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Bacterial carbohydrate-degrading capacity in foal faeces: changes from birth to pre-weaning and the impact of maternal supplementation with fermented feed products

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Abstract

The present study aimed at (1) describing age-related changes in faecal bacterial functional groups involved in carbohydrate degradation and in their activities in foals (*n* 10) from birth (day (d) 0) to 6 months (d180) and (2) investigating the effect of maternal supplementation (five mares per treatment) from d − 45 to d60 with fermented feed products on response trends over time of the foal bacterial carbohydrate-degrading capacity. Maternal supplementation with fermented feed products stimulated foal growth from d0 to d60 and had an impact on the establishment of some digestive bacterial groups and their activities in foals from d0 to d5 but not in the longer term. Irrespective of the maternal treatment, total bacteria, total anaerobic, lactate-utilising and amylolytic bacteria were established immediately after birth ($P<0{\cdot}05$) and were active as shown by the significant increase in total volatile fatty acids. In the foals of supplemented mares, total anaerobes and lactate utilisers were established rapidly between d0 and d2 ($P=0{\cdot}021$ and 0·066, respectively) and the increase in the percentage of propionate occurred earlier ($P=0{\cdot}013$). Maternal supplementation had no effect on the establishment of fibrolytic bacteria and their activity. Cellulolytic bacteria and *Fibrobacter succinogenes* first appeared at d2 and d5, and increased progressively, reaching stable values at d30 and d60, respectively. From the second week of life, the increase in the molar percentage of acetate and the ratio (acetate + butyrate):propionate ($P<0{\cdot}05$) suggested that fibrolytic activity had begun. From d60, only minor changes in bacterial composition and activities occurred, showing that the bacterial carbohydrate-degrading capacity was established at 2 months of age.

Key words: Bacterial establishment: Carbohydrate degradation: Suckling foals: Maternal supplementation: Fermented feed products

In adult horses, one of the primary roles of the bacterial ecosystem in the large intestine is to break down parietal carbohydrates of plant cell walls, and to ferment them into volatile fatty acids (VFA) that, in turn, are absorbed across the intestinal wall, providing energy to the horse[1]. *Ruminococcus flavefaciens*, *R. albus* and *Fibrobacter succinogenes* have been described as the major cellulolytic bacterial species in the equine hindgut[2–5]. In addition to cellulolytic bacteria, amylolytic and lactate-utilising bacteria are other major functional groups in the hindgut of horses[6] involved in the degradation of cytoplasmic carbohydrates that escape enzymatic digestion in the small intestine. The balance between the main functional bacterial groups is essential for optimising fibre degradation and depends greatly on the horse diet[7].

In suckling foals, fibre rapidly becomes one major source of nutrients as suggested by the linear increase in grazing time with age while suckling activity decreases[8]. With respect to ruminants[9], the bacterial degradation of fibre probably establishes the bacterial colonisation of the foal's hindgut. Information on the establishment of the foal intestinal ecosystem is sparse but some data are available from birth to 3 months of age. Meconium has been reported to be free of bacteria[10]. Diverse maternal and environmental bacterial populations, such as enterococci, Enterobacteriaceae, Clostridia, lactobacilli and streptococci, have been shown to establish rapidly after birth[10,11]. Using fingerprint techniques[12], the bacterial pattern resembled the adult one within the first month of age, suggesting that the sequence of bacterial colonisation was complete. Although cellulolytic bacteria were detected between the third and fifth days of life in foal faeces and reached adult values at 2 months of age[13], the age at which fibre-degrading capacity appears in the foal's hindgut is actually unknown. Previous studies[11,14] reported that VFA were detectable from 1 d of life and peaked at 7 d of life in the faeces of foals, suggesting that bacterial fermentation occurred very rapidly in the hindgut. An *in vitro* study suggested that

Abbreviations: CFU, colony-forming units; VFA, volatile fatty acids.

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the capacity for fibre degradation in the foal by 1 month of age was similar to that in the mare[15], but this was not confirmed *in vivo*.

As suggested in other species[16,17], the establishment of the bacterial ecosystem in foals could have an impact on intestinal bacterial composition in adult horses and thus on digestive efficiency. Therefore, strategies increasing the beneficial microbial count and activity in the foal's hindgut during the neonatal period could optimise the bacterial ecosystem composition and activity later in life. As the maternal digestive microflora dominates the microbial environment in early life[18], one strategy could consist of modifying the intestinal microflora of the mare[19]. In mice[20,21] and pigs[22,23], maternal dietary supplementation during gestation and lactation may influence in a beneficial way both the intestinal microflora of the dam and the establishment of bacterial communities in offspring, but this information is actually unknown in horses.

Microbial fermented feed products are used in pig nutrition to improve zootechnical performances[24–28]. Such products contain feed materials that are obtained by the fermentation of one or more microbes on specific media. In pigs supplemented with microbial fermented feed products, recent investigations reported an increase in bacterial richness and diversity[26], especially the *Bacteroidetes* and *Lactobacillus* populations[27]. These findings could suggest that microbial fermented products could have an impact on the intestinal microflora in mares, but no controlled studies have been reported in the peer-reviewed literature.

The present study aimed at (1) describing the age-related changes in bacterial functional groups involved in carbohydrate degradation and in their activities in foal faeces from birth to pre-weaning and (2) determining the effect of maternal supplementation with fermented feed products on response trends over time of the foal bacterial carbohydrate-degrading capacity. This effect was tested during the period of supplementation (from birth to 2 months of age) and for a longer term (from 2 to 6 months).

Materials and methods

The experiment was conducted at the Animal Research Facility of AgroSup Dijon under experimental approval from the ethics committee of Burgundy University (agreement no. B1110).

Animals and management

A total of ten Haflinger mares (age: 10 (SD 4) years; body weight at 300 d of gestation: 571 (SD 35) kg) were included in the experiment from 45 d before foaling (d−45) until pre-weaning (180 d after foaling, d180). The day of foaling (d0) was determined by Ca determination in pre-foaling mammary secretion[29] and foaling was induced by micro-injection of oxytocin[30]. The foals were used for the experiment at d0. From d−45 to d14, the animals were kept in indoor individual free stalls with straw bedding during the night and in sandy paddocks during the day. From d14 to d180, mares and foals were maintained in sandy paddocks day and night. Mares and foals were wormed with Fenbendazole (Panacur®; Intervet) at d14 and d120 and at d90 and d150, respectively. From d0 to d14, foals were checked daily for signs of diarrhoea. Foals were weighed at d1, then every week from d5 to d60, at d75, and once a month from d90 to d180.

Experimental design and dietary treatment

According to their age, parity, expected foaling date, body weight and intake level of hay at 10 months of gestation, mares were divided into two homogeneous groups in a block design. In the first group, mares (n 5, supplemented mares) were supplemented with two fermented feed products FAP® (Original Process): EquiProcess® pellets (360 g/d from d−44 to d14; 240 g/d from d15 to d60 in the morning concentrate meal) and FloreProcess® oral paste (20 g at 30 min after foaling and every day at 11.00 hours from d1 to d3). Fermented feed products FAP® contain feed materials obtained during the germination and fermentation of barley with eight deposited strains of lactic bacteria (National Collection of Micro-organisms Cultures at Institut Pasteur, France) through a specific process. Their composition is given in Table 1. These products contained none of the bacteria that was used during the process.

