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Molecular profiling of *Lactobacillus*, *Streptococcus*, and *Bifidobacterium* species in feces of active racehorses

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(Received March 1, 2007; Accepted May 9, 2007)

Diversity and compositions of the *Lactobacillus*, *Streptococcus*, and *Bifidobacterium* group in the feces of six healthy, actively racing horses (Thoroughbreds) were analyzed by using PCR-denaturing gradient gel electrophoresis (DGGE) and real-time PCR with primer sets specific for each group. PCR-DGGE analysis of the feces showed that *Lactobacillus equi*, *Lactobacillus johnsonii*, a phylogenetic relative of *Lactobacillus salivarius*, a phylogenetic relative of *Lactobacillus gastricus*, and *Weissella confusa* were predominant in almost all of the feces tested, and *Streptococcus bovis*/*Streptococcus equinus* was predominant in the *Streptococcus* group. The *Bifidobacterium* group was not detected by single-PCR but atypical species of the group were found in three of the six Thoroughbreds tested by nested-PCR. Calculation and estimation of lactic acid bacteria and bifidobacteria revealed that lactic acid bacteria were predominant in the feces and bifidobacteria were minor. These results indicate that the community of lactic acid bacteria and bifidobacteria in horse feces are unique because of the presence of specific species for horse feces and a minority of the *Bifidobacterium* group. Repeated tests of the feces from the same horse over 3 months showed that the diversity and composition of lactic acid bacteria and bifidobacteria in the feces was basically stable throughout the test period.

Key Words—*Bifidobacterium*; horse feces; *Lactobacillus*; PCR-DGGE; real-time PCR; *Streptococcus*

Introduction

The gastrointestinal tract of animals harbors microbial flora. Lactic acid bacteria and bifidobacteria are members in the flora of many animals. The organisms have been reported to have several health-promoting effects on host animals (Nagao et al., 2000; Ouwehand et al., 2002), and thus the flora of the organisms in feces and/or gastrointestinal contents have been ac-

tively studied on many animals (Gueimonde et al., 2004; Mitsuoka and Kaneuchi, 1977; Walter et al., 2001).

Yuyama reported a beneficial aspect of lactic acid bacteria for horses. The administration of the living cell mixture of *Lactobacillus salivarius*, *L. reuteri*, *L. crispatus*, *L. johnsonii*, and *L. equi* prevented diarrhea and enhanced the growth of horses treated with probiotics, compared with placebo-controlled horses (Yuyama et al., 2004). A negative aspect of lactic acid bacteria was that *Streptococcus bovis* and five different *Lactobacillus* spp. affected horse laminitis (Bailey et al., 2003; Mungall et al., 2001). Bifidobacteria are predominant in gastrointestinal microbial flora of many animals (Mitsuoka and Kaneuchi, 1977), and promote the health of hosts, such as prevention of diarrhea, short-

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Abbreviation: DGGE, denaturing gradient gel electrophoresis.

ening of its duration, and immune modulation (Isolauri et al., 2000; Ouwehand et al., 2002). However, despite its importance for intestinal lactic acid bacteria and bifidobacteria, information about the bacterial flora of the organisms in feces and/or intestinal contents of horses is limited, compared with pet animals and farm animals (Mentula et al., 2005; Orban et al., 1997; Petr and Rada, 2001; Rada et al., 2006). Little has been known about lactic acid bacteria (Bailey et al., 2003; Yuyama et al., 2004), and particularly bifidobacteria (Mitsuoka and Kaneuchi, 1977). Moreover, all the knowledge has derived from data obtained only by the culturing technique. Monitoring the diversity of intestinal bacteria by the culturing technique is not only time-consuming but also difficult because of the presence of yet-to-be cultured bacteria or selection bias of media used. According to the estimates, up to 70% of microbes in the human intestine are considered to be uncultured (Suau et al., 1999). In contrast, culture-independent molecular techniques have recently emerged as reliable methods to analyze the bacterial diversity in feces and gastrointestinal contents. PCR-denaturing-gradient-gel-electrophoresis (PCR-DGGE) is a culture-independent technique to enable us to analyze microbial flora in environmental samples, and is frequently used to analyze the fecal flora (Heilig et al., 2002; Requena et al., 2002). Real-time PCR is also a culture-independent technique and is a precise quantification PCR through the measurement of the amount of PCR products by using a fluorescent probe in each cycle, and the real-time PCR with species, group, or genus-specific primers demonstrates targeted bacterial populations in samples (Gueimonde et al., 2004; Matsuki et al., 2004a; Selim et al., 2005). Moreover, a good correlation has been shown between the results determined by real-time PCR and by the culturing technique (Matsuki et al., 2004a).

