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Comparison of the bacterial community structure within the equine hindgut and faeces using Automated Ribosomal Intergenic Spacer Analysis (ARISA)

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The horse's hindgut bacterial ecosystem has often been studied using faecal samples. However few studies compared both bacterial ecosystems and the validity of using faecal samples may be questionable. Hence, the present study aimed to compare the structure of the equine bacterial community in the hindgut (caecum, right ventral colon) and faeces using a fingerprint technique known as Automated Ribosomal Intergenic Spacer Analysis (ARISA). Two DNA extraction methods were also assessed. Intestinal contents and faeces were sampled 3 h after the morning meal on four adult fistulated horses fed meadow hay and pelleted concentrate. Irrespective of the intestinal segment, Principal Component Analysis of ARISA profiles showed a strong individual effect ($P < 0.0001$). However, across the study, faecal bacterial community structure significantly ($P < 0.001$) differed from those of the caecum and colon, while there was no difference between the two hindgut communities. The use of a QIAamp[®] DNA Stool Mini kit increased the quality of DNA extracted irrespective of sample type. The differences observed between faecal and hindgut bacterial communities challenge the use of faeces as a representative for hindgut activity. Further investigations are necessary to compare bacterial activity between the hindgut and faeces in order to understand the validity of using faecal samples.

Keywords: equine, gut, bacterial community, ARISA

Implications

The use of faeces for representing hindgut content in term of bacterial ecosystem is important. However the validity of using faeces may be questionable. This is why this study aimed to compare the bacterial communities' structure between the cecum, the right ventral colon and the faeces of alive horses. Faecal bacterial community structure differed from those of the caecum and colon, but the two hindgut communities were similar. Further investigation comparing bacterial composition and activity between large intestine compartments and faeces are required in order to state whether the faecal ecosystem is representative of different intestinal segments.

Introduction

The horse possesses a large intestinal (caecum and colon) microbial ecosystem essential for efficiently degrading fibre and thus providing nutrients from its naturally forage-based diet.

This microbial ecosystem has been well studied, in particular the bacterial community, and is now recognized to play a major role in equine nutrition and digestive health. Thus, a greater understanding of this community may allow for improved feed efficiency and horses health. In many studies, the large intestinal microbial ecosystem has been assessed by analysing faecal samples (Kobayashi *et al.*, 2006; Vörös, 2008; Yamano *et al.*, 2008; Willing *et al.*, 2009) due to their ease of collection. However, extrapolating results obtained from faeces to the hindgut situation is questionable. Indeed, it has yet to be demonstrated whether the faecal bacterial ecosystem is representative of that of the caecum and/or colon. Few studies have compared the bacterial community diversity of the equine caecum, colon and faeces (de Fombelle *et al.*, 2003; Hastie *et al.*, 2008; Dougal *et al.*, 2011 and 2012; Schoster *et al.*, 2013). Of these few studies, all of them concluded that the faecal bacterial community was not representative of that of the caecum. However, when comparing bacterial communities in the colon and faeces, differences in sampling location and/or the analytical techniques may have contributed to contradictory results. de Fombelle *et al.* (2003) and Hastie *et al.* (2008) quantified

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specific bacterial community in the right ventral v. left dorsal colon using culturing techniques and ventral v. dorsal colon using quantitative PCR. Recently, Dougal *et al.* (2011 and 2012) and Schoster *et al.* (2013) assessed the impact of gut location at the level of bacterial community using terminal restriction fragment length polymorphism (TRFLP). Dougal *et al.* (2011 and 2012) found that the bacterial diversity of the right dorsal colon was similar to that of the faeces, which could be expected since, anatomically, the right dorsal colon is the closest to the rectum. Interestingly, Schoster *et al.* (2013) observed, also by TRFLP, that the bacterial diversity of the pelvic flexure (between left ventral and left dorsal colon) was different from that of the faeces. In order to contribute to the faecal model statement, the present work compared the equine faecal bacterial community to those of the caecum and the right ventral colon by using a fingerprint profiles analysis: the Automated Ribosomal Intergenic Spacer Analysis (ARISA). As underlined by Kovacs *et al.* (2010) ARISA has become a very useful tool for comparing community structure across multiple samples based on profile patterns. Additionally, this fingerprint technique has a high-resolution, is highly reproducible and is very robust for discriminating between microbial communities based on the length polymorphism of the intergenic spacer (IGS) between the small (16S) and large (23S) subunit rRNA genes in the ribosomal RNA (*rrn*) operon (Ranjard *et al.*, 2001). ARISA like most molecular biology tools requires a DNA extraction step, which is hard to process from equine gut content due to components, such as humic acid, potentially inhibiting the PCR reaction (Yu and Morrison, 2004). Hence, the efficiency of different DNA extraction methods of equine caecal, colonic and faecal contents was also evaluated in the present paper.