In the second group, mares (n 5, control mares) did not receive any supplementation (control). The basal diet of mares (Table 2) was made up of meadow hay, a commercial pelleted feed (DP Evasion; InVivo NSA) and a soyabean meal. Hay was offered *ad libitum* and its intake level was measured during twenty-four consecutive hours every 2 weeks from d−45 to d90, and then each month until d180. Daily amounts of the pelleted feed and soyabean meal were adjusted every 2 weeks depending on the average intake of hay to cover nutritional requirements of late pregnancy[31] and lactation[32]. Concentrate feeds were provided in two equal meals at 08.00 and 17.30 hours until d105, and then in one meal at 08.00 hours until d180. From d−45 to d180, mineral pellets (150 g/d, Prelac Star; InVivo NSA) were provided in the morning meal of concentrate.

Table 1. Chemical composition of EquiProcess® pellets and FloreProcess® paste

	EquiProcess®	FloreProcess®
DM (g/kg)	880·0	229·4
OM (g/kg DM)	973·0	871·8
Nutrients (g/kg DM)		
CP	116·0	48·0
Fat	35·0	ND
CF	54·0	49·7
Neutral-detergent fibre	163·7	130·3
Resistant starch	10·9	ND
Starch	537·4	131·9
Free sugars*	49·3	ND
Organic acids (mmol/kg DM)		
Lactic acid	37·5	467·7
Acetic acid	9·5	1071·9
Propionic acid	1·6	149·4

OM, organic matter; CP, crude protein; ND, not detected; CF, crude fibre.
* Free sugars = glucose + fructose + sucrose + maltose.

Table 2. Chemical composition of hay, mare and foal pelleted food and soyabean meal

	Hay (g/kg)	Mare pelleted food* (g/kg)	Foal pelleted food† (g/kg)	Soyabean meal (g/kg)
DM	879·8	889·9	885·9	885·2
OM‡	934·2	919·2	920·2	926·5
CP‡	75·7	130·6	188·3	517·6
Fat‡	NA	33·7	39·5	NA
CF‡	321·8	153·4	107·5	55·5
Neutral-detergent fibre‡	639·8	419·7	303·8	119·8
Starch‡	NA	151·0	178·0	NA
Net energy (MJ/kg)‡§	4·88	8·52	9·29	7·35

OM, organic matter; CP, crude protein; NA, not analysed; CF, crude fibre.
* The pelleted food for mares had the following composition on a DM basis (%): wheat bran (32 %); wheat shorts (16 %); barley (15 %); dehydrated alfalfa (10 %); straw (9·4 %); soyabean husks (5 %); sugar cane molasses; pomace; vitamin; mineral premix.
† The pelleted food for foals had the following composition on a DM basis (%): wheat bran (38 %); barley (18 %); soyabean meal (11 %); alfalfa (8 %); wheat shorts (10 %); sugar beet molasses (5 %); oat husks; malt sprouts; vitamin; mineral premix.
‡ DM basis.
§ Calculating from the nutritional composition[68].

Suckling foals did not receive any supplementation. From d0 to d105, they had no access to the mares' concentrate and had limited access to the mares' hay provided in elevated hayracks (>1·1 m) in order to limit ingestion of hay. From d105 to d180, suckling foals had free access to hay offered *ad libitum* and received a commercial pelleted feed (DP Evolution; InVivo NSA; Table 2) distributed in one meal at 17.00 hours (0·3 kg DM/d from d105 to d150; 0·6 kg DM/d from d150 to d180) to cover the energy and protein requirements of suckling foals[33]. Water and a block of salt were offered free-choice to all animals.

Collection procedure and sampling

In mares, faecal samples were collected manually from the rectum using single-use, but not sterile, gloves before foaling at d−45 (1 d before the beginning of the dietary treatment), d−30 and d−1 (within the last 3 weeks before foaling) and after foaling at d1, d5, d10, d14, d30 and d60. A subsample of 1 g of faecal material was immediately diluted under O_2-free CO_2 in 9 ml of an anaerobic mineral solution[34] for microbial analysis. A second subsample of about 100 g was filtered (Blutex 100 μm). The filtered content was divided into aliquots and frozen for further determination of VFA concentrations (1 ml mixed with 0·1 ml of a preservative solution consisting of 4·25 % (v/v) H_3PO_4 and 1·0 % (w/v) $HgCl_2$).

In foals, a rectal smear was obtained with a sterile swab (IMV Technologies) after cleaning the anus with a 10 % (v/v) iodine solution (Vétédine solution, Vétoquinol) within 20 min after birth to check the presence of total anaerobes. Meconium (d0) was collected in the rectum of foals using sterile gloves within 3 h maximum after birth. Faecal samples were taken directly from the rectum of foals using single-use, but not sterile, gloves, every day from d1 to d7, at d10, and every 2 weeks from d30 to d90 at d14, and then every month until d180. A subsample of 1 g of faecal material was immediately diluted in 9 ml of an anaerobic mineral solution[34] in the same conditions as described for mares and used for microbial analysis. Furthermore, one part of the faecal dilution was also divided into two aliquots and immediately frozen (−20°C) for further determination of VFA concentrations as described for mares. A second subsample of about 10 g of faecal material was immediately frozen at −20°C in hermetically sealed plastic boxes for further molecular analysis.

Analysis of bacterial functional groups

Serial decimal dilutions of the faecal samples were prepared under O_2-free CO_2 in an anaerobic mineral solution[34] for inoculation on specific media.

Total viable anaerobic bacteria and lactate-utilising bacteria were enumerated following the roll-tube procedure under O_2-free CO_2 in a non-selective medium[3,35] and in a selective medium containing 2 % (v/v) lactate as the sole energy source[36], respectively. Bacterial numbers were determined after 48 h of incubation at 38°C from three replicate roll tubes prepared per dilution.

Amylolytic bacteria were deeply inoculated on an adapted medium containing 1 % (w/v) soluble starch as the main energy source[37]. For each dilution, three replicate Petri plates were inoculated. For bacterial counts, iodine solution was added to Petri plates after 48 h of incubation at 38°C.

Cellulolytic bacteria were enumerated in a complex liquid medium[3,38] containing a strip of filter paper (Whatman no. 1) as the sole energy source. After an incubation of 2 weeks of three replicate tubes per dilution at 38°C, the most probable number of cellulolytic bacteria in the faecal sample was determined using McGrady's tables[39].

DNA extraction and real-time quantitative PCR

Faecal samples obtained from foals at d0, d1, d3, d5, d10, d14, d30 and then every month until d180 were used for real-time PCR quantification of total bacteria and *F. succinogenes*.

