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GENERAL ARTICLE

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Faecal microbiota and antimicrobial resistance gene profiles of healthy foals

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Abstract

Background: The human and domestic animal faecal microbiota can carry various antimicrobial resistance genes (ARGs), especially if they have been exposed to antimicrobials. However, little is known about the ARG profile of the faecal microbiota of healthy foals. A high-throughput qPCR array was used to detect ARGs in the faecal microbiota of healthy foals.

Objectives: To characterise the faecal microbiota and ARG profiles in healthy Australian foals aged less than 1 month.

Study design: Observational study.

Methods: The faecal microbiota and ARG profiles of 37 Thoroughbred foals with no known gastrointestinal disease or antimicrobial treatment were determined using 16S rRNA gene sequencing and a high-throughput ARG qPCR array. Each foal was sampled on one occasion.

Results: *Firmicutes* and *Bacteroidetes* were dominant in the faecal microbiota. Foals aged 1-2 weeks had significantly lower microbiota richness than older foals. Tetracycline resistance genes were the most common ARGs in the majority of foals, regardless of age. ARGs of high clinical concern were rarely detected in the faeces. The presence of ARGs was associated with the presence of class I integron genes.

Main limitations: Samples were collected for a case-control study so foals were not sampled longitudinally, and thus the development of the microbiota as individual foals aged could not be proven. The history of antimicrobial treatment of the dams was not collected and may have affected the microbiota of the foals.

Conclusion: The ARGs in foal faeces varied concomitantly with age-related microbiota shifts. The high abundance of tetracycline resistance genes was likely due to the dominance of *Bacteroides* spp.

KEYWORDS

antimicrobial resistance gene, faecal microbiota, farm environment, horse

1 | INTRODUCTION

The gastrointestinal tract of neonatal foals is colonised in the first few days of life by bacteria from the mare's microbiota (vaginal, perineal and milk) and the foal's environment, and later by core members from the adult faecal microbiota through coprophagy.[1,2] Progressive, age-dependent changes in the foal faecal microbiota have been identified[1-4] and, after 2 months, the foal faecal microbiota becomes relatively stable and closely resembles the adult faecal microbiota.[3,4] Bacteria associated with fibre digestion (eg *Lachnospiraceae*, *Ruminococcaceae* and *Fibrobacteres*) increase proportionally with age in the faecal microbiota of the foal to become dominant.[3,4] Changes in the faecal microbiota have been associated with age, diet,[5] temperature,[6] diarrhoea[7] and antimicrobial exposure.[8] Use of trimethoprim-sulfadiazine in healthy horses has been found to result in a decrease in faecal microbiota diversity and a reduction in *Verrucomicrobia* for at least 9 days after treatment.[8]

Bacteria within the microbiota of humans and other animals can harbour antimicrobial resistance genes (ARGs).[9,10] Many studies of ARGs associated with horse intestinal bacteria have been limited to the readily culturable bacteria, such as *Escherichia coli* and *Enterococcus* species.[11-14] Although these bacteria are useful indicators for monitoring ARGs in faeces, they only represent a small fraction of the bacteria in the equine microbiota. The abundance of ARGs in the total faecal microbiota from horses remains largely unexplored. Culture independent approaches, such as deep sequencing and high-throughput qPCR (HT-qPCR), bypass the limitations of culture and enable a holistic evaluation of ARGs in the microbiota. High-throughput qPCR has been used to investigate a broad range of ARGs in faecal samples of various animal species.[9,15,16]

The aim of this study was to characterise the faecal microbiota and ARG profiles of farm-raised foals less than 30 days of age that had had no direct exposure to antimicrobial agents. Factors with a potential to affect the microbiota and the ARG profile were also analysed.

2 | MATERIALS AND METHODS

2.1 | Sample collection and DNA extraction

Foal faecal samples and medical records were collected as part of a previous age-matched case-control study of diarrhoea.[17] Only samples from control foals aged less than 30 days were included in the current study. A single faecal sample was obtained from each of 37 Thoroughbred foals on four stud farms in the Hunter Valley, New South Wales, Australia, between September and December 2010 (Table S1). The foals had no known history of diarrhoea in the week preceding sampling and there was no record of prior antimicrobial treatment.

Freshly voided faeces were collected from foals while their dams were being examined for breeding purposes. Faecal samples were collected into sterile containers and kept overnight at −20°C, then stored at −70°C until processing. DNA was extracted from 0.2 g of faeces using the DNeasy PowerSoil kit (Qiagen) following the manufacturer's instructions. A FastPrep bead homogeniser (MP Biomedicals) was used at 5.5 m/s for 30 s to break the bacterial cells open. The quality and quantity of DNA were determined using a Nanodrop ND-1000 (NanoDrop Technologies). DNA was stored at −20°C until analysis.

2.2 | Bacterial 16S rRNA gene sequencing and bioinformatic analysis

The V3-V4 region of the bacterial 16S rRNA genes was amplified using primers 341F′(CCTAYGGGRBGCASCAG) and 806R′(GGACTACNNGGGTATCTAAT).[18,19] Amplicons were sequenced on an Illumina Miseq platform. Sequences were analysed through a QIIME2 pipeline (Version qiime2-2018.11).[20] DADA2 was used to trim the de-multiplexed reads to 250 bp and they were de-noised, paired and grouped into amplicon sequence variants (ASV).[21] A pre-trained Naïve Bayes classifier and the Greengenes database (2013 August release) were used for taxonomic assignment.[22,23] ASVs only present in a single sample or classified as non-bacterial were removed. Sequences from each foal were randomly subsampled to the same depth for fair comparison. The minimum number of reads that would not affect coverage or result in the discard of any samples was used. Good's coverage was calculated for each sample to assess the adequacy of the reads. Sequence data are available in the NCBI database under accession number PRJNA638757.

2.3 | Detection and quantification of ARGs in foals

ARGs were detected using the SmartChip Real-time PCR system (Takara Bio USA). Forty-four primer pairs were used to amplify ARGs encoding resistance to the following groups of antimicrobials: aminoglycosides, beta lactams, quinolones and phenicols (FC), macrolide-lincosamide-streptogramin B (MLSB), sulphonamides/trimethoprim, tetracyclines, virginiamycin and streptothricin. Primer pair "blaCTX-M-04" amplified the group 2 type $bla_{CTX\text{-}M}$ genes, such as $bla_{CTX\text{-}M\text{-}2}$. Two primer pairs were used to amplify the class I integron markers *int*I1 and *qac*EΔ, and one primer pair was specific for the bacterial 16S rRNA gene (Table S2).[9,24] The primers used were a subset of 295 primers validated in previous studies.[9,24,25] Primers for intrinsic resistance genes were excluded and genes detected in livestock in previous studies were included.[9,10,26]

The qPCR conditions were previously described by Wang et al.[24] A cycle threshold (Ct) of 27 was used as the limit of detection.[9] The abundance of each ARG was normalised to the 16S rRNA gene abundance in each sample using the equation:$\text{relative abundance} = \frac{E(16S)^{Ct(16S)}}{E(ARG)^{Ct(ARG)}}$, where E(16S) was the efficiency of amplification of the 16S rRNA gene, E(ARG) was the efficiency of amplification of each ARG and Ct was the threshold cycle.[27,28]

