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Lactobacillus and *Bifidobacterium* Diversity in Horse Feces, Revealed by PCR-DGGE

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Abstract *Lactobacillus equi*, *Lactobacillus hayakitensis*, *Lactobacillus johnsonii*, and *Weissella confusa/cibaria* were the dominant species in 12 South African horses. The *Bifidobacterium*-group was detected in the feces of only one of the 12 horses. Sequencing of the nested-PCR amplicon identified the *Bifidobacterium*-group as *Parascardovia denticolens*. Cell numbers of *L. equi*, *L. hayakitensis*, and *W. confusa/cibaria* were consistent in all samples. *P. denticolens*, *Bifidodobacterium pseudolongum*, and a phylogenetic relative of *Alloscardovia omnicolens* were rarely detected. *L. equigenerosi*, a dominant species in Japanese horses, was detected in the fecal samples of only one horse.

Introduction

Since the first health benefits encountered by individuals that consumed certain strains of lactic acid bacteria in fermented milk [14], many lactic acid bacteria with probiotic properties have been described [17]. The probiotic application for humans varies from the prevention of infectious diseases [19], curing of irritable bowel syndrome, alleviation of allergies, digestion of lactose, and lowering of serum cholesterol levels [2], to the prevention of cancer [9]. In general, probiotic lactic acid bacteria do not cause immunological side effects [20]. However, *Lactobacillus casei* has been associated with symptoms of fever, arthritis, and hepatobiliary lesions in humans [22]. Symptoms such as these may be caused by cell wall components such as peptidoglycans that elicit cytokines [15]. Immunological side effects are often caused by cells that invade epithelial cells, migrate through mucus [24], and degrade mucus [20].

The human and animal gastrointestinal tracts are host to hundreds of bacterial species. Most research on intestinal microbiota has been done on humans [20] and ruminants [23, 28], but little is known about the microbiota in the equine gut, despite their important role in digestion. The bacteria, fungi, protozoa, and archaea in the equine hindgut are mostly anaerobic with strong cellulolytic activity. Celluloses are fermented to soluble sugars, which in turn are fermented to short chain fatty acids (SCFA) such as acetate, propionate, and butyrate. These SCFA are absorbed across the large intestinal epithelium and provide 60–70% of the horse's energy [5].

Although *Lactobacillus* and *Bifidobacterium* spp. are regarded as beneficial to the general health of the host [17], not all lactic acid bacteria fall in the same category. *Lactobacillus delbrueckii*, *L. fermentum*, *L. mucosae*, *L. reuteri*, and *L. salivarius* are known to decarboxylate amino acids and have been implicated in equine laminitis [3, 16]. *Lactobacillus pentosus* WE7, isolated from the intestinal tract of horses, failed to prevent neonatal diarrhea in foals [27]. Instead, the strain stimulated the development of diarrhea and additional clinical abnormalities [27].

Bacteria–host interactions are specific. *Lactobacillus rhamnosus* strain GG (LGG), isolated from human feces and a well known probiotic, does not colonize in the intestinal tract of horses [26]. *Lactobacillus salivarius*, isolated from horse stomach, adhered to horse stomach tissue but not rat stomach [29]. Yuyama et al. [30] have shown that treatment of horses with *L. crispatus*, *L. equi*, *L. johnsonii*, and *L. reuteri*, which originated from horses, prevented diarrhea and enhanced growth. The 16S rRNA

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sequences recorded for 89% of the bacteria isolated from horses did not correspond to any known sequence, suggesting that the equine intestine may contain a number of yet-to-be cultured bacteria [5].

Culture-independent techniques based on PCR and PCR-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) yield much more information on the diversity of microbiota. Furthermore, by performing real-time PCR (RT–PCR), the numbers of cells present for each genus or species can be determined. The aim of this study was to determine the *Lactobacillus* and *Bifidobacterium* population in horse feces by using PCR-DGGE and RT-PCR.

Materials and Methods

Reference Strains and Culture Conditions

The following strains were used as reference strains: *Lactobacillus equi* U6, *Lactobacillus equigenerosi* NRIC 0697^T, *Lactobacillus hayakitensis* U46, *Lactobacillus johnsonii* NCFB 2241^T, and *Weissella confusa* SU2. All these strains, except *L. johnsonii* NCFB 2241^T, were isolated from horse feces [8] and were grown in MRS broth (Biolab, Biolab Diagnostics, Midrand, South Africa) at 37°C.

