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Effects of concentrate levels on intestinal fermentation and the microbial profile in Japanese draft horses

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In racehorses, feeding a high-concentrate diet could cause abnormal fermentation in the hindgut. This feeding management regime is not suitable for the nutritional physiology of horses. However, studies on the hindgut environment have yet to be reported in Japanese draft horses, so feeding management needs to be investigated in these horses. Therefore, the objective of this study was to investigate the effects of a high-concentrate diet on hindgut fermentation in Japanese draft horses. Feces were collected from 20 male Japanese draft horses managed by two stables with different feeding designs (65% weight ratio of concentrate feed, HC; 50% weight ratio of concentrate, MC), and fecal metabolic characteristics and the microbiome were analyzed. Higher lactate concentrations and lower fecal pH levels were observed in the HC group (P=0.0011, P=0.0192, respectively). Fecal microbiome analysis revealed a decrease in microbial diversity (P=0.0360) and an increase in the relative abundance of Streptococcus lutetiensis/equinus/infantarius (P=0.0011) in the HC group. On the other hand, fibrolytic bacteria in the MC group had similarities with Clostridium sacchalolyticum and Ruminococcus albus. This study revealed that overfeeding of concentrates induced abnormal fermentation in the hindgut of Japanese draft horses. This suggests that the establishment of a feeding design based on not only the chemical compositions of feeds but also microbial dynamics is needed.

Key words: *concentrate diet, hindgut fermentation, Japanese draft horse, microbiome, nutrition*

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The Japanese draft horse is a crossbreed of other heavy horse breeds, such as the Percheron, Belgian, and Breton [10]. One of the characteristics of this horse is its body size; it has a body height of approximately 1.6 to 1.8 m and almost double the body weight (approximately 1,000 kg) of light breed horses like Thoroughbreds (approximately 500 kg), so it is one of the biggest types of horse in the world [10]. Due to its large body size, the Japanese draft horse was used for farm work and wood transportation and contributed to the development of Hokkaido since the 19th century [8]. However, due to the widespread use of agricultural machinery, draft horse production decreased in the 20th century. Nowadays, it is bred as a racehorse to preserve its cultural and historical roots, and it plays an important role as a tourism resource in Ban'ei horse racing, the only form of horse racing in the world in which draft horses pull an iron sled, at Obihiro Racecourse in Hokkaido, Japan.

Horses, including Japanese draft horses, are hindgut fermenting animals with a huge cecum and colon suitable for fiber degradation and fermentation. There are extremely

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complex and diverse microorganisms that coexist in the hindgut and degrade and ferment some feeds eaten by their host. In racehorses, however, to meet the demand for high performance in races, the horses are fed a high-concentrate diet [28]. This type of feed management could lead to a microbial imbalance in the hindgut due to the influx of starch and, consequently, the onset of laminitis and colic [2, 18]. This basic information has already been utilized to establish feeding standards for Thoroughbreds to prevent metabolic disorders. On the other hand, a feed management system for Japanese draft horses has yet to be established. Although Japanese draft horses are also fed a high-concentrate diet, no study has reported the relationship between feed composition and hindgut microbial fermentation in Japanese draft horses. The objective of this study was to investigate the effects of the proportion of concentrate in diet on the hindgut environment in Japanese draft horses.

Materials and Methods

Animals and feeding management

All procedures related to animal management and sampling described below were approved by the Animal Care and Use Committee of Obihiro University of Agriculture and Veterinary Medicine. Twenty male Japanese draft horses managed by two different stables at Obihiro Racecourse were used for this experiment. The horses' ages ranged between 2 and 8 years old, and 10 horses were selected from each stable (Table 1).

