating bacillus [46], *Butyricimonas*, which decomposes dietary fiber and produces SCFAs [41], *Odoribacter*, which is known to produce butyrate [42], *Acutalibacter*, which is a member of the family Oscillospiraceae [47,48], *Helicobacter*, which belong to the phylum Campylobacterota (previously called Epsilonproteobacteria) [49], *Amedibacillus*, which is Gram-positive, obligately anaerobic, non-spore forming, and non-motile [50], *Colidextribacter*, which has been reported to increase in humans and mice treated with cancer immunotherapy using anti-PD-1 or anti-PD-L1 antibodies [51], and *Anaerotaenia*, which increases in premenstrual disorders (PMDs; PMS and PMDD) [52], all significantly increased in FOS mice compared to Lac mice, while *Dorea*, which belongs to the phylum Firmicutes, family Lachnospiraceae, and produces SCFAs, especially butyrate [53,54], and *Criibacterium*, which is classified as a new genus of bacteria in the family Pep-

tostreptococcus [55].

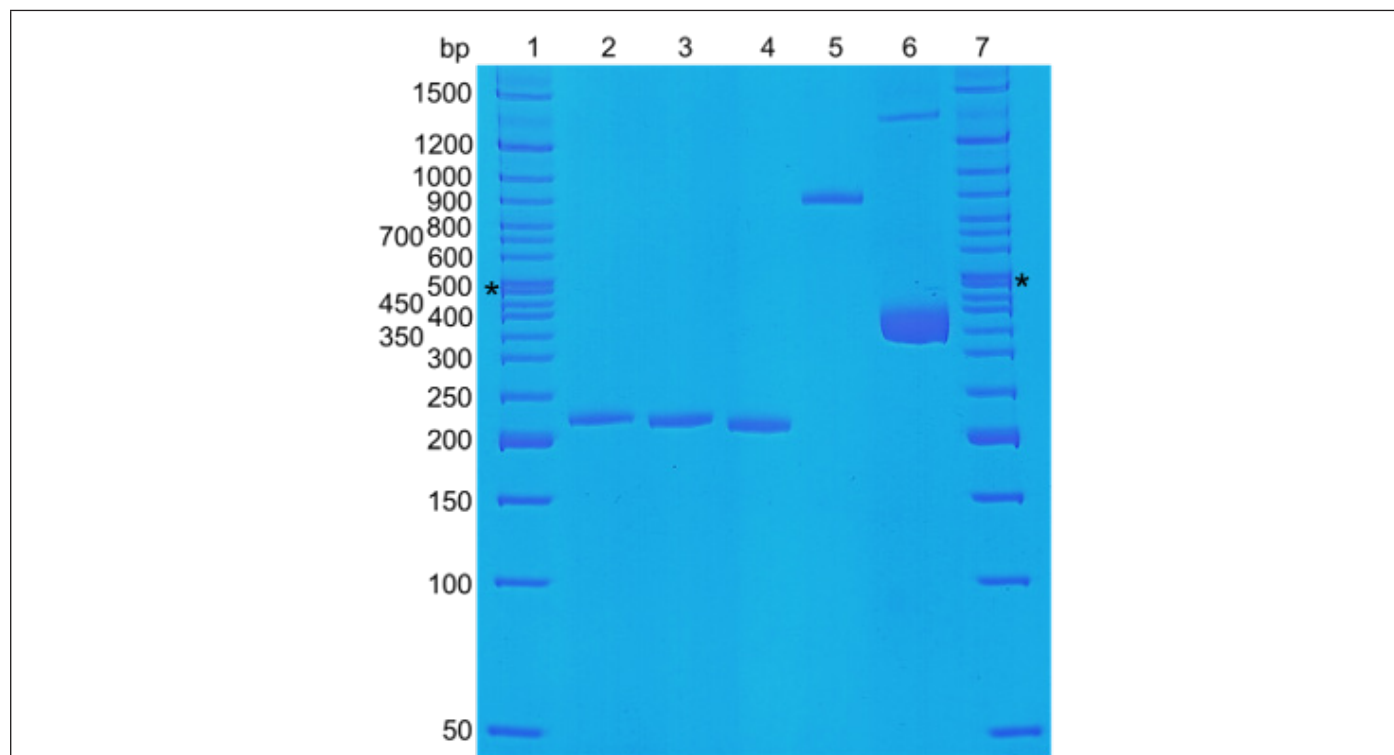
Significantly decreased (see also Table S6). Contrary to our expectations, *Oscillibacter*, whose relative amount is known to increase in C57BL/6 mice that have become obese due to a high-fat diet [56], and *Dysosmobacter*, whose bacteria may act defensively against obesity and changes in glucose metabolism associated with obesity [57] also significantly increased. The control [CE-2 mice (n = 4)] indicated a normal gut microbiome. As our metagenomic studies detected sufficient reads of genera sequences but not of species sequences using the RDP (Ribosomal Database Project) database [34], we examined listed species of four detected genera that increased after FOS administration in HFD-fed ICR mice at <https://www.ncbi.nlm.nih.gov/guide/taxonomy/>. We were able to list four species (*Clostridium acidisoli*, *C. chauvoei*, *C. perfringens*, and *C. tertium*) in *Clostridium sensu stricto*, 11 species (*Bacteroides acidifaciens*, *B. caecimuris*, *B. cellulosilyticus*, *B. faecichinchillae*, *B. finnegoldii*, *B. intestinalis*, *B. oleiciplenus*, *B. rodentium*, *B. stercoris*, *B. uniformis*, and *B. xylanisolvens*) in *Bacteroides*, two species (*Butyricimonas synergistica* and *B. virosa*) in *Butyricimonas*, and one species (*Odoribacter laneus*) in *Odoribacter* from the database. We detected limited reads of *C. chauvoei*, *C. tertium*, and *B. virosa* in our metagenomic analysis using the ENKI v3.2 database for microbial identification (TechnoSuruga Laboratory) [58,59].