Material and methods

Animal management and sample collection

Four adult crossbred geldings (average BW of 470 ± 17 kg), each fitted with polyvinyl chloride cannulas (30 mm i.d.) in the caecum and right ventral colon (Drogoul *et al.*, 2000), were individually housed in 3×3.5 m free stalls bedded with wood shavings. Horses were fed meadow hay (75% of dry matter intake) and pelleted fibrous concentrate (25% of dry matter intake) (DP Evasion, Invivo NSA, 71 500 Louhans, France; Table 1). The hay was offered at 1.15 kg of dry matter intake/100 kg BW per day). The ration was calculated to meet maintenance energy requirements according to French recommendations (net energy, UFC) (Martin-Rosset *et al.*, 1994). Hay and pellets were offered daily at 1000 and 1600 h, and 0800 and 1730 h, respectively.

Digestive contents were collected in duplicate, 3 h after the morning meal of concentrate (i.e. 1100 h). Caecal and colonic digesta samples were collected by gravity via the cannulas. Faecal grab samples were collected from the rectum. All samples were collected in Eppendorf tubes, placed on ice immediately after sampling and stored at -20°C until analysis.

Table 1 Chemical composition of hay and pelleted food

Item	Hay (g/kg)	Pelleted food (g/kg) ¹
Dry matter	880	890
Organic matter ²	934	919
CP ²	76	131
Fat ²	na	34
Crude fiber ²	322	153
NDF ²	640	420
Starch ²	na	151
Net energy (MJ/kg) ^{2,3}	4.88	8.52

¹The pelleted food had the following composition on a dry matter basis (%): wheat bran (32%), wheat shorts (16%), barley (15%), dehydrated alfalfa (10%), straw (9.4%), soya bean husks (5%), sugar cane molasses, pomace, vitamin and mineral premix.

²Dry matter basis.

³Calculating from nutritional composition (Martin-Rosset *et al.*, 2006).

The study was conducted under license from the Department of Health and Animal Care of the French Veterinary Authority.

DNA extraction and ARISA

Total DNA was extracted using two different methods: Yu and Morrison (2004) and Boom *et al.* (1990). In bacterial diversity studies of digestive contents, these methods alone comprise an initial step with a bead beating procedure allowing the highest bacterial diversity to be obtained (Salonen *et al.*, 2010). After this mechanical cell disruption, each method had its own specific step for nucleic acid purification. The Yu and Morrison (2004) method uses purification by QIAamp[®] columns (Qiagen, Valencia, Canada), whereas Boom *et al.* (1990) uses a Guanidium isothiocyanate/silica matrix.

One ml of caecal and colonic content and 0.25 g of faeces were used for both methods. DNA was quantified using a spectrophotometer (Eppendorf, Hamburg, Germany) and its purity was assessed by calculating $A_{260/280}$ ratios. The $A_{260/280}$ ratio is an indication of contamination by proteins and should be in the region of 2.0 for pure DNA and should exceed 1.7 for nearly pure nucleic acid extractions from environmental samples.