Feed type and design were based on each stable's respective feeding management guidelines. Stable I fed 8.4 kg of oats, 5.2 kg of bran, and 3.2 kg of molasses-coated oats as the concentrate diet as well as 5.6 kg of alfalfa hay and 3.6 kg of timothy hay as roughage per day on an as-fed basis. Stable II fed 8.4 kg of compound feeds and 4.4 kg of bran as the concentrate diet; 0.8 kg of alfalfa hay, 0.4 kg of alfalfa silage, and 10.4 kg of timothy hay as roughage feed; and 0.2 kg of minerals per day per head on an as-fed basis. All horses were fed those feeds in four separate manners each day. The weight ratio of the concentrate was 65% for stable I and 50% for stable II. In this study, the horses from stable I were considered the high concentrate (HC) group, and those from stable II were considered the medium concentrate (MC) group.

Table 1. Ages and weights of the horses

	Stable I (n=10)	Stable II (n=10)	*P*-value
Age	4.6 ± 0.8	4.4 ± 0.7	0.8522
Body weight (kg)	979.8 ± 18.8	1,016.0 ± 30.5	0.3275

All horses were between the ages of 2 and 8 years old. Data were compared by Student's *t*-test.

Feed samples were collected from each stable, and the chemical compositions were analyzed. First, the feed samples were dried at 60°C over two days and ground using a Heavy-Duty Cutting Mill SM 2000 (Retsch GmbH, Haan, Germany). The ground feeds were mixed in accordance with the feed design of each stable, and the chemical composition was determined by the Tokachi Federation of Agricultural Cooperatives (Obihiro, Hokkaido, Japan), which analyzes the chemical composition of feed materials.

Fresh fecal samples were collected from each stable and immediately transported to the laboratory to measure fecal pH and short-chain fatty acid (SCFA) concentrations. The remaining samples were stored at −80°C until DNA extraction.

Measurement of pH and SCFA concentrations

The pH and SCFA concentrations of fecal samples were measured by the following method. Approximately 3 g of fecal samples were transferred to sterile tubes, mixed with 6 m*l* of distilled water (1:2 dilution ratio), and vortexed for 5 min. Each sample was centrifuged (10,000 × *g* for 5 min at 4°C), and supernatant was transferred into a sterile microtube to measure SCFAs. The pH of the residual supernatant was measured using a pH meter (LAQUA F-72, Horiba, Kyoto, Japan). The microtubes for SCFA quantification were centrifuged (16,000 × *g* for 5 min at 4°C) again, and the supernatant was filtered through a 0.45 μl filter (Sartorius, Goettingen, Germany). Filtrated samples were subjected to high-performance liquid chromatography (Shimadzu Corp., Kyoto, Japan) to measure SCFA concentrations.

Bacterial DNA extraction and sequencing

Fecal bacterial DNA extraction and 16S rRNA gene sequencing were performed as reported by Nagata *et al.* [24]. Bacterial DNA from the lysates of the fecal samples was extracted by the RBB+C method [33] and purified using a QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA, USA). The variable region V3–V4 of the 16S rRNA gene was amplified using the following bacterial overhang adapters and universal primers: the forward primer 341F (5′ - TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG - 3′) and reverse primer 805R (5′- GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G GAT TAC HVG GGT ATC TAA TCC - 3′). For subsequent index PCR, Illumina sequencing adapters and Dual-index barcodes were added to the amplicon targets using a Nextera XT Index Kit (Illumina, San Diego, CA, USA). The concentration of the PCR products was measured using a Quantus™ Fluorometer and QuantiFluor® dsDNA System (Promega Corp., Madison, WI, USA). The final products were pooled into one tube in equal volumes. The

16S rRNA gene sequencing was performed using the Illumina MiSeq platform (Illumina).

Analysis of the 16S rRNA gene sequence

The original 16S rRNA gene data were analyzed by Quantitative Insights Into Microbial Ecology (QIIME) software (version 1.9.1) [1]. Sequences with a Phred quality score of less than 20 were excluded. The remaining high-quality reads were assigned to one operational taxonomic unit (OTU) by PyNAST with a sequence similarity threshold against the Greengenes core set database of more than 97%. Beta diversity, alpha diversity, and relative abundance of the bacterial taxonomic groups at the phylum and OTU levels were compared between the HC and MC groups using the Calypso software (version 8.84) [34]. The sequences were assessed using NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to estimate the nearest related bacterial species of the OTU sequences that showed significant differences between the groups.

