# Development of a novel feeding method for Japanese black calves with thermophile probiotics at postweaning

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# Development of a novel feeding method for Japanese black calves with thermophile probiotics at postweaning.

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Running Head: Feeding calves with thermophiles

**Abstract**

**Aims:** Probiotic effects of compost containing thermophiles on productivity have been reported in domestic animals, although not cattle. We evaluated the effects of administering *Caldibacillus hisashii*, a thermophile contained in compost, on growth, blood components, faecal organic acid concentrations and microbiota population in Japanese black calves.

**Methods and results:** Calves were administered *C. hisashii* from 3 to 5 mo of age. Administering *C. hisashii* decreased feed intake without affecting body weight, indicating that feed efficiency is improved by administration. Administering *C. hisashii* decreased plasma insulin concentration without affecting glucose and nonesterified fatty acid concentrations. Chao1 was decreased by exposure at 5 mo of age. Similarly, weighted- and unweighted UniFrac distances were affected by treatment at 5 mo of age. Faecal abundance of the phylum Bacteroidetes tended to be increased by exposure. Faecal propionic acid concentration was correlated positively with faecal abundance of phylum Bacteroidetes but negatively with that of Firmicutes. Interestingly, the population of the genus *Methanobrevibacter*, representing the majority of methanogens, was lowered by exposure and was negatively correlated with faecal propionic acid concentration.

**Conclusion:** Administration of *C. hisashii* has the potential to improve growth performance of Japanese black calves and to contribute to reducing environmental load, which may be associated with altered endocrine kinetics and gut microbial populations.

**Significance and impacts of the study:** This study revealed that isolated thermophiles included in compost may exert probiotic effects on calves.

**Key words:** Agriculture; Bacillus; Metabolism; Probiotics; Diversity

## Introduction

The mammalian gastrointestinal (GI) microbial community, which consists of 100 trillion ($10^{14}$) microorganisms (Ley et al. 2006), has important roles in energy efficiency in the host, including energy intake, transport, conversion, and storage (Angelakis et al. 2013). Microbial fermentation of carbohydrates in the hindgut is responsible for 5 to 10% of total-tract carbohydrate digestion in cattle (Gressley et al. 2011). In addition to luminal nutrient metabolism, the enterobacterial community has been shown to be related to glucose metabolism via alterations in insulin resistance (Gomes et al. 2018). Insulin exerts anabolic actions in muscle and fat tissues; thus, alteration of its circulating concentration or sensitivity may be associated with the growth of calves (Schäff et al. 2016). Furthermore, it has been recently reported that the synthesis of insulin-like growth factor 1 (IGF-1) is modulated by intestinal short-chain fatty acids (SCFAs) produced by the gut microbiota to influence growth (Yan and Charles 2018). Therefore, ensuring the function of the hindgut, as well as that of the rumen, is of importance to ruminants such as cattle.

In domestic calves, natural weaning occurs 7 to 14 months (mo) after birth, with great

individual variation (Reinhardt 1981). Conversely, in conventional feeding regimens, weaning is usually abrupt and earlier than the natural process (Enríquez et al. 2011). Early weaning promotes rumen development through an increase in solid feed intake (Khan et al. 2007). However, an increase in concentrate feed intake was not enough to compensate for the decreased milk intake, resulting in lower energy intake (Bittar et al. 2020). In addition, it has been reported that solid feed intake is decreased immediately after weaning due to a lack of adaptation of the GI tract to solid feeds (Fukumori et al. 2012), likely leading to suboptimal growth performance of dairy and beef calves. Thus, a novel feeding regimen is necessary to counterbalance the impaired growth performance of calves postweaning.

To solve the above problem, we focused on probiotics. The term probiotics was defined as "live microorganisms, which when administered in adequate amounts, confer a health benefit on the host" by the Food and Agriculture Organization of the United Nations and the WHO (FAO/WHO, 2001) and adopted by the International Scientific Association for Probiotics and Prebiotics (Hill et al., 2014). There is increasing evidence of the beneficial effects of probiotic administration on the health and growth of calves via optimization of the enteric microbiota (Uyeno et al. 2015). Administration of probiotics was reported to increase body weight (Al-Saiady et al., 2010) and improve feed efficiency (Timmerman et al., 2005) in the previous studies using calves. Based on these findings, a feeding strategy using probiotics is likely effective in improving growth

performance through modification of the intestinal environment.

Recently, the oral administration of a compost containing thermophiles has been reported to improve fecundity and quality through modification of the intestinal bacterial population in flatfish, chickens, and pigs (Tanaka et al. 2010; Miyamoto et al. 2012; Satoh et al. 2012; Miyamoto et al. 2013b; Ito et al. 2016; Tanaka et al. 2016). In mice, administration of an isolated strain related to *Bacillus thermoamylovorans* increased body weight compared to the administration of potable water, which was associated with better feed efficiency (Miyamoto et al. 2013a). These findings suggest that administration of thermophile-containing products to calves beneficially affects growth performance via alterations in the gut microbiota population. Miyamoto et al. isolated a probiotic candidate, *Caldibacillus hisashii* (BP-863, international deposited No., which was registered by the National Institute of Technology and Evolution) (Miyamoto et al. 2013a; Nishida et al. 2015), which was shown to be closely related to *Bacillus thermoamylovorans* in 2013 (Miyamoto et al. 2013a), and which was registered as *Bacillus hisashii*, a new strain of N-11$^T$ (=NRBC10226$^T$ and LMG28201$^T$), in 2015 (Nishida et al. 2015) and thereafter was recategorized as a bacterium belonging to *Caldibacillus*, a new genus (Gupta et al. 2020). Here, the aim of our study was to assess host-microbiota interactions in animals after exposure to *C. hisashii*. The relationship between their growth and the faecal microbiota after application of *C. hisashii* was analysed in calves. Our present study revealed that administering isolated

thermophiles may exert probiotic effects on the performance of beef calves via alterations in the gut microbial population. Furthermore, the faecal abundance of the genus *Methanobrevibacter*, representing the majority of methanogens, was reduced by administration. Therefore, this study is the first to report the beneficial impact of probiotics on the reduction in environmental loading as well as on the productivity of animals.

## Materials and Methods

### *Animals and diet*

This study was conducted at a Kuju agricultural research centre that raises Japanese black cattle in Oita Prefecture, Japan. The procedures used in the present study were performed according to ARRIVE guidelines and the Guidelines for Animal Experiments by the Faculty of Agriculture at Kyushu University and with the approval of the Kyushu University Laboratory Animal Care and Use Committee (Approval number: A29-085-0). Measuring BW and feed intake and collecting blood and faecal samples were routine procedures in the daily management of cattle on the farm. A total of eight Japanese black female calves weaned at 3 mo of age were randomly assigned to two treatment groups (CON group: n = 4, initial BW = 114.6 ± 3.5 kg; BP group: n = 4, initial BW = 99.0 ± 10.1 kg). Calves in the CON group were fed concentrate feed (crude protein, 18%; crude fat, 1%; total digestible nutrients, 70%) and hay (ad libitum) throughout the experimental period.

*C. hisashii* (BP-863) was cultivated as previously described (Miyamoto et al., 2013a; Nishida et al., 2015). In this experiment, the strain stocked with glycerol was cultivated at 50 ℃ for 48 hr, centrifuged twice, and then the precipitate was incubated at 75℃ for 30 min (Sermas Co., Ltd., Japan). The cultivated strain was adjusted to be $10^5$ spores per 5g dried potato starch, a material of feed (Keiyo Gas Energy Solution Co., Ltd., Japan). Calves in the BP group were fed the same diets as those used for the CON group, but the adjusted dried potato starch containing *C. hisashii* was supplemented in the concentrate feed at 5 g ($10^5$ spore of *C. hisashii* per 5g) per kg of concentrate feed for 2 mo at 3 to 5 mo of age, delivering $10^2$ spore of *C. hisashii* per d.