The ribosomal InterGenic Sequence (IGS) present between the 16S and the 23S rDNA gene of bacteria was amplified by PCR using primers S-D-Bact-1522-b-S-20 (5'-TCGGGCTGGATGACCTCCTT-3') and L-D-Bact-132-a-A-18 (5'-CCGGGTTCCCATTCGC-3'), the forward primer was labelled at the 5' end with IRD800 fluorochrome (Ranjard *et al.*, 2001). The PCR mixture (25 μl) contained $1 \times$ PCR buffer, 25 $\mu\text{mol/l}$ of each dNTP, 0.5 $\mu\text{mol/l}$ of each primer, 0.6 μg T4 gene 32 protein (MP Biomedicals, Santa Ana, CA, USA), 3.75 U of Taq DNA polymerase (MP Biomedicals) and 2.5 ng/ μl of purified sample DNA. Amplification was performed in a Perkin Elmer Cetus 2400 after a hot start at 94°C for 3 min, followed by 30 cycles consisting of 94°C for 1 min, 55°C for 30 s, and 72°C for 1 min, extension of incomplete products for 5 min at 72°C . PCR products were purified by MinElute kit (Qiagen).

ARISA fragments were resolved on 3.7% polyacrylamide gels. A pre-run was done in $0.8 \times$ TBE buffer for 1 h 30 min at

20°C using a fixed voltage of 2500 V. Then, ~2.5 ng/μl of denatured purified PCR product were applied per well and electrophoresed for 15 h at 45°C using a fixed voltage of 2500 V on an 4300 DNA Analyzer (LI-COR Biosciences, Lincoln, USA). The data were analysed using the 1D-Scan software (ScienceTec) (Ranjard *et al.*, 2003).

Statistical analysis

The DNA extraction data were processed by ANOVA using the MIXED procedure of SAS v9 (SAS Institute Inc., Cary, NC, USA). The statistical model included DNA extraction method, gut segment and gut segment × DNA extraction method. Effects of individual animals were considered as random variables. Effects were regarded as statistically different at $P < 0.05$.

ARISA statistical analyses were carried out using R version 2.9.2 (<http://www.r-project.org>). Data obtained from the 1D-Scan software were converted into a table summarizing the band presence (i.e. peak) and intensity (i.e. area of peak) using the PrepRISA program (Ranjard *et al.*, 2001). One hundred bands were integrated for each ARISA profile with 2 bp resolution to ensure a robust analysis (Ranjard *et al.*, 2003). The data were then analysed using a principal component analysis (PCA) performed on the covariance matrix of the samples (samples as rows and bands as columns describing the bacterial community). This method provided an ordination of the samples according to their bacterial community genetic structure. The relevant number of principle components was determined according to their eigenvalue which displays the amount of variance explained by each axis (Brocard *et al.*, 2011). In the present analysis, the first three components contributed to 20%, 15% and 13% of the total variance of equine hindgut bacterial communities, whereas the remaining variance was distributed along the 35 other components (Table 2). Therefore, the first three axes were considered in the analysis and in the permutation tests. According to this, the samples were plotted on the factorial map according to their scores on the first two principle components. The third principle component was not represented on the figure to limit the number of data. Differences between gut segments, DNA extraction methods and gut segment × DNA extraction method were tested by a Monte-Carlo permutation test (1000 permutations). All these analyses were performed with the R software using the ADE-4 package (Thioulouse and Dray, 2007).

Results

Effect of DNA extraction method on equine gut bacterial community

Irrespective of gut segment, the DNA yield and purity were significantly higher when samples were extracted using the Yu and Morrison (2004) method ($P < 0.0001$; Table 3) compared with the Boom *et al.* (1990) method. Irrespective of DNA extraction method, DNA yield from faecal samples was higher and purity was lower ($P < 0.0001$; Table 3) that those of caecum and right ventral colon samples.

Table 2 Eigenvalues and the contribution of principal components (PC) to the total variance in equine hindgut bacterial communities

Items	Eigenvalue	Proportion of total variance (%)	Cumulative variance (%)
PC1	0.008	20	20
PC2	0.006	15	35
PC3	0.005	13	48
∑PC4 to PC38	0.02	52	100

ARISA fingerprints of bacterial communities provided complex profiles (Figure 1) and some bacterial community profiles are unavailable, likely due to the presence of inhibitors during the PCR amplifications (Figure 1). Irrespective of animal or gut segment, no difference was observed in equine gut bacterial community structure due to DNA extraction method ($P > 0.05$; Figure 1). PCA and the Monte-Carlo permutation test of ARISA profiles showed no differences in bacterial community structure between duplicates ($P > 0.05$; Figure 1).

