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Molecular monitoring of the bacterial community structure in foal feces pre- and post-weaning

Céline Faubladier*, Sophie Sadet-Bourgeteau, Christelle Philippeau, Emmanuel Jacotot, Véronique Julliand

Agrosup Dijon, USC-INRA Nutrition du cheval athlète, Dijon, France

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ABSTRACT

This study assessed the time-scale variability of bacterial community structure in foal feces from birth to 365 days of age using Automated Ribosomal Intergenic Spacer Analysis (ARISA). Fecal samples were collected from five foals 2 h after birth (meconium) and in the morning at days 1, 2, 5, 10, 30, 60, 120, 179, 183, 194 and 365. The ARISA profiles were compared using an analysis of similarity (ANOSIM). Although both the age effect and the foal effect were highly significant ($P < 0.010$), the *R*-ANOSIM value for the foal effect was very low (*R*-ANOSIM = 0.089), while that of the age effect was much higher (*R*-ANOSIM = 0.309). Significant age-related changes were detected between days 0 and 2 (*R*-ANOSIM = 0.500), days 2 and 10 (*R*-ANOSIM = 0.475) and days 10 and 30 (*R*-ANOSIM = 0.519). No further shifts between consecutive times of sampling were detected in the bacterial community after day 30 and no changes were observed at weaning (day 180). These results show that the establishment of the intestinal bacterial community in foals is a sequential process, which reaches its climax state at around one month of age. Further studies using new generation sequencing based methods could be conducted to identify which bacterial genera are establishing in foals during the first month of life.

1. Introduction

As in other monogastric herbivores, the complex bacterial ecosystem inhabiting the hindgut of horses is essential for degrading dietary plant fiber and providing energy to the animal [1]. This intestinal bacterial ecosystem has been mostly investigated in adult horses [2,3], with few studies focusing on foals [4–6]. Studies of other species [7,8] have shown that the establishment of the bacterial ecosystem during early life may impact on the bacterial community structure in the adult and thus on its digestive efficiency. In addition, the bacterial ecosystem in the digestive tract of the neonate could play an important role in the post-natal maturation of the intestine, especially its immune system, as demonstrated in other species [9–11].

The process of microbial succession in the digestive tract has been reported to be remarkably similar between species [12]. In foals, most studies have been conducted using culture-based methods and have shown that diverse maternal and environmental bacterial populations were established rapidly after birth [4–6]. Recently, Denaturing Gradient Gel Electrophoresis (DGGE) analysis was used to compare the bacterial communities in the feces of mares and their foals and showed that the similarity between foals and mares was 71% at 6 weeks of life and remained stable until 12 weeks of life [13]. However, the fecal bacterial community in the foal has never been investigated after the first three months, yet weaning in this species generally occurs at between four and six months of age. The cessation of milk is associated with strong environmental and social changes for the foal [14]. Weaning could therefore impact on the intestinal microbiota, as shown in other monogastric animals, such as the rabbit [15], and the piglet [16,17]. After weaning, the intestinal bacterial community converges to a more adult-like steady state after a period of time, which varies in relation to species and feeding habits. In the rabbit, the cecal microbiota reaches its adult state six weeks after weaning [18], whereas in piglets, the intestinal bacterial community was reported to be stable after one week [17].

Molecular approaches have been developed to complement culture-based methods for a more complete assessment of intestinal bacterial diversity. Among these molecular approaches, fingerprint techniques have been used in fecal samples to monitor

* Corresponding author. USC-INRA Nutrition du cheval athlète, 26 bd Dr Petitjean, 21 000 Dijon, France. Tel.: +33 3 80 77 25 60; fax: +33 3 80 77 25 84.
E-mail addresses: c.faubladier@gmail.com, christelle.philippeau@agrosupdijon.fr (C. Faubladier), veronique.julliand@agrosupdijon.fr (V. Julliand).

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the overall dynamics of the bacterial community in the individual, both for human neonates [19–21] and growing animals [7,22,23]. The Automated Ribosomal Intergenic Spacer Analysis (ARISA) previously developed to improve both resolution and analysis of the bacterial communities in freshwater [24] and soil [25], allows rapid and simultaneous investigation of numerous samples. It has been successfully adapted to equine digestive samples [26]. This study aimed at monitoring age-related changes in the bacterial community structure in foal feces at pre- and post-weaning using ARISA.

2. Materials and methods

2.1. Animals and fecal samples

This experiment was conducted on five foals (birth body weight: 47 ± 5.4 kg), that were part of a study reported previously [27]. It consisted in two experimental periods, from birth (day 0) to day 194 and from day 330 to day 365, conducted at the animal research facility of Agrosup Dijon, under experimental approval from the Burgundy University ethical committee. During the first experimental period, all mares received the same diet consisting of hay *ad libitum* and concentrate feed, with quantities regularly adjusted to cover the nutritional requirements of lactation [28]. From day 0 to day 14, foals were kept with their respective dams in a sandy paddock during day time and were housed in individual stalls with straw bedding during night-time. From day 15 to day 180, mares and foals were maintained in a sandy paddock all the time. Before day 105, foals did not receive concentrate and had limited access to hay. From day 105, in accordance with practices commonly used by French equine breeders, foals received meadow hay *ad libitum* supplemented with concentrate (DP Evolution, Invivo NSA, Louhans, France) (Table 1). Concentrate feed was distributed at 16:00 h and the quantities were adjusted every month to foal bodyweight (0.36 kg DM/100 kg BW) to cover the nutritional requirements of growing foals [29]. Water and a block of salt were offered free-choice to all animals. Foals were abruptly weaned from their dams at 180 days of age. From day 180 to day 194, foals were kept in indoor individual free-stalls with wood shavings and were allowed access to a collective dry paddock for 1 h per day.

