Hindgut microbes, fermentation and their seasonal variations in Hokkaido native horses compared to light horses

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Running title: Hindgut microbes in native horses

Abstract: Fecal bacteria and protozoa of Hokkaido native horses and light horses were enumerated to compare seasonal variation in hindgut microbes and fermentation between the two breeds. Fecal samples were collected in winter and summer from eight horses (4 for each breed) that had been reared together under the same conditions after birth (on woodland pasture in winter and on grassland pasture for the rest of the year). Total fecal bacteria counts for both breeds showed temporal variation, with the highest levels occurring in summer ($P<0.05$). For both breeds, Gram negative rods were the major constituents (58-69%) and showed higher counts in winter ($P<0.05$) than in summer. Total protozoa counts in both breeds were lower in winter than in summer ($P<0.05$). The proportion of large cellulolytic protozoa such as *Cochliatoxum periachtum* was increased ($P<0.05$) in winter, and this tended to be more pronounced in native horses. Although total volatile fatty acids (VFA) in feces were lower in winter ($P<0.05$), the reduction was smaller in native horses ($P<0.05$). Fecal VFA pattern showed a shift toward more acetate and less propionate production in winter regardless of the horse breed. Evaluation of digestive tract organs in 12 animals showed that the relative weight of the colon in body weight or total digestive tract weight is larger in native horses than in light horses ($P<0.05$). The present results suggest that hindgut microbial adaptation to winter diets occurs to a greater extent in native horses, as partly characterized by advantages in anatomical structure.

Key words: Hokkaido native horse; hindgut microbes; seasonal change; cellulolytic protozoa; colon

Introduction

Microbes in the large intestine of horses are responsible for fiber digestion. In addition, these symbiotic microbiota, consisting of bacteria, protozoa and fungi, produce volatile fatty acids (VFA) as a means of supplying energy to the host animal (Stewart 1996). Population sizes of specific microbial groups vary in response to differences in feed type, and intestinal digestion changes to reflect feed type (Goodson et al. 1988). However, the factors influencing microbial populations and fermentation in the equine hindgut have not been clearly described. Feed type and intake level alter the retention time of digesta in horses (Dorogoul et al. 2000a), consequently affecting hindgut microbiota composition. In addition, the establishment of microbiota could depend on the hindgut capacity of horses as a reflection of body size or even horse breed.

Comparative studies on digestion among horse breeds, particularly breeds of different body sizes, are quite limited. Although ponies are small-bodied, some have been thought to have higher digestive capability for diets of low quality forage (Kondo 2001). Kawai et al. (1998) showed that Hokkaido native horses, one of the seven native pony breeds in Japan, tend to have higher digestibility of neutral detergent fiber (NDF) and acid detergent fiber (ADF) than light horses. Lower body weight reduction in Hokkaido native horses compared to that in light horses on winter woodland pasture was also reported (Shingu et al. 2000). Our preliminary observation suggested that the relative size of the large intestines is greater in native horses than the reported value in Thoroughbred (Stewart 1996). In horses, having a proportionally large sized large intestine is considered to be advantageous for longer retention of feed and microbes,

which could in turn promote fiber digestion.

We studied the differences in hindgut microbe and fermentation characteristics between Hokkaido native horses and light horses that had been raised together in a same manner since birth. This study may clarify the digestive capability of the native horses from the viewpoint of hindgut microbes and fermentation. We also attempted to discuss hindgut microbes in relation to anatomical characteristics, *i.e.* relative weight of large intestine in horses.

Although there is no detailed comparative study, we assumed that fecal microbiota could reflect hindgut microbiota to some extent, since no significant difference in microscopic enumeration was observed between the rectum and colon of horses (Koike et al. 2000). This was also reported by Kern et al. (1974) who investigated microbes of the terminal colon (close to rectum) and cecum of the pony. Similarly, fecal sample was used as an alternative to hindgut sample to study hindgut protozoa in the horse (Ike et al., 1985).

Methods

Animals, pastures, samplings and study area

A total of eight horses, consisting of 4 Hokkaido native horses (native horses) and 4 light horses (75% Thoroughbred and 25% other light breed; light horses), were included in our study. Average body weights with SD at the beginning of the experiment were 355±41 and 575±49 kg for native horses and light horses, respectively. All were born at the experimental livestock farm of the Northern Biosphere Field Science Center of Hokkaido University, Shizunai, Japan (42°25' N, 142°28' E) and raised under the

exactly same conditions. The animals were in direct contact with each other for a long period of time, allowing ample chance for microbial transfer to occur. The animals were conventionally reared with grazing on grassland pasture from spring to autumn (from April through November) and on woodland pasture in winter (from December through March). The grassland pasture was primarily occupied by timothy, while the undergrowth of the woodland pasture was bamboo grass (*Sasa nipponica*). On winter woodland pasture, the horses also had access to bark and twigs when the bamboo grass was depleted or covered with snow.