Effect of gut segment on equine gut bacterial community

Visual comparison of the ARISA profiles (Figure 1) showed that equine gut bacterial community structure was subject to a strong individual effect, as confirmed by PCA and the Monte-Carlo permutation test ($P < 0.0001$; Figure 2).

The PCA results for the ARISA profiles of caecal, colonic and faecal bacterial communities are presented according to the gut location without (Figure 2) or with inclusion of inter-animal variation (Figure 3). Faecal bacterial community was separated from those of the caecum and colon on both axes, which explained 20% and 15% of the total variability on axis 1 and 2, respectively (Figures 2 and 3). A permutation test confirmed that gut segment was an important factor in the discrimination of the equine hindgut bacterial community structure: the faecal bacterial community appeared significantly different from those of the caecum and colon ($P < 0.0001$; Figures 2, and $P < 0.01$; Figure 3). Except for horse D ($P < 0.01$; Figure 3), bacterial community structure from the caecum and right ventral colon were not different ($P > 0.05$; Figure 2).

Discussion

This is the first report that simultaneously compares the bacterial community structure from different gut segments (caecum, colon and faeces) *in vivo* using the DNA fingerprint technique, ARISA. A key issue in the success and sensitivity of the ARISA process is the purity of DNA extracted from the intestinal content matrix. Compared with blood or other clinical samples, DNA extraction from samples of intestinal content, especially faeces, has been reported to be particularly difficult due to the variation in consistency, as well as endogenous and dietary components of this matrix (McOrist *et al.*, 2002;

Table 3 Comparison of DNA yield and purity extracted by Yu and Morrison (2004) or Boom *et al.* (1990) method (mean values with their standard errors (s.e.m.))

DNA extraction method	Gut segment				s.e.m.	Significance ¹		
	Caecum	Colon	Faeces	M		S	M × S	
DNA yield (µg/g)	Yu and Morrison	22.5 ^{aA}	43.5 ^{aA}	84.0 ^{aB}	7.9	***	***	ns
	Boom <i>et al.</i>	4.5 ^{bA}	12.5 ^{bA}	55.0 ^{bB}				
DNA purity (260/280 ratio)	Yu and Morrison	1.8 ^{aA}	1.8 ^{aA}	1.5 ^{aB}	0.1	***	***	ns
	Boom <i>et al.</i>	1.4 ^{bA}	1.3 ^{bA}	1.2 ^{bB}				

^{a,b}Values within an item and a column are different if superscript differs ($P < 0.05$).

^{A,B}Values within a row are different if superscript differs ($P < 0.05$).

¹Significance of effects of DNA extraction method (M), the anatomic segment of the hindgut (S) and of the interaction (M × S).