2.4 | Data analysis

Statistical analyses were completed using QIIME2 (version qiime2-2018.11)[20] and R (version 3.6.3).[29] Foals were grouped by sex, farm or age. The impact of these factors on the faecal microbiota and ARG profiles was tested using non-metric multidimensional scaling (NMDS) based on a Bray-Curtis distance matrix calculated from genus-level ASVs and ARG relative abundance respectively. The similarity of the faecal microbiota and ARG profiles between groups was tested by permutational multivariate analysis of variance (PERMANOVA) using the 'adonis' function in the R package vegan.[30] The *P* value of pairwise PERMANOVA was adjusted using the Benjamini-Hochberg method using the R package RVAideMemoire.[31]

Differences in the abundance of bacterial taxa, at family and genus level, between the age groups were tested using the analysis of composition of microbiomes method (ANCOM).[32] Alpha diversity of the faecal microbiota of each foal was measured based on ASVs. Richness indices Chao1 and ACE were used to estimate the number of ASVs in the sample, and the Shannon and Simpson diversity indices were used to estimate the richness and evenness of the microbiota of each sample. Differences in Chao1, ACE and Shannon indices between age groups were tested using one-way ANOVA with *post-hoc* analysis by Tukey's Honest Significant Difference (HSD) test. Differences in the Simpson index between age groups were tested using the pairwise Wilcoxon rank sum test, with *P* values adjusted using the Benjamini-Hochberg correction method.[33] A $P < .01$ was considered significant.

Differences in the relative abundance of ARGs between foal age groups were tested with the pairwise Wilcoxon rank sum test, with the *P* value adjusted using the Benjamini-Hochberg correction method.[33] A $P < .01$ was considered significant. The patterns of co-occurrence of ARGs were analysed using the Spearman rank correlation using the 'rcorr' function in the R package Hmisc.[34] Correlations were represented as networks with only strongly co-occurring ARGs (Spearman's rho ≥0.75, $P < .01$) shown. To avoid false positives, ARGs detected in fewer than 30% of samples were excluded. *P* values were adjusted using a Benjamini-Hochberg correction method.[33] The network visualisation was performed using Gephi.[35]

3 | RESULTS

3.1 | Impact of sex, farm and age on the faecal microbiota profile

A total of 1 855 588 reads from 37 foals passed all quality filters and were partitioned into ASVs. The original read depth of all samples ranged from 22 443 to 454 745. A subsample of 22 443 reads was randomly selected for each sample to enable fair comparisons. The rarefied reads were partitioned into 2085 ASVs. The Good's coverage index based on ASV ranged from 0.99 to 1, indicating a sample depth of 22 443 reads was sufficient for accurate estimates of the microbiota richness and evenness.

The foals were grouped based on their sex, farm of origin or age: 23 foals were female and 14 were male; 13 were from farm A, 10 from farm C, 4 from farm D and 10 from farm E. They were categorised

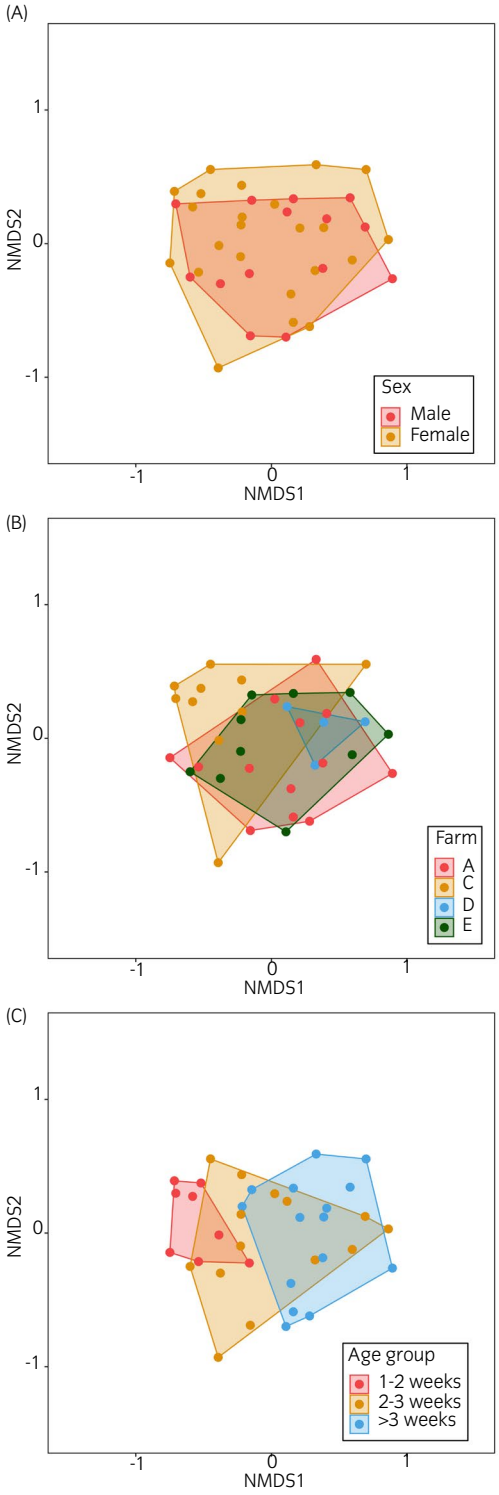

FIGURE 1 Similarity of the faecal microbiota of foals grouped by sex (A), farm (B) and age (C). NMDS analysis with Bray-Curtis distances was based on genus-level ASVs (NMDS stress = 0.18)

into three age groups: 1-2 weeks (n = 8, aged 8-14 days), 2-3 weeks (n = 14, aged 15-21 days) and >3 weeks old (n = 15, aged 23-29 days). The differences in the microbiota profiles between the three age groups are shown in Figure 1A-C. No significant separation was seen between the sex or farm groups (Figure 1A,B, Table S3). Significant separation was seen between foals aged 1-2 and >3 weeks ($P = .003$), with foals aged 2-3 weeks intermediate, with no significant separation from the other two groups (Figure 1C, Table S3).