The experiment was comprised of three sampling periods: the first sampling in January, 2000 (1st winter), the second in August, 2000 (summer) and the last in January, 2001 (2nd winter). During these periods, freshly deposited feces were collected for microbial and fermentation analyses. The samples were taken from the interior of the feces to avoid contamination by soil microorganisms and kept in an icebox until further processing. According to climate data for this area during the experimental period, the average temperature ranged from 20.4°C in August to -4.4°C in February. Snowfall occurred from November until March with largest accumulation of 37 cm in January, 2000.

Microbial enumeration and VFA analysis

Fecal samples were homogenized with saline containing 20% chloroform and preserved at room temperature until analysis. Microscopic observation was made to morphologically enumerate fecal bacteria according to Suto's methods (Suto 1973). In brief, homogenized feces (1g) were diluted with saline ($\times 10^{2-3}$), a part of which was fixed on a glass plate after mixing with agar solution. Then following Gram staining,

counts with morphological classification for selected 16 fields per sample were made under a light microscope (BX51, Olympus, Tokyo, Japan) fitted with a micrometer (×1,000). Bacteria were classified into four categories: rods and cocci being either Gram positive or Gram negative. For analysis of protozoa, feces (1g) were gently mixed with MFS (Methylgreen Formalin Saline) solution (4ml) and enumerated (×400) and counted (×100) under a microscope. The enumerative counting was performed twice per sample. These procedures and protozoa identification were carried out according to Ogimoto and Imai (1981).

VFA analysis and pH determination was carried out with the supernatant of feces that were homogenized in distilled water and centrifuged (10,000×g). VFA analysis was made by gas chromatography (GC14B, Shimazu, Kyoto, Japan) with total VFA being expressed in terms of fecal dry matter (Suto 1973). All the chemicals used were of reagent grade (Wako, Osaka, Japan).

Collection of anatomical data

Weights of digestive organs were determined on a total of 12 horses, 6 native horses (191±28 kg in body weight; 1-2 y of age) and 6 light horses (100% Thoroughbred; 562 ±30 kg; 14-15 y), which were killed at a local slaughterhouse. All of these horses had been raised mainly on a timothy hay/wheat bran diet in the same area of Shizunai. Alimentary tracts for all horses were dissected, washed, weighed and separated into cecum, colon and others. The separated cecum and colon were also weighed. Since fiber digestion occurs mainly in the ventral and dorsal colon (Hintz *et al.* 1971; Drogoul *et al.* 2000b), the distal small colon was excluded from the “colon” in this study.

Statistical analyses

Data were expressed as means with standard errors of four replications (microbial and fermentation data) or six replications (anatomical organ data) within each breed. The microbial and fermentation data were analyzed using a 3 x 2 factorial arrangement using the GLM procedure of SAS with the factors of season (1st winter vs. summer vs. 2nd winter), breed (native horse vs. light horse), and their interaction. When a significant difference among the seasons was detected, multiple comparisons between seasons were made using the least significant difference (LSD) test. The anatomical data were compared between the breeds (native horse vs. light horse) by Student's t-test.

Results

Bacteria

Total fecal bacteria counts and morphological classifications of bacteria are shown in Table 1. Total counts varied with season, with highest counts in summer ($p<0.05$). There were no breed-specific changes in total bacterial counts.

Gram negative rods formed the majority of the bacteria (ranging from 58.1 to 69.1%) followed by Gram negative cocci (26.3 to 37.0%), Gram positive rods (1.5 to 6.4%) and Gram positive cocci (1.2 to 3.5%). There were consistent seasonal changes in the proportions of Gram negative cocci and rods; higher proportions of Gram negative rods were noted in both breeds in the winters ($p<0.05$), while lower proportions of Gram negative cocci were recorded in the corresponding periods. There were no significant differences in the proportions of enumerated bacterial groups between native horses and light horses.

Protozoa

Seasonal changes in total fecal protozoa counts and generic composition in native horses and light horses are presented in Table 2. There was an apparent seasonal shift in total protozoa counts in native horses, with an increase from the first winter to summer ($p<0.05$) and a reduction in the second winter ($p<0.05$). For light horses, an increase in summer was not significant, while a decrease in the second winter was apparent ($p<0.05$).