In the present study, we demonstrated the diversity and the composition of the *Lactobacillus* group and the *Streptococcus* group in feces of six actively racing horses, Thoroughbreds, by using PCR-DGGE and real-time PCR with group-specific primer sets. Furthermore, the diversity and the composition of the *Bifidobacterium* group in the feces of the horses was also demonstrated, about which little had been known. The intestinal flora of lactic acid bacteria and bifidobacteria among the horses was compared. We also determined the stability of the intestinal flora of the same horse over 3 months.

Materials and Methods

Bacterial strains and culture conditions. The following bacterial strains were used as reference strains: *L. johnsonii* NRIC 0220^T, *Streptococcus equinus* NRIC 1139^T, and *Bifidobacterium breve* NRIC 0267. These strains were obtained from NODAI Culture Collection Center, Tokyo University of Agriculture, Japan. *L. johnsonii* NRIC 0220^T and *S. equinus* NRIC 1139^T were grown in MRS broth (Oxoid, Basingstoke, United Kingdom) at 37°C. *B. breve* NRIC 0267 was grown in BL broth (Eiken Kagaku, Tokyo, Japan) under anaerobic conditions at 37°C. After culturing, the cells of each strain were counted with a SLGC Bacteria Counter (Minato Medical, Tokyo, Japan) and a phase-contrast microscope.

Fecal samples. Fecal samples were collected from six healthy Thoroughbreds (subjects A through F) of different ages (2 to 7 years old) and sexes (one male and five females) at four stables from October through December in 2003. The Thoroughbreds were actively being raced when the samples were collected. Feces of Thoroughbred A were collected four times (A¹: October 3, A²: October 24, A³: November 12, and A⁴: December 18 in 2003). The Thoroughbreds were foraged two times a day, and the forage mainly consisted of oats, fresh apples, and fresh carrots. None of the Thoroughbreds had been suffering from any known gastrointestinal disease, and none had received antibiotics, probiotics, or prebiotics during the 2 weeks before sampling. Feces were collected immediately after defecation and brought to our laboratory under anaerobic conditions. The samples were stored at -80°C until use.

DNA extraction. DNA extraction from the feces and known amounts of cells of reference strains were performed by the method as described by Matsuki et al. (2004b), with a slight modification. In short, the feces (100 mg) or the cells of reference strains were washed three times with 1 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0) in order to remove PCR inhibitors. The washed sample was resuspended in a solution containing 250 µl of extraction buffer (100 mM Tris-HCl, 40 mM EDTA; pH 9.0), 50 µl of 10% sodium dodecyl sulfate, and 150 µl of benzyl chloride. One hundred milligrams of glass beads (0.1 mm in diameter) was added to the suspension, and the mixture was beaten at 2,500 rpm for 30 min with a Microincubator (Taitec, Saitama, Japan). One hundred fifty microliters

Table 1. Primers used in this study.