Statistical analysis

The obtained data, except for principal coordinate analysis, are presented as the mean ± standard error of the mean (SEM). Calypso was used to test the similarity of bacterial compositions using the Bray-Curtis index and ADONIS test, and other items were analyzed using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). Differences in fecal pH and SCFA concentrations were compared by unpaired t-test. Bacterial composition at the OTU level was visualized by principal coordinate analysis using the Bray-Curtis index, and the similarity was compared using the ADONIS test. The differences in body weight, age, and fecal characteristics were compared by Student's *t*-test, and alpha diversity indices and microbial communities were compared by the Mann-Whitney test. A *P*-value less than 0.05 was considered statistically significant.

Results

Feed chemical composition

The neutral detergent fiber and acid detergent fiber levels were higher in the MC group, while the non-fiber carbohydrate and starch levels were higher in the HC group. On the other hand, the crude protein, ether extract, and ash composition levels were similar between the groups (Table 2).

Fecal pH and SCFA concentrations

Fecal characteristic data are shown in Table 3. In this study, the fecal pH in the HC group was significantly lower than that in the MC group (P=0.0220). Among the quantified organic acids, the HC group's lactate concentration was significantly higher (P=0.0031). The remaining parameters were not significantly different between the groups.

Fecal microbiome analysis

Beta diversity: Figure 1 shows bacterial composition (OTU level) of feces presented in the principal coordinate analysis (PCoA) based on the Bray-Curtis index. The plot shows that OTUs were densely clustered in both experimental groups and that two distinct clusters were formed for each group. In addition, the ADONIS test showed a significant difference between the HC group and MC group (P=0.0003).

Alpha diversity: Alpha diversity indices of the fecal microbiome are shown in Table 4. The Shannon and Simpson

Table 2. Chemical compositions of the feeds used in this study (% dry matter)

Composition	HC	MC
Neutral detergent fiber	43.1	51.0
Acid detergent fiber	23.7	26.5
Non-fiber carbohydrate	38.9	31.9
Starch	23.9	16.8
Crude protein	13.3	12.7
Ether extract	2.8	2.6
Ash	5.2	6.0

HC, high concentrate; MC, medium concentrate.

Table 3. Fecal pH and short-chain fatty acid properties in the Japanese draft horses

Parameter	All horses (n = 20)	HC (n = 10)	MC (n = 10)	*P*-value
pH	6.89 ± 0.08	6.68 ± 0.08	6.97 ± 0.08	0.0220
Acetate (mM)	21.78 ± 1.80	22.38 ± 1.74	21.19 ± 3.24	0.4813
Propionate (mM)	6.17 ± 0.61	5.82 ± 0.76	6.52 ± 0.99	0.7959
Butyrate (mM)	0.94 ± 0.27	0.84 ± 0.31	1.03 ± 0.46	0.9700
Lactate (mM)	0.79 ± 0.27	1.58 ± 0.41	0.00 ± 0.00	0.0031
Total (mM)	29.68 ± 2.53	30.62 ± 2.71	28.74 ± 4.40	0.4359

Data were compared by Student's *t*-test. HC, high concentrate; MC, medium concentrate.