Bacteroides are normally commensals in the gut microbiota, but these organisms can also cause infections associated with severe morbidity and mortality [60]. *C. chauvoei* and *B. virosa* are pathogenic bacteria [61, 62]. Consequently, we were interested in *C. tertium* as a safe butyrate producer. The whole genome sequence of *C. tertium* ATCC 14573 was recently published at <https://genomes.atcc.org>. The gene encoding phosphate acetyltransferase (*pta*; a gene involved in acetate production) starts from 337633 to 338541 in the ATCC 14573 genome. The *pta* nucleotide sequence was identified with the *ptb* gene from the NCBI reference sequence of *C. tertium* (NZ_OBJV01000001.1). The downstream genes of *pta* were two *buk* genes (*buk-buk*) in the ATCC 14573 strain. The *buk-buk* is also identified with *buk-buk* nucleotide sequence of NCBI reference sequence. Vital et al, discovered 225 bacteria with the potential to produce butyrate by screening 3184 sequenced bacterial genomes

and their individual genes. They identified butyrate producers, including *C. butyricum* 5521 and *C. butyricum* E4 str. BoNT E BL5262, with *buk* as the terminal gene [15]. To determine whether *C. tertium* is a butyrate producer or not, we verified the presence of *ptb-buk-buk* in *C. tertium* ATCC 14573 using PCR (Figure 3).

As shown in Fig. 3, we designed five primer pairs for the *ptb* and *buk* genes in *C. tertium* ATCC 14573 to confirm the existence of the *ptb-buk-buk* region in the genome. P1F/P1R, P2F/P2R, and P3F/P3R were used to amplify Amp1 (*ptb* partial sequence), Amp2 (upstream *buk* partial sequence), and Amp3 (downstream *buk* partial sequence) (Fig. 3 and Table 1). P4F/P4R was used to amplify Amp4, which spans upstream and downstream *buk*. P5F/P5R was used to amplify Amp5 and Amp5'. PCR using five primer sets (Table 1) identified all five cases (Fig. 4). We detected two bands in lane 6 using the P5F/P5R primers (Figure 4).