The ingredients of concentrate feed are shown in Table S1. In both groups, concentrate feed was offered at an amount required to achieve 1 kg of daily gain (DG) according to the Japanese Feeding Standard for Beef Cattle (Japan Agriculture and Food Research Organization 2000). Feed intake was recorded daily, and body weight was measured at 3 (initiation of this study), 4, 5 and 6 mo of age.f

***Sample collection***

Schedule for feeding and sample collection is shown in Figure S1. Blood was collected from the jugular vein using heparinized tubes (Venoject II VP-H100K with heparin sodium; Terumo Corp., Tokyo, Japan) before the morning feeding at 5 and 6 mo of age. Blood samples were

centrifuged at 2330 × g and 4 °C for 20 min, and the plasma was stored at -80 °C until analysis. Faecal samples were collected at 3, 4, 5, and 6 mo of age before the morning feeding and stored at -20 °C until analysis.

***Analyses of plasma metabolites and hormones***

Plasma samples were analysed for plasma glucose, nonesterified fatty acid (NEFA), insulin, and IGF-1 concentrations. Plasma hormone concentrations were measured according to the time-resolved fluoroimmunoassay technique, which has been previously described (Sugino et al. 2004). The plasma concentration of insulin was measured using a solid-phase competition immunoassay with europium-labelled bovine insulin and polystyrene microtiter strips coated with anti-guinea pig γ-globulin (Inabu et al. 2018). The plasma IGF-1 concentration was analysed using a solid-phase competition immunoassay as previously reported (Phomvisith et al. 2017). The plasma glucose concentration was measured by the glucose oxidase enzymatic method (glucose B‐test; Wako Pure Chemical, Osaka, Japan). The plasma NEFA concentration was measured by the acyl‐CoA synthetase‐acyl‐CoA oxidase enzymatic method (FFAC; Wako Pure Chemical).

***Analysis of the faecal bacterial population***

DNA extraction of faecal samples was performed using a QIAGEN QIAamp PowerFecal

DNA Kit according to the manufacturer's protocol. DNA concentrations were evaluated using a Quant-iTTM PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific). The V4 region of the bacterial 16S rRNA gene (515F-806R) was sequenced according to previous work (Jinnohara et al. 2017). Approximately 290-bp amplified fragments were sequenced on an Illumina MiSeq according to the manufacturer's instructions. We obtained 250-bp paired-end reads. 16S rRNA reads were analysed using QIIME (version 1.6); fasta, quality files and a mapping file indicating the barcode sequence corresponding to each sample were used as input, and 10,000 filter-passed reads were obtained from each faecal sample. Faecal samples were subjected to sequencing analysis. The filtered sequences were clustered into operational taxonomic units (OTUs) defined by 97% similarity. The OTUs were assigned a taxonomy by comparison to the Greengenes database using the RDP classifier (Wang et al. 2007; McDonald et al. 2012). Indices for α-diversity, namely, community richness (Chao1) and diversity (Shannon and Simpson), were calculated (Chao 1984; Magurran 2004), and indices for β-diversity were also estimated using UniFrac analysis with weighted and unweighted PCoA (Lozupone and Knight 2005; Kim et al. 2013). All 16S rRNA gene datasets were deposited in the GenBank Sequencing Read Archive database (accession number: DRA010943).

***Analysis of the faecal organic acid concentration***

The sample faeces were diluted with distilled water 10 times and shaken at 1500 rpm for 10 min at 4 °C (MSC-100; Allsheng). Thereafter, the suspension was centrifuged at 9057 x g for 15 min at room temperature (MX-300; Tomy), and all of the supernatant was filtered through a 0.45-μm filter (Millex-HA Filter Unit SLHA025NB; Merck) to obtain nonresidue supernatant that was ready to be applied for high-performance liquid chromatography (HPLC). Organic acids (lactic acid, acetic acid, propionic acid, butyric acid, valerianic acid, isovalerianic acid and phosphoric acid) in the faecal supernatant were determined using a specific HPLC system (Organic Acid Analyser; Shimadzu, Kyoto, Japan) equipped with an ion exclusion column (Shim-pack SCR-102H; Shimadzu) at 40 °C and with an electric conductivity detector (CDD-10AVP; Shimadzu). Mobile phases A (5 mM p-toluenesulfonic acid) and B (20 mM bis(2-hydroxyethyl)imino tris(hydroxymethyl)methane, 5 mM p-toluenesulfonic acid, 0.2mM EDTA-4H) were used, each at a flow rate of 0.8 mL $min^{-1}$ (Tashiro et al. 2013; Tashiro et al. 2016) according to the manufacturer's instructions.

***Statistical analysis***

Data for concentrate feed and hay intake, body weight (BW), DG, plasma concentrations of metabolites and hormones, abundance of the phyla Firmicutes and Bacteroidetes, and faecal organic acid concentrations were analysed using the fit model procedure of JMP® 14 (SAS

Institute Inc., Cary, NC) according to the following model:

$Y_{ijk} = \mu + Trt_i + Time_j + Calf_k + Trt \times Time_{ij} + e_{ijk}$,

where $Y_{ijk}$ is the dependent variable, μ is the overall mean, $Trt_i$ is the fixed effect of treatment, $Time_j$ is the fixed effect of age used as a repeated measure, $Trt \times Time_{ij}$ is the fixed effect of treatment by age interaction, $Calf_k$ is the random effect of the calf, and $e_{ijk}$ is the error term. A simple main effect test was performed to detect the differences between treatment groups at the same time point when Trt × Time $P \leq 0.05$.

Comparison of the abundance of faecal genera between treatment groups was performed by paired t-test using R software (version 3.6.2). Correlations of faecal organic acid concentrations and phylum abundance of Firmicutes and Bacteroidetes in faeces were analysed by Pearson's correlation method of JMP® 14. Spearman's correlation between the faecal abundance of genera and organic acid concentrations was analysed by the construction of a correlation heatmap using R software (version 3.6.2). Data for the genus composition of faecal bacteria were transformed into Z-scores and clustered according to their Euclidean distance.

Significance was declared when $P < 0.05$, and tendencies were declared at $0.05 \leq P < 0.10$.

## Results

### *Growth performance*

Growth performance data are shown in Figure 1. Hay (Figure 1A), concentrate feed (Figure 1B), and total feed intake (hay intake + concentrate feed intake; Figure 1C) increased through 3–6 mo of age regardless of dietary treatments (Time $P < 0.01$). No treatment effect was observed for concentrate feed intake, whereas hay intake was lower (Trt $P = 0.02$) for the BP group (2116 ± 102 g $d^{-1}$) than for the CON group (2564 ± 102 g $d^{-1}$). This resulted in lower (Trt $P = 0.02$) total feed intake for BP (5554.8 ± 166 g $d^{-1}$) compared to CON (6158 ± 166 g $d^{-1}$). In both treatment groups, BW and DG increased throughout the experimental period (Time $P < 0.05$; Figure 1D and 1E, respectively). Despite the lower feed intake for BP, BW and DG did not differ between treatment groups. The BW gain (g)/feed intake (g) ratios through 3 to 5 mo of age (22.7 ± 1.5 and 17.2 ± 1.5 for the BP group and CON group, respectively; P = 0.04) and 3 to 6 mo of age (21.1 ± 1.1 and 16.7 ± 1.1 for the BP group and CON group, respectively; P = 0.03) were significantly greater for calves administered *C. hisashii* (Figure S2).