***Significant ($P < 0.0001$); ns, not significant.

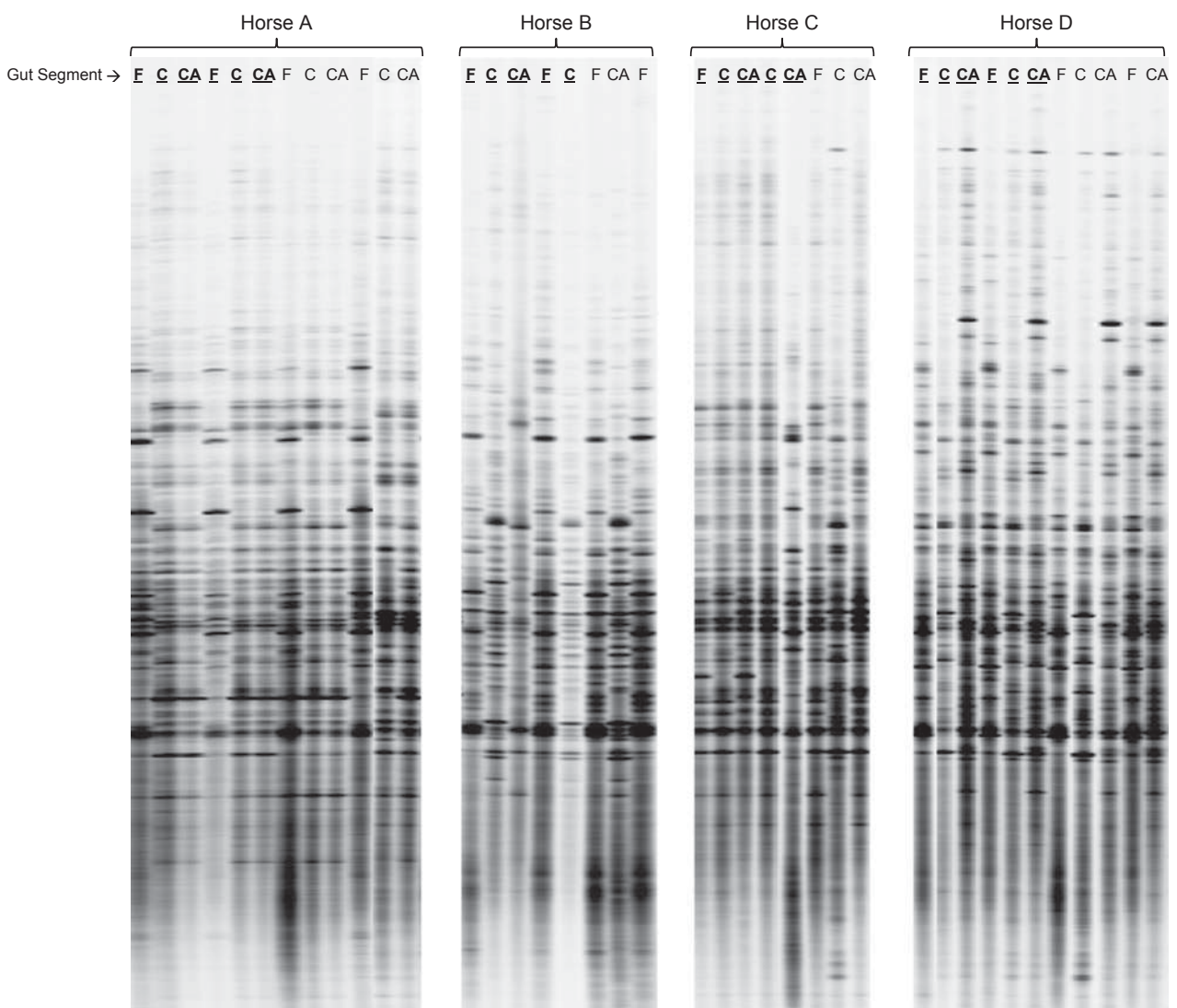


Figure 1 Typical Automated Ribosomal Intergenic Spacer Analysis gel (ARISA) profiles of bacterial communities present in the caecum, colon and faeces (CA, C and F, respectively) of four horses. Both DNA extraction methods are represented with the Yu and Morrison (2004) method underlined in bold.

Yu and Morrison, 2004). Hence, this study also investigated the efficiency of two different DNA extraction methods, as well as the variation in structure of bacterial communities from faeces and different regions of the gut.

Equine gut bacterial ecosystem: effect of DNA extraction methods

For both DNA extraction methods, the difference in DNA yield between faeces *v.* the caecum and colon could