Primer	Sequence (5' to 3')	Target	Reference
Lac1	AGCAGTAGGGAATCTTCCA	<i>Lactobacillus</i> group 16S rDNA	Walter et al., 2001
Lac2GC	CGCCCGGGGCGCGCCCCGGGCGGCCCGGG GGCACCGGGGG-ATTYCACCGCTACACATG	Lactic acid bacterial 16S rDNA	Walter et al., 2001
Lac3	AGCAGTAGGGAATCTTCGG	<i>Streptococcus</i> group 16S rDNA	Endo and Okada, 2005
Lac2	ATTYCACCGCTACACATG	Lactic acid bacterial 16S rDNA	Walter et al., 2001
Bif164-f	GGGTGGTAATGCCGGATG	<i>Bifidobacterium</i> group 16S rDNA	Kok et al., 1996; Langendijk et al., 1995
Bif662-GC-r	CGCCCGCCGCGCGCGGGCGGGCGGGCGGG GGCACGGGGGG-CCACCGTTACACGGGAA	<i>Bifidobacterium</i> group 16S rDNA	Kok et al., 1996; Langendijk et al., 1995
Im26-f	GATTCTGGCTCAGGATGAACG	<i>Bifidobacterium</i> group 16S rDNA	Kaufmann et al., 1997
Im3-r	CGGGTGCTICCCACTTTCATG	<i>Bifidobacterium</i> group 16S rDNA	Kaufmann et al., 1997

of 3 M sodium acetate was added to the beaten sample, and the sample was cooled on ice for 15 min. The sample was centrifuged at $15,000\times g$ for 10 min. After centrifugation, the supernatant was transferred to a new tube, and DNA was precipitated with isopropanol. The precipitated DNA was suspended in 50 μ l of the TE buffer. For the feces, the DNA solution was diluted 10 times with TE buffer, and the dilution was used for PCRs.

PCR amplification. All PCR primers used in the present study are listed in Table 1. PCRs were carried out with a PTC-200 DNA Engine (MJ Japan, Tokyo, Japan). The diversity of the *Lactobacillus* group in the feces was analyzed with Lac1 and Lac2GC primers. This primer set is specific for the 16S rDNA of the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Weissella* (Walter et al., 2001). The group of these four genera was regarded as the *Lactobacillus* group in the present study. The reaction mixture was prepared according to Endo and Okada (2005) and the amplification program was as described by Walter et al. (2001). The diversity of the *Streptococcus* group in the samples was analyzed with Lac3 and Lac2GC primers. This primer set is specific for the 16S rDNA of the genera *Streptococcus*, *Lactococcus*, *Enterococcus*, *Tetragenococcus*, and *Vagococcus* (Endo and Okada, 2005). The group of these five genera was regarded as the *Streptococcus* group in the present study. The reaction mixture and amplification program were as described previously (Endo and Okada, 2005). The diversity of the *Bifidobacterium* group in the samples was analyzed with Bif164-f and Bif662-GC-r primers. The reaction mixture and amplification program were as described by Satokari et al. (2001). In addition,

nested-PCR was also carried out for analysis of the bifidobacterial flora in the samples. Im26-f and Im3-r primers were used for the first-round PCR, and Bif164-f and Bif662-GC-r primers were used for the second-round of PCR. The reaction mixtures and the amplification programs for both the primer sets were as described by Satokari et al. (2001). After the first-round of amplification (using Im26-f and Im3-r), PCR amplicons were purified by using Wizard SV Gel and a PCR Clean-up system (Promega, Madison, Wis.). Purified products were diluted 10 times in TE buffer, and 1 μ l of the diluted DNA was used as a template for the second-round PCR.

DGGE analysis and excision of DNA fragment. DGGE analysis of each PCR product was carried out with a DCode System (Bio-Rad, Hercules, Calif.). The DGGE analyses of the *Lactobacillus* group, the *Streptococcus* group, and the *Bifidobacterium* group were carried out by using 8% (wt/vol) polyacrylamide gels with denaturing gradients ranging from 35% to 50%, from 37.5% to 52.5%, and from 45% to 60%, respectively, and the electrophoresis were performed in Tris-acetate-EDTA buffer for 14 h at constant voltages of 60, 80, and 80 V for each group. A 100% denaturant corresponds to 7 M urea and 40% formamide. After electrophoresis, gel staining and excision of bands in the gels were carried out by the methods as described previously (Endo and Okada, 2005). The DNAs in the bands were reamplified by using the same primer sets as used for generating the DGGE samples.

Sequence analysis. The PCR products derived from the DGGE bands were purified and sequenced by the method as described previously (Endo and Okada, 2005). Similarities between sequences of the

Table 2. Similarities of bands obtained by DGGE profiles of horse feces.