***Plasma metabolites and hormone concentrations***

Plasma metabolite and hormone concentrations at 5 and 6 mo of age are shown in Figure 2. Time effect and treatment by time interaction were not observed for any metabolite and hormone concentrations. Treatment effects were not observed for plasma glucose, NEFA or IGF-1 concentrations. Conversely, the plasma insulin concentration was lower (Trt $P = 0.01$; Figure

2C) for BP (0.51 ± 0.08 mg $dL^{-1}$) than for CON (0.91 ± 0.08 mg $dL^{-1}$).

***Faecal organic acid concentrations***

Data for faecal organic acid concentrations are shown in Figure 3. There was no difference in organic acid concentrations between treatment groups. The propionic acid concentration decreased with age regardless of treatment (time $P = 0.01$). The faecal lactic, butyric, valerianic, and isovalerianic acid concentrations were lower than the least detectable level (hence, data not shown).

***Microbial populations in the faeces***

Data for α- and β-diversity indices are shown in Figure 4. A time effect was not observed for α-diversity indices. Although the observed OTUs and Shannon and Simpson indices were not affected by treatment, Chao1 was lower for the BP group (1182.0 ± 72.1) than for the CON group (1481.4 ± 72.1) at 5 mo of age (Trt × time $P = 0.04$; Figure 4B). Weighted UniFrac distances (Figure 4E) and unweighted UniFrac distances (Figure 4F) were affected by treatment at 5 mo of age ($R^2 = 0.43$, $P = 0.03$ and $R^2 = 0.20$, $P = 0.05$, respectively; Figure 4G). These data show that the bacterial population was altered by treatment at 5 mo of age.

We analysed the faecal abundance of phyla to evaluate the treatment effect on the faecal

microbial population in detail (Figure 5). In both treatment groups, the phyla Firmicutes and Bacteroidetes were the predominant bacteria (Figure 5A). In the CON group, the abundance of the phylum Firmicutes increased from 45 to 58% at 3 to 5 mo of age. However, an increase in the abundance of the phylum Firmicutes was not observed at 5 mo of age in the BP group. The proportions of the phyla Firmicutes and Bacteroidetes in faeces are shown in Figure 5B and 5C, respectively. The abundance of the phylum Firmicutes tended to be lower (Trt $P$ = 0.06) for the BP group (46.7 ± 2.0%) than for the CON group (52.2 ± 2.0%) throughout the experimental period. Conversely, the abundance of the phylum Bacteroidetes tended to be higher (Trt $P$ = 0.08) for the BP group (46.0 ± 2.0%) than for the CON group (41.0 ± 2.0%). Consequently, the Firmicutes (%)/Bacteroidetes (%) ratio (F/B) was significantly lower (Trt $P$ = 0.03; Figure 5D) in the BP group (1.05 ± 0.11) than in the CON group (1.40 ± 0.11).

***Correlation analysis of the faecal bacteria and SCFA***

The correlation between the abundance of the phyla Firmicutes and Bacteroidetes vs. faecal organic acid concentrations throughout the experimental period is shown in Figure 6. The faecal acetic acid concentration tended to be negatively correlated with the Firmicutes content (r = -0.30, $P$ = 0.09). The faecal propionic acid concentration was negatively correlated with the abundance of Firmicutes (r = -0.59, $P$ < 0.01) but was positively correlated with that of Bacteroidetes (r = 0.55,

$P < 0.01$).

To analyse the above relationship in more detail, correlations between genera and faecal organic acid concentrations were determined, as shown in the heatmap (Figure 7). Genera were roughly divided into two clusters: Clusters 1 and 2. Cluster 1 was primarily composed of the phylum Firmicutes, whereas Cluster 2 contained more of the phylum Bacteroidetes. Consistent with Figure 6, the propionic acid concentration was negatively correlated with many genera of Cluster 1 and positively correlated with those of Cluster 2, and a similar tendency was observed for acetic acid concentration. In particular, within the phylum Firmicutes in Cluster 1, the genera *Unc._Eryspipelotrichaceae*, *Unc._Lachnospiraceae*, *Streptococcus*, *Butyrivibrio*, *Ruminococcus*, *Butyriciococcus*, *Clostridiaceae_Clostridium*, *Roseburia*, and *Lachnobacterium* were strongly correlated with propionic acid concentration. In addition, the genera *Anaeroplasma* and *Unc._Anaeroplasmataceae*, belonging to the phylum Tenericutes, and the genus *Methanobrevibacter,* belonging to the phylum Euryarchaeota, were negatively correlated with propionic acid. Among Bacteroidetes in Cluster 2, propionic acid concentration was positively correlated with the genera *Odouribacter*, *Parabacteroides*, *Bacteroides*, *Sharpea*, and *Paludibacter*.

Principal component analysis (PCoA) of genera and the faecal concentrations of acetic and propionic acids are shown in Figure 8A, which indicates that the CON group has high abundances

of the phyla Firmicutes and Euryarchaeota, whereas the BP group has a high abundance of the phylum Bacteroidetes and high faecal acetic and propionic acid concentrations at 5 mo of age. These results show that treatment caused alteration in the bacterial population and its relationship to SCFA production at 5 mo of age. Figure 8B shows a heatmap of quantified genera with $P < 0.10$ between CON and BP. Within the phylum Bacteroidetes, the abundance of the genera *Prevotella* and *Prevotella*-allied bacteria tended to be higher for BP than CON. Conversely, the abundances of the genera *Paraprevotella* and *5-7N15,* belonging to phylum Bacteroidetes, were lower in BP than in CON. The genus *Methanobrevibacter* was significantly lower in the BP group than in the CON group ($P < 0.05$). Within the phylum Firmicutes, the abundances of the genera Unc._*Erysipelotrichaceae*, *Coprobacillus*, and Unc._*Ruminococcaceae* were lower ($P < 0.05$) in the BP group than in the CON group. Among the phyla Proteobacteria and Tenericutes, the abundances of Unc._*Acetobacteraceae* and Unc._*Anaeroplasmataceae* were lower ($P < 0.05$) in the BP group than in the CON group.

## Discussion

The current study reported for the first time that a novel feeding method for Japanese black calves using an isolated thermophile, *C. hisashii,* decreased feed intake without affecting growth performance immediately after weaning as shown in Figure 1, indicating that feed efficiency was

likely improved by *C. hisashii* administration. In support of this hypothesis, the BW gain (g)/feed intake (g) ratios through 3 to 5 mo of age and 3 to 6 mo of age were greater for calves administered *C. hisashii* (Figure S2). Our results did not contradict previous findings: feed efficiency was improved in germ-free mice offered isolated thermophilic bacteria (N-11 strain = *C. hisashii*) compared to those offered potable water as a control (Miyamoto et al. 2013a). The difference in energy content between treatments was negligible because the amount of potato starch supplemented was lower than 0.5% of the total amount of diet per day in the current study. Thus, administration of *C. hisashii* may alter entero-nutrient digestion and absorption via modification of the entero-bacterial population, which is likely associated with a possible improvement of feed efficiency. The reason for the decreased feed intake by *C. hisashii* is unclear. However, it is possible that the energy requirements and appetite of calves could be satisfied with a lower nutrient intake if the feed efficiency was improved.