3.2 | Faecal bacterial composition of foals in different age groups

Bacterial taxa with relative abundances above 0.5% within a sample, and detected in at least 33% of samples, were considered to represent the main taxa (Figure 2A-C). *Firmicutes* and *Bacteroidetes* were dominant in the faecal microbiota and comprised up to 80% of the reads from each age group (Figure 2A). *Verrucomicrobia*

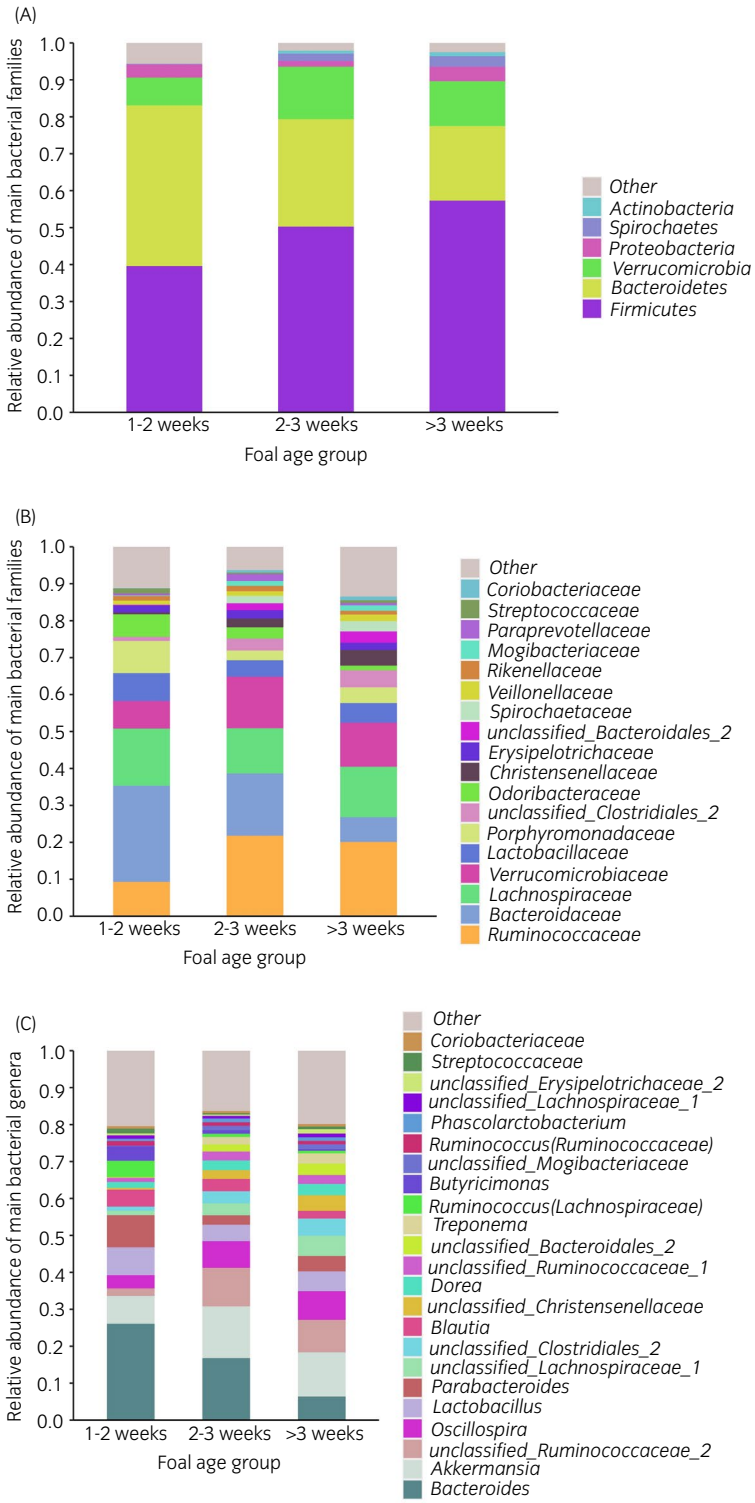

FIGURE 2 Relative abundance of dominant bacterial phyla (A), families (B) and genera (C) in the faecal microbiota of foals in each age group. Only taxa with a relative abundance above 0.5% in more than 33% of samples are shown. Phyla, families and genera below the cut-off were assigned to "Other"

TABLE 1 Taxa with significant differences in abundance between different age groups of foals

	ANCOM W[a]	Relative abundance (%)		Abundance ratio (>3 wk/1-2 wk)
		1-2 wk	>3 wk	
1-2 vs >3 wk				
Family				
Christensenellaceae	40	0.48	4.27	8.9
Odoribacteraceae	37	6.13	1.24	0.2
Genus				
Unclassified Christensenellaceae	78	0.48	4.27	8.9
Butyricimonas	68	3.92	0.41	0.1
Unclassified Clostridiales 1	64	0.02	0.46	23
Eubacterium	62	0.43	0.04	0.1
Treponema	61	0.02	2.78	139
	ANCOM W[a]	**Relative abundance (%)**		**Abundance ratio (>3 wk/2-3 wk)**
		2-3 wk	**>3 wk**	
2-3 vs >3 wk				
Family				
Odoribacteraceae	34	3.05	1.24	0.4
Christensenellaceae	33	2.38	4.27	1.8
Genus				
Butyricimonas	65	1	0.41	0.4
Unclassified Christensenellaceae	64	2.38	4.27	1.8

Abbreviation: wk, weeks of age.

[a]Differing abundances of taxa between age groups were assessed using ANCOM, adjusting for farm. For each of the n taxa, the mean abundance between two age groups was compared to the data for the other n−1 taxa. W is the number of null hypotheses rejected for the taxa (which can range from 0 to n−1). Taxa with a proportion of rejected hypotheses >0.7 were considered to have a significantly different abundance between age groups. The ANCOM comparison was performed using 86 genera and 47 families. ANCOM does not assign a *P* value for the final result, but *P* values with a Benjamini-Hochberg correction were used in the pipeline to calculate W.

was the third most abundant phylum, and comprised 8%, 14% and 12% of the total reads from foals aged 1-2, 2-3 and >3 weeks respectively.

At the family level (Figure 2B), *Ruminococcaceae*, *Bacteroidaceae*, *Lachnospiraceae* and *Verrucomicrobiaceae* were abundant in all age groups, and comprised more than 50% of the reads from each age group. At the genus level (Figure 2C), *Bacteroides*, *Akkermansia*, *Lactobacillus*, *Blautia*, *Dorea*, *Parabacteroides* and *Oscillospira* were abundant in all age groups, with each genus comprising at least 1% of the reads from each age group (Figure 2C).

Differences in the abundances of bacterial taxa at family and genus level between age groups are shown in Table 1. Foals aged >3 weeks had a significantly higher abundance of genus *Treponema* than the foals aged 1-2 weeks. Moreover, the family *Christensenellaceae* was significantly more abundant in foals aged >3 weeks than in the younger foals. On the other hand, the abundance of the genus *Butyricimonas*, as well as its family, *Odoribacteraceae*, was significantly lower in foals aged >3 weeks than in foals in the other two age groups. No family or genus had a significantly different abundance between foals aged 1-2 and 2-3 weeks.

3.3 | Comparison of alpha diversity of faecal microbiota between age groups

Four alpha diversity indices were calculated for the faecal microbiota in each group of foals (Figure 3). Foals aged 2-3 and >3 weeks had significantly higher faecal microbiota richness than foals aged 1-2 weeks, as indicated by the Chao1 richness estimator ($P < .01$) and the ACE index ($P < .01$) (Figure 3A,B). No significant difference was found between the age groups in any of the diversity indices (Figure 3C,D).