Figure	Band	Closest relatives	% similarity	Accession no.
1	1	<i>L. johnsonii</i>	99	AB250236
	2	<i>L. equi</i>	100	AB250237
	3	<i>W. confusa</i> , <i>W. cibaria</i>	99	AB250238
	4 ^a	Uncultured eubacterium clone (AY212750)	99	AB250239
		<i>L. salivarius</i>	99	
	5	<i>L. gastricus</i>	98	AB250240
2	6 ^b	Not identified	—	AB250241
	7	<i>E. casseliflavus</i>	99	AB250242
	8 ^b	Not identified	—	AB250243
	9	<i>S. bovis</i> , <i>S. equinus</i> , <i>S. infantarius</i> , <i>S. criceti</i> , <i>S. hyointestinalis</i>	96	AB250244
	10	<i>S. bovis</i> , <i>S. equinus</i> , <i>S. macedonicus</i> , <i>S. alactolyticus</i> , <i>S. gallolyticus</i> , <i>S. intestinalis</i> , <i>S. pasteurii</i>	96	AB250245
	11	<i>S. equi</i>	99	AB250246
	12	<i>S. bovis</i> , <i>S. equinus</i>	99	AB250247
	13	<i>S. bovis</i> , <i>S. equinus</i>	100	AB250248
	14	<i>S. bovis</i> , <i>S. equinus</i>	99	AB250249
3	15	<i>B. urinalis</i>	97	AB250250
	16	<i>B. urinalis</i>	97	AB250251
	17	<i>P. denticolens</i>	99	AB250252
	18	<i>P. denticolens</i>	99	AB250253
	19	<i>B. boum</i>	99	AB250254

L.: *Lactobacillus*, *W.*: *Weissella*, *E.*: *Enterococcus*, *S.*: *Streptococcus*, *B.*: *Bifidobacterium*, *P.*: *Parascardovia*.

The number of the bands corresponds to those in Figs. 1 through 3.

^aAs the closest relative in GenBank was an uncultured eubacterial clone (AY212750), similarity to *L. salivarius*, which was the most-related known species, was also shown.

^bThe closest relatives and sequence similarity were not able to be shown because of too low similarity with all sequences in Database.

isolated DNAs and those deposited at GenBank were determined by using Blast analysis (Altschul et al., 1990). When the similarity was less than 98% between the sequence that we determined and those of the most phylogenetically related species, the determined sequence is regarded as a phylogenetic relative of a known species.

Nucleotide sequence accession numbers. Accession numbers of the DNA sequences in the DGGE bands derived from the feces are listed in Table 2.

Calculation of the cell number of each group. The cell number of the *Lactobacillus* group and the *Streptococcus* group was determined on the samples by using real-time PCR assay with a LightCycler-Fast-Start DNA Master SYBR Green I (Roche Diagnostics, Mannheim, Germany). Lac1 and Lac2 primers were

used for real-time PCR of the *Lactobacillus* group, and Lac3 and Lac2 primers were used for the *Streptococcus* group. Each reaction mixture (10 µl) was prepared according to the manufacturer's instructions but for 3 mM MgCl₂. The amplification conditions comprised initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 61°C for 5 s, and 72°C for 15 s. DNAs extracted from known amounts of cells of *L. johnsonii* NRIC 0220^T and *S. equinus* NRIC 1139^T were used as references for making standard curves for the *Lactobacillus* group and the *Streptococcus* group.

The cell number of the *Bifidobacterium* group in the samples was estimated from the detection limits of the two types of PCR performed for PCR-DGGE analysis of the *Bifidobacterium* group. In short, the detection

limits for single-PCR (using Bif164-f and Bif662-GC-r) and nested-PCR (using Im26-f-Im3-r and Bif164-f-Bif662-GC-r) were determined by using the DNA extracted from the known cell number of *B. breve* NRIC 0267. The reaction mixtures and amplification programs were as the same as those used for DGGE analysis. The amplification products were subjected to gel electrophoresis in 1.5% agarose, followed by ethidium bromide staining.