At the end of the first experimental period, two foals were withdrawn from the experiment by their owners. The remaining three foals were kept in a pasture with meadow hay *ad libitum* between the two experimental periods. At day 316, they were maintained in a sandy paddock all the time. They received the same diet as described in the first period for five weeks before fecal sampling at day 365. No foals were ill during the study period and did receive any antimicrobials treatments.

All fecal samples were collected in the rectum of foals. Meconium samples were collected using sterile gloves, on average 2 h after birth. Further fecal samples were collected using single-use gloves between 09:00 and 11:00 h at days 1, 2, 5, 10, 30, 60, 120, 179, 183, 194 and 365 and a subsample of around 30 g was stored at −20 °C until molecular analysis.

Table 1
Dry matter, chemical composition and energy content of the foal diet (g/kg of dry matter unless stated otherwise).[a]

	Hay	Pelleted food[b]
Dry matter (g/kg as fed basis)	893.7 (±20.0)	890.5 (±10.2)
Organic matter	932.5 (±8.2)	921.3 (±2.6)
Crude protein	79.8 (±13.6)	186.7 (±3.8)
Crude fiber	321.0 (±11.9)	105.3 (±3.7)
Neutral detergent fiber	622.0 (±26.6)	314.0 (±23.8)
Fat	nd	39.5[c]
Starch	nd	184.2 (±1.3)
Net energy[d] (MJ/kg DM)	4.89 (±0.16)	9.35 (±0.08)

nd, not determined.
[a] Values are means ± standard deviation ($n = 17$ samples for hay, $n = 8$ samples for pellets).
[b] The pelleted food had the following composition on a DM basis (%): wheat bran (38%), barley (18%), soybean meal (11%), alfalfa (8%), wheat shorts (10%), sugar beet molasses (5%), oat husks, malt sprouts, vitamine and mineral premix.
[c] Given by the producer.
[d] Calculating from nutritional composition [47].

2.2. DNA extraction and amplification

As fecal samples may contain inhibitory substances, the bead-beating procedure with subsequent DNA purification by QIAamp® columns (QIAamp DNA Stool MiniKit, QIAGEN, Courtaboeuf, France) was used to extract total genomic DNA from 0.25 g of frozen feces [30]. The extracted DNA was stored at −20 °C. Two independent DNA extractions were carried out for each fecal sample.

The 16S–23S intergenic spacer regions of the bacterial rRNA were amplified by PCR using 12.5 ng of template DNA with the universal primers ARISA-1552f (5′-TCGGGCTGGATGACCTCCTT-3′) and ARISA-132r (5′-CCGGGTTTCCCCATTCGG-3′) (IDT, Leuven, Belgium) [31]. The forward primer was labeled at its 5′ end with the IRD800 fluorochrome. Amplification was carried out in a total volume of 25 μL, containing 2.5 μL of 10× dilution buffer, 25 mM of each dNTP, 12.5 pM of each primer, 0.625 μg of T4 gene 32 protein (MP Biomedicals, Illkirch, France) and 3.75 U of Taq polymerase (MP Biomedicals, Illkirch, France). Amplification was performed in a thermocycler (Mastercycler ep gradient, Eppendorf, Odil, Dijon, France) after an initial denaturation step at 94 °C for 3 min followed by 30 cycles (94 °C for 1 min, 55 °C for 30 s, 72 °C for 1 min). A final elongation step at 72 °C for 5 min was performed before cooling at 4 °C. All PCR products were purified by using a QIAGEN MinElute PCR purification kit (QIAGEN, Courtaboeuf, France) as recommended by the manufacturer.

2.3. ARISA fingerprinting of individual fecal samples

The concentration of labeled PCR products was estimated and 2 μL of the diluted PCR product was added to 1 μL of deionized formamide and denatured at 92 °C for 2 min. ARISA fragments were resolved on 4.0% polyacrylamide gels and run under denaturing conditions for 16 h at 1500 V/80 W on a LiCor IR^2 DNA sequencer (ScienceTec, Courtaboeuf, France).

The digitized gel images were analyzed using One-D-Scan software (ScienceTec, Courtaboeuf, France). Data were converted into electrophoregrams, where peaks represented the PCR fragments, and the heights of peaks were the relative proportion of the fragments in the total product. Lengths (in base pairs) of PCR fragments were estimated by comparison with size standard, with bands ranging from 200 to 700 bp. Data were then converted using the PrepRISA software [25] into a table summarizing band presence (i.e. peaks) and intensity (i.e. area of peak).

2.4. Statistical analyses

Statistical analyses on ARISA profiles were carried out using StatFingerprint version 2.0 [32] running on R version 2.10.0 [33]. Similarity indices between ARISA profiles were calculated using the Pearson correlation coefficient. Firstly, the analysis of similarity (ANOSIM) was performed to measure the effect of duplicate on the bacterial profiles. No difference was found between duplicates, as shown by the *R*-ANOSIM value (*R*-ANOSIM = 0.015, $P = 0.952$), this is why only one of the two duplicates was used for further statistical analyses. Secondly, a new ANOSIM was performed to measure