Total DNA was extracted from 0·25 g of pure bacterial and faecal samples according to the bead-beating procedure[40]. DNA concentration was determined by measuring absorbance at 260 nm.

DNA amplifications were performed using the Biorad iCycler iQ5 (Bio-Rad) with fluorescence detection of SYBR Green dye. Primer sequences were as follows: total bacteria (forward, 5′-CGGCAACGAGCGCAACCC; reverse, 5′-CCATTGTAGCACGTGTGTTAGCC) and *F. succinogenes* (forward, 5′-GTTCGGAATTACTGGGCGTAAA; reverse, 5′-CGCCTGCCCCTGAACTATC)[41]. All DNA samples from the faeces and pure bacterial samples were diluted 100-fold in sterile re-distilled water before amplification to reduce inhibition. Amplification was carried out in a total volume of 25 μl containing 1 × IQ SYBR Green Super Mix (Bio-Rad), forward and reverse primers

(1 μM each) and DNA template (5 μl). All the samples were prepared in duplicate. After an initial denaturation step at 95°C for 3 min, amplification and quantification programmes were repeated forty-five times (95°C for 30 s, 60°C for 30 s and 72°C for 1 min). Amplification was followed by the melting curve programme (72–95°C for 23 min) with a continuous fluorescence measurement. Negative controls without a DNA template were run with every assay. For each measurement, the mean threshold cycle (C_t) value was calculated.

F. succinogenes S85 was grown during 48 h in cellobiose (liquid medium)[42] at 38°C. The most probable number of *F. succinogenes* was determined using McGrady's tables[39] before DNA extraction. The extracted DNA of *F. succinogenes* cells was diluted in serial decimal dilution and further quantified by real-time PCR using the primer set targeting *F. succinogenes* species. The standard curve was generated by plotting the observed C_t values *v.* bacterial concentration ($\log_{10}$ cells/ml). The standard curve for the absolute quantification of total bacteria was generated using the same procedure with the primer set targeting total bacteria. Standard curves were linear over the range of cell concentrations from 2·4 $\log_{10}$ to 6·4 $\log_{10}$ cells/ml (data not shown). When PCR was performed on unknown faecal samples, we used these standard curves to quantify each bacterial population. The extracted DNA of faecal samples was diluted 100 times for the PCR process, the detection limit being 4·4 $\log_{10}$ cells/g fresh faeces.

pH measurement and volatile fatty acid analysis

All samples were analysed in duplicate. Immediately after sampling, pH was determined using a pH meter (pH 510; EUTECH Instruments Europe B.V.), which was calibrated using certified pH 4, 7 and 10 buffer solutions on fresh faecal matter in foals and on filtered faecal content in mares.

VFA concentrations in faecal samples were determined by GLC (Clarus® 500 Gas Chromatograph; PerkinElmer) following the procedure described by Jouany[43]. The ratio (acetate + butyrate):propionate was calculated[44].

Statistical analyses

Before statistical analyses, the means of replicate were calculated for all data and logarithmic transformations were performed for bacterial concentrations.

For all data, we used mixed models with a repeated-measures design to test the main effects of maternal supplementation and the time of sampling and the interaction between maternal supplementation and the time of sampling using PROC MIXED on the Statistical Analysis System statistical software package[45]. With the REPEATED statement, we used the compound symmetric structure[46]. Time of sampling was the repeated measure and the animal nested in maternal supplementation was the subject. When the interaction or fixed effects were significant, least-squares means were separated using the pairwise *t* test (PDIFF option in the LSMEANS statement). Statistical significance was accepted at $P<0·05$ and a trend was considered at $P<0·1$. All presented data are expressed as least-squares means with their standard errors.

A first statistical analysis was performed on data obtained in foals from d0 to d60. A second statistical analysis was then done on foal data obtained after the end of maternal supplementation, from d60 to d180, to assess the long-term effect of maternal supplementation.

Results

Zootechnical performances of foals

The ten foalings spread from 16 April to 7 June. Every foal was viable at birth and had a normal status with regard to first standing, suckling and colostrum intake. During the first 2 weeks of life, one foal of the control mare group presented minor diarrhoea during 1 d and five foals of the supplemented mares during 1·8 (SD 0·4) d, but did not need any specific medical care.

Foals suckling the supplemented mares were heavier at d19 ($P=0·048$), d26 ($P=0·028$), d40 ($P=0·041$), d54 ($P=0·005$) and d60 ($P<0·001$) than those from the control mares (Fig. 1).

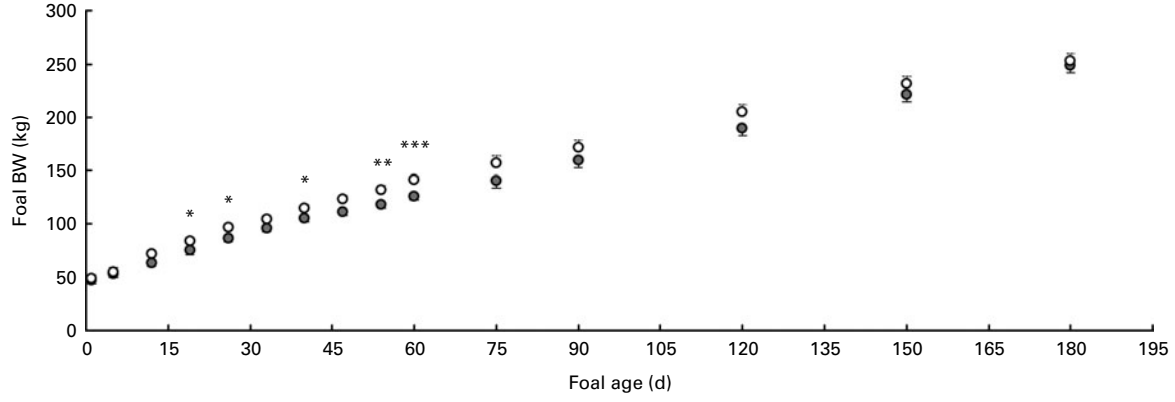

Fig. 1. Body weight (BW) of the foals of control mares (*n* 5, ●) and supplemented mares (*n* 5, ○) from 1 to 180 d of life. Values are adjusted means, with their standard errors represented by vertical bars. From day (d) 1 to d60, there was a significant maternal supplementation × age interaction ($P<0·05$). From d60 to d180, there was no significant maternal supplementation × age interaction ($P>0·05$), no effect for maternal supplementation ($P>0·05$), but a significant effect for age ($P<0·001$). Mean values were significantly different for the same age between the groups of foals: * $P<0·05$, ** $P<0·01$, *** $P<0·001$.

The average daily gain was 1·55 (SD 0·15) and 1·34 (SD 0·19) kg/d in the foals of supplemented mares and in the foals of control mares, respectively. From d60, maternal supplementation had no effect on the body weight of foals. The average daily gain was on average 0·98 (SD 0·11) kg/d.