Fecal Samples

Fecal samples were collected from 12 healthy horses (5 stallions and 7 mares, ranging from 4 to 15 years old), stabled at Welgevallen experimental farm, Stellenbosch, South Africa. All horses were free from intestinal infections and did not receive antibiotics, probiotics, or prebiotics. The horses were fed barley hay, supplemented with commercial feed containing bran, maize, oat, and minerals. All animals were fed twice a day (9 h apart). Six horses were selected from which fecal samples were collected over a period of three months. Feces were collected immediately after defecation and were stored at −20°C until used.

DNA Extraction

DNA extraction from fecal samples and reference strains was performed by the method described previously [7], modified by using FastPrep FP120 (Savant Instruments, Farmingdale, NY, USA) for cell disruption, according to instructions of the supplier. DNA extracted from fecal samples was diluted ten times with TE buffer before subjected to PCR amplification.

PCR Amplification

All PCR primers used in this study are listed in Table 1. The *Lactobacillus* group community was analyzed with Lac1 and Lac2GC primers described by Walter et al. [25]. The reaction mixture was prepared as described by Endo and Okada [6]. DNA amplification was done according to the method of Walter et al. [25]. The community of the *Bifidobacterium*-group was analyzed with single-PCR and nested-PCR, as described previously [7]. For single-PCR, primers bif164-f and bif662-GC-r were used. For nested-PCR, primers Im26-f and Im3-r were used for the first-round PCR, and primers bif164-f and bif662-GC-r for the second-round PCR. After the first-round PCR, amplicons were purified by using the QIA quick PCR purification kit (QIAGEN Inc., Valencia, USA). Purified products were diluted ten times with TE buffer and the purified DNA was used as a template for the second-round PCR. The reaction mixture and the amplification program used for PCR of the *Bifidobacterium* group were as described by Satokari et al. [21].

DGGE Analysis and Excision of DNA Fragments

DGGE analysis of each PCR product was conducted with a DCode System (Bio-Rad Laboratories, Hercules, CA, USA) as described previously [7]. The denaturant gel gradient ranged from 35 to 50% for the *Lactobacillus*-group and from 45 to 60% for the *Bifidobacterium*-group. Electrophoresis was performed in Tris–acetate–EDTA

Table 1 Primers used in this study

Primer	Sequence (5′ to 3′)	Reference
Lac1	AGCAGTAGGGAATCTTCCA	[25]
Lac2GC	CGCCCGGGGCGCGCCCCGGGCGGCCCGGGGGCACCGGGGG–ATTYCACCGCTACACATG	[25]
Bif164-f	GGGTGGTAATGCCGGATG	[13]
Bif662-GC-r	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG–CCACCGTTACACCGGGAA	[21]
Im26-f	GATTCTGGCTCAGGATGAACG	[12]
Im3-r	CGGGTGCTICCCACTTTCATG	[12]
S-G-Lab-0677-a-A-17	CACCGCTACACATGGAG	[10]

buffer for 14 h at a constant voltage at 60 V. Gels were stained for 30 min with SYBR green I nucleic acid gel stain (BioWhittaker Molecular Applications, Rockland, ME, USA). Excision of bands from the gel was performed using sterilized toothpicks as described previously [6]. For the *Lactobacillus*-group, PCR amplicons of *L. equi*, *L. equigenerosi*, *L. hayakitensis*, *L. johnsonii*, and *W. confusa* with Lac1 and Lac2GC primers were used as markers of migration distances. Bands produced at the same migration distances to the reference strains were identified as the species. DNA bands that did not match the migration pattern were re-amplified by using the same primer set as used for generating the DGGE samples.

Sequence Analysis

The PCR products of the re-amplification products were purified by using a QIA quick PCR purification kit (QIAGEN Inc., Valencia, USA). Sequencing was according to the method described previously [6]. Blast analysis was used to determine similarities between sequences of the isolated DNA and those deposited at GenBank [1]. Accession numbers for the sequences were AB491611 to AB491613.

Calculation of the Number of Cells in the *Lactobacillus*-Group

The number of cells in the *Lactobacillus*-group present in each sample was determined by using RT-PCR with SYBR Green JumpStart™ Taq ReadyMix™ (Sigma, Missouri, USA) in a LightCylcler (Roche Diagonostics, Mannheim, Germany). The primer set Lac1 and S-G-Lab-0677-a-A-17 described by Rinttilä et al. [18] was used for the *Lactobacillus* group. The reaction mixture was prepared according to instructions of the supplier. Amplification was as follows: initial denaturation at 95°C for 30 s and 40 cycles of 94°C for 0 s, 58°C for 10 s, and 72°C for 15 s. DNA extracted from known amounts of cells of *L. johnsonii* NCFB 2241^T was used as reference in preparing a standard curve. Cell counts of *L. johnsonii* NCFB 2241^T was determined by serial dilution in saline and plating on MRS agar. The plates were incubated at 37°C for 3 days under anaerobic conditions using a gas generating kit (Anaerobic system BR0038B, Oxoid Ltd., Basingstoke, Hants, UK).