Results

DGGE analysis of the diversity of the *Lactobacillus* group in the feces

The diversity of the *Lactobacillus* group in the feces was determined by using PCR-DGGE. The four samples from Thoroughbred A (Fig. 1, lanes from A1 through A4) yielded similar band patterns for the samples A², A³, and A⁴ (Fig. 1, lane A2 to A4), but those for A¹ varied slightly. Sample A¹ lacked band nos. 3 and 4 on the DGGE gel (Fig. 1, lane A1), but these bands were detected with high intensity on the samples from A² through A⁴ (Fig. 1, lanes A2 to A4). Sequence analyses of the DNAs extracted from the two bands indicated that one was a phylogenetic rela-

tive of *L. salivarius* and the other was *Weissella confusa*/*Weissella cibaria* (Table 2). *L. johnsonii*, *L. equi*, and a phylogenetic relative of *Lactobacillus gastricus* were also detected on the samples originating from Thoroughbred A (Fig. 1, Table 2). In particular, *L. equi* and a phylogenetic relative of *L. gastricus* were detected on all samples tested of Thoroughbred A. The Thoroughbreds from B through F yielded DGGE profiles similar to those of Thoroughbred A (Fig. 1), with the same species detected. Moreover, a phylogenetic relative of *L. gastricus* was detected on all the feces tested.

DGGE analysis of the diversity of the *Streptococcus* group in the feces

The diversity of the *Streptococcus* group in the feces was determined by using PCR-DGGE. Sequence data and the DGGE profiles demonstrated that *S. bovis*/*S. equinus* was predominant in most of the feces of Thoroughbred A (Fig. 2, Table 2). Because of the difficulty in the classification between *S. bovis* and *S. equinus* by using 16S rDNA sequences, we were unable to identify the DNA originating from *S. bovis*/*S. equinus* precisely. *S. bovis*/*S. equinus* was also predominant in the feces of Thoroughbreds B through F (Fig. 2), and a

Fig. 1. DGGE profiles of the PCR products of the *Lactobacillus* group amplified with Lac1 and Lac2GC primers from the feces tested.

Lanes from A1 through A4 correspond to the feces from A1 through A4, and lanes B through F correspond to the feces of Thoroughbreds B through F, respectively. The bands labeled 1 through 5 are described in Table 2.

Fig. 2. DGGE profiles of the PCR products of the *Streptococcus* group amplified with Lac3 and Lac2GC primers from the feces tested.

Lanes A1 through A4 correspond to the feces from A1 through A4, and lanes B through F correspond to the feces of Thoroughbreds B through F, respectively. The bands labeled 6 through 14 are described in Table 2.

phylogenetic relative of *S. bovis*/*S. equinus* group (Fig. 2, band no. 9) was detected on some of the samples tested.

DGGE analysis of the diversity of the *Bifidobacterium* group in the feces

An analysis of the diversity of the *Bifidobacterium* group in the feces was attempted by using DGGE after single-PCR with Bif164-f and Bif662-GC-r. However, no amplicons were produced from any samples tested by using the single-PCR (data not shown), and it would be due to the detection limit of the single-PCR. Nested-PCR conjugated with DGGE analysis is often used for detection of minor organisms with an increase of detection sensitivity (Nielsen et al., 2003). Therefore, the nested-PCR was carried out for analysis of the diversity of the *Bifidobacterium* group in the feces. Primers Im26-f and Im3-r were used for the first-round PCR, and primers Bif164-f and Bif662-GC-r were used for the second-round PCR. As a result, amplicons were yielded from six of the nine samples tested by the nested-PCR. The PCR products were subjected to DGGE analysis. All samples of Thoroughbred A yielded a high-intensity band (Fig. 3, band no. 17), and