3.4 | Detection of ARGs in foal faeces

Foal samples were screened for a total of 44 ARGs using qPCR. Most ARGs were detected in at least one foal, with the exception of *sul*1, *lnu*B, *mph*C, *msr*A, *qnr*A, *tet*K, *vat*E and *vgb*B. The relative abundances of ARGs in individual foals are presented in Figure 4 as proportions relative to the 16*S* rRNA gene. A total of 10 ARGs were detected in 30/37 of samples (81%) (Table 2). The tetracycline

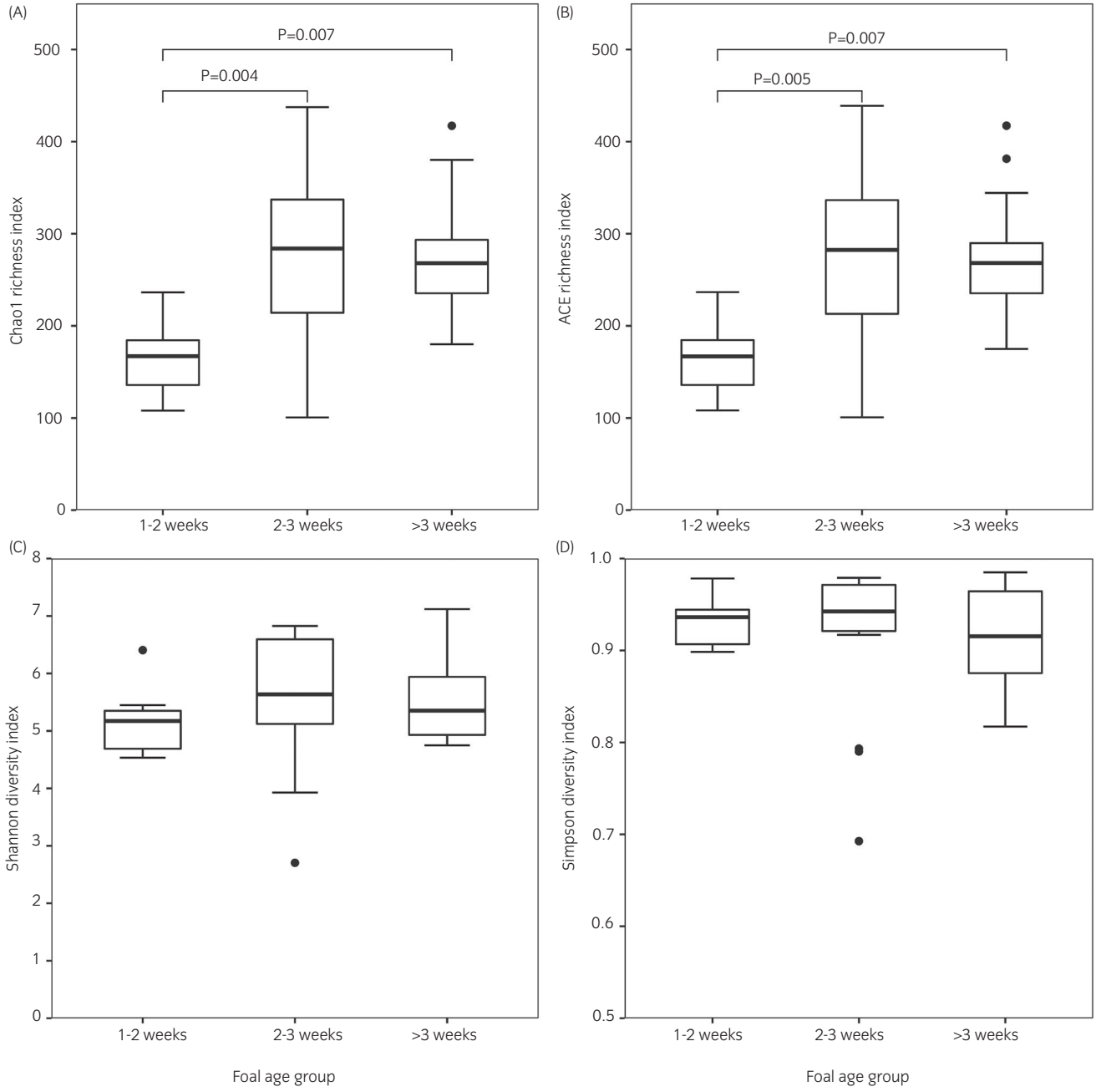

FIGURE 3 Alpha diversity of the faecal microbiota of foals in each age group. The richness indices Chao1 (A) and ACE (B), and the Shannon (C) and Simpson (D) diversity indices are shown as box plots, where the upper and lower ends of the boxes indicate the interquartile range, and the whiskers indicate 1.5 times the interquartile ranges. Individual dots indicate outliers. *P* values were calculated by one-way ANOVA with post-hoc analysis by Tukey's Honest Significant Difference (HSD) test

resistance genes *tet*Q, *tet*O and *tet*W contributed most to the total ARG abundance in the faecal microbiota, with median relative abundances of 2.3×10^{-2}, 2×10^{-2} and 7.9×10^{-3}, respectively. Other abundant ARGs were *aph*A3, *sat*4 and *mef*A, which had median relative abundances above 3.9×10^{-3}.

The similarities of the ARG profiles were compared between foals grouped by sex, farm and age. There was no significant separation of foals grouped by sex or farm (Figure 5A,B, Table S3), but foals aged 1-2 weeks had significantly different ARG profiles from those of the older foals ($P < .01$) (Figure 5C, Table S3).

The relative abundance of each ARG was compared between the age groups. Foals aged 1-2 weeks had a significantly greater abundance of *aph*A3, *sat*4, *tet*M and *tet*X than the older foals ($P < .01$). ARG abundances did not differ significantly between foals aged 2-3 weeks and those >3 weeks. This is demonstrated in Figure 4, with *aph*A3 representative of the aminoglycoside resistance genes, *sat*4 representative of the streptothricin resistance genes and the *tet* genes representative of the tetracycline resistance genes.