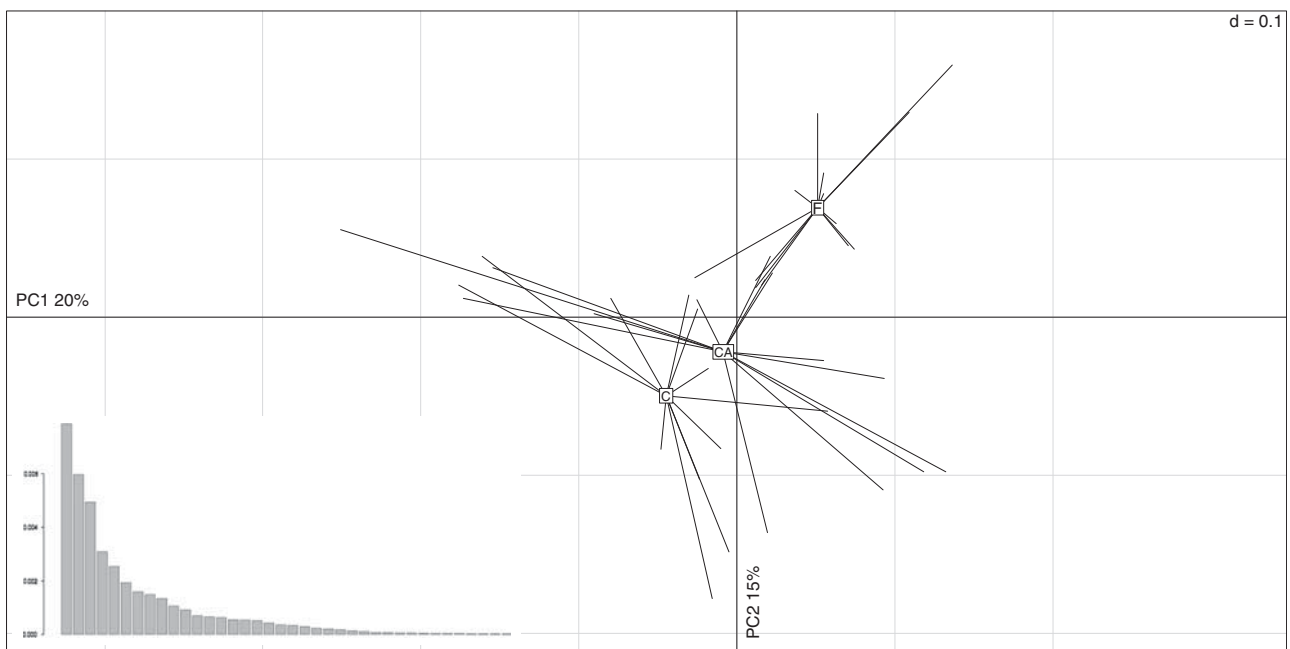


Figure 2 Comparison of Automated Ribosomal Intergenic Spacer Analysis variability performed by principal component analysis (PCA) of profiles obtained from different equine gut segments. The eigenvalues are represented by the graphic at bottom left of PCA. CA = caecum; C = colon; F = faeces.