sequence analysis revealed that the band was derived from *Parascardovia denticolens* (Table 2). This species had been formerly classified as *Bifidobacterium denticolens* (Crociani et al., 1996), and it was later reclassified as *P. denticolens* (Jian and Dong, 2002). However, as the partial 16S rDNA of *P. denticolens* is amplified by using PCR with Bif164-f and Bif662-GC-r (Satokari et al., 2001), this species is regarded as a member of the *Bifidobacterium* group in the present study. The DGGE profiles indicated that *P. denticolens* was predominant in the *Bifidobacterium* group in the feces of Thoroughbred A. *P. denticolens* was also detected with high-intensity on the sample of Thoroughbred C. In addition, two kinds of phylogenetic relatives of *Bifidobacterium urinalis* were obtained from the samples A³ and A⁴, and *Bifidobacterium boum* was from Thoroughbred F (Fig. 3, Table 2). Compared with determined sequences of the two kinds of phylogenetic relatives of *B. urinalis* (Fig. 3, band nos. 15 and 16), eight base mismatches were found between them. Therefore, these two bands would have been derived from two different phylogenetic relatives of *B. urinalis*.

Quantification of the *Lactobacillus* group and the *Streptococcus* group in the feces by real-time PCR assay

In order to quantify the cell number of the *Lactobacillus* group, DNA extracted from serial dilutions from 10^0 to 10^8 cells per ml of *L. johnsonii* NRIC 0220^T was used as a reference. The standard curve was found to be linear over the range of cell concentrations from 10^2 to 10^8 cells per ml (data not shown). This result indicated that the linear range for the procedures used in the present study is $>10^4$ cells per gram of the feces because 100 mg of the feces were used for DNA extraction, and the extracted DNA was diluted 10 times for PCR. Almost identical data were obtained for the *Streptococcus* group by using the DNA extracted from a known cell number of *S. equinus* NRIC 1139^T.

As shown in Table 3, the cell number of the *Lactobacillus* group (mean $\pm$ standard deviation) was $8.47 \pm 0.62 \log_{10}$ cells per gram of the feces (wet weight) which was similar to that for the *Streptococcus* group ($8.50 \pm 0.43 \log_{10}$). The predominance of *S. bovis*/*S. equinus* in almost all the samples tested (Fig. 2 and Table 2) means that *S. bovis*/*S. equinus* accounts for most of the *Streptococcus* group in the feces tested. The cell number of the *Lactobacillus* group in the sample A¹ was slightly less than those of the other sam-

Fig. 3. DGGE profiles of the PCR products of the *Bifidobacterium* group amplified by using nested-PCR from the feces tested.

Lanes A1 through A4 correspond to samples from A1 through A4, and lanes B through F correspond to the feces of the Thoroughbreds B through F, respectively. The bands labeled 15 through 19 are described in Table 2.

ples of Thoroughbred A. The cell number of the *Lactobacillus* group and the *Streptococcus* group determined by using real-time PCR was similar to those obtained by the culturing technique, which were $8.16 \pm 1.90 \log_{10}$ and $8.5 \pm 0.8 \log_{10}$, respectively (Mitsuoka and Kaneuchi, 1977; Yuyama et al., 2004).

Estimation of the population of the *Bifidobacterium* group in the feces

The cell number of the *Bifidobacterium* group in the feces was estimated by determination of the detection limits of both the single- and the nested-PCR assays. The DNA extracted from a known cell number of *B. breve* NRIC 0267 was used as a reference. Figure 4a and 4b show that the detection limits for the single- and nested-PCR assays were 10^1 and 10^3 cells per ml, respectively, corresponding to 10^3 and 10^5 cells per gram of the feces. Considering the calculated detection limits in the present study, the cell number of the *Bifidobacterium* group per gram of the feces (wet weight) ranged from 10^3 to 10^5 among the samples from Thoroughbreds A, C, and F (Table 3) because of the non-detection by using single-PCR and detection by using nested-PCR as described above. Furthermore, the cell number of the *Bifidobacterium* group in the samples from Thoroughbreds B, D, and E was estimated to be $<10^3$ cells per gram of the feces (wet weight; Table 3) because they were not detected by either PCR assay.

Discussion

In the present study, we demonstrated the diversity and the composition of lactic acid bacteria and bifidobacteria in the feces of the 6 Thoroughbreds by

using molecular techniques.