Faecal bacterial ecosystem of mares

Supplementation did not affect the faecal bacterial concentrations of total anaerobic, amylolytic, lactate-utilising and cellulolytic bacteria from d−45 to d60. Total anaerobic, amylolytic and cellulolytic bacterial counts were stable from d−45 to d60, and were on average 8·2 (SD 0·48) $\log_{10}$ colony-forming units (CFU)/g fresh faeces, 5·5 (SD 0·68) $\log_{10}$ CFU/g fresh faeces and 6·2 (SD 0·77) $\log_{10}$ most probable number/g fresh faeces, respectively. From d−45 to d0, lactate-utilising bacterial counts were stable and were on average 6·1 (SD 0·58) $\log_{10}$ CFU/g fresh faeces. Then, they increased significantly from d−1 to d1 (+0·6 $\log_{10}$ CFU/g fresh faeces, $P=0·035$) and tended to decrease from d5 to d60 (−0·5 $\log_{10}$ CFU/g fresh faeces, $P=0·053$).

Faecal pH and total VFA concentration were not affected by the supplementation or the day of sampling from d−45 and d60, and were on average 6·6 (SD 0·33) and 64·0 (SD 16·57) μmol/ml, respectively (data not shown). The VFA profile was not affected by the day of sampling from d−45 to d60; however, irrespective of the day of sampling, supplemented mares had a higher molar proportion of acetate than the control mares ($P=0·046$; Table 3).

Faecal bacterial communities of foals

During maternal supplementation (from day 0 to day 60). Rectal smears were performed within 20 min after birth and contained less than 10 CFU per swab for eight foals, and 33·6 and 647 CFU total anaerobes per swab for one foal of control mares and supplemented mares, respectively. Meconium collected on average 2·0 (SD 1·0) h after birth was not sterile irrespective of the method used (culture *v.* molecular method; Figs. 2 and 3 and Table 4). No cellulolytic bacteria were detected in the meconium.

Data of d4 and d6 are not presented on the plotted points for clarity in the figures.

There was a significant interaction between the age of foal and maternal supplementation for the total anaerobic counts from d0 to d60 ($P=0·021$; Fig. 2). This interaction tended to be significant for lactate-utilising bacterial counts from d0 to d60 ($P=0·066$; Fig. 3). In both groups of foals, the concentration of total anaerobes and lactate utilisers increased from d0 to d2 ($P<0·01$). The magnitude of the increase between d0 and d1 was greater in the foals of supplemented mares than in the foals of control mares (+6·64 *v.* +4·14 $\log_{10}$ CFU/g fresh faeces for total anaerobes and +7·18 *v.* +4·55 $\log_{10}$ CFU/g fresh faeces for lactate utilisers). The concentration of total anaerobes and lactate utilisers was significantly higher at d1 ($P<0·05$) and numerically higher at d2 ($P>0·05$) in the foals of supplemented mares compared with the controls. No difference was detected between the groups from d3. Thereafter, in both groups of foals, total anaerobes and lactate utilisers were stable until d10 and d14, respectively, and decreased until d60 ($P<0·05$). The mean concentrations of cellulolytic and amylolytic bacteria or their establishment from d0 to d60 were not affected by maternal supplementation (Figs. 2 and 3, respectively). Amylolytic bacteria increased significantly from d0 to d2 ($P<0·05$). From d3 to d60, a minor variation in amylolytic bacterial concentration occurred and was on average 6·81 (SD 0·91) $\log_{10}$ CFU/g fresh faeces. By the culture method, cellulolytic bacteria were first detected at d2 and found to increase significantly between d4 and d5 ($P=0·013$). Thereafter, cellulolytic bacterial concentration increased progressively, reaching stable values at d30 ($P<0·05$).

Real-time quantitative PCR revealed that maternal supplementation did not affect the mean concentration of *F. succinogenes* and total bacteria and their establishment.

Table 3. Molar proportions of the main volatile fatty acids (VFA) and (acetate + butyrate):propionate in the fresh faeces of mares from 45 d before foaling (day (d) −45) to 60 d after foaling (d60) depending on the supplementation with fermented feed products*

(Adjusted means values with their highest standard errors)

	Days										*P*†	
Items and maternal groups	d−45	d−30	d−1	d1	d5	d10	d14	d30	d60	SEM	S	T
Acetate:total VFA (%)												
Control	74·3	74·9	74·6	72·8	73·8	72·3	74·8	74·6	75·5	1·22	0·046	0·820
Supplemented	76·7	77·3	76·3	76·3	77·1	76·7	74·6	76·2	76·3			
Propionate:total VFA (%)												
Control	15·7	16·5	16·5	18·4	16·5	17·0	16·6	16·7	15·9	0·87	0·061	0·072
Supplemented	14·4	14·3	14·1	16·1	15·2	15·2	16·7	15·6	14·4			
Butyrate:total VFA (%)												
Control	4·9	4·4	4·2	4·5	4·4	4·7	4·3	4·1	4·1	0·51	0·673	0·286
Supplemented	4·9	4·6	5·0	4·8	4·3	4·5	5·0	4·2	4·7			
(Acetate + butyrate):propionate												
Control	5·1	4·9	4·8	4·3	4·8	4·7	4·8	4·8	5·1	0·36	0·059	0·117
Supplemented	5·9	5·9	6·0	5·1	5·4	5·4	4·9	5·2	5·7			

S, supplementation; T, time.

* Five mares per group.

† There was no significant interaction between supplementation and time on the fermentative parameters in mares ($P>0·05$).

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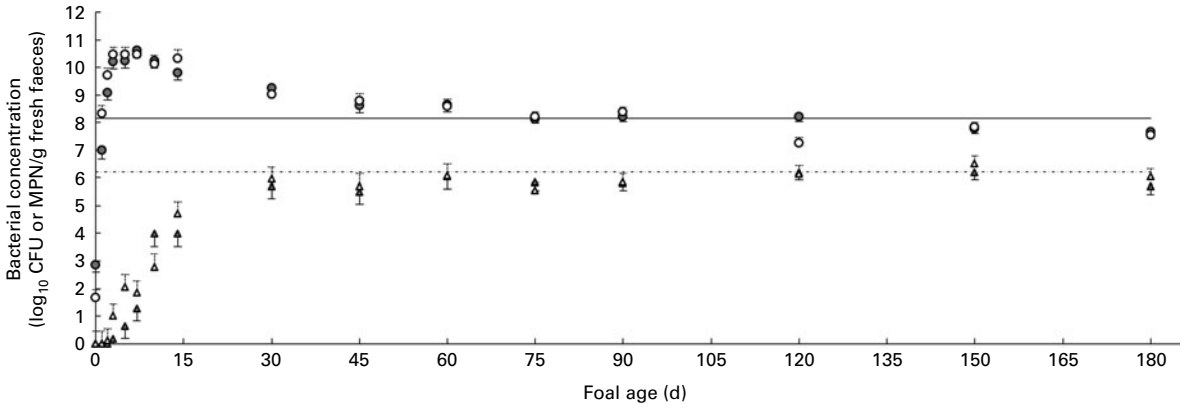