The relative abundance of Firmicutes tended to be decreased and that of Bacteroidetes tended to be increased by administering *C. hisashii*, resulting in a significantly lower F/B in calves administered *C. hisashii* (Figure 5). In numerous studies in humans, it has been reported that F/B was increased in obese people compared to lean people (Barlow et al. 2015; Mathur and Barlow 2015). Similar to the present results, it was previously reported that administering *C. hisashii* reduced the accumulation of visceral fat in mice (Miyamoto et al. 2018). This phenomenon may

be explained by the fact that the phylum Firmicutes is more effective as an energy source than Bacteroidetes, resulting in the more efficient absorption of calories and increasing the subsequent weight gain (Turnbaugh et al. 2006; Krajmalnik-Brown et al. 2012). This is inconsistent with our present findings because feed efficiency might be higher despite the lower abundance of Firmicutes in the calves offered *C. hisashii*. However, it has been shown that an increase in the relative abundance of Bacteroidetes and an enrichment of genes linked to carbohydrate metabolism were found in the microbiomes of lean people compared to those in obese people (Turnbaugh et al. 2009). Similarly, it was reported that Bacteroidetes showed a high genomic content of carbohydrate hydrolytic enzymes (Bertucci et al. 2019). These findings indicate that an increase in the phylum Bacteroidetes is associated with the improvement of carbohydrate digestion in the intestine. Although the precise mechanism is still unknown, it is possible that an altered F/B ratio is associated with improved feed efficiency via altered carbohydrate digestion because the primary nutrients ingested by cattle are carbohydrates, such as starch and cellulose. As mentioned previously, organic acids, including SCFAs, are primary end-products of intestinal microbiota and are subsequently utilized by the host as substrates for metabolic energy production (Martin-Gallausiaux 2021). Therefore, SCFAs produced by lower GI tract fermentation contribute to the overall quality and quantity of energy used by animals, thus improving feed efficiency. Although the enterobacterial population was affected by treatment, as indicated by

altered α- and β-diversity indices (Figure 4) and the abundance of phyla and genera, there was no difference in faecal organic acid concentrations between treatment groups as shown in Figure 3. However, it is noteworthy that the faecal propionic acid concentration was positively correlated with the abundance of the phylum Bacteroidetes and negatively correlated with that of the phylum Firmicutes (Figure 6). This finding may support the above conjecture regarding the relationship between the F/B ratio and carbohydrate digestion because propionic acid is primarily produced by carbohydrate fermentation in both the human gut (Hosseini et al. 2011) and cow rumen (Khan et al. 2008). It has been well established that luminal SCFAs are rapidly absorbed from the intestinal wall (Engelhardt 1995). Thus, we cannot exclude the possibility that administration of *C. hisashii* affects intestinal organic acid production via alteration of the gut bacterial population in our study. Within the phylum Bacteroidetes, the abundance of the genera *Prevotella* and *Prevotella*-related bacteria tended to be increased at 5 mo of age by treatment (Figure 8B). It has been shown that some *Prevotella* spp. in the rumen possess pathways for propionate production (Mitsumori et al. 2012). Although we did not measure the rumen microbiota population, a previous study reported that a number of bacterial genera were strongly associated with either rumen or faecal samples (Holman and Gzyl 2019). Therefore, it is possible that administration of *C. hisashii* promotes gut propionic acid production by increasing the *Prevotella* spp. population.

Nutrient intake might be reduced in calves offered *C. hisashii,* as feed intake was decreased by

treatment, whereas plasma concentrations of glucose and NEFA were not affected by treatment (Figure 2A,B). As described above, modification of the enterobacterial population caused by *C. hisashii* administration may be associated with altered entero-nutrient digestion or absorption. Thus, we suggest that improvements in nutrient digestion and uptake compensate for the decreased feed intake, resulting in no difference in circulating metabolite concentrations. Similar to blood metabolites, the plasma IGF-1 concentration was not affected by *C. hisashii* exposure (Figure 2D). The circulating IGF-1 concentration is associated with nutrient status. For example, the circulating IGF-1 concentration is low in the negative energy balance in cattle (Zulu et al. 2002). In the present study, the glucose and NEFA concentrations did not differ among treatment groups, suggesting that nutrient status was not dramatically affected by treatment and resulted in no difference in IGF-1 concentration. Although glucose and NEFA concentrations did not differ, the plasma insulin concentration was decreased by *C. hisashii* exposure (Figure 2C) despite the glucose-lowering action of insulin (Inabu et al. 2018). This is possibly explained by the improved insulin sensitivity in calves administered *C. hisashii*. It has been established that alterations in the gut microbiota are associated with obesity and insulin resistance (Vrieze et al. 2012). A previous study reported that alteration of the abundances of both Firmicutes and Bacteroidetes affected insulin resistance in mice (Hwang et al. 2015). In addition, it has been shown that decreased abundance of the phylum Firmicutes due to the oral administration of antibiotics was associated

with improved insulin sensitivity in humans (Vrieze et al. 2014). Therefore, no differences in plasma glucose and NEFA irrespective of altered insulin concentration may be attributed to improved insulin sensitivity by the administration of *C. hisashii*, which was presumably caused by reduced abundance of the phylum Firmicutes. Insulin enhances tissue growth via the stimulation of anabolic processes in muscle and fat tissue in calves (Schäff et al. 2016); thus, increased circulating concentrations or improved sensitivity to insulin may promote the growth performance of animals. Therefore, it is possible that improved insulin sensitivity in calves administered *C. hisashii* partly contributes to compensating for decreased feed intake in the current study.

Interestingly, the abundance of the genus *Methanobrevibacter*, a methanogenic archaea, was decreased by *C. hisashii* administration at 5 mo of age (Figure 8B) and increased after the end of treatment (at 6 mo of age) (data not shown). In addition, *Methanobrevibacter* was negatively correlated with faecal propionic acid concentration (Figure 7). *Methanobrevibacter* is a major methanogenic bacterium accounting for nearly 2/3 of rumen archaea (Janssen and Kirs 2008). Furthermore, the genus *Methanobrevibacter* was found in nearly every rumen, rumen epithelial and faecal sample (Holman and Gzyl 2019). It has been reported that inhibition of methanogenesis in the rumen results in increased ruminal propionic acid production via an increase in the population of hydrogen-consuming *Prevotella* spp. (Mitsumori et al. 2012). As described above, the abundance of the genus *Prevotella* was increased by treatment, consistent

with previous findings. Furthermore, studies in animal models have suggested a potential role for *Methanobrevibacter smithii* in the development of obesity. In humans, it was found that increased methane concentration in a breath test was associated with a greater BMI, suggesting that methanogens may be one of the factors contributing to levels of obesity (Basseri et al. 2012). In addition, introduction of both a *Bacteroides* species (*Bacteroides thetaiotaomicron*) and *M. smithii* to germ-free mice increased body weights compared with *B. thetaiotaomicron* alone. As described above, calves administered *C. hisashii* exhibited a characteristic that was observed in lean people (Barlow et al. 2015; Mathur and Barlow 2015). Thus, it is possible that decreased abundance of the genus *Methanobrevibacter* is associated with a decreased F/B ratio upon *C. hisahsii* exposure. Enteric methane emissions are a problem because of energy loss and adverse effects on animal productivity, as well as environmental issues (Ohene-Adjei et al. 2007; Subepang et al. 2019). Methane emissions from beef and dairy cows cause a loss of enteric methane energy, accounting for 2% to 12% of gross energy intake (Gerber et al. 2013). Therefore, methane energy loss reduces the efficiency of feed energy utilization and beef cattle productivity (Chaokaur et al. 2015). Although the amount of methane produced was not measured in the current study, it is possible that the reduced abundance of *Methanobrevibacter* is associated with a possible decrease in energy loss, resulting in no differences in BW and DG despite the lower feed intake in calves offered *C. hisashii*.

In the present study, we show for the first time that a novel feeding method with *C. hisashii* alters the faecal organic acid concentrations, faecal bacterial community and physiological kinetics, which is likely associated with the possible improvement of feed efficiency. Interestingly, we found that administration of *C. hisashii* affected the *Methanobrevibacter* population in faeces, which likely contributed to not only the prevention of global warming but also the possible improvement of feed efficiency via the reduction of energy loss. Overall, our study shows that *C. hisashii* is an effective probiotic to improve the growth of Japanese black calves, which has significant implications from the viewpoint of the Sustainable Development Goals and warrants further study.