The bla_{SHV} and group 2 type $bla_{CTX\text{-}M}$ extended spectrum beta lactamase (ESBL) genes, as well as the fluoroquinolone resistance

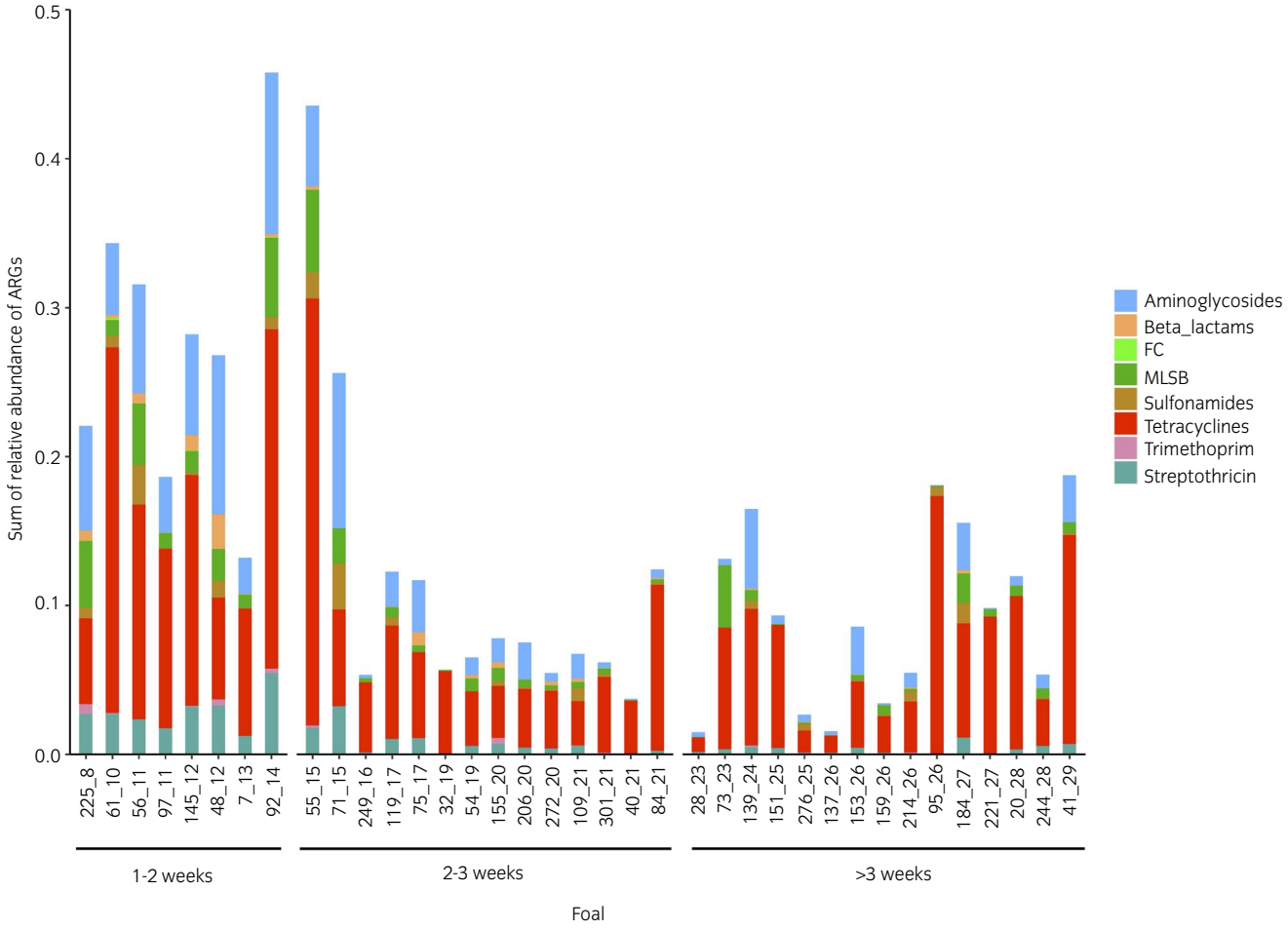

FIGURE 4 Sum of the relative abundances of ARGs (in proportion to the 16S rRNA gene) in each sample. The number after the underscore is the age in days. FC, quinolone/phenicol; MLSB, macrolide-lincosamide-streptogramin B

gene *qnr*B, were detected in fewer than 10 foals. The relative abundances of these genes were low, from ~10^{-6} to 10^{-4}.

3.5 | Co-occurrence of ARGs in foal faeces

The patterns of co-occurrence of ARGs were analysed using Spearman's rank correlation and examined as networks (Figure 6, Table S4). A cluster comprised of ARGs encoding resistance to the aminoglycosides, beta lactams and trimethoprim, and the class I integron markers *int*I1 and *qac*EΔ, was detected. Other clusters contained *aph*A3, *aad*E, *sat*4 and *tet*M, *str*B and *sul*2, and *erm*F and *tet*X.

4 | DISCUSSION

This study described the faecal microbiota and ARG profiles of foals younger than 1 month of age that were being raised on commercial stud farms. The influence of sex, farm and age on the microbiota and the ARG profiles were examined, and only age was found to distinguish between the groups.

Foals were not sampled longitudinally in this study, so the data do not conclusively prove that the changes in the faecal microbiota were a result of ageing, but the features detected in the microbiota of the foals in different age groups were consistent with previous longitudinal studies.[3,4] Foals aged 1-2 weeks were found to have significantly lower microbiota richness than the older foals, suggesting that colonisation of the gastrointestinal tract with new bacterial species increased with age. Previous studies have shown that the number of operational taxonomic units (OTUs) increases in the faecal microbiota of foals as they age from 7 to 28 days and then 60 days,[4] and that foals in the first month of life had fewer OTUs than older foals.[3]

Our results confirm previous studies that have shown that *Treponema* species are abundant in the adult horse faecal microbiota[36,37] and are likely to interact with cellulolytic bacteria to promote the digestion of fibre.[38,39] Members of the phylum *Spirochaetes* have been shown to increase in abundance in the horse faecal microbiota when haylage is introduced into the diet.[6] Thus, the increase in abundance of *Treponema* species in foals >3 weeks old may be a result of the increasing proportion of forage in their diet. The family *Christensenellaceae* (phylum *Firmicutes*) was also more abundant in foals >3 weeks old. The role of members

TABLE 2 Relative abundances of ARGs detected in more than 80% samples

Gene	Relative abundance		
	Min	Max	Median
*aad*E	4.3×10^{-5}	2.9×10^{-2}	1.5×10^{-3}
*aph*A3	1.8×10^{-4}	7.7×10^{-2}	9.6×10^{-3}
*str*B	1.4×10^{-5}	4.3×10^{-2}	2×10^{-3}
*mef*A	8.9×10^{-5}	5×10^{-2}	3.9×10^{-3}
*sul*2	9.6×10^{-6}	3.1×10^{-2}	1.4×10^{-3}
*tet*M	2.3×10^{-5}	2.5×10^{-2}	8.1×10^{-4}
*tet*O	1.2×10^{-3}	3.1×10^{-2}	7.9×10^{-3}
*tet*Q	1.7×10^{-3}	2.4×10^{-1}	2.3×10^{-2}
*tet*W	4.6×10^{-3}	1.2×10^{-1}	2×10^{-2}
*sat*4	2.3×10^{-4}	5.5×10^{-2}	5.1×10^{-3}

of the *Christensenellaceae* is less well understood, but their abundance has been found to differ between lean and obese horses.[40]

The phyla *Firmicutes* and *Bacteroides* were the two most abundant phyla in the majority of foals, consistent with previous observations in foals older than 1 week of age.[2,4] Other studies have found *Verrucomicrobia* or *Proteobacteria* to be among the most abundant phyla.[3,7] Although these earlier reports used the same Illumina MiSeq platform, only the V4 region of the bacterial 16S rRNA gene was sequenced, while our study targeted the V3-V4 region, perhaps contributing to these differences.[41] Differences in horse breed, diet and environmental factors could also have affected the faecal microbiota profiles.