Results

Separation of PCR amplicons by DGGE revealed several DNA bands for each of the 12 fecal samples (Fig. 1). Five of the DNA bands corresponded with the migration profile of the standard DNA (lane M), indicating that *L. johnsonii*, *L. equi*, and *W. confusa/cibaria* are the predominant species present in the fecal samples analyzed. *L. hayakitensis* was detected in seven out of 12 samples, and *L. equigenerosi* in only one sample (sample no. 9). Similar fecal microbiota was recorded among individual horses.

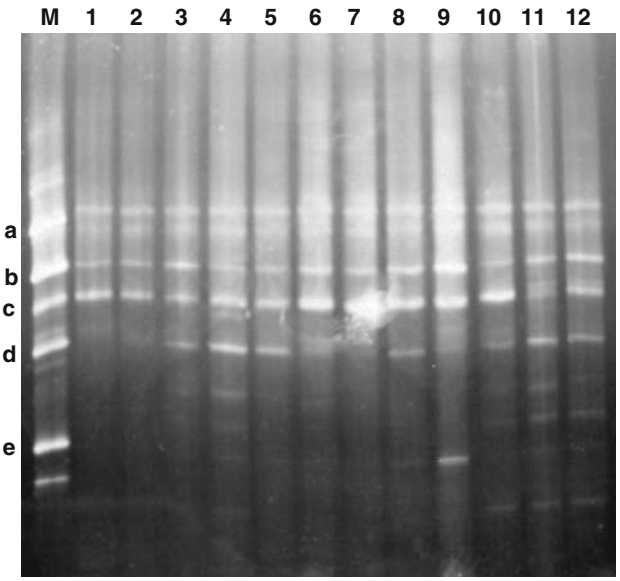

Fig. 1 DGGE profiles of the *Lactobacillus* group in horse feces (numbered 1–12). *M* a combination of DNA from *L. equi*, *L. equigenerosi*, *L. hayakitensis*, *L. johnsonii*, and *W.cibaria.* Labeled bands with letters "*a*" to "*e*" were allotted to the following species: *a*: *L. johnsonii*, *b*: *L. equi*, *c*: *W. cibaria*, *d*: *L. hayakitensis*, *e*: *L. equigenerosi.* The gel was stained with SYBR Green I nucleic acid gel stain (BioWhittaker Molecular Applications)

No PCR amplicons were generated for bifidobacteria by single-PCR. However, nested-PCR produced an amplicon from sample no. 6 and DGGE profile generated a single band from the sample. The DNA band in the DGGE gel was excised with sterile toothpicks and re-amplified by PCR using the bif164-f and bif662-GC-r primer set. Sequence analysis of PCR amplified DNA revealed the amplicon originated from *Parascardovia denticolens* (Accession no. AB491611).

Concluded from the DGGE profiles, the *Lactobacillus* flora in the six horses remained stable during the 3-month test period. *L. equi*, *L. hayakitensis*, and *W. confusa/cibaria* were detected in more than 80% of samples analyzed. *L. johnsonii* and *L. equigenerosi* were detected in 40 and 20% of samples analyzed, respectively. Other *Lactobacillus* spp. have not been detected.

P. denticolens was detected in one or two time-course samples from horses numbered 2, 3, 6, 9, and 11 as bifidobacteria by DGGE analysis. *Bifidobacterium pseudolongum* was detected in a time-course sample of horse no. 7 (Accession no. 491613). A phylogenetic relative of *Alloscardovia omnicolens* (98% sequence similarity) was

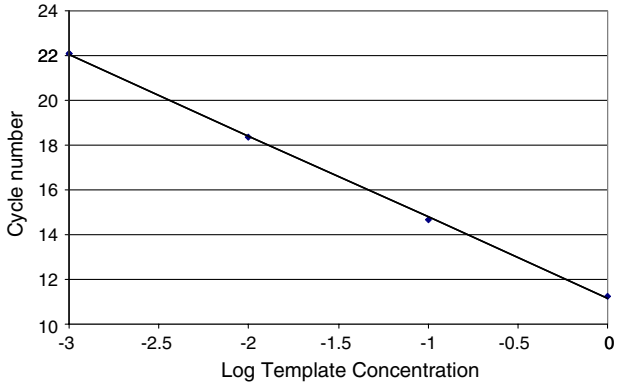

Fig. 2 Standard curve of real-time PCR. *L. johnsonii* NCFB 2241^T was used as a reference strain. $y = -3.624x + 11.159$

detected in a time-course sample of horse no. 11 (Accession no. 491612), which had a 100% similarity to the DNA sequence detected from the feces of a Japanese horse (Accession no. AB250251) [7].