Fig. 2. Age-related changes in the viable cell number of total anaerobic (log_{10} colony-forming units (CFU)/g) and cellulolytic bacteria (log_{10} most probable number (MPN)/g,) from birth to 180 d of age in the fresh faeces of foals (●, total anaerobes, foals of control mares; ○, total anaerobes, foals of supplemented mares; ▲, cellulolytic bacteria, foals of control mares; △, cellulolytic bacteria, foals of supplemented mares). Values are adjusted means, with their standard errors represented by vertical bars. For total anaerobes, there was a significant maternal supplementation × age interaction: $P=0{\cdot}021$, from day (d) 0 to d60; $P=0{\cdot}042$, from d60 to d180. For cellulolytic bacteria from d0 to d180, there was no significant maternal supplementation × age interaction ($P>0{\cdot}05$), no effect for maternal supplementation ($P>0{\cdot}05$), but a significant effect for age ($P<0{\cdot}001$). For cellulolytic bacteria from d60 to d180, there was no significant maternal supplementation × age interaction ($P>0{\cdot}05$), and no effects for age and maternal supplementation ($P>0{\cdot}05$). The mean value of mares was reported on the graph to represent the adult level of total anaerobes (—) and cellulolytic bacteria (·····).

This is why mean concentrations were averaged over maternal supplementation in Table 4. Total bacteria increased from d0 to d3 ($P<0{\cdot}001$), reaching a stable plateau until d60. *F. succinogenes* was first detected at d5 and increased significantly from d30 to d60 ($P=0{\cdot}012$).

After the end of maternal supplementation (from day 60 to day 180). There was a significant interaction between the age of foal and maternal supplementation only for the total anaerobic counts from d60 to d180 ($P=0{\cdot}042$; Fig. 2). In both groups of foals, total anaerobes slowly decreased from d60 until d180, but the bacterial count at d120 was lower in the foals of supplemented mares compared with the foals of control mares ($P<0{\cdot}001$). Irrespective of the group of foals, the counts of lactate utilisers slowly decreased between d60 and d180 ($P=0{\cdot}002$; Fig. 3), whereas cellulolytic bacterial counts remained stable and were on average 6·06 (SD 0·63) log_{10} most probable number/g fresh faeces (Fig. 2). Amylolytic bacteria slowly decreased between d60 and d180 ($P<0{\cdot}001$; Fig. 3) and irrespective of the day of sampling, except at d75, their mean concentration was lower in the foals of supplemented mares than those of control mares ($P=0{\cdot}008$).

Real-time quantitative PCR revealed that maternal supplementation did not affect the mean concentrations of total bacteria and *F. succinogenes* after d60. Total anaerobes and *F. succinogenes* were stable after d60 and were on average 8·79 (SD 0·48) and 7·40 (SD 0·58) log_{10} CFU/g fresh faeces, respectively (data not shown).

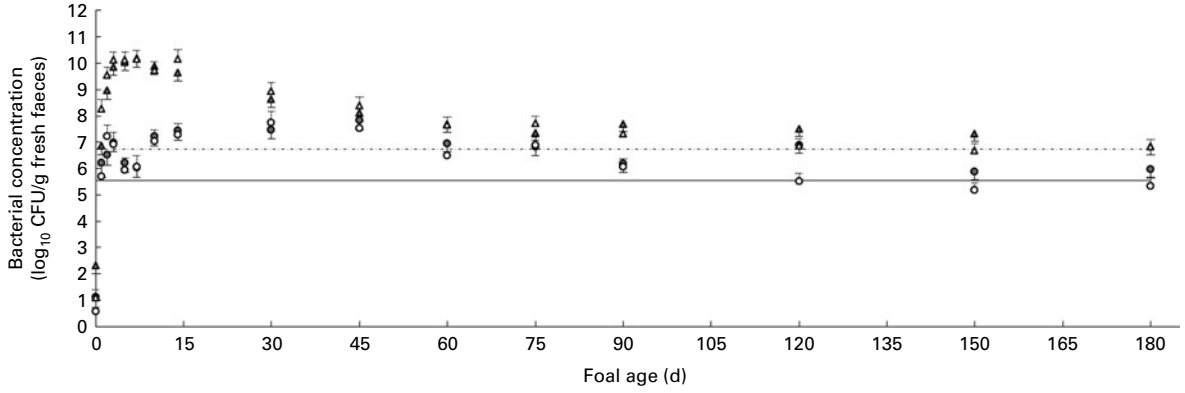

Fig. 3. Age-related changes in the viable cell number of amylolytic and lactate-utilising bacteria from birth to 180 d of age in the fresh faeces of foals (●, amylolytic bacteria, foals of control mares; ○, amylolytic bacteria, foals of supplemented mares; ▲, lactate utilisers, foals of control mares; △, lactate utilisers, foals of supplemented mares). Values are adjusted means, with their standard errors represented by vertical bars. For amylolytic bacteria from day (d) 0 to d60, there was no significant maternal supplementation × age interaction ($P>0{\cdot}05$), no effect for maternal supplementation ($P>0{\cdot}05$), but a significant effect for age ($P<0{\cdot}001$). For amylolytic bacteria from d60 to d180, there was no significant maternal supplementation × age interaction ($P>0{\cdot}05$), but there were significant effects for maternal supplementation ($P=0{\cdot}008$), and borderline significant for age ($P<0{\cdot}001$). For lactate utilisers from d0 to d60, there was a maternal supplementation × age interaction ($P=0{\cdot}066$). For lactate utilisers from d60 to d180, there was no significant maternal supplementation × age interaction ($P>0{\cdot}05$), no effect for maternal supplementation ($P>0{\cdot}05$), but a significant effect for age ($P=0{\cdot}033$). The mean value of mares was reported on the graph to represent the adult level of amylolytic (—) and lactate–utilising bacteria (·····). CFU, colony-forming units.

Table 4. Quantification of total bacteria and *Fibrobacter succinogenes* using real-time quantitative PCR in the fresh faeces of foals from birth (day (d) 0) to 60 d of age (d60)*

(Adjusted mean values with their highest standard errors)

	Days									*P*
Items†	d0	d1	d3	d5	d10	d14	d30	d60	SEM	T
Total bacteria (log_{10} cells/g fresh faeces)	4·89	6·25	8·29	8·34	8·25	8·05	8·56	8·14	0·249	<0·001
F. succinogenes (log_{10} cells/g fresh faeces)	NE	NE	NE	5·11	5·55	5·97	5·99	6·85	0·554	0·017

T, age of foals; NE, non-estimated.
* For ten foals.
† As there was no effect of maternal supplementation and no significant interaction between maternal supplementation and the age of foal, means were averaged over the maternal groups.

Faecal fermentative parameters in foals

During maternal supplementation (from day 0 to day 60). Data of d4 and d6 are not presented on the plotted points for pH and total VFA concentration and in tables for molar percentages of VFA for clarity in the tables and figures.