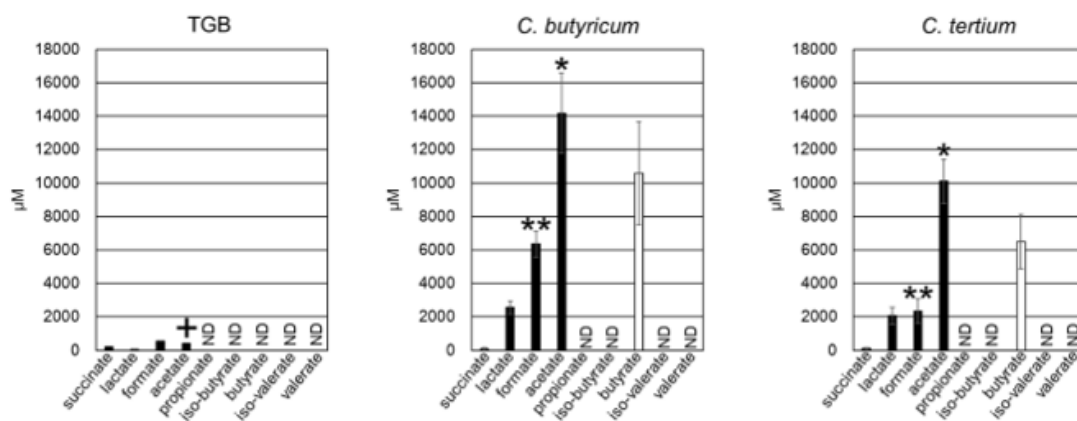


Note*: PCR products of ~250, ~800, ~400, and ~1500 bp were obtained from *C. tertium* gDNA. The molecular size markers consisted of a 50-bp ladder (Lane 1 and Lane 7). Lane 2 indicates Amp1 in Fig. 3 (*ptb*); Lane 3 (Amp2, upstream *buk*); Lane 4 (Amp3, downstream *buk*); Lane 5 (Amp4, *buk-buk*: 776 bp); Lane 6 (Amp5 and Amp5', *buk-buk*: 369 and 1461 bp). PAGE separated a thickened reference band containing two fragments. These are a doublet band at around 500 bp (shown by *).

Figure 4: Polyacrylamide gel electrophoresis (PAGE) of PCR fragments.

Next, we examined the organic acid production of *C. tertium* and *C. butyricum* (Fig. 5). Organic acids (succinate, lactate, formate, acetate, propionate, iso-butyrate, butyrate, iso-valerate, and valerate) in bacterial cultures were measured using high-performance liquid chromatography (HPLC). A low background level of production was observed in the medium control (TGB), and lactate,

formate, acetate, and butyrate were observed in *C. butyricum* and *C. tertium* (Fig. 5). Compared to *C. butyricum* ($n = 4$), formate and acetate from *C. tertium* were significantly different. The results for succinate ($P = 0.812$), lactate ($P = 0.161$), and butyrate ($P = 0.058$) were not significantly different (Figure 5).



Note*: *C. tertium* produces lactate, formate, acetate, and butyrate, as well as *C. butyricum*. TGB medium was also quantified as control. *C. butyricum* and *C. tertium* were cultured with TGB medium. “+” indicates that acetate was detected but that impurities were also present. The limit of detections (LODs) of succinate, lactate, formate, acetate, propionate, iso-butyrate, butyrate (n-butyrate), iso-valerate, and valerate (n-valerate) were 43.3 μM, 55.5 μM, 217.2 μM, 83.3 μM, 67.5 μM, 113.5 μM, 113.5 μM, 97.9 μM, and 97.9 μM, respectively. ND indicates no detection. The number of bacterial cells with a Petroff-Hauser counting chamber. The numbers were $6.9 \pm 2.6 \times 10^6$ of *C. butyricum* and $6.6 \pm 1.0 \times 10^6$ of *C. tertium*, respectively. Error bars indicate the SEM. *P < 0.05, **P < 0.0005.

Figure 5: The concentration of organic acids (μM) in the culture supernatant of bacteria.