Forty-four ARGs encoding resistance to the major antimicrobial classes were screened for using a HT-qPCR array. The tetracycline resistance genes *tet*Q, *tet*O and *tet*W contributed greatly to the total abundance of ARGs. These genes encode cytoplasmic proteins that protect ribosomes from tetracycline, and have been found in a broad range of bacteria, including genera dominant in the foal faecal microbiota (eg *Bacteroides*, *Lactobacillus*, *Ruminococcus* and *Streptococcus*).[42] In addition, these *tet* genes are often associated with mobile genetic elements that can transfer between bacterial species.[43] Metagenomic studies have shown that the ribosomal protection *tet* genes are commonly detected at a high abundance in the faecal microbiota of humans and several livestock species.[10,44]

Analyses of gene co-occurrence revealed that the abundances of some ARGs could be correlated with class I integron genes. Although analyses of co-occurrence are not sufficient to prove a physical linkage in the genome, they do indicate the potential for horizontal spread of ARGs within the bacterial microbiota.

The co-occurrence of *str*B and *sul*2 may be a result of carriage of the *sul*2-*str*A-*str*B cluster on plasmids. RSF1010-like plasmids carrying this gene cluster are widespread in the environment.[45,46] The formation and dissemination of this ARG cluster can occur without the selective pressure of antimicrobial agents,[47] suggesting a natural prevalence of these genes in the foal faeces. The co-occurrence of *aph*A3, *sat*4 and *aad*E could be a result of carriage of the gene cluster *aph*A3-*sat*4-*aad*E on Tn5404-like elements and members of the Tn1545/Tn6003 family, which have been found in Gram-positive bacteria.[48,49]

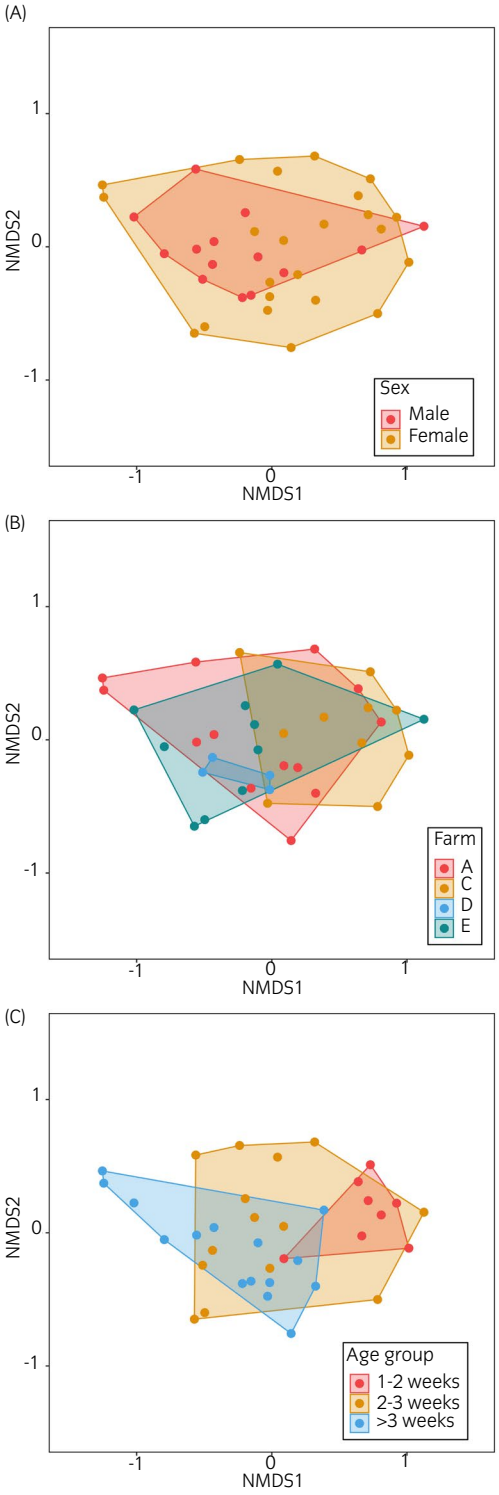

FIGURE 5 Similarity of the ARG profile between foals grouped by sex (A), farm (B) and age (C). NMDS analysis was based on Bray-Curtis distances derived from the relative abundance of ARGs (NMDS stress = 0.15)

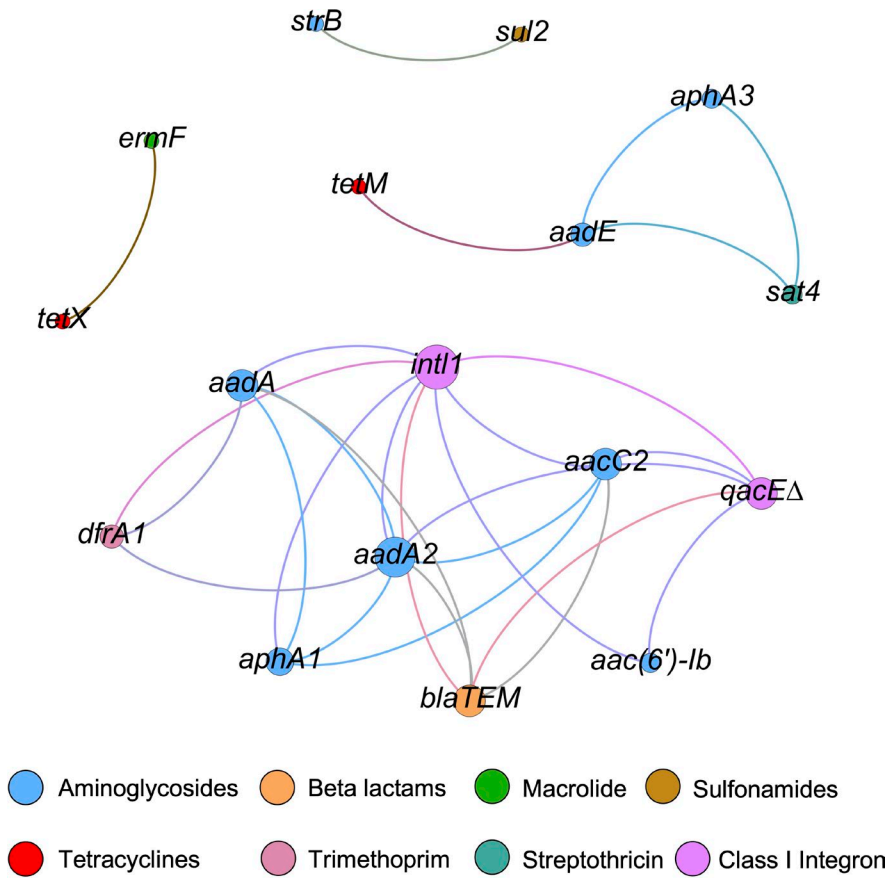

FIGURE 6 Network of co-occurrence patterns of ARGs detected in foals (Spearman's rho ≥0.75, $P < .01$). The size of each node is proportional to the number of connections