An interaction between the age of the foal and maternal supplementation was detected for faecal pH from d0 to d60 ($P=0·018$; Fig. 4(a)). In the foals of supplemented mares, faecal pH first decreased from d1 to d2 ($P=0·010$) and then increased between d2 and d3 ($P=0·023$). In the foals of control mares, similar significant changes were observed with a delay of 1–2 d. This resulted in lower faecal pH at d2 ($P=0·005$) and higher faecal pH at d4 ($P=0·009$) in the foals of supplemented mares compared with the foals of control mares. From d5, faecal pH remained stable until d30 and then decreased from d30 to d45 in both groups of foal ($P<0·05$).

Maternal supplementation did not affect the concentration of total VFA from d0 to d60 (Fig. 4(b)), but had an effect on the age-related change in the VFA profile (Table 5). The total VFA concentration first increased from d2 to d3 ($P=0·007$), reaching a high-level plateau until d10. Thereafter, total VFA concentration slowly decreased until d60 ($P<0·001$). At d0 and d1, the molar percentage of acetate was very high and then decreased from d1 and d5 whereas the molar percentages of propionate and butyrate increased ($P<0·05$). These changes occurred earlier in the foals of supplemented mares than in those of control mares, resulting in a lower percentage of acetate at d2 and d3 ($P=0·031$ and $P<0·001$, respectively) and a higher percentage of propionate at d3 and d4 ($P=0·002$ and $P=0·004$, respectively) in the foals of supplemented mares compared with the controls. After d5, in both groups of foals, molar percentages were stable until d14. Then, the molar percentage of acetate slowly increased

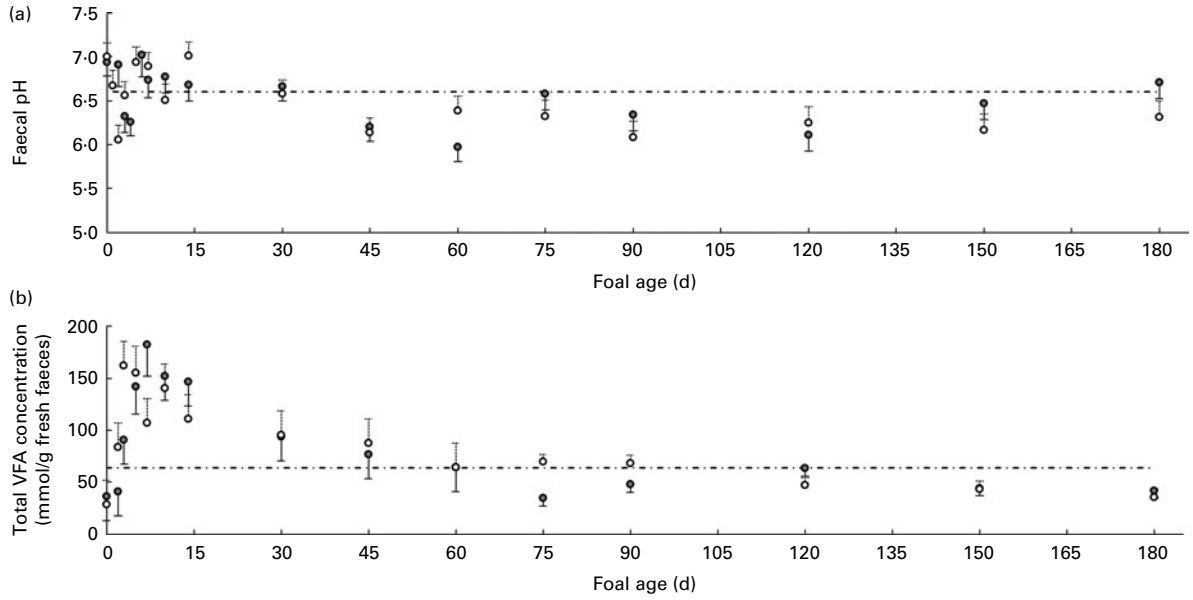

Fig. 4. Age-related changes in (a) pH and (b) total volatile fatty acid (VFA) concentration from birth to 180 d of age in the fresh faeces of foals of control mares (*n* 5, ●) and supplemented mares (*n* 5, ○). Values are adjusted means, with their standard errors represented by vertical bars. For pH from day (d) 0 to d60, there was a significant maternal supplementation × age interaction ($P=0·018$). For pH from d60 to d180, there was no significant maternal supplementation × age interaction, and no effects for maternal supplementation and age ($P>0·05$). For total VFA concentration from d0 to d60, there was no significant maternal supplementation × age interaction ($P>0·05$), no effect for maternal supplementation ($P>0·05$), but there was a significant effect for age ($P<0·001$). For total VFA concentration from d60 to d180, there was a significant maternal supplementation × age interaction ($P=0·004$). For each parameter, the mean value of mares was reported on the corresponding graph (·····) to represent the adult level.

Table 5. Molar proportions of the main volatile fatty acids (VFA) and (acetate + butyrate):propionate ratio in the fresh faeces of foals from birth (day (d) 0) to 60 d of age (d60) depending on maternal supplementation with fermented feed products*
(Adjusted means values with their highest standard errors)

	Days											*P*		
Items and maternal treatments	d0	d1	d2	d3	d5	d7	d10	d14	d30	d45	d60	SEM	S	T	S × T
Acetate:total VFA (%)															
Control	89·2	91·0	90·9[a]	79·3[a]	57·2	52·3	55·6	67·5	69·9	72·1	74·0	4·10	0·079	<0·001	0·002
Supplemented	88·0	92·4	80·5[b]	58·6[b]	53·3	50·6	56·9	59·3	67·9	70·0	71·5				
Propionate:total VFA (%)															
Control	4·6	4·8	4·1	11·8[a]	19·6	22·1	21·7	16·4	15·5	16·1	16·8	2·07	0·322	<0·001	0·013
Supplemented	7·3	2·4	6·6	19·4[b]	21·9	20·1	18·8	17·8	15·2	17·2	18·2				
Butyrate:total VFA (%)															
Control	5·2	3·4	5·0	6·7	11·6	10·5	9·0	7·6	7·0	5·4	5·3	1·87	0·325	<0·001	0·374
Supplemented	3·0	4·6	8·9	11·1	9·8	10·8	11·1	11·3	8·2	6·7	5·3				
(Acetate + butyrate) :propionate															
Control	15·3	12·3[b]	13·7	8·2[a]	3·7	2·9	3·7	4·7	5·0	4·9	4·9	1·61	0·405	<0·001	<0·001
Supplemented	13·8	28·4[a]	15·9	3·7[b]	2·9	3·3	3·8	4·2	5·0	4·5	4·3				

S, maternal supplementation; T, age of foal; S × T, interaction between maternal supplementation and the age of the foal.
[a,b] Mean values within a column with unlike superscript letters were significantly different ($P<0·05$).
* Five foals per maternal group.

until d60, whereas the molar percentages of propionate and butyrate slowly decreased ($P<0·05$).