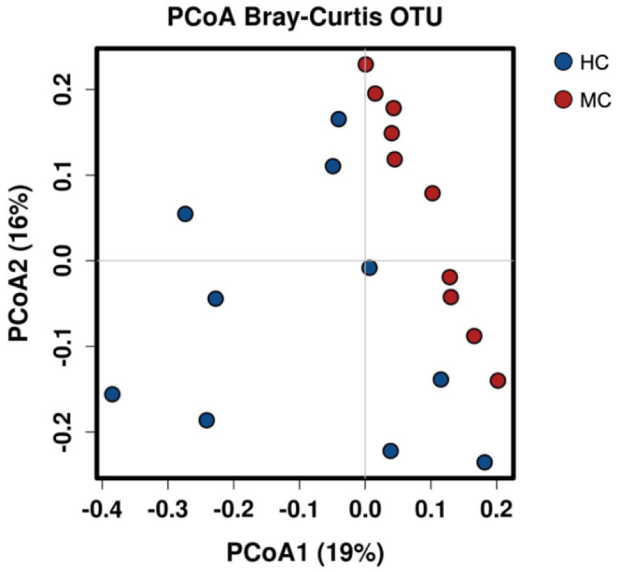

Fig. 1. Analysis of the beta diversity of the microbial community by principal coordinate analysis at the operational taxonomic unit level. Blue dots represent the high concentrate horses, and red dots represent the medium concentrate horses.

indices were significantly lower in the HC group compared with the MC group (P=0.0089, P=0.0095, respectively). The remaining indices were not significantly different between the groups.

Bacterial composition at the phylum level

The microbial composition at the phylum level is shown in Table 5. Firmicutes predominated (62.8%), followed by Bacteroidetes (21.6%), Verrucomicrobia (5.2%), Spirochaetes (3.8%), Fibrobacteres (2.0%), and Actinobacteria (1.2%). The abundances of all the phylum groups did not differ between the HC and MC groups.

Major OTU groups in Japanese draft horses

In this study, approximately 900 to 1,200 OTUs were detected per individual, and a total of 1,389 OTUs were detected in the 20 horses. The top 30 OTUs accounted for 25% of all the OTUs in terms of relative abundance. Table 6 shows the nearest relative bacterial species for the top 30 OTUs and their relative abundances. Among the major 30 OTUs, OTU 303161 (the nearest relative: *Streptococcus lutetiensis/equinus/infantarius*, 5.93%) was the most abundant, followed by new reference OTU 1336 (the nearest relative: *Clostridium sacchalolyticum*, 1.34%) and OTU 102910 (the nearest relative: *Colidextribacter massiliensis*, 1.33%). Furthermore, the OTUs nearest to *S. lutetiensis/equinus/infantarius* and *C. massiliensis* among the 30 OTUs were significantly higher in the HC group (P=0.0011, P=0.0232, respectively). In contrast, the OTUs nearest to *C. sacchalolyticum*, *Akkermansia glycaniphila*, *Ruminococcus albus*, and *Butyrivibrio fibrisolvens*/*Pseudobutyrivibrio ruminis* were significantly higher in the MC group (P=0.0433, P=0.0355, P=0.0052, P=0.0003, respectively).

Discussion

This study aimed to clarify the relationship between feed and the hindgut environment in Japanese draft horses by comparing the chemical characteristics and bacterial compositions of feces of horses managed by two stables with different feed concentrate ratios. For racehorses expected to achieve high levels of performance in racing, it is difficult to meet their daily energy requirements with only roughage; therefore, they are also fed a concentrate which degrades faster and has more nutrients than forage [28]. However, when fed a diet high in concentrates, starch can escape digestion in the small intestine and flow into the hindgut, causing lactate accumulation and decreased pH there [5, 6]. Therefore, attention should be paid to the starch content in the feed and to starch intake in order to maintain proper hindgut fermentation while meeting the energy requirements for racing.

The maximum amount of starch that can be digested in the small intestine in Thoroughbreds is 0.2%/kg of body weight/meal [9]. In this study, the feed designs were consistent for each horse regardless of body weight, but the starch amount was within the allowable range (minimum body weight = 915 kg, recommended starch amount ≤1.83 kg/meal, and actual amount = 1.40 kg/meal for the HC group; minimum body weight = 813 kg, recommended starch amount ≤1.63 kg/meal, and actual amount = 0.92 kg/meal for the MC group). The fecal analysis showed no lactate in the horses belonging to the MC group (Table 3). It is well known that lactate levels detected in the cecum or feces are quite low when horses are fed only hay or the recommended starch amount [5, 14, 35]. Therefore, it is suggested that most of the dietary starch was digested in the small intestine in the MC group. However, lactate was detected in seven horses belonging to the HC group, indicating that these horses had insufficient starch degradation in the foregut and that undigested starch may have flowed into the hindgut. Even though the starch content in the HC group was higher than in the MC group (Table 2), the starch intake in the HC group was probably within the recommended range that can be digested in the foregut in Thoroughbreds [9]. This suggests that starch digestibility in the foregut in Japanese draft horses may be lower than in Thoroughbreds.

