Characterising the equine faecal microbiota in health and disease

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Contents

Authorship Certificate

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Jillian Bell

Chapters 3, 4 and 5 have been accepted and published in peer-reviewed veterinary journals as outlined below. Jillian Bell was responsible for composing the manuscripts, however the collaborating authors contributed to the critical revision of the article and approved the final manuscript for publication.

I, Kristopher Hughes, sign on behalf of the collaborating authors to confirm the above statement and consent to the inclusion of these publications in this thesis.

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Kristopher Hughes

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Ethics Approval

Animal Care and Ethics

Animal care and ethics approval was obtained prior to initiation of all projects. The Charles Sturt University Animal Care and Ethics Committee approved all study protocols.

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Publications

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Bell, J., Raidal, S.L. and Hughes, K.J. The effect of anthelmintic treatment and efficacy on the faecal microbiota of healthy adult horses. *Veterinary Parasitology.* 2024. 330. p. 110241. doi:10.1016/j.vetpar.2024.110241.

Abstract

Maintenance of the gastrointestinal microbiota is understood to play a critical part in health and disease in horses and foals. Changes to the gastrointestinal microbiota have been associated with a several factors including diet, geography, transport, medication administration, general anaesthesia and diseases of the gastrointestinal tract including causes of colic, colitis and diarrhoea. Anthelmintics are commonly used in the husbandry of horses, and administration of certain anthelmintics can cause changes to the gastrointestinal microbiota. Further characterisation of these changes could assist in an improved overall understanding of the host-parasite-microbiota relationship and influence recommendations for anthelmintic use in the future. Diarrhoea is an important cause of clinical disease in foals and is a common presenting problem in veterinary practice. The treatment of diarrhoea in foals currently is non-specific supportive care, with no current recommendations for targeted treatment of the gastrointestinal microbiota and dysbiosis. Faecal microbiota transplantation (FMT) has been used by equine veterinarians with anecdotal success as a treatment to aid restoration of the gastrointestinal microbiota in adult horses and foals with colitis and diarrhoeas. Recently, FMT administration in horses with diarrhoea has been reported, with a beneficial impact on clinical recovery. The efficacy of FMT is yet to be assessed in foals. Solutions for FMT are typically prepared immediately prior to administration and lack of access to donor horses may pose limitations on its use. Storage of equine FMT preparation and the impact of storage conditions on the microbiota has received limited attention.

The aims of the research undertaken in this thesis were to (i) investigate the effects of FMT administration to foals with diarrhoea and evidence of systemic inflammation (ii) investigate the effects of storage of FMT in conditions of 4°C and -20°C (iii) investigate the effects of anthelmintic administration to horses with cyathostomin infection.

The results of (i) showed that administration of FMT was associated with improvement of some clinical and clinicopathological variables in foals but did not result in increased resolution of diarrhoea or survival.

The results of (ii) showed that storage of FMT at 4°C for 72 hoursand -20°C for 28 days had minimal effect on the microbiota composition.

The results of (iii) showed that changes to the faecal microbiota were limited following anthelmintic administration. Removal of cyathostomins resulted in greater changes to the microbiota compared to maintenance of cyathostomin burden.

The thesis provides important contributions to the understanding of the outcomes associated with FMT in foals and the effects of storage on equine FMT solution. This thesis also contributes to understanding of the effects of anthelmintic treatment on the faecal microbiota of healthy horses. The research improved the current knowledge of the effects of use of anthelmintics with and without removal of cyathostomins and reported the first clinical trial of FMT administration as a targeted treatment for diarrhoea in foals. It is also the first equine study investigating the effects of short term storage on the microbiota of equine FMT.

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Glossary of abbreviations

ABA	Abamectin
AM	Arithmetic mean
Amplicon sequence variants	ASV
ANOSIM	Analysis of Similarity
ANOVA	Analysis of Variance
CI	Confidence interval
CL	Confidence limit
DNA	Deoxyribonucleic acid
Epg	Eggs per gram
ESBL	extended spectrum B lactamase
FEC	Faecal egg count
FECR	Faecal egg count reduction
FECRT	Faecal egg count reduction test
FMT	Faecal microbiota transplantation
GI	Gastrointestinal
GIT	Gastrointestinal tract
KEGG	Kyto Encyclopedia of Genes and Genomes
LDA	Linear discriminate analysis
LefSe	Linear discriminant analysis effect size
OXF	Oxfendazole
PCA	Principal component analysis
PCoA	Principal coordinate analysis
PCV	Packed cell volume
PCR	Polymerase chain reaction
PICRUSt	Phylogenic Investigation of Communities by Reconstruction of Unobserved States
QIIME2	Quantitative Insights Into Microbial Ecology