The bla_{SHV} and group 2 type $bla_{CTX\text{-}M}$ ESBL genes, and the fluoroquinolone resistance gene *qnr*B, were rarely detected in the foals, suggesting that young foals without direct antimicrobial exposure were unlikely to carry these genes in their faecal microbiota. A limitation of this study is that the group 1 type $bla_{CTX\text{-}M}$ genes were not included in the HT-qPCR assay. These genes have often been detected in ESBL-producing *E. coli* from horses and could be added in future studies.[11,50] If comprehensive coverage of ARGs is required, then alternative sequencing strategies could be used, for example, metagenomic sequencing.[10]

In our study, the overall faecal microbiota of foals did not differ between farms. Differences in farm management, such as the amount of access to pasture and the housing conditions, have been identified as factors influencing the faecal microbiota in other studies,[4,51] but an evaluation of the impact of management practices on the microbiota was not a primary aim of this study. While the majority of mares and foals were kept in grass paddocks, detailed information on this variable was not collected. In addition, the small number of foals from each farm evaluated in this study restrained the statistical power of any investigations of potential differences in the microbiota attributable to differences in farm management. This should be a fruitful area for future research. Another limitation was the lack of any history about antimicrobial treatment of the dams of the foals. A study in humans demonstrated that antimicrobial treatment of mothers during delivery may have affected the composition of the microbiota and the level of ARGs and mobile genetic elements in the infant gut.[52] This, in addition to the coprophagical behaviour of foals, may have influenced our results. Therefore, future studies that aim to describe the influences of age and farm management on the development of the faecal microbiota of healthy foals should have a larger sample size and record farm management practices in greater detail. This will allow investigation of any variation in the foal faecal microbiota and ARG profiles associated with differences in farm management.

In conclusion, this study analysed faecal microbiota and ARG profiles in Thoroughbred foals aged from 8 to 29 days raised on commercial stud farms in Australia. Age was found to influence the richness and composition of the faecal microbiota. The tetracycline resistance genes *tet*Q, *tet*O and *tet*W were the largest contributors to the total abundance of ARGs in the faeces, probably because of the high concentrations of potential host genera for these genes in the normal foal faecal microbiota. The observation of correlations between specific ARGs and class I integron genes suggested indirect exposure to antimicrobials on the farms.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST

No competing interests have been declared.

AUTHOR CONTRIBUTIONS

Y. Liu, laboratory work, data analysis and interpretation and manuscript preparation; K. E. Bailey, sample collection, data interpretation and manuscript review. M. Dyall-Smith, M. S. Marenda, L. Y. Hardefeldt and G. F. Browning, study design, data interpretation and manuscript review; J. R. Gilkerson, sampling study design and manuscript review; H. Billman-Jacobe, study design, project supervision, data interpretation and manuscript review.

ETHICAL ANIMAL RESEARCH

Research ethics committee oversight not currently required by this journal: the study was performed on material obtained noninvasively.

OWNER INFORMED CONSENT

Approval for inclusion of the foals in the study was obtained from the stud farm managers prior to commencing the study.

DATA ACCESSIBILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

PEER REVIEW

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REFERENCES

1. Quercia S, Freccero F, Castagnetti C, Soverini M, Turroni S, Biagi E, et al. Early colonisation and temporal dynamics of the gut microbial ecosystem in Standardbred foals. Equine Vet J. 2018;51:231–7.
2. Husso A, Jalanka J, Alipour MJ, Huhti P, Kareskoski M, Pessa-Morikawa T, et al. The composition of the perinatal intestinal microbiota in horse. Sci Rep. 2020;10:441.
3. Costa MC, Stämpfli HR, Allen-Vercoe E, Weese JS. Development of the faecal microbiota in foals. Equine Vet J. 2016;48:681–8.
4. De La Torre U, Henderson JD, Furtado KL, Pedroja M, Mora A, Pechanec MY, et al. Utilizing the fecal microbiota to understand foal gut transitions from birth to weaning. PLoS One. 2019;14:e0216211.
5. Dougal K, de la Fuente G, Harris PA, Girdwood SE, Pinloche E, Geor RJ, et al. Characterisation of the faecal bacterial community in adult and elderly horses fed a high fibre, high oil or high starch diet using 454 pyrosequencing. PLoS One. 2014;9:e87424.
6. Salem SE, Maddox TW, Berg A, Antczak P, Ketley JM, Williams NJ, et al. Variation in faecal microbiota in a group of horses managed at pasture over a 12-month period. Sci Rep. 2018;8:8510.
7. Schoster A, Staempfli H, Guardabassi L, Jalali M, Weese J. Comparison of the fecal bacterial microbiota of healthy and diarrheic foals at two and four weeks of life. BMC Vet Res. 2017;13:144.
8. Costa MC, Stämpfli HR, Arroyo LG, Allen-Vercoe E, Gomes RG, Weese JS. Changes in the equine fecal microbiota associated with the use of systemic antimicrobial drugs. BMC Vet Res. 2015;11:19.
9. Zhu Y-G, Johnson TA, Su J-Q, Qiao M, Guo G-X, Stedtfeld RD, et al. Diverse and abundant antibiotic resistance genes in Chinese swine farms. Proc Natl Acad Sci USA. 2013;110:3435–40.
10. Li B, Yang Y, Ma L, Ju F, Guo F, Tiedje JM, et al. Metagenomic and network analysis reveal wide distribution and co-occurrence of environmental antibiotic resistance genes. ISME J. 2015;9:2490–502.
11. Dolejska M, Duskova E, Rybarikova J, Janoszowska D, Roubalova E, Dibdakova K, et al. Plasmids carrying *bla*CTX-M-1 and *qnr* genes in *Escherichia coli* isolates from an equine clinic and a horseback riding centre. J Antimicrob Chemother. 2011;66:757–64.
12. Moura I, Radhouani H, Torres C, Poeta P, Igrejas G. Detection and genetic characterisation of *van*A-containing *Enterococcus* strains in healthy Lusitano horses. Equine Vet J. 2010;42:181–3.
13. Ahmed MO, Clegg PD, Williams NJ, Baptiste KE, Bennett M. Antimicrobial resistance in equine faecal *Escherichia coli* isolates from North West England. Ann Clin Microbiol Antimicrob. 2010;9:12.
14. Moura I, Torres C, Silva N, Somalo S, Igrejas G, Poeta P. Genomic description of antibiotic resistance in *Escherichia coli* and *Enterococci* isolates from healthy Lusitano horses. J Equine Vet Sci. 2013;33:1057–63.
15. Qian X, Gu J, Sun W, Wang X-J, Su J-Q, Stedfeld R. Diversity, abundance, and persistence of antibiotic resistance genes in various types of animal manure following industrial composting. J Hazard Mater. 2018;344:716–22.
16. Muziasari WI, Pitkanen LK, Sorum H, Stedtfeld RD, Tiedje JM, Virta M. The resistome of farmed fish feces contributes to the enrichment of antibiotic resistance genes in sediments below Baltic sea fish farms. Front Microbiol. 2016;7:2137.
17. Bailey KE. Investigation of the Infectious Causes of Diarrhoea in Australian Thoroughbred Foals. 2017. PhD Thesis, The University of Melbourne, Melbourne.
18. Yu Y, Lee C, Kim J, Hwang S. Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction. Biotechnol Bioeng. 2005;89:670–9.
19. Thijs S, Op De Beeck M, Beckers B, Truyens S, Stevens V, Van Hamme JD, et al. Comparative evaluation of four bacteria-specific primer pairs for 16S rRNA gene surveys. Front Microbiol. 2017;8:494.
20. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat Biotechnol. 2019;37:852–7.
21. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: high-resolution sample inference from Illumina amplicon data. Nat Methods. 2016;13:581–3.
22. Bokulich NA, Kaehler BD, Rideout JR, Dillon M, Bolyen E, Knight R, et al. Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. Microbiome. 2018;6:90.
23. DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, et al. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl Environ Microbiol. 2006;72:5069–72.
24. Wang FH, Qiao M, Su JQ, Chen Z, Zhou X, Zhu YG. High throughput profiling of antibiotic resistance genes in urban park soils with reclaimed water irrigation. Environ Sci Technol. 2014;48:9079–85.
25. Looft T, Johnson TA, Allen HK, Bayles DO, Alt DP, Stedtfeld RD, et al. In-feed antibiotic effects on the swine intestinal microbiome. Proc Natl Acad Sci USA. 2012;109:1691–6.
26. Liu Y, Dyall-Smith M, Marenda M, Hu HW, Browning G, Billman-Jacobe H. Antibiotic resistance genes in antibiotic-free chicken farms. Antibiotics. 2020;9:120.
27. Muller PY, Janovjak H, Miserez AR, Dobbie Z. Processing of gene expression data generated by quantitative real-time RT-PCR. Biotechniques. 2002;32:1372–9.
28. Muller PY, Janovjak H, Miserez AR, Dobbie Z. Erratum. Biotechniques. 2002;33:514.
29. R Core Team. R: A language and environment for statistical computing. 2020. https://www.R-project.org/