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**Author Contribution Statement:** Conceived and designed the experiments: M.H. and T.H. Experimental work, data analysis, data interpretation, and manuscript preparation: I.Y., T.A., M.H., and T.H. Assisted with experimental work: T.Y. F.R., S.Y., and E.T. NGS analysis: T.A., M.M., and K.T. HPLC analysis: T.N. Contributed to providing reagents/materials/analysis tools and revising

manuscript: O.T. and U.M. Revised the manuscript: K.H. and O.H.

**Data Availability Statement:** The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

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## FIGURE LEGENDS

Figure 1. Intake of hay (A), concentrate feed (B) and total feed (hay + concentrate feed) (C), body weight (BW) (D), and daily BW gain (DG) (E) in calves fed concentrate feed containing *C. hisashii* (BP-863) at 0 (CON; ○) or 5 g $kg^{-1}$ (BP; ●). mo = months.

Figure 2. Plasma glucose (A), non‐esterified fatty acid (NEFA) (B), insulin (C), and insulin-like growth factor 1 (IGF-1) (D) concentrations in calves fed concentrate feed containing *C. hisashii* (BP-863) at 0 (CON; ○) or 5 g $kg^{-1}$ (BP; ●). mo = months.

Figure 3. Faecal acetic acid (A), propionic acid (B) and phosphoric acid concentrations (C) in calves fed concentrate feed containing *C. hisashii* (BP-863) at 0 (CON; ○) or 5 g $kg^{-1}$ (BP; ●). mo = months.

Figure 4. α-Diversity (A-D) and β-diversity (E-G) indices for calves administered concentrate feed containing *C. hisashii* (BP-863) at 0 (CON; □) or 5 g $kg^{-1}$ (BP; ■). mo = months. *$P < 0.05$ between treatments within a time point.

Figure 5. Phylum abundance of gut microbiota (A), abundances of the phyla Firmicutes (B) and Bacteroidetes (C), and the Firmicutes/Bacteroidetes ratio (F/B) (D) in calves fed concentrate feed containing *C. hisashii* (BP-863) at 0 (CON; □) or 5 g $kg^{-1}$ (BP; ■). mo = months.

Figure 6. Correlation coefficients between faecal organic acid concentrations and abundances of the phyla Firmicutes and Bacteroidetes (n = 8).

Figure 7. Heatmap of Spearman's correlations between faecal organic acid concentrations and the genus abundance of the gut microbiota (n = 8). The suggested annotations for some families or genera are enclosed in square brackets.

Figure 8. Principal component analysis of genera and faecal organic acid (A) and heatmap of the quantified abundance of genera in calves fed concentrate feed containing *C. hisashii* (BP-863) at 0 (CON) or 5 g $kg^{-1}$ (BP) at 5 mo of age (B). Genera shown in the heatmap are $P < 0.10$ between CON and BP. The suggested annotations for some families or genera are enclosed in square brackets. mo = months. *$P < 0.05$ between CON and BP.

**SUPPORTING INFORMATION**

Figure S1. Schedule for feeding and sample collection in the CON and BP groups. Calves fed concentrate feed containing *C. hisashii* (BP-863) at 0 (CON) or 5 g $kg^{-1}$ (BP). Hay was fed ad libitum. Concentrate feed was offered at an amount required to achieve 1 kg of daily body weight gain according to the Japanese Feeding Standard for Beef Cattle. Faecal samples were collected monthly from 3 to 6 mo of age. Blood samples were collected at 5 and 6 mo of age.

Figure S2. Body weight gain (g)/feed intake (g) through 3 to 5 mo of age (A) and 3 to 6 mo of age (B) in calves fed concentrate feed containing *C. hisashii* (BP-863) at 0 (CON; □) or 5 g $kg^{-1}$ (BP; ■). *$P < 0.05$ between CON vs. BP.

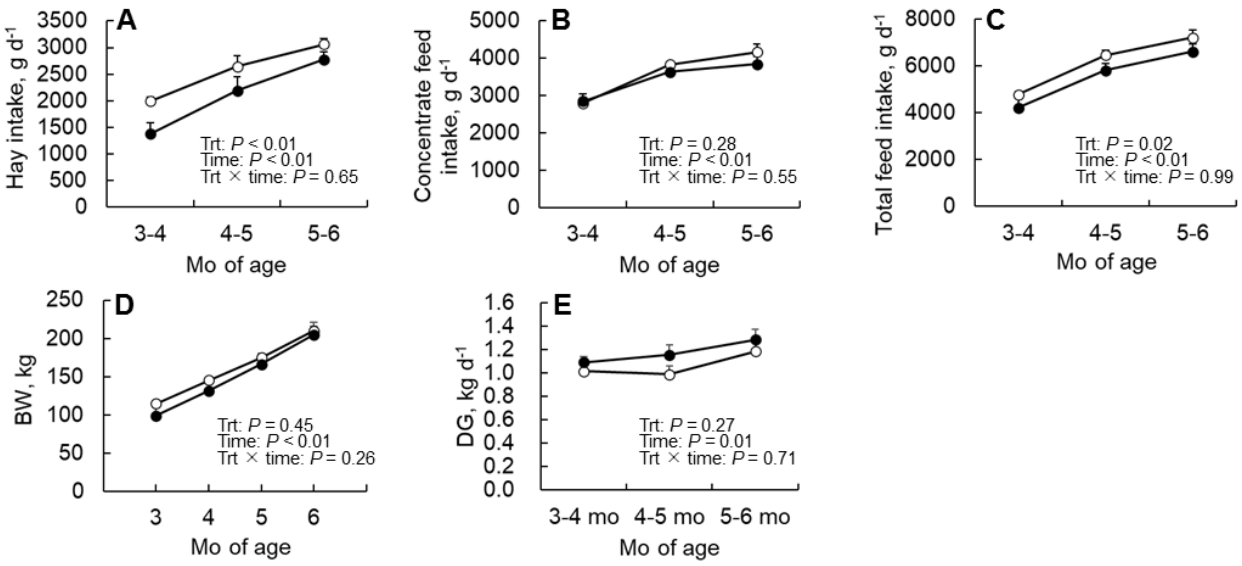

A
Hay intake, g d-1
3500
3000
2500
2000
1500
1000
500
0
Trt: P < 0.01
Time: P < 0.01
Trt × time: P = 0.65
3-4
4-5
5-6
Mo of age
B
Concentrate feed intake, g d-1
5000
4000
3000
2000
1000
0
Trt: P = 0.28
Time: P < 0.01
Trt × time: P = 0.55
3-4
4-5
5-6
Mo of age
C
Total feed intake, g d-1
8000
6000
4000
2000
0
Trt: P = 0.02
Time: P < 0.01
Trt × time: P = 0.99
3-4
4-5
5-6
Mo of age
D
BW, kg
250
200
150
100
50
0
Trt: P = 0.45
Time: P < 0.01
Trt × time: P = 0.26
3
4
5
6
Mo of age
E
DG, kg d-1
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Trt: P = 0.27
Time: P = 0.01
Trt × time: P = 0.71
3-4 mo
4-5 mo
5-6 mo
Mo of age

Figure 1.