Bundleia postciliata was the most common protozoa species (39.2-54.7%) in the hindgut of the horses examined here, irrespective of breed, followed by *Didemis quadrata* (5.8-17.2%) and *Ditoxum funinucleum* (2.0-15.8%). These major species showed no significant changes with season, but showed great variation among individual animals. Meanwhile, a fibrolytic, large-sized protozoon *Cochliatoxum periachtum* showed a typical seasonal change. *C. periachtum* was present in higher proportions in winters compared to the summer ($P<0.05$). This species was undetectable in light horses in summer, and as a result, the abundances of this species were significantly higher in native horses ($P<0.05$). Active engulfment of plant fragments by this large protozoon was frequently observed, especially in native horses (Fig. 1).

Fermentation

Table 3 shows fecal pH, total VFA concentration and molar proportions of VFA. Fecal pH decreased in summer and increased in winters, corresponding to the opposite changes observed for total VFA concentrations ($P<0.05$). There were differences in the total VFA concentrations between the horse breeds, with higher values observed in

native horses (P<0.05). Fermentation patterns showing more acetate and less propionate were observed in winters, irrespective of horse breed.

Anatomical parameters

Body weight and anatomical data for the digestive tract are given in Table 4. Absolute weights of the whole body, total digestive tract, cecum and colon were lower in native horses (P<0.001). When tissue weights were expressed as percentages per body weight and per weight of total digestive tracts in order to normalize for the different body sizes for the two breeds, the relative colon weight was higher in native horses than in light horses (P<0.05). Meanwhile, the relative weight of cecum did not differ significantly between the two breeds. The relative weight of the colon plus cecum was higher in native horses compared to light horses (P<0.01).

Discussion

Hokkaido native horses have been considered to be able to digest lower quality forage compared to light breeds such as Thoroughbreds (Kondo 2001). Although their behavioral advantage in grazing on woodland pasture had been reported (Shingu *et al.* 2000), evaluations from a nutritional viewpoint had not been undertaken.

The superior digestive ability in native horses was suggested to be due to anatomical advantages such as a relatively enlarged colon (Table 4). This allows plant fibers to be held for longer periods of time in the hindgut, thus promoting fiber digestion. These anatomical advantages, also promote longer residence time of hindgut microbes, which could facilitate the establishment of fibrolytic microbes in the hindgut since they require

longer doubling times than other microbes (Van Soest 1994). Although no reports on the differences of digesta passage between native horses and light horses are available, the above anatomical differences given in terms of body weight could produce differing retention times of digesta and microbes since animals are generally fed based on body weight. The colon is considered to be the main site of fibrolysis in horses (Hintz *et al.* 1971; Drogoul *et al.* 2000b). The present results show a larger relative weight for the colon in native horses, suggesting that they may have superior fiber digestion.

Since bacterial enumeration was performed, based on Gram staining and morphology, it is not possible to make inferences on taxonomy. However, there was a clear shift in the proportion of Gram negative rods, showing significant increases in the winter seasons (Table 1). Koike et al. (2000) found that Hokkaido native horses have a bacterial adaptation to low-quality roughage diets on winter woodland pasture in their increased levels of *Fibrobacter succinogenes*, one of the most potent fibrolytic species present in the equine hindgut. They also observed an increase in Gram negative rods in winter (Koike *et al.* 2000). Since *F. succinogenes* is categorized with the Gram negative rods, the increased proportion of this group in winters of the present study (Table 1) may be partly explained by the increase of *F. succinogenes* in winter up to 1.0-4.4% of total bacteria (Koike et al 2000).

Although Julliand et al. (1999) pointed out the significance of *Ruminococcus flavefaciens* in fiber digestion of horses, we have already confirmed that this species is minor in Hokkaido native horses (Koike *et al.* 2000). Instead of *R. flavefaciens*, novel groups of *F. succinogenes* in the equine hindgut (Lin & Stahl 1995) should be explored in our case. Even though no direct comparisons were made for cellulolytic bacterial counts between native horses and light horses, reported values show that Shetland

ponies, similar to our native horses in body size, have higher numbers of cellulolytic bacteria than light horses (10^7 vs. 10^6/g) (Kern et al. 1973, 1974; Mackie & Wilkins 1988; Moore & Dehority 1993).

Total bacterial counts showed seasonal variations, with higher levels in summer than in the winter seasons, as was also noted in the preceding report (Koike *et al.* 2000) in which the winter feed supply was thought to be lacking in both quantity and quality. These situations could limit the growth of a wide variety of bacterial species. This could result in the selection of fibrolytic species, possibly accompanying a reduction in the passage rate of digesta.