In addition, fecal pH was significantly lower in the HC group (Table 3). This could have been due to the lactate accumulation because there was no significant difference

Table 4. Alpha diversity indices of the fecal microbiome in the Japanese draft horses

Diversity index	All horses (n=20)	HC (n=10)	MC (n=10)	*P*-value
Observed OTU	1,105.68 ± 15.63	1,089.49 ± 22.44	1,121.87 ± 21.66	0.3527
Chao1	1,180.73 ± 14.72	1,169.50 ± 21.39	1,191.96 ± 20.73	0.7541
Shannon	8.42 ± 0.16	8.09 ± 0.27	8.74 ± 0.11	0.0089
Simpson	0.98 ± 0.01	0.97 ± 0.01	0.99 ± 0.00	0.0095
Phylogenetic diversity	56.20 ± 0.58	56.01 ± 0.83	56.40 ± 0.84	0.7959
Good's coverage	0.99 ± 0.00	0.99 ± 0.00	1.00 ± 0.00	0.1958

Data were compared by the Mann-Whitney test. HC, high concentrate; MC, medium concentrate; OTU, operational taxonomic unit.

Table 5. Relative abundances of all phylum groups in the Japanese draft horses

Phylum	All horses (n=20)	HC (n=10)	MC (n=10)	*P*-value
Firmicutes	62.80 ± 1.31	64.83 ± 2.11	60.77 ± 1.37	0.1051
Bacteroidetes	21.61 ± 0.89	21.47 ± 1.62	21.75 ± 0.87	0.8534
Verrucomicrobia	5.16 ± 0.57	4.16 ± 0.42	6.16 ± 0.99	0.1655
Spirochaetes	3.82 ± 0.35	3.93 ± 0.51	3.71 ± 0.49	0.7239
Fibrobacteres	2.00 ± 0.31	1.96 ± 0.43	2.04 ± 0.46	0.8711
Actinobacteria	1.22 ± 0.21	0.87 ± 0.07	1.58 ± 0.38	0.0603
Proteobacteria	1.05 ± 0.63	0.52 ± 0.32	1.58 ± 1.23	0.9853
TM7	0.53 ± 0.08	0.49 ± 0.12	0.57 ± 0.12	0.6988
WPS2	0.46 ± 0.11	0.46 ± 0.16	0.46 ± 0.17	0.7814
Planctomycetes	0.42 ± 0.04	0.40 ± 0.06	0.44 ± 0.06	0.7251
Synergistetes	0.24 ± 0.03	0.27 ± 0.05	0.21 ± 0.04	0.4692
Cyanobacteria	0.20 ± 0.03	0.17 ± 0.03	0.22 ± 0.05	0.516
Euryarchaeota	0.17 ± 0.04	0.12 ± 0.02	0.21 ± 0.07	0.4459
Armatimonadetes	0.15 ± 0.03	0.15 ± 0.05	0.15 ± 0.03	0.6681
Tenericutes	0.15 ± 0.03	0.18 ± 0.04	0.12 ± 0.04	0.183
Lentisphaerae	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.02	0.6596

Data were compared by the Mann-Whitney test. HC, high concentrate; MC, medium concentrate.

in the concentrations of acetate, propionate, butyrate, and total SCFAs between the groups. In fact, the mean fecal pH of the seven horses in the HC group in which lactate was detected was 6.67, and the values of two horses were close to the value regarded as acidosis reported by Richards *et al*. [28]. Since low pH in the gastrointestinal tract is associated with intestinal mucosal injury [12, 13] and the development of laminitis [19], the horses fed a high-concentrate diet could be susceptible to these diseases. The results of the fecal microbiome analysis showed that beta diversity was significantly different between the groups. This is considered to be related to the difference in feed design between the groups. In addition, the Shannon and Simpson indices, which indicate the species richness and evenness, were significantly lower in the HC group than in the MC group. These decreases in microbial diversity could be the result of lactate accumulation and the drop in pH, as previously reported in ruminants fed high-concentrate diets [15].