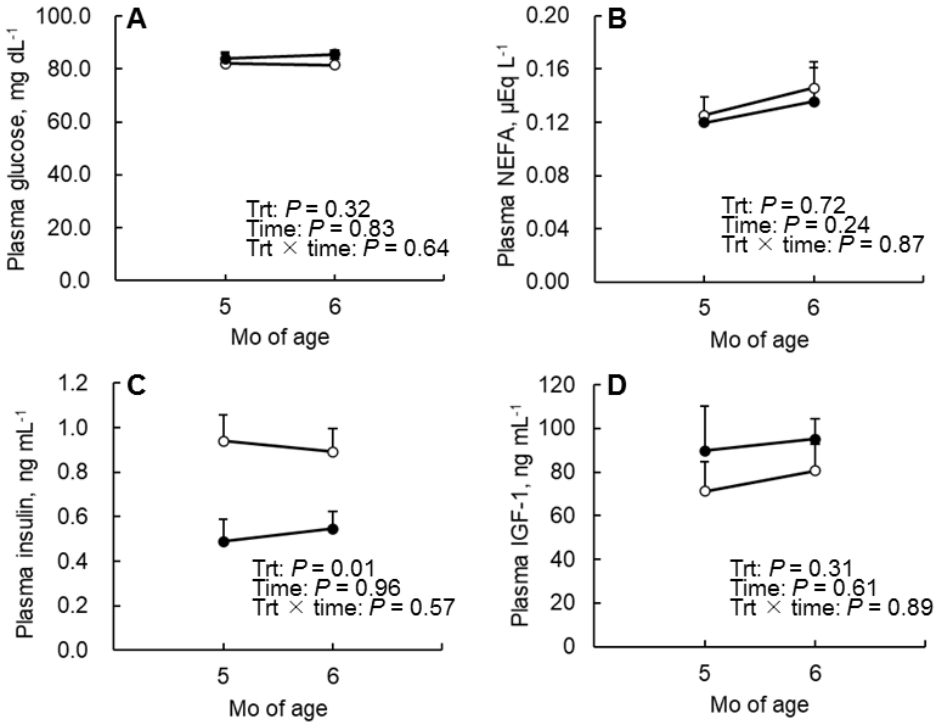



Figure 2.

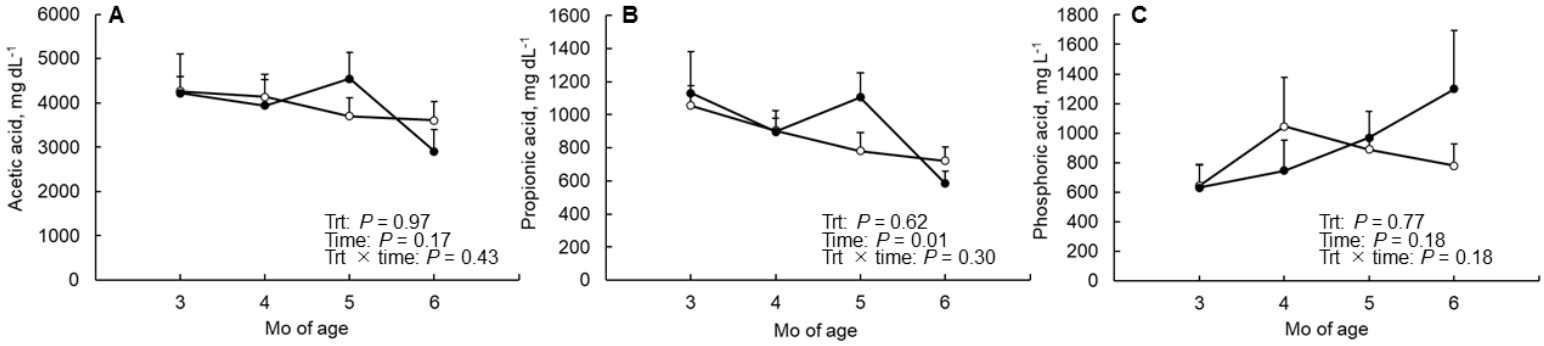
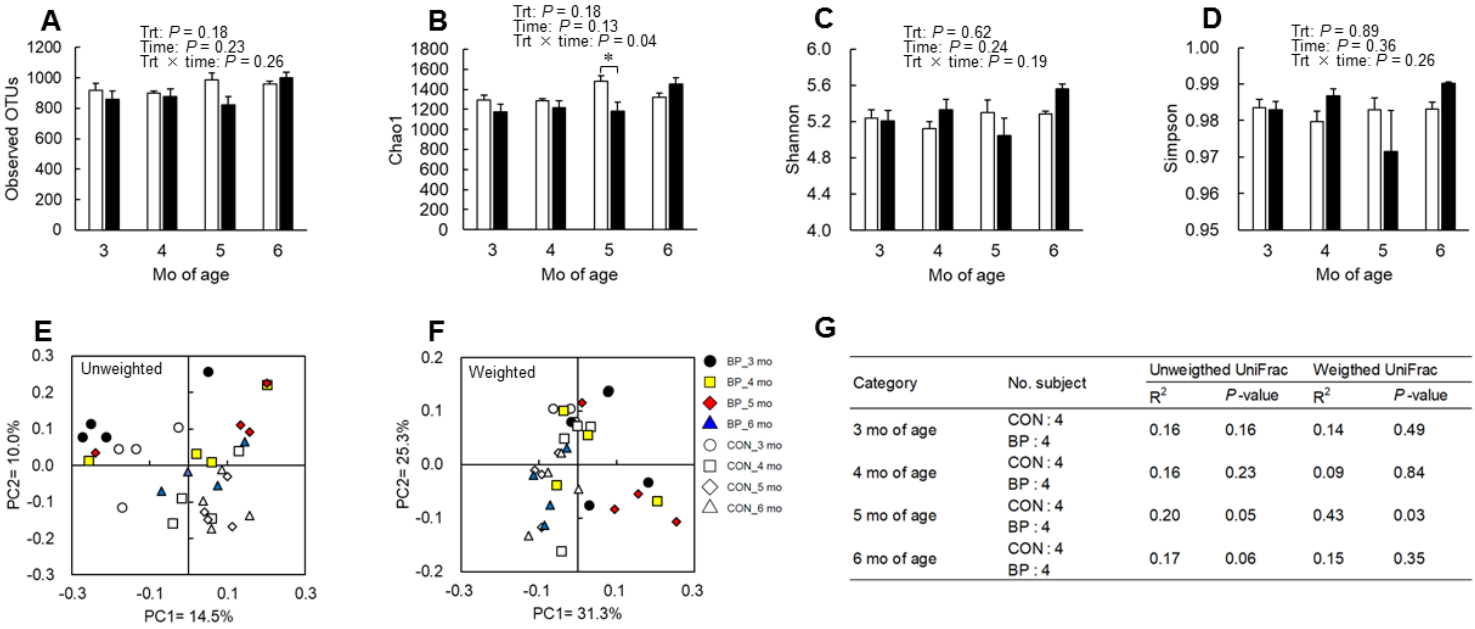

A
Acetic acid, mg dL-1
6000
5000
4000
3000
2000
1000
0
3
4
5
6
Mo of age
Trt: P = 0.97
Time: P = 0.17
Trt × time: P = 0.43
B
Propionic acid, mg dL-1
1600
1400
1200
1000
800
600
400
200
0
3
4
5
6
Mo of age
Trt: P = 0.62
Time: P = 0.01
Trt × time: P = 0.30
C
Phosphoric acid, mg L-1
1800
1600
1400
1200
1000
800
600
400
200
0
3
4
5
6
Mo of age
Trt: P = 0.77
Time: P = 0.18
Trt × time: P = 0.18

Figure 3.