PCR-DGGE with group-specific primers revealed

(a). Single-PCR

(b). Nested-PCR

Fig. 4. Detection limits of the *Bifidobacterium* group with specific single-PCR (a) and nested-PCR (b) assays, as determined by using DNA extracted from *Bifidobacterium breve* NRIC 0267.

Lane M, 100 bp DNA ladder; lane 1, 10^8 cells per ml; lane 2, 10^7 cells per ml; lane 3, 10^6 cells per ml; lane 4, 10^5 cells per ml; lane 5, 10^4 cells per ml; lane 6, 10^3 cells per ml; lane 7, 10^2 cells per ml; lane 8, 10^1 cells per ml; lane 9, 10^0 cells per ml.

Table 3. The cell number of each lactic acid bacterial group in horse feces.

	Log ₁₀ cells/g of feces (wet weight) for horses									Mean±SD
	A ¹	A ²	A ³	A ⁴	B	C	D	E	F	
<i>Lactobacillus</i> group ^a	7.50	8.91	8.72	8.63	9.03	9.27	7.62	8.55	8.03	8.47±0.62
<i>Streptococcus</i> group ^a	8.66	8.79	8.60	8.67	7.72	8.87	7.81	8.83	8.52	8.50±0.43
<i>Bifidobacterium</i> group ^b	3≤cells≤5	3≤cells≤5	3≤cells≤5	3≤cells≤5	<3	3≤cells≤5	<3	<3	3≤cells≤5	— ^c

^a We used real-time PCR to determine the cell number of the *Lactobacillus* group and the *Streptococcus* group.

^b We used the detection limits of single- and nested-PCR assays to estimate the cell number of the *Bifidobacterium* group.

^c —, not determined.

that the predominant members of the *Lactobacillus* group community in the feces tested were those shown in Fig. 1 and Table 2. Of the five species detected of the *Lactobacillus* group, to our knowledge, *L. equi* has been isolated only from horse feces (Morotomi et al., 2002), a phylogenetic relative of *L. salivarius* has been detected only on horse manure (Simpson et al., 2004; accession no. AY212750), and a phylogenetic relative of *L. gastricus* has never been isolated from or detected on any other samples. Moreover, the phylogenetic relative of *L. gastricus* was detected on all of the samples tested (Fig. 1, Table 2). These findings would indicate that the *Lactobacillus* group community in horse feces is not similar to those in any other animal feces. *Lactobacillus agilis*, *L. crispatus*, *L. mucosae*, and *L. reuteri* have been isolated from feces and gastrointestinal contents of horses by culturing techniques (Bailey et al., 2003; Morotomi et al., 2002), but they were not detected by PCR-DGGE in the present study. This discrepancy may be due to a difference of techniques used or individual differences in horses. In fact, of the above four *Lactobacillus* species reported, only *L. mucosae* was detected in the two of six horses tested by *L. mucosae*-specific-PCR analysis (data not shown).

Of the *Streptococcus* group, *S. bovis*/*S. equinus* was predominantly detected on almost all samples tested (Fig. 2, Table 2). *S. bovis* is a normal inhabitant in gastrointestinal contents of many animals (Hungate, 1966), and the organism has been reported to represent 2% of the total clones in a cloning analysis of 16S rDNA in horse colonic contents (Daly et al., 2001), and predominantly isolated from horse cecal contents (Bailey et al., 2003). Our data would support these reports. In the present study, several bands originating from *S. bovis*/*S. equinus* (Fig. 2, bands nos. 12, 13, and 14) were found on the DGGE gel. This finding has been reported previously (Meroth et al., 2003).

Little is currently known about the bifidobacterial community in feces and/or gastrointestinal contents of horses. We were unable to detect the *Bifidobacterium* group by using single-PCR but detected it in three of the six horses tested by using nested-PCR. We confirmed that nested-PCR DGGE analysis was 100 times more sensitive than single-PCR DGGE analysis (Fig. 4). Of the four species in the *Bifidobacterium* group, *P. denticolens* was detected with high-intensity in two of the six horses tested (Fig. 3, Table 2). Moreover, this species was detected on all samples of Thoroughbred

A (Fig. 3). This might suggest that *P. denticolens* colonized in the gastrointestinal tract of Thoroughbred A. Only one previous study addressed isolation of the bifidobacteria in horse feces at the genus level (Mitsuoka and Kaneuchi, 1977). The present study is the first to seek the organism at the species level, and we have found that nested-PCR DGGE with group-specific primers is a useful tool for studying the bifidobacterial diversity in horse feces.