30. Oksanen J, Blanchet F, Friendly M, Kindt R, Legendre P, McGlinn D. vegan: community ecology package. 2019. https://CRAN.R-project.org/package=vegan
31. Hervé M. RVAideMemoire: testing and plotting procedures for biostatistics. 2019. https://CRAN.R-project.org/package=RVAideMemoire
32. Mandal S, Van Treuren W, White RA, Eggesbø M, Knight R, Peddada SD. Analysis of composition of microbiomes: a novel method for studying microbial composition. Microb Ecol Health Dis. 2015;26:27663.
33. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J Roy Stat Soc Ser B (Stat Method). 1995;57:289–300.
34. Harrell FE Jr. Hmisc: harrell miscellaneous. 2019. https://CRAN.R-project.org/package=Hmisc
35. Bastian M, Heymann S, Jacomy M. Gephi: an open source software for exploring and manipulating networks. 2009. In: *International AAAI Conference on Weblogs and Social Media*.
36. O'Donnell M, Harris H, Jeffery I, Claesson M, Younge B, O'Toole P, et al. The core faecal bacterial microbiome of Irish Thoroughbred racehorses. Lett Appl Microbiol. 2013;57:492–501.
37. Metcalf JL, Song SJ, Morton JT, Weiss S, Seguin-Orlando A, Joly F, et al. Evaluating the impact of domestication and captivity on the horse gut microbiome. Sci Rep. 2017;7:15497.
38. Kudo H, Cheng K-J, Costerton J. Interactions between *Treponema bryantii* and cellulolytic bacteria in the *in vitro* degradation of straw cellulose. Can J Microbiol. 1987;33:244–8.
39. Stanton T, Canale-Parola E. *Treponema bryantii* sp. nov., a rumen spirochete that interacts with cellulolytic bacteria. Arch Microbiol. 1980;127:145–56.
40. Biddle AS, Tomb J-F, Fan Z. Microbiome and blood analyte differences point to community and metabolic signatures in lean and obese horses. Front Vet Sci. 2018;5:225.
41. Bukin YS, Galachyants YP, Morozov I, Bukin S, Zakharenko A, Zemskaya T. The effect of 16S rRNA region choice on bacterial community metabarcoding results. Sci Data. 2019;6:190007.
42. Roberts MC, Schwarz S, Aarts HJ. Erratum: acquired antibiotic resistance genes: an overview. Front Microbiol. 2012;3:384.
43. Chopra I, Roberts M. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiol Mol Biol Rev. 2001;65:232–60.
44. Hu Y, Yang X, Qin J, Lu N, Cheng G, Wu N, et al. Metagenome-wide analysis of antibiotic resistance genes in a large cohort of human gut microbiota. Nat Commun. 2013;4:2151.
45. Yau S, Liu X, Djordjevic SP, Hall RM. RSF1010-like plasmids in Australian *Salmonella enterica* serovar Typhimurium and origin of their *sul*2-*str*A-*str*B antibiotic resistance gene cluster. Microb Drug Resist. 2010;16:249–52.
46. Partridge SR. Analysis of antibiotic resistance regions in Gram-negative bacteria. FEMS Microbiol Rev. 2011;35:820–55.
47. Okubo T, Ae R, Noda J, Iizuka Y, Usui M, Tamura Y. Detection of the *sul*2–*str*A–*str*B gene cluster in an ice core from Dome Fuji Station, East Antarctica. J Glob Antimicrob Resist. 2019;17:72–8.
48. Werner G, Hildebrandt B, Witte W. Linkage of *erm*(B) and *aad*E-*sat4*-*aph*A-3 in multiple-resistant *Enterococcus faecium* isolates of different ecological origins. Microb Drug Resist. 2003;9:9–16.
49. Palmieri C, Mingoia M, Massidda O, Giovanetti E, Varaldo PE. *Streptococcus pneumoniae* transposon Tn1545/Tn6003 changes to Tn6002 due to spontaneous excision in circular form of the *erm*(B)-and *aph*A3-containing macrolide-aminoglycoside-streptothricin (MAS) element. Antimicrob Agents Chemother. 2012;56:5994–7.
50. Walther B, Klein K-S, Barton A-K, Semmler T, Huber C, Wolf SA, et al. Extended-spectrum beta-lactamase (ESBL)-producing Escherichia coli and Acinetobacter baumannii among horses entering a veterinary teaching hospital: The contemporary "Trojan Horse". PLoS One. 2018;13:e0191873.
51. Kaiser-Thom S, Hilty M, Gerber V. Effects of hypersensitivity disorders and environmental factors on the equine intestinal microbiota. Vet Q. 2020;40:97–107.
52. Pärnänen K, Karkman A, Hultman J, Lyra C, Bengtsson-Palme J, Larsson DGJ, et al. Maternal gut and breast milk microbiota affect infant gut antibiotic resistome and mobile genetic elements. Nat Commun. 2018;9:3891.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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