After the end of maternal supplementation (from day 60 to day 180). From d60 to d180, faecal pH was not affected by maternal supplementation and was stable over the period, and was on average 6·31 (SD 0·43) (Fig. 4(a)).

Maternal supplementation had an effect on total VFA concentration from d60 to d180 ($P=0·004$; Fig. 4(b)), but did not affect the VFA profile (Table 6). In both groups of foals, total VFA concentration decreased between d60 and d180 ($P<0·05$) but VFA concentration was higher at d75 ($P=0·002$) and tended to be higher at d90 ($P=0·055$) in the foals of supplemented mares compared with that of controls. Irrespective of the maternal treatment, the molar percentage of propionate tended to increase and the (acetate + butyrate):propionate ratio decreased from d90 to d120 ($P=0·054$ and $P=0·014$, respectively), whereas the molar percentages of acetate and butyrate were stable from d60 to d180.

Discussion

The process of bacterial establishment in the digestive tract of young animals plays an important role for host health and nutrition. Among several strategies developed to improve this process, we tested in the present study the impact of mare supplementation with fermented feed products around foaling. The present data confirmed previous data on the bacterial establishment in the foals' digestive tract from birth to 3 months of life, and provided new information about the bacterial carbohydrate-degrading capacity until 6 months of age, corresponding to the pre-weaning period. Moreover, this was the first investigation demonstrating the effect of maternal supplementation with fermented feed products around foaling on bacterial functional groups and their activities in foals.

Establishment of the bacterial carbohydrate-degrading capacity in foals

To characterise the establishment of the bacterial groups involved in carbohydrate degradation in foals, we used both conventional microbial methods and non-culture-dependent methods. Using culture techniques, we focused on cellulolytic, amylolytic and lactate-utilising bacteria, which are the main functional bacterial groups involved in carbohydrate degradation reported in adult horses[6]. As species-level detection was impossible using culture-based methods, we used real-time PCR quantification to focus on *F. succinogenes* that is one of the major fibrolytic bacterial strains in adult horses[2,5]. Bacterial quantifications were completed by VFA determination in order to assess indirectly the fermentative activity of bacteria.

In accordance with the sterility of the digestive tract *in utero*[47,48], the rectum contained no total anaerobes at birth. In contrast, the meconium collected 2 h after birth contained on average 4·91 (SD 0·18) $\log_{10}$ cells of total bacteria per g fresh faeces that were active as shown by the small amounts

Table 6. Molar proportions of the main volatile fatty acids (VFA) and (acetate + butyrate):propionate ratio in the fresh faeces of foals from 60 d of age (d60) to 180 d of age (d180) depending on maternal supplementation with fermented feed products*
(Adjusted means values with their highest standard errors)

	Days							*P*		
Items and maternal treatments	d60	d75	d90	d120	d150	d180	SEM	S	T	S × T
Acetate:total VFA (%)										
Control	74·0	75·4	75·7	74·3	73·1	73·1	1·83	0·319	0·600	0·894
Supplemented	71·5	74·2	71·8	73·7	71·5	72·5				
Propionate:total VFA (%)										
Control	16·9	17·3	16·9	18·3	19·0	20·1	1·15	0·625	0·013	0·442
Supplemented	18·2	16·9	17·4	19·7	21·0	18·9				
Butyrate:total VFA (%)										
Control	5·3	5·5	4·3	4·6	5·4	5·2	0·85	0·733	0·991	0·571
Supplemented	5·3	5·2	6·2	5·2	4·8	5·1				
(Acetate + butyrate):propionate										
Control	4·9	4·7	4·8	4·4	4·3	4·0	0·36	0·545	0·044	0·743
Supplemented	4·3	4·8	4·7	4·0	3·7	4·2				

S, maternal supplementation; T, age of foal; S × T, interaction between maternal supplementation and the age of the foal.
* Five foals per maternal group.

of VFA (25·6 (SD 17·32) μmol/g fresh faeces). Although different from previous data in foals[10], this was not completely surprising. Meconium has been reported to be non-sterile in human neonates[49,50]. During the first 3 d of life, a rapid and early establishment of total bacteria and strict anaerobes occurred, as reported previously in foals[10,11,13]. The present results showed that amylolytic and lactate-utilising bacteria were the first functional groups involved in carbohydrate degradation that were established in foals during the first 2 weeks of life. Amylolytic bacteria enumerated on Petri plates were probably streptococci or lactobacilli, as previously reported in foals[13]. Because no starch is available in milk, these bacteria were probably involved in the degradation of substrates other than starch such as lactose and other oligosaccharides that may escape the precaecal enzymatic digestion and enter the foal's hindgut, as reported in human subjects[51]. Shifts in fermentation end products measured during the first days of life could be explained by bacterial metabolic cross-feedings. During the first 3 d, amylolytic bacterial activity could result in the production of acetate (about 88·6 % of total VFA) and probably in lactate, as reported in previous studies in foal[10,11] and calf faeces[52]. This could explain the acidification of pH. From d3, the increase in the molar proportion of propionate and butyrate could suggest that lactate utilisers were then involved efficiently in the conversion of lactate to propionate and butyrate[53,54]. Concomitantly, faecal pH reached neutrality, confirming the decrease in lactate concentration, as reported in the faeces of calves[52], known as a strong acid ($pK_a = 3{\cdot}86$). Moreover, the decrease in acetate concomitantly with the increase in butyrate could also result from the conversion of acetate by butyrate-producing bacteria[55].

In accordance with the literature[8], we observed foals eating forage from the second day of life. Forage intake is actually unknown but probably increased with age, as suggested by the grazing time that increased linearly while sucking activity decreased[8]. In suckling lambs, the intake of solid food increased significantly from the end of the first month of life[56,57]. In parallel with the enhancement of forage intake rich in complex parietal carbohydrates, we observed the establishment of fibrolytic bacteria, as in the rumen of lambs[56]. In accordance with previous data in foal faeces[13] and lamb rumen[58], cellulolytic bacteria first appeared at d2 and increased progressively, reaching stable values at d30. From the second week of life, the molar proportion of acetate and the ratio (acetate + propionate):butyrate increased. Such modifications in the VFA profile could result from cellulolytic activity and suggest that the bacterial fibre-degrading capacity appeared in the foal's hindgut. This is in agreement with observations in the rumen of milk-fed calves where fibrolytic activity was detected during the first week of life as shown by the increase in xylanase activity from d4, reaching a maximal value at d7[57]. When foals were 2 months old, the VFA profile remained constant and cellulolytic bacterial concentration was comparable with adult values similarly to what has been reported in the rumen of young ruminants[9]. In accordance with Rey *et al.*[57] reporting that the main ruminal fermentative and enzymatic activities are stabilised approximately 1 month of age in calves, the present data could suggest that the fibre-degrading capacity in foals was established at 2 months of age.