The cell number of the *Lactobacillus* group and the *Bifidobacterium* group ranged from 10^7 to 10^9 and from 10^3 to 10^5 or $<10^3$ cells per gram of the feces, respectively. Total bacterial count ranged from 10^9 to 10^{10} cells per gram of horse feces (Yuyama et al., 2004). Considering our data and the preceding report together, the *Lactobacillus* group would account for a major part of fecal bacterial flora of horses, and the *Bifidobacterium* group for a minor part. The cell number of fecal total bacteria of various pet animals and farm animals, such as calves, chickens, dogs, and pigs, was similar to that of horses (Mentula et al., 2005; Orban et al., 1997; Rada et al., 2006; Selim et al., 2005), and the cell number of the *Lactobacillus* group of the animals was similar to or slightly higher than that of horses. However, the *Bifidobacterium* group accounted for a major part of the fecal bacterial flora of the animals (Mentula et al., 2005; Orban et al., 1997; Petr and Rada, 2001; Rada et al., 2006), and this finding is quite different from result of the horses determined in the present study. This difference may be due to the absence of bifidus factor(s) in milk and/or intestinal mucin of horses. In fact, two oligosaccharides originating from human milk and/or mucin have been recently reported to have a possibility of bifidus factors in human intestinal tracts (Katayama et al., 2005; Kitaoka et al., 2005).

Active racehorses take different forages to alter energy intake and to maintain their body weight before/after racing. Thoroughbred A was entered in races two times and the forage was changed during our sampling of the feces. The diversity and the composition of the *Lactobacillus* group, the *Streptococcus* group, and the *Bifidobacterium* group remained stable throughout the test period, except for the *Lactobacillus* group of sample A¹. Both the number of species detected and the cell number of the *Lactobacillus* group on sample A¹ was lower than A² to A⁴ (Fig. 1, Table 3). These results might reflect a difference in the forage during the four sampling times.

Recently, there have been a few attempts to prevent diarrhea in horses or to shorten its term by using lactic acid bacteria as probiotics (Parraga et al., 1997; Weese and Rousseau, 2005). However, most of these trials have lacked a significant beneficial effect (Parraga et al., 1997; Weese and Rousseau, 2005). It may be caused by the lack of microbial knowledge of feces and/or gastrointestinal contents of horses. We believe that particularly the knowledge of the community of lactic acid bacteria is essential for effective probiotic treatments because our study leads to the conclusion that the diversity and the composition of the *Lactobacillus* group and the *Bifidobacterium* group in horse feces were not similar to those of any other animals.

In the present study, we studied the diversity and the composition of the *Lactobacillus* group, the *Streptococcus* group, and the *Bifidobacterium* group in the feces of the actively raced Thoroughbreds by using PCR-DGGE and real-time PCR with each group-specific primer set. Knowledge of lactic acid bacterial and bifidobacterial populations, particularly bifidobacterial diversity, has been limited for horse feces until now. Thus, the findings of the diversity and the composition of these organisms in the feces determined in the present study will give a great deal of information on further application, such as probiotics for horses.

Acknowledgments

We thank all concerned parties of the Seishi, Yuasa, and Setoguchi stables of the Japan Racing Association and the Muneishi stable of the Kochi Racing Association, as well as Masatake Iida, owner of Chiyoda Farm, Minoru Matsumoto of Japan Race Horse Registry, Keisuke Shimamura of KK Best-sellers, and Masahiro Nagayama of Kochi Racing Association for their help on collection of horse fecal samples. We also thank Kazuo Komagata for valuable discussions and Chihiro Obata (NODAI Culture Collection Center, Faculty of Applied Bioscience, Tokyo University of Agriculture) for technical assistance.

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