Hindgut fauna, mainly comprised of small-sized protozoa, is considered to play a minor role in feed digestion in the equine hindgut, as indicated in a defaunation study (Moore & Dehority 1993). However, the present results on seasonal protozoal variation suggest that large-sized fibrolytic protozoa may support some extent of forage digestion, especially in native horses (Table 2). In fact, fibrolytic *C. periachtum* (Ogimoto & Imai 1981) increased its relative proportion in winter in native horses (Table 2). Abundance of this species in the hindgut was able to fluctuate in native horses so as to adapt to the more fibrous diet in winter. This hypothesis is partly supported by the direct observation of plant fragments being actively ingested by this protozoon (Fig.1). This is not the case for light horses, probably due to the lower relative capacity of the hindgut on total digestive tract weight basis (Table 4), which does not allow a longer residence of large-sized and slow-growing fibrolytic protozoa (Van Soest 1994). In fact, *C. periachtum* is found at lower levels in light horses (even becoming undetectable level in summer) (Table 2).

Lower fecal VFA levels with proportionally more acetate and less propionate in

winter (Table 3) explain the seasonal microbial selection due to diet changes from pasture grass (summer) to bamboo, bark and others (winter) discussed above, as fibrolytic bacteria and protozoa are acetate producers (Stewart 1996). Higher VFA levels, especially higher acetate levels, in native horses even during winter (Table 3) may suggest that microbial adaptation to fibrous diet is greater in native horses than in light horses. A similar shift of fermentation pattern is reported in the rumen of wild sika deer ingesting highly fibrous foods such as bark in snowy winter (Ichimura et al. 2004). Floral shift of horse hindgut in the present study (more Gram negative rod in winter, Table 1) was opposite to that of deer rumen (less Gram negative rod but more Gram negative cocci in winter, Ichimura et al. 2004), implying the difference in floral members between these two animal species. Thus, microbial adaptation could be microbial community shift to maximize feed digestion in a certain situation.

The present study indicates the alteration of hindgut microbiota with season (dietary condition) and horse breed. In particular, shifts in micro-fauna as well as micro–flora, corresponding to seasonal changes, are more profound in Hokkaido native horses, which have larger relative colon volumes than light horses. This anatomical character in native horses could be advantageous to fibrolytic bacteria and protozoa, which contribute to the digestion of low quality roughage while producing more acetate. According to digestion trials using a double indicator technique, NDF digestibility of bamboo grass during grazing was higher, but not significantly so, in native horses than in light horses (38.6 vs. 32.1%) (Shingu *et al.* 2000).

Adaptation of gut microbiota in wildlife to seasonal or dietary conditions has been described for the ruminants deer (Orpin *et al.* 1985; Dehority & Demarais 1999; Ichimura *et al.* 2004) and sheep (Hobson *et al.* 1975). Our results suggest that this

adaptation is also present in the equine hindgut, the extent of which would be greater in Hokkaido native horses than in light horses. Further nutritional implications of this phenomenon need to be more closely examined in digestion trials involving the determinations of microbes, fermentation and digesta passage.

The differences in anatomical data between native horses and light horses should be carefully discussed, considering difference of age (1.5 vs. 14.5 years of age). In fact, the slaughtered native horses were not fully matured. They may have higher relative weight of digestive tract on body weight basis when compared with matured animals, since bone and digestive tract development comes first, followed by muscle development and fat deposition (Gill & Oldham 1993). Meanwhile, Cunha (1991) describes that development of large intestine is completed by 15-24 months old that is the age of the slaughtered native horses in the present study. Based on these, it might be true that at least the relative weight of hindgut on total tract weight basis is a valid index to mention anatomical difference between native horses and light horses even in different maturity.

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Table 1 Fecal bacteria and morphological enumeration in Hokkaido native horses and light horses.

Breed	Season	Total bacteria ($x10^{10}$/g)	Gram positive cocci (%)	Gram positive rods (%)	Gram negative cocci (%)	Gram negative rods (%)
Native horses	1st Winter	1.2 a	1.2	1.5	28.2 a	69.1 a
	Summer	9.2 c	1.8	3.1	37.0 b	58.1 b
	2nd Winter	3.8 b	1.8	3.0	31.3 a	63.9 a
Light horses†	1st Winter	1.3 a	3.5	6.4	26.3 a	63.8 a
	Summer	14.9 b	1.5	3.4	34.9 b	60.2 b
	2nd Winter	3.2 c	2.9	5.2	27.1 a	64.8 a
Statistics						
	Breed	NS	NS	NS	NS	NS
	Season	**	NS	NS	*	*
	Breed x Season	NS	NS	NS	NS	NS

Each value represents the average from 4 horses.
a, b: Values with different superscripts within the same column for each breed differ significantly (P<0.05).
†: Light horses are 75% Thoroughbred and 25% other light horse breeds.
*: $P<0.05$; **: $P<0.01$

Table 2 Fecal protozoa and generic enumeration in Hokkaido native horses and light horses.