For quantifying specific fibrolytic bacterial strains in the faeces of foals, we used a complementary approach to the conventional culture-based method: real-time PCR technology based on the molecular analysis of the bacterial *16S* rRNA gene. In adult horses, the population of *F. succinogenes* was successfully estimated using competitive PCR assays[5] or semi-quantitative real-time PCR[2]. In the present study, we used the real-time PCR assay with standard curves based on bacterial DNA from pure culture to perform the absolute quantification of *F. succinogenes*[41]. Similarly to cellulolytic bacteria establishment, *F. succinogenes* was first detected at d5 and increased significantly from d30 to d60 and reached high stable values (about 6·85 $\log_{10}$ cells/g fresh faeces),

suggesting that *Fibrobacter* could be one of the most abundant fibrolytic bacteria established in foals, as reported in calves[59,60]. Nevertheless, other fibrolytic bacterial strains, such as *R. albus* and *R. flavefaciens* that have been reported in adult horses[2,3,5], may be present in foals.

In human infants, the structure of faecal bacterial communities shows a relative stability after 30 d of life and the bacterial composition may fluctuate slightly until weaning[61]. Similarly to this observation, only minor changes occurred in the foal faecal bacterial communities and activities after 2 months of age and were probably correlated with changes in the feeding behaviour of the foals. Indeed, milk consumption decreased following the decrease in mare milk production after the second month of lactation[62]. This could explain why counts of bacteria involved in lactate metabolism significantly decreased until 6 months of age. Moreover, we observed an increase in the molar percentage of propionate and a decrease in the (acetate + butyrate):propionate ratio from d90 to d120. This probably resulted from the degradation of starch contained in pellets into lactate by amylolytic bacteria, which was then converted to propionate by lactate-utilising bacteria. The addition of pellets in the diet of foals aimed at covering the high nutrient requirement that exceeds the level of energy and protein supply provided by both ingested milk and forage at this age[33]. Such changes in bacterial activities have also been reported in the hindgut of adult horses, when the proportion of cereals increased in the ration[63] and in the rumen of weaned lambs when concentrate intake increased[57].

At 6 months of age, the bacterial composition in foal faeces was similar to that of mares. Nevertheless, few differences persisted in bacterial fermentative activities as suggested by the (acetate + butyrate):propionate ratio that remained lower in foals compared with mares. As foals still suckled their mother before weaning, lactose and other oligosaccharides may have reached the hindgut and enhanced the activity of lactate-producing bacteria. As lactate could then be converted to propionate by lactate-utilising bacteria, this could explain the higher proportion of propionate that we measured in foals compared with mares. We also measured a higher proportion of butyrate and a lower proportion of acetate in foals compared with mares. This could be partly due to the conversion of lactate and acetate by butyrate-producing bacteria[55]. This could also be explained by a less efficient fibre-degrading capacity in foals than in adult horses. Among the explanatory factors, the intake level of forage was certainly lower in foals compared with mares, as suggested by the time spent in grazing, which has been reported to be lower in foals compared with mares: approximately 48 and 80 % of time, respectively, in animals with free access to pasture[8]. Another explanatory factor could be the retention time in the digestive tract of foals that may be shorter than in mares. Nevertheless, it is actually unknown in the suckling foal. Further investigations are needed to determine when the bacterial carbohydrate-degrading capacity in the growing horse is similar to that of the adult horse.

Effect of maternal supplementation on the bacterial digestive ecosystem in foals

In the present study, maternal supplementation with fermented feed products had an effect on the early establishment of total anaerobes and lactate utilisers from d0 to d3. This result was correlated with significant effects of maternal supplementation on the age-related change in VFA profile and pH from d1 to d5. We hypothesised that these differences between the groups of foals could be due to (1) the modification of the bacterial ecosystem in mares and/or (2) an alteration in milk production.

In supplemented mares, no change in the bacterial composition was detected, but the molar proportion of acetate and the (acetate + butyrate):propionate ratio were higher, suggesting an improvement in bacterial fibrolytic activity. As reported in the faeces of weaned pigs supplemented with yeast fermentation products using real-time PCR[27], it is probable that the composition of the intestinal microbiota was altered in mares. However, these changes could not be detectable using the conventional culture technique. Although little is known about bacterial colonisation modes of the foal's hindgut, the maternal microflora could be directly transmitted to the foal during and after the birth conditioned by the close contact between the newborn and its mother[64]. Nevertheless, another contamination mode of the foal's hindgut could be the mare's milk. Indeed, it was recently demonstrated in a mouse model that bacterial migration from the digestive tract lumen via blood cells to the breast occurred in lactating mothers[65], suggesting that a mother-to-child efflux may exist during lactation and play a role in bacterial establishment in the digestive tract in neonates. This process could be confirmed by the presence of bacterial DNA detected in the milk of the mother[66]. Bacterial diversity has never been investigated in mare's milk. It could be an important source of maternal bacteria and/or bacterial DNA in the hindgut of suckling foals. To confirm or invalidate this hypothesis, further investigations on bacterial diversity in milk compared with that in foal and mare faeces are required.

In foals fed a commercial colostrum replacer at birth and once every 2 weeks during the first 3 months, fewer cellulolytic bacteria and fewer lactobacilli were counted in the faeces during the first 2 weeks of life[13], suggesting that milk composition could have an impact on bacterial intestinal establishment in foals. As milk is the main nutrient source for foals in the neonatal period, a change in milk composition and/or in quantity could also explain the higher rate of growth in foals of supplemented mares, as reported in suckling piglets of sows supplemented with the *Saccharomyces cerevisiae* fermentation product[28]. In supplemented mares, as previously explained, the fibrolytic activity of the microflora could be improved. This could lead to a better utilisation of the daily ration and thus an increase in milk production, as suggested in sows supplemented with the *S. cerevisiae* fermentation product[28]. Nevertheless, no energetic placebo was used in the present experimental control mares and fermented feed products provided higher energy in the basal

diet of mares (+3·8 MJ/d), and thus may have increased the energy composition of milk[67].

After the end of maternal supplementation with fermented feed products, no difference in growth rate was observed between the two groups of foals. Moreover, only slight modifications in total anaerobic and amylolytic bacterial counts and VFA concentrations were detectable. In the rumen of lambs[16], differential bacterial establishment in early life has shown to lead to differences in the structure of the bacterial communities that persisted after weaning. In the present study, it is not possible to explain the changes in the composition of the microbial communities in growing foals with regard to the differences observed in early bacterial establishment. Further investigations are required to explain these differences between the two groups of foals after 2 months of age.

Conclusion

The present study investigated for the first time the establishment of bacterial carbohydrate-degrading capacity in foals from birth to pre-weaning. The present results showed that the established bacterial communities were able to degrade fibre from 2 months of age, suggesting that suckling foals became herbivorous at a very early age, as young ruminants. Nevertheless, at the pre-weaning period, bacterial carbohydrate-degrading capacity in foals seemed not yet to be that of the adult. Moreover, the present results showed that maternal supplementation with fermented feed products stimulated foal growth and had an impact on the early establishment of some bacterial functional groups and their activities from d0 to d5 in foals but not in the longer term. The stimulation of growth could not be explained as such by a change in the microflora in foals.

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