In comparing the relative abundances of the major 30 OTUs among the groups, it was found that OTU 303161 and OTU 102910 were significantly more abundant in the HC group and that the nearest related bacterial species were *Streptococcus lutetiensis*/*equinus*/*infantarius* and *Colidextribacter massiliensis*, respectively. *S. lutetiensis, S. equinus*, and *S. infantarius* are major amylolytic bacteria belonging to the *S. bovis*/*S. equinus* complex [26, 31] and found in equine and human feces [30]. Although *S. lutetiensis*/*equinus*/*infantarius* was hardly detected in the MC group, it accounted for up to 34% in the HC horses. This supports the assertion that the difference in the extents of starch digestion in the foregut between the groups is the factor that impacted the amount of starch allowed to flow into the hindgut. The accumulation of lactic acid might be caused by *Streptococcus* spp., which are known to be lactate producer [17, 18, 29, 32], resulting in a decline in pH, and the accompanying reduction in microbial diversity in the HC group was caused by this bacteria group. Moreover, Milinovich *et al*. suggested that *S. lutetiensis*, *S. equinus*, and *S. infantarius* cause laminitis [17, 18], and Mungall *et al*. reported that equine lamellar hoof explants separate

Table 6. Relative abundances and nearest relatives of major operational taxonomic units identified by 16S rRNA gene sequence similarity