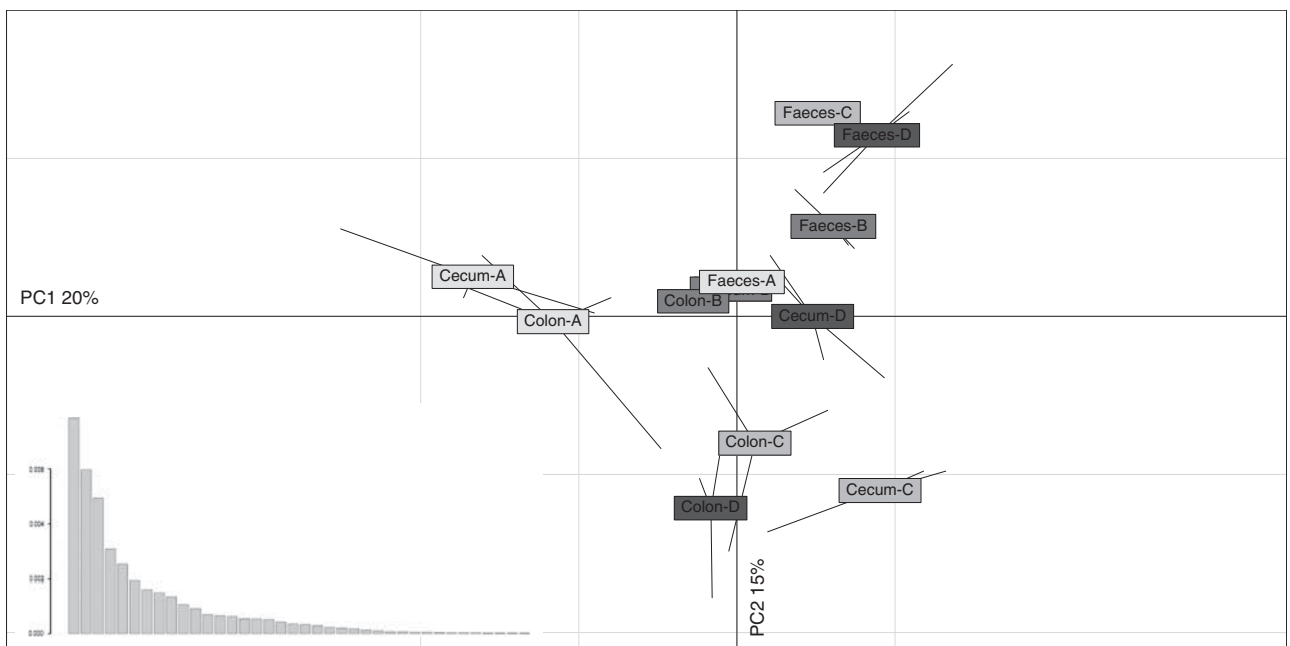


Figure 3 Comparison of Automated Ribosomal Intergenic Spacer Analysis variability performed by principal component analysis of profiles obtained from different equine gut segments (caecum, colon and faeces) taking into account the inter-animal variation (horses A, B, C and D). The eigenvalues are represented by the graphic at bottom left of principal component analysis (PCA).

partly be explained by the higher water content of the caecal and colonic samples possibly diluting the quantity of extracted DNA.

For faecal samples, the DNA yield we obtained using the Yu and Morrison (2004) method was in accordance with that found in the original article (Yu and Morrison, 2004). No such comparison could be made with the Boom *et al.* (1990) method as it primarily extracts DNA from human serum and

urine, which are not comparable to intestinal contents. Moreover, these authors did not give any data about the DNA yield obtained by their protocol. Additionally, McOrist *et al.* (2002) who used the Boom *et al.* (1990) method to extract DNA from human faecal samples did not provide any data about the DNA yield. The Yu and Morrison (2004) method had the better DNA yield, suggesting a higher efficiency of cell disruption, DNA precipitation and elution.

According to Salonen *et al.* (2010), DNA yield alone may be an unreliable measure for extraction efficiency and should be complemented by other parameters, such as DNA purity and bacterial diversity. Therefore, in this study, DNA purity was assessed spectrophotometrically. The Yu and Morrison (2004) method yielded more pure DNA extract compared with Boom *et al.* (1990). No difference was observed between ARISA profiles whatever the DNA extraction method, suggesting that neither method induced bias in DNA bacterial community extraction. To our knowledge neither DNA extraction method has been assessed using a finger-print technique.

According to McOrist *et al.* (2002), use of the QIAamp kit in the extraction of bacterial DNA from faeces gave superior downstream performance in PCR. Other studies found that this kit was effective for overcoming problems of coextracted PCR inhibitors from faecal samples (Foltan *et al.*, 2005; Nechvatal *et al.*, 2008). In the present study, even though the DNA extraction methods have no impact on bacterial community profiles obtained using ARISA, we chose to extract equine gut content microbial DNA using the Yu and Morrison (2004) method as it provided a better yield and purity of DNA allowing a superior downstream performance in PCR. This type of decision could prove important in similar future studies, where inhibitors of PCR are present (McOrist *et al.*, 2002; Yu and Morrison, 2004). Moreover, the Yu and Morrison (2004) protocol does not utilize highly toxic components, such as guanidium isothiocyanate and may, therefore, be more suitable than Boom *et al.* (1990) in terms of health and safety.

Equine bacterial community structure: hindgut v. faeces

The strong individual effect observed, irrespective of the intestinal segment, confirmed the significant individual variation reported previously in equine gut contents using temperature gradient gel electrophoresis (Faubladier *et al.*, 2006), TRFLP (Schoster *et al.*, 2013) or denaturing gradient gel electrophoresis analyses (Grønvold *et al.*, 2010). This high inter-animal variation probably contributed to the low PCA axes discrimination (20% and 15%) observed.

In the present study, we compared gut bacterial community structure averaged across all animals, as well as on an individual animal basis. With the exception of Schoster *et al.* (2013), within-horse and between-horse comparisons have not been accounted for when equine bacterial ecosystems in different segments of the gastrointestinal tract have been compared. Thus, published data have generally been averaged across animals with inter-animal variation nor discussed (de Fombelle *et al.*, 2003; Hastie *et al.*, 2008; Dougal *et al.*, 2011 and 2012).