A standard curve for real-time PCR, obtained with reference strain *L. johnsonii* NCFB 2241^T is shown in Fig. 2. The number of cells ($\log_{10}$) recorded for *Lactobacillus* present in samples numbered 1–12 were 8.25, 7.34, 7.13, 6.95, 7.78, 8.12, 6.83, 7.85, 8.11, 7.12, 7.07, and 7.35 cells g^{-1} of feces, respectively. The mean ± standard deviation (SD) was 7.49 ± 0.50. The numbers of cells ($\log_{10}$) detected in time-course samples were stable and ranged from 6.85 to 7.30 for horse no. 2, from 6.64 to 7.28 for no. 3, from 7.47 to 8.17 for no. 6, from 6.57 to 7.15 for no. 7, from 7.43 to 7.77 for no. 9, and from 6.86 to 7.33 for no. 11.

Discussion

The diversity of *Lactobacillus* and *Bifidobacterium* spp. in horse feces is described using group-specific primers. PCR-DGGE with each primer set revealed target bacterial diversity. The *Lactobacillus* population in the feces of each horse was almost identical. *L. johnsonii*, *L. equi*, *L. hayakitensis*, and *W. confusa/cibaria* were predominant. These species have also been reported as dominant in feces of Japanese racehorses [7]. *L. equigenerosi*, a dominant species in Japanese horses [8], is a minority species in South African horses and was detected in the feces of only one of the 12 horses by DGGE. Cell numbers ($\log_{10}$) recorded for the *Lactobacillus* group ranged from 6.83 to 8.25 (mean SD = 7.49 ± 0.50). This was slightly lower than that the numbers recorded for Japanese horses (8.47 ± 0.62) [7]. This may be due to differences in forages and the environment. The Japanese horses were racehorses kept in stables and had several hard trainings [7].

No species allocated to the *Bifidobacterium* group were found by single-PCR, but a few species were detected by nested-PCR, suggesting that cell number of bifidobacteria was $<10^3$ g^{-1} of feces or $10^3 <$ cells $< 10^5$ g^{-1} of feces. This conclusion is based on limitations in the detection of single-PCR (10^3 cells g^{-1} of feces) and nested-PCR (10^1 cells g^{-1} of feces) as described previously [7]. *P. denticolens* was detected as a *Bifidobacterium*-group species in fecal samples from horse no. 6. *P. denticolens* was also detected in the feces of time-course samples collected from horse nos. 2, 3, and 6. This species has been formerly classified as *Bifidobacterium denticolens* [4], and it was later reclassified as *P. denticolens* [11]. Strains of the species have been detected in two out of six fecal samples collected from Japanese horses [7], suggesting that *P. denticolens* is a normal inhabitant of the horse GIT. This is an interesting characteristic, since *P. denticolens* had previously only been detected in human oral cavities. In time-course samples taken from horse no. 11, a phylogenetic relative of *A. omnicolens* was detected. The sequence shared 100% similarity to the sequence detected in feces of Japanese horse (Accession no. AB250251) [7]. This suggests that the species is a normal inhabitant of horses. *B. pseudolongum* was found in horse feces for the first time.

L. delbrueckii, *L. fermentum*, *L. mucosae*, *L. reuteri*, and *L. salivarius*, associated with equine laminitis [3], were not detected in the present study. These species are recorded in horses that have been on a high carbohydrate diet [3]. An excess of forage may thus lead to the disruption of complex and well-balanced microbiota, resulting in laminitis.

Lactobacillus spp. detected in the feces of South African horses have also been described in the feces of Japanese horses [7], suggesting that certain species are predominantly present, irrespective of the breed, forage, or environmental conditions. Further research is needed to develop strains of *L. johnsonii*, *L. equi*, *L. hayakitensis*, and *W. confusa/cibaria* into probiotics. *B. pseudolongum*, *P. denticolens*, and the phylogenetic relative of *A. omnicolens* are in the minority and may not play any role as probiotics.

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