OTU ID	Nearest relative (NCBI accession number)	Similarity (%)	All horses (n=20)	HC (n=10)	MC (n=10)	*P*-value
303161	***Streptococcus lutetiensis* (MK331880)**	**99.8**	**5.93 ± 2.47**	**11.62 ± 4.30**	**0.24 ± 0.16**	**0.0011**
	***Streptococcus equinus* (LR134292)**	**99.8**				
	***Streptococcus infantarius* (KY801940)**	**99.8**				
New reference OTU 1336	***Clostridium sacchalolyticum* (FJ957875)**	**96.4**	**1.34 ± 0.25**	**0.90 ± 0.18**	**1.77 ± 0.44**	**0.0433**
102910	***Colidextribacter massiliensis* (NR_147375)**	**92.5**	**1.33 ± 0.42**	**2.22 ± 0.71**	**0.43 ± 0.25**	**0.0232**
580121	*Pseudoflavonifractor* sp. (MK287742)	93.2	1.30 ± 0.14	1.11 ± 0.18	1.50 ± 0.19	0.1903
New reference OTU 2229	*Oscillibacter* sp. (MK287653)	93.7	1.20 ± 0.13	1.20 ± 0.21	1.21 ± 0.17	0.9118
446153	*Sarcina maxima* (NR_026147)	99.3	1.17 ± 0.27	1.68 ± 0.45	0.66 ± 0.20	0.0892
321606	***Akkermansia glycaniphila* (LT629973)**	**95.5**	**0.97 ± 0.31**	**0.47 ± 0.11**	**1.48 ± 0.59**	**0.0355**
562408	***Ruminococcus albus* (AY445592)**	**94.9**	**0.91 ± 0.14**	**0.54 ± 0.12**	**1.27 ± 0.19**	**0.0052**
New reference OTU 275	*Faecalitalea cylindroides* (LT223666)	94.6	0.67 ± 0.13	0.70 ± 0.16	0.64 ± 0.21	0.4359
	Eubacterium cylindroides (FP929041)	94.6				
316092	*Prevotella* sp. (EU728713)	92.2	0.63 ± 0.09	0.48 ± 0.10	0.78 ± 0.13	0.1051
314935	*Pseudoflavonifractor capillosus* (MH282440)	93.0	0.62 ± 0.09	0.67 ± 0.14	0.56 ± 0.12	0.5288
	Lawsonibacter asaccharolyticus (LC371917)	93.0				
	Oscillibacter valericigenes (NR_074793)	93.0				
352570	*Fibrobacter* sp. (KY463343)	97.2	0.55 ± 0.14	0.61 ± 0.19	0.48 ± 0.21	0.2799
356407	*Blautia* sp. (MK217409)	96.8	0.57 ± 0.11	0.39 ± 0.13	0.76 ± 0.17	0.1424
354566	*Bacteroidales oral* (AF481207)	89.6	0.51 ± 0.13	0.65 ± 0.22	0.38 ± 0.14	0.2799
	Porphyromonas-like sp. (AY005071)	89.6				
346525	*Ruminococcus gauvreauii* (NR_044265)	95.2	0.52 ± 0.08	0.35 ± 0.05	0.69 ± 0.14	0.0753
301621	*Opitutus* sp. (KY039339)	79.0	0.50 ± 0.07	0.43 ± 0.05	0.57 ± 0.13	0.6842
296811	*Cephaloticoccus capnophilus* (NR_151906)	79.8	0.49 ± 0.06	0.44 ± 0.06	0.54 ± 0.10	0.5787
New reference OTU 1730	*Fibrobacter* sp. (KY463343)	99.3	0.44 ± 0.13	0.51 ± 0.22	0.38 ± 0.15	0.4813
340727	*Bacteroides luti* (MG428915)	85.2	0.47 ± 0.10	0.28 ± 0.11	0.67 ± 0.14	0.0630
New reference OTU 3377	*Clostridium fallax* (NR_044714)	83.6	0.45 ± 0.11	0.45 ± 0.16	0.44 ± 0.16	0.6409
100041	*Lactobacillus hayakitensis* (MG694669)	99.4	0.49 ± 0.13	0.49 ± 0.21	0.49 ± 0.15	0.5288
578649	*Clostridium* sp. (KC331165)	96.8	0.45 ± 0.03	0.45 ± 0.04	0.46 ± 0.04	0.8534
353085	*[Clostridium] aerotolerans* (NR_119068)	96.1	0.44 ± 0.09	0.43 ± 0.15	0.45 ± 0.11	0.5787
New reference OTU 756	*Porphyromonas pogonae* (MH894204)	83.9	0.42 ± 0.08	0.43 ± 0.10	0.41 ± 0.13	0.9118
813654	*[Clostridium] xylanolyticum* (MF188188)	97.5	0.42 ± 0.04	0.35 ± 0.05	0.49 ± 0.07	0.1230
	Clostridium aerotolerans (AB910753)	97.5				
346659	*Sphingobacterium lactis* (MH715156)	86.5	0.40 ± 0.14	0.62 ± 0.26	0.17 ± 0.10	0.2176
334555	*Kurthia massiliensis* (NR_118218)	99.6	0.39 ± 0.33	0.02 ± 0.01	0.77 ± 0.66	0.4343
319237	*Limisphaera ngatamarikiensis* (NR_134756)	79.4	0.39 ± 0.05	0.31 ± 0.03	0.48 ± 0.09	0.2176
573124	*Prolinoborus fasciculus* (MF077142)	98.7	0.39 ± 0.38	0.02 ± 0.03	0.77 ± 0.76	0.5960
	Acinetobacter lwoffii (JQ815203)	98.7				
589852	***Butyrivibrio fibrisolvens* (KF156791)**	**99.3**	**0.39 ± 0.03**	**0.28 ± 0.02**	**0.50 ± 0.04**	**0.0003**
	***Pseudobutyrivibrio ruminis* (JN619348)**	**99.3**				

Data were compared by the Mann-Whitney test. Bolded letters represent OTUs with significant differences between groups. OTU, operational taxonomic unit;.HC, high concentrate; MC, medium concentrate.

when cultured with streptococcal pyrogenic exotoxin B (SpeB), which contains in *S. bovis* [21]. These results indicate that the *S. bovis*/*S. equinus* complex is associated with horse laminitis when horses are fed diets too high in concentrates. Therefore, further investigation into the relationship between these bacterial groups and horse laminitis is required to improve the health of Japanese draft horses.