This study demonstrated a difference between the faecal and hindgut (caecal and colonic) bacterial community structures both averaged across all animals and for individual horses. This finding is interesting and suggests that the inter-segment variation is stronger than the inter-animal variation. The difference observed between the bacterial community structure from the caecum and faeces is in accordance with

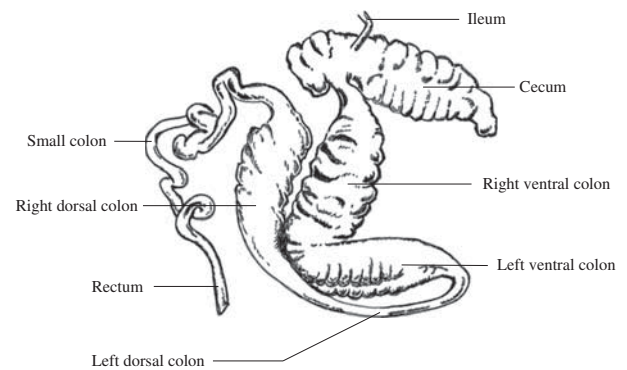


Figure 4 Different segments of the horse large intestine.

previous studies using either culturing (de Fombelle *et al.*, 2003) or molecular biological techniques (Hastie *et al.*, 2008; Dougal *et al.*, 2011 and 2012; Schoster *et al.*, 2013). Conversely, the bacterial community structure from the right ventral colon showed no difference compared with that from the caecum but was different from that from the faeces. This is consistent with previous studies comparing bacterial communities between the caecum and right ventral colon (Daly *et al.*, 2001) or the pelvic flexure (Schoster *et al.*, 2013), or between the right ventral colon or the pelvic flexure and faeces (de Fombelle *et al.*, 2003; Faubladiet *et al.*, 2006; Müller *et al.*, 2008; Schoster *et al.*, 2013). More precisely, according to de Fombelle *et al.* (2003), the total anaerobic bacterial counts were higher in the faeces than in the right ventral colon. Additionally, faecal samples have been reported to have a higher concentration of lactate-utilizing (Müller *et al.*, 2008) and lactate-producing bacteria than samples collected from the right ventral colon (de Fombelle *et al.*, 2003; Müller *et al.*, 2008). Interestingly, Dougal *et al.* (2011 and 2012) sampled intestinal contents from the right dorsal (and not right ventral) colon and found no difference between colonic and faecal bacterial communities, however, they highlighted a difference when comparing caecal and colonic contents. Anatomically, the right ventral colon is closer to the cecum than the right dorsal colon (Figure 4). Conversely, the right dorsal colon is closer to the faeces than the right ventral colon (Figure 4). These differences in microbial communities according to anatomical locations could be related to selective pressures acting on the microorganisms at the different levels of the alimentary tract (Camp *et al.*, 2009). In addition the presence of different microbial communities along the hindgut of horse suggests a specific microbial activity according to the anatomical locations.

However, there is disagreement among studies comparing the bacterial community from the right ventral colon with that from faeces (de Fombelle *et al.*, 2003; Hastie *et al.*, 2008; Sadet-Bourgeteau *et al.*, 2011). There are various factors that could potentially explain these contradictions. Dietary effects could be implicated, however, there is insufficient dietary composition information in the study of Hastie *et al.* (2008) to draw any conclusions. Differences in

analytical methodology used to study bacterial diversity could also be a source of variation. de Fombelle *et al.* (2003) used culturing techniques and found that the faecal bacterial community was different from that of the colon, whereas Hastie *et al.* (2008) and Sadet-Bourgeteau *et al.* (2011), using molecular techniques, reported that the colonic bacterial community was not different from that of the faeces. The different techniques used (culturing *v.* molecular) could explain the discordance in results obtained.

Several studies reporting on the equine gut microbiota have used faecal samples and results have been extrapolated to the hindgut. The present work underlined that the bacterial community structure appears different in the faeces compared with the caecum and right ventral colon. Thus, faeces could probably not be used in the field as a marker of the hindgut in terms of bacterial community structure. However, studying the structure of equine large intestine bacterial communities is not enough for concluding on the validity of faecal samples. Further investigation is necessary to compare bacterial composition and activity between large intestine compartments and faeces in order to assess more precisely the effect of individual variation and to state whether the faecal ecosystem is representative of different intestinal segments.

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