G

| Category | No. subject | Unweigthed UniFrac | | Weigthed UniFrac | |
|---|---|---|---|---|---|
| | | $R^2$ | *P*-value | $R^2$ | *P*-value |
| 3 mo of age | CON : 4<br>BP : 4 | 0.16 | 0.16 | 0.14 | 0.49 |
| 4 mo of age | CON : 4<br>BP : 4 | 0.16 | 0.23 | 0.09 | 0.84 |
| 5 mo of age | CON : 4<br>BP : 4 | 0.20 | 0.05 | 0.43 | 0.03 |
| 6 mo of age | CON : 4<br>BP : 4 | 0.17 | 0.06 | 0.15 | 0.35 |

Figure 4.

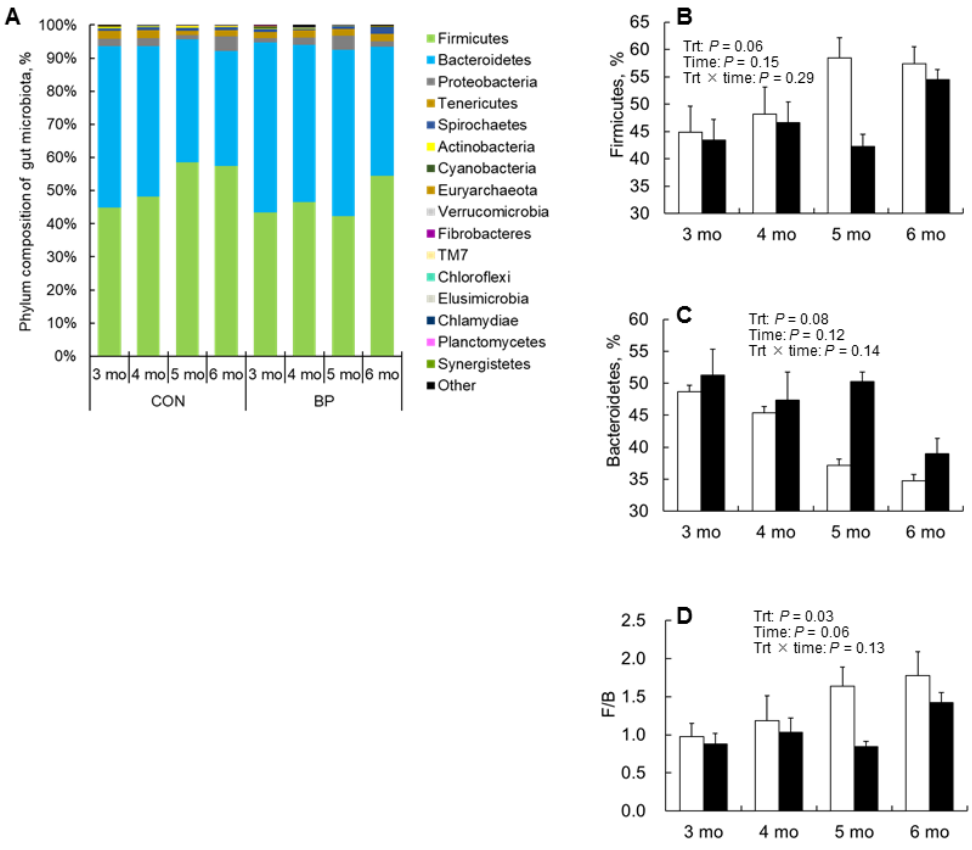

A
Phylum composition of gut microbiota, %
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
3 mo
4 mo
5 mo
6 mo
3 mo
4 mo
5 mo
6 mo
CON
BP
Firmicutes
Bacteroidetes
Proteobacteria
Tenericutes
Spirochaetes
Actinobacteria
Cyanobacteria
Euryarchaeota
Verrucomicrobia
Fibrobacteres
TM7
Chloroflexi
Elusimicrobia
Chlamydiae
Planctomycetes
Synergistetes
Other
B
Firmicutes, %
Trt: P = 0.06
Time: P = 0.15
Trt × time: P = 0.29
C
Bacteroidetes, %
Trt: P = 0.08
Time: P = 0.12
Trt × time: P = 0.14
D
F/B
Trt: P = 0.03
Time: P = 0.06
Trt × time: P = 0.13

Figure 5.

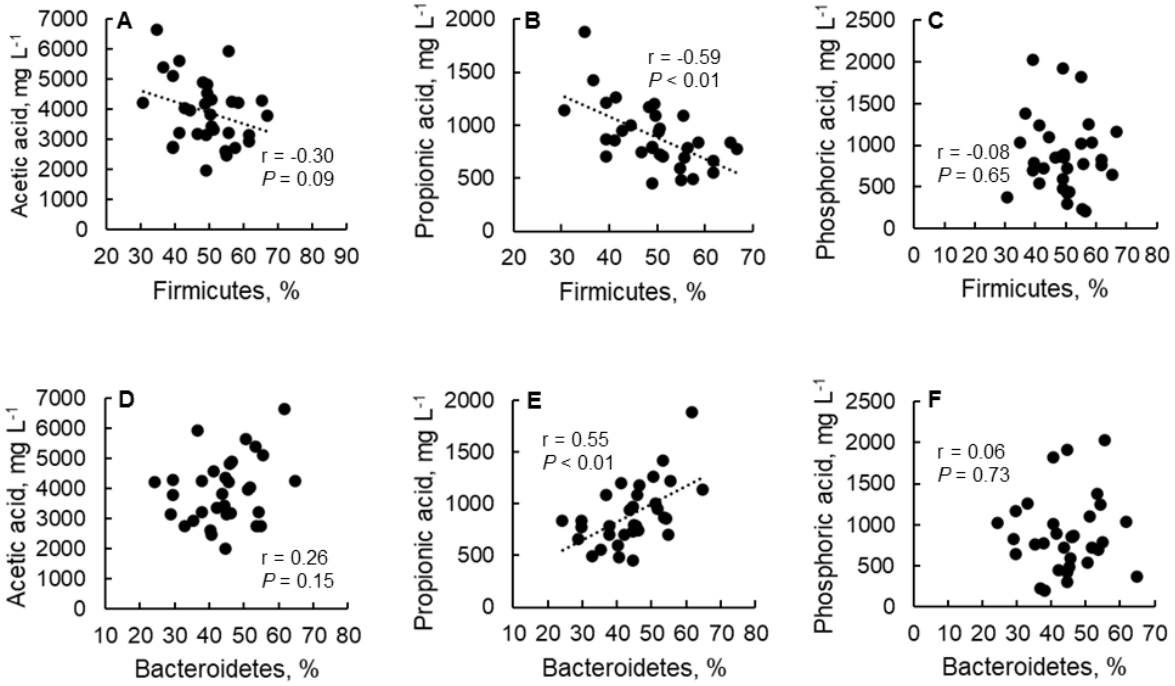

A
Acetic acid, mg L-1
7000
6000
5000
4000
3000
2000
1000
0
20 30 40 50 60 70 80 90
Firmicutes, %
r = -0.30
P = 0.09
B
Propionic acid, mg L-1
2000
1500
1000
500
0
20 30 40 50 60 70
Firmicutes, %
r = -0.59
P < 0.01
C
Phosphoric acid, mg L-1
2500
2000
1500
1000
500
0
0 10 20 30 40 50 60 70 80
Firmicutes, %
r = -0.08
P = 0.65
D
Acetic acid, mg L-1
7000
6000
5000
4000
3000
2000
1000
0
10 20 30 40 50 60 70 80
Bacteroidetes, %
r = 0.26
P = 0.15
E
Propionic acid, mg L-1
2000
1500
1000
500
0
10 20 30 40 50 60 70 80
Bacteroidetes, %
r = 0.55
P < 0.01
F
Phosphoric acid, mg L-1
2500
2000
1500
1000
500
0
0 10 20 30 40 50 60 70
Bacteroidetes, %
r = 0.06
P = 0.73

Figure 6.