Discussion

A phosphate acetyltransferase (pta) of three pta genes in sequenced *C. tertium* ATCC 14573 gDNA has at time of writing been corrected to a ptb gene at <https://genomes.atcc.org>. Each pta gene was located 337633~338541, 933320~934309, and 2359827~2358826 (compliment) in the ATCC 14573. BLASTN searches identified the pta 933320~934309 and 2359827~2358826 with pta. BLASTN searches identified the pta (337633~338541) with ptb. The pta genes 933320~934309 and 2359827~2358826 are equivalent to the pta genes located in 357490~358591 and 422872~423959 of the NCBI reference sequence of *C. tertium* (NZ_JAGGJY010000001.1), respectively. *C. tertium* is rare in that it possesses two buk genes in tandem in its genome (Fig. 3 and Fig. 4). We examined its butyrate production (Fig. 5). We investigated the consensus sequences of bacteria 31 genomes for putative butyrate producing genes, using <https://www.ncbi.nlm.nih.gov/genome/> (Table 2). We found that all bacterial species possess buk except *L. monocytogenes* (table 2) [15,63]. Although the buk gene is missing in the reference genome of *L. monocytogenes*, *L. monocytogenes* 10403S contains ptb and buk genes [64]. We confirmed that the pta-buk gene was present in the sequenced genome of *L. monocytogenes* ATCC 15313 at <https://genomes.atcc.org>. Table 2 shows 13 gene arrangements, with ptb-buk and pta, present on 11 reference genomes (Table 2; *Butyrivibrio crossotus*, *Butyrivibrio proteoclasticus*, *C. butyricum*, *C. perfringens*, *Clostridium tetani*, *Coprococcus comes*, *Coprococcus eutactus*, *Eubacterium ventriosum*, *Enterococcus faecalis*, *Shuttleworthia satellites* and *Subdoligranulum variabile*), representing a particularly frequent arrangement. Two bacterial reference genomes have dif-

ferent patterns, ptb-buk, buk, and pta (Table 2; *C. acetobutylicum* and *Clostridium saccharobutylicum*). *Clostridium beijerinckii* and *Natrananaerobius thermophilus* have ptb-buk, two buk genes, and pta (Table 2); *Caldanaerobacter subterraneus* and *Clostridium sporogenes* have buk-ptb-buk and pta (Table 2); *Acetoanaerobium sticklandii* has four buk genes in the genome (buk-ptb-buk and two buk genes) (Table 2); and *Thermoanaerobacter wiegelii* has three buk genes (buk-ptb-buk, buk and pta) (Table 2).

Butyrate has been found previously in *C. tertium*, but the amounts produced were not quantified. Gas-liquid chromatography showed that *C. tertium* produced acetate and butyrate; however, the authors did not show the production of acetate and butyrate [65]. *C. tertium* produced butyrate after cultivation in Wilkins-Chalgren anaerobe agar-based liquid medium [66]. Candelieri et al, reported that *C. tertium* produces butyrate from mucin during bacterial fermentation, however, while acetate, propionate, and iso-valerate were produced in abundance, butyrate was not [67]. We found *C. tertium* produces organic acids, including butyrate (Fig. 5). We detected 6.5 ± 1.6 mM butyrate in the culture supernatant of *C. tertium* (Fig. 5). It appears that *C. tertium* produces normal levels of butyrate. Butyrate concentrations are approximately 5 mM in the colonic lumen of mice and 10–70 mM in the colonic lumen of humans [68]. *C. butyricum* produces butyrate (1.95 ± 0.19 – 11.77 ± 0.37 mM) during in vitro fermentation [69]. In our study, *C. butyricum* produced 10.6 ± 3.1 mM of butyrate (Fig. 5). Butyrate production is downregulated by cysteine in *C. difficile* when added to peptone yeast extract cultures [70]. The authors showed that *C. difficile* produced 0.1–10.0 mM of butyrate with or without downregulation of cysteine. The butyrate levels were 10- to 100-fold lower in *C. diffi-*

cile than in *C. acetobutylicum* [70].