C. massiliensis is an anaerobic bacterium isolated from the human proximal colon [27], and its characteristics are not yet well understood; therefore, it will require more research. *C. sacchalolyticum* was isolated from a cellulose-enriched culture of sewage sludge [23]. It cannot digest cellulose but is able to utilize cellobiose and xylose [23]. Furthermore, it has been reported that coculturing *C. sacchalolyticum* with *Bacteroides cellulosolvens,* which is known as cellulolytic bacterium, resulted in the enhancement of cellulose degradation by 33% compared with *B. cellulosolvens* alone [22]. Considering these reports, *C. sacchalolyticum* appears to contribute to fiber degradation and fermentation, and new reference OTU 1336 could play a similar role in the

equine hindgut. *Ruminococcus* is a major bacterial group frequently detected in various herbivore animals, such as the bovine, ovine, equine, and leporine [4, 7, 11, 16]. It has been reported that *R. albus* possesses multiple glycoside hydrolase (GH) families that degrade cellulose (GH5, 9, and 48) and hemicellulose (GH10, 11, 26, 30, 43, and 76) [3]. Moreover, enzymes produced by *R. albus* contain a carbohydrate-binding module (CBM) that displays broad substrate-binding affinities, and it is important for effective fiber degradation because multiple GH families attach to the cell surface [3]. Based on these reports, OTU 562408 could contribute to fiber degradation in the equine hindgut. Therefore, the significantly high abundance of new reference OTU 1336 and OTU 562408 in the MC group is thought to be due to the high proportion of hay and NDF in the feed. In addition, since the growth of *C. sacchalolyticum* and *R. albus* is suppressed in a low pH environment [20, 23], it is conceivable that the low abundance of these species is related not only to the fiber content in the feed but also to lower fecal pH. *A. glycaniphila* is an obligate anaerobic bacteria belonging to *Verrucomicrobiae* subdivision I, which was isolated from reticulated python feces, and is known as a mucin degrader [25]. Thus, OTU 321606 could be involved in mucin degradation in the hindgut of horses, but further studies are needed to determine why this bacterial group was abundant in the MC group. *Butyrivibrio* and *Pseudobutyrivibrio* are frequently detected in ruminants, such as the bovine, ovine, and caprine [7]. Although it has been reported that *B. fibrisolvens* and *P. ruminis* have multiple GH families to degrade various polysaccharides, such as cellulose and hemicellulose, the most frequently encoded GH in their genomes is GH13, which is known for its amylolytic function [32]. Hence, these bacterial groups could have contributed to starch degradation in the MC horses and competed with other amylolytic bacteria, such as *Streptococcus* spp., in degrading the same substrate. Interestingly, since *B. fibrisolvens* and *P. ruminis* have not been reported as laminitis-causative bacteria, in contrast to *S. lutetiensis*, *S. equinus*, and *S. infantarius*, exploring the conditions that activate these bacterial groups in the hindgut could be one of the ways to prevent the development of laminitis.

Conclusion

This study revealed that a high-concentrate diet (65%) leads to increased levels of *S. lutetiensis/equinus/infantarius* in the feces and consequently leads to lactate accumulation, lower pH, and a reduction of fibrolytic bacteria in the hindgut of Japanese draft horses. This abnormal state may induce colic or laminitis. Therefore, it is necessary to establish a feed design focusing on the chemical compositions of feeds and microbial dynamics to prevent gastrointestinal disorders. In addition, starch digestibility in the foregut of Japanese draft horses may be lower than in Thoroughbreds. Thus, specific guidelines for the starch content of the total diet for this breed are expected.

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