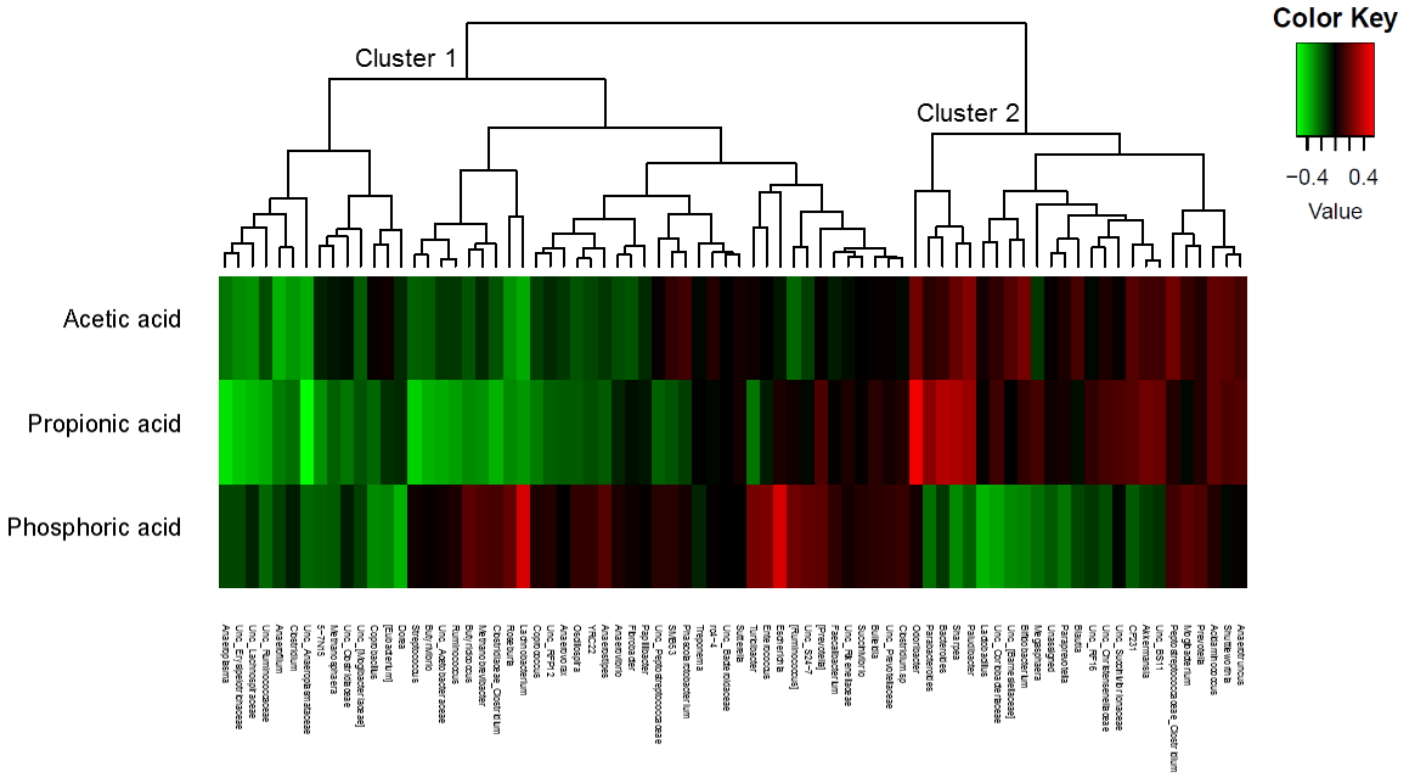

Color Key
−0.4 0.4
Value
Cluster 1
Cluster 2
Acetic acid
Propionic acid
Phosphoric acid

Figure 7.

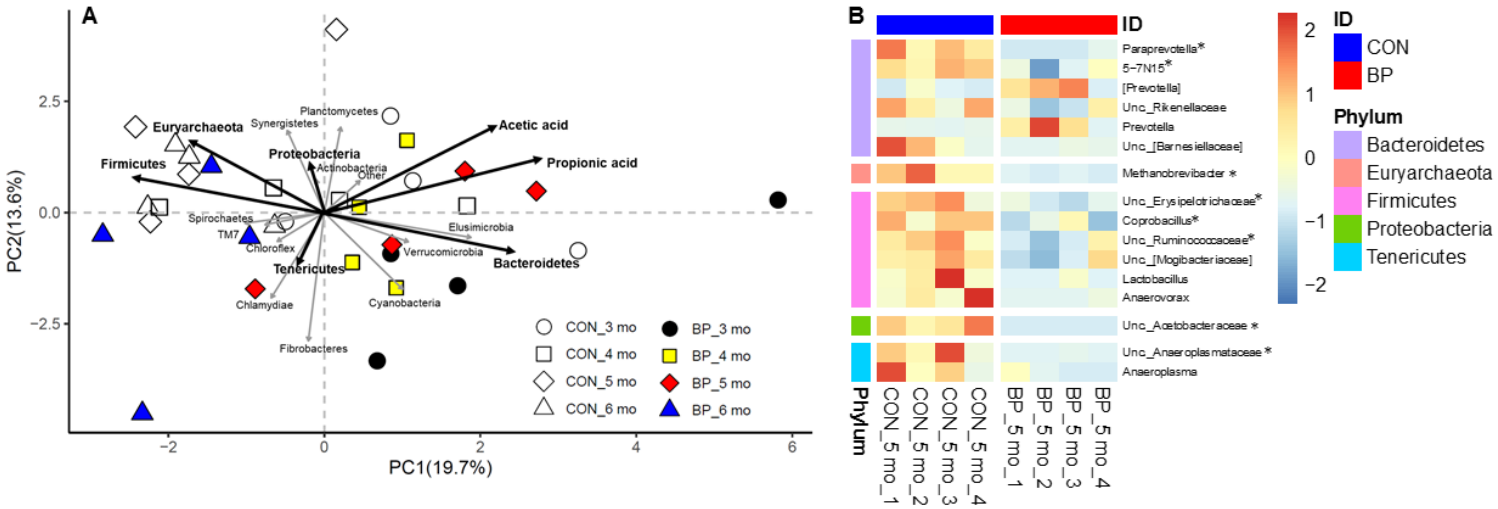

A
PC2(13.6%)
PC1(19.7%)
Euryarchaeota
Firmicutes
Proteobacteria
Synergistetes
Planctomycetes
Actinobacteria
Other
Acetic acid
Propionic acid
Spirochaetes
TM7
Chloroflex
Tenericutes
Chlamydiae
Fibrobacteres
Elusimicrobia
Verrucomicrobia
Cyanobacteria
Bacteroidetes
CON_3 mo
CON_4 mo
CON_5 mo
CON_6 mo
BP_3 mo
BP_4 mo
BP_5 mo
BP_6 mo
B
ID
Paraprevotella*
5−7N15*
[Prevotella]
Unc_Rikenellaceae
Prevotella
Unc_[Barnesiellaceae]
Methanobrevibacter *
Unc_Erysipelotrichaceae*
Coprobacillus*
Unc_Ruminococcaceae*
Unc_[Mogibacteriaceae]
Lactobacillus
Anaerovorax
Unc_Acetobacteraceae *
Unc_Anaeroplasmataceae*
Anaeroplasma
Phylum
CON_5 mo_1
CON_5 mo_2
CON_5 mo_3
CON_5 mo_4
BP_5 mo_1
BP_5 mo_2
BP_5 mo_3
BP_5 mo_4
ID
CON
BP
Phylum
Bacteroidetes
Euryarchaeota
Firmicutes
Proteobacteria
Tenericutes

Figure 8.