Exogenous acetate restores clostridial glucose metabolism. Li et al, clearly showed that *C. tyrobutyricum* growth decreased with the increasing exogenous butyrate [71]. Butyrate inhibited the cell growth due to severe inhibition of clostridial substrate (glucose) consumption [71]. While butyrate inhibited clostridial growth, acetate avoided glucose consumption by butyrate and resulted in alleviated growth inhibition [71]. Yu et al, summarized that Neutral Red (NR) and Benzyl Viologen (BV) as Exogenous Electron Mediators (EEMs) have been successfully employed to enhance the production of butyrate [72]. BV increased butyrate production of *C. tyrobutyricum* from glucose by ~50% while acetate production was completely inhibited [73]. Exogenous acetate and BV may stimulate butyrate production of *C. tertium* under anaerobic conditions in culture medium. While glucose concentration is thought to be low in the colon due to small intestinal absorption, the human colon still interacts with glucose, expressing glucose transporters like Glucose Transporter 2 (GLUT2), GLUT5 and Sodium-dependent Glucose Transporter 1 (SGLT1) [74]. Acetic acid bacteria (acetogenic bacteria) will support the alleviated growth inhibition of *C. tertium* similar to *C. tyrobutyricum* that were germinated from spore form. Like *C. butyricum* spores [75], *C. tertium* spores might resist the low pH and high oxidation-reduction potential of the stomach and small intestine. Thus, acetogenic bacteria should be anaerobic bacterium if *C. tertium* and acetogenic bacteria are to be co-administered. It was reported that *Acetobacterium woodii*, *Clostridium acetum*, *C. ljungdahlii*, *Sporomusa ovata*, *Sporomusa sphaeroides* and *Moorella thermoacetica* produce acetate [76,77].

These bacteria were cultured under anaerobic conditions [76,77]. Of the six acetogenic bacteria, *C. acetum*, *S. ovata*, *S. sphaeroides* and *M. thermoacetica* have been reported to form spores and the other two not to [78-83]. These four bacteria are classified as Biosafety Level 1 (BSL-1) organisms as well as *C. tertium*. Therefore, we expect that combined with *C. tertium*, they could be developed as probiotics. While *C. tertium* produced acetate (Fig. 5), if spores of *C. tertium* and these acetogenic bacteria are administered orally as probiotics in the future, they will be able to supply abundant acetate in the colon. Indeed, sufficient amounts of acetate from the four acetogenic bacteria would help bacterial growth and produce a therapeutically effective amount of butyrate from *C. tertium* in the colon. Recently, metagenomic analysis has provided a wealth of insight into the distribution of gut bacteria [84-86]. In this study, we focused on four bacteria (*Clostridium sensu stricto*, *Butyricimonas*, *Odoribacter*, and *Bacteroides*) through metagenomic analysis, but we were unable to plan to culture all of these candidate bacteria. Because the purpose was to use them as probiotics, we selected *C. tertium*, a bacterium of the genus *Clostridium sensu stricto* that can be handled at BSL-1, as our research subject. We hope that this study will contribute to the development of probiotics to address growing global health awareness and the need for alternatives to antibiotics [87-92].

Data Summary

The authors confirm all supporting data within the article or through supplementary data files. Sequence reads have been deposited in the NCBI Sequence Read Archive under BioProject accession PRJNA1198297. Individual sample SRA accession numbers are SRR31716405, SRR31716406, SRR31716407.

Repositories

The data analysed has been represented in the manuscript and raw data has been submitted to NCBI under the BioProject PRJNA1198297.

Author Statements

Author Contributions

Conceptualization: M.M., N.K., N.H., and H.I. Data curation: M.M. and N.K. Formal analysis: M.M., N.K., N.H., and H.I. Funding acquisition: M.M., N.K., H.I. Methodology: H.I. Visualization: N.H., H.I. M.M. wrote the original draft of the manuscript, with input from N.K. and H.I. All authors have reviewed and approved the final manuscript.

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Ethical Approval

Protocols for all animal experiments were approved by the ethics committee of Yamamuragakuen school corporation and Yamamurakokusai high school, permit number YKA013, and were based on the guidelines for proper conduct of animal experiments by the Science Council of Japan (SCJ) at <https://www.scj.go.jp/en/animal/>.

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Conflicts of Interest

The authors declare that there are no conflicts of interest.

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