Breeds	Season	Total protozoa	*Bundleia postciliata*	*Ditoxum funinucleum*	*Triadinium caudatum*	*Tripalmaria dogieri*	*Didesmis quadrata*	*Cycloposuhium bipalmatum*	*Cochliatoxum periachtum*	*Blepharocorys uncinata*
		($x10^4$/g)	(%)							
Native horses	1st Winter	1.2 a	48.5	15.8	2.4 ab	6.5	8.7	6.9	5.9 b	5.3
	Summer	1.9 b	49.7	2.0	5.7 b	4.8	7.8	5.8	1.1 a	23.2
	2nd Winter	1.0 a	39.2	6.6	0.6 a	16.4	10.0	12.2	5.6 b	9.4
Light horses†	1st Winter	1.3 ab	54.6	11.5	1.1 a	5.0	17.2	3.2	2.9	4.5
	Summer	1.7 b	45.7	10.3	8.3 b	11.0	5.8	11.3	ND	7.6
	2nd Winter	0.9 a	54.7	10.1	0.1 a	6.5	13.7	8.6	2.1	4.3
Statistics										
	Breed	NS	NS	NS	NS	NS	NS	NS	*	NS
	Season	*	NS	NS	*	NS	NS	NS	*	NS
	Breed x Season	NS	NS	NS	NS	NS	NS	NS	NS	NS

Each value represents the average for 4 horses. ND: not detected.
a, b: Values with different superscripts within the same column for each breed differ significantly ($P<0.05$).
†: Light horses are 75% Thoroughbred and 25% other light horse breeds.
*: $P<0.05$

Table 3 Fecal pH and volatile fatty acids in Hokkaido native horses and light horses.

Horse breeds	Seasons	pH	Volatile fatty acids				
			Total	Acetate	Propionate	Butyrate	Others
			(m moles/g DM)	(molar %)			
Native horses	1st Winter	6.79 a	0.09 a	68.2 a	21.5 a	7.0	3.3
	Summer	6.37 b	0.16 b	62.1 b	26.6 b	7.4	3.9
	2nd Winter	6.59 ab	0.10 ab	66.5 a	22.9 a	7.4	3.2
Light horses†	1st Winter	6.85 a	0.05 a	69.1 a	20.8 a	6.5	3.6
	Summer	6.35 b	0.15 b	62.5 b	27.6 b	6.9	3.0
	2nd Winter	6.70 a	0.06 a	67.8 a	21.9 a	7.0	3.3
Statistics							
	Breed	NS	*	NS	NS	NS	NS
	Season	*	*	*	*	NS	NS
	Breed x Season	NS	NS	NS	NS	NS	NS

Each value represents the average for 4 horses.

a, b: Values with different superscripts within the same column for each breed differ significantly (P<0.05).

†: Light horses are 75% Thoroughbred and 25% other light horse breeds.

*: $P<0.05$

Table 4 Comparison of digestive organs between Hokkaido native horses and light horses.

Parameter of digestive tissue	Breed				Difference
	Native horses (n=6)		Light horses† (n=6)		
Body weight (kg)	190.8	(34)	561.8	(100)	***
Total digestive tract (kg)	12.8	(40)	31.8	(100)	***
Cecum (kg)	2.0	(47)	4.3	(100)	***
Colon (kg)	5.4	(52)	10.3	(100)	***
% per body weight					
Cecum	1.1	(138)	0.8	(100)	NS
Colon	2.9	(161)	1.8	(100)	*
Cecum plus colon	4.0	(154)	2.6	(100)	*
% per total tract weight					
Cecum	15.6	(116)	13.5	(100)	NS
Colon	42.2	(130)	32.4	(100)	*
Cecum plus colon	57.8	(126)	45.9	(100)	**

Values in parentheses are relative percentages when the weights of light horses are expressed as 100.
†: Light horses are 100% Thoroughbred.
*: $P<0.05$; **: $P<0.01$; ***: $P<0.001$

Fig. 1.
Cochliatoxum periachtum, a protozoon notably present in feces of Hokkaido native horses, actively ingesting plant fragments (light microscopy, magnification: ×400; bar scale: 100μm).