RR	Respiratory rate
TPP	Total plasma protein
SAA	Serum amyloid A
WBC	White blood cell

Chapter 1: Introduction

The equine gastrointestinal (GI) tract is a sizable organ system that broadly consists of the stomach, small and large intestines. The equine GI microbiota is a complex environment composed of bacteria, fungi, viruses, archaea and protozoa that inhabit these regions. The microbiota plays an important role in digestion, metabolism, immune function and protection against pathogens (Stewart, et al. 2018). Throughout the equine GIT the microbiota of different compartments changes associated with the function of each particular environment (Costa, et al. 2015a). Characterisation of the normal equine microbiota (Costa, et al. 2015a) (Costa, et al. 2012b) has been described, with differences between geographic region (Garber, et al. 2020), feed type (Arnold, et al. 2021) (Fernandes, et al. 2014) and individual variation observed. Age has also been associated with differences in the microbiota, notably in foals compared to adult horses up until the age of weaning (De La Torre, et al. 2019).In adult horses of various ages, no significant differences of the microbiota have been detected (McKinney, et al. 2020b; Mshelia, et al. 2018).

Change in the gastrointestinal microbiota has been associated with gastrointestinal disease (Costa, et al. 2012b), administration of medications (Cerri, et al. 2020; Kunz, et al. 2019; Whitfield-Cargile, et al. 2018) and other interventions including travel and general anaesthesia (Perry, et al. 2018). An imbalance and disruption of the microbiota is referred to as dysbiosis. The presence of dysbiosis can lead to alterations in functional and metabolic activities, precipitating disease in affected animals.

Anthelmintics are commonly used drugs in equine husbandry. These medications are fundamental in the management of helminthic infestations in horses. Previous studies assessing the effect of anthelmintic treatment have reported that i) removal of intestinal

parasites from the gastrointestinal tract is associated with transient alterations in the faecal microbiota (Peachey, et al. 2018) and ii) administration of anthelmintics to horses with low egg shedding is associated with changes in the faecal microbiota (Kunz, et al. 2019). The effects of anthelmintic administration on the faecal microbiota include influences on taxa (Kunz, et al. 2019; Peachey, et al. 2018; Walshe, et al. 2019), alpha diversity (Kunz, et al. 2019; Walshe, et al. 2019) and beta diversity (Walshe, et al. 2019). In addition to this, (Walshe, et al. 2019) showed that treatment with anthelmintics and removal of adult cyathostomins results in an inflammatory phenotype in horses. This dramatic shift in intestinal parasite infestation and change to the intestinal microbiota may also play a role in disease including acute larval cyathostominosis (Walshe, et al. 2019). A better understanding of the impact of anthelmintic administration on the microbiota would provide further information that could shape anthelmintic regimen recommendations. Further recommendations may also assist in the formulation of an approach to anthelmintic resistance.

Diarrhoea is a common disease of foals (Grinberg, et al. 2009). Diarrhoea in foals can be caused by variety of infectious agents and non-infectious factors. Diarrhoea also causes changes to the microbiota, with the microbiota of foals with diarrhoea being different to healthy aged matched peers (Schoster, et al. 2017). Treatment of diarrhoea in foals is mostly non-specific and supportive with no effective treatment targeting the gastrointestinal microbiota. Probiotics have been used in foals, with conflicting results (Schoster, et al. 2016a; Ströbel, et al. 2018; Tanabe, et al. 2014a; Urubschurov, et al. 2019). More recent investigation of these effects have shown no improvement with probiotic treatment and in some cases the administration has resulted in worsening of diarrhoea (Ströbel, et al. 2018) and caused diarrhoea in healthy foals (Weese and Rousseau 2005). In addition to this, probiotic treatment does not have a significant effect on the faecal microbiota of foals (Urubschurov, et al. 2019).

No other targeted treatments are available to assist in the re-establishment of the GI microbiota of foals.

Faecal microbiota transplantation (FMT) in used in humans for the treatment of diarrhoea and ulcerative colitis (Costello, et al. 2017), recurrent *Clostridium difficile* infection (Singh, et al. 2022) and appears beneficial in the management of irritable bowel syndrome (Ianiro, et al. 2019). As changes in the gastrointestinal microbiota are associated with diarrhoea, it is reasonable for one of the goals of treatment to be to re-establish the normal flora. FMT has been used to treat diarrhoea in horses by equine clinicians for decades; however, studies investigating its effects have only recently been undertaken. There are mixed reports of clinical success, with some studies describing a positive clinical effect of treatment (Dias, et al. 2018; McKinney, et al. 2021; McKinney, et al. 2020a) and normalisation of the faecal microbiota (McKinney, et al. 2020a). Other studies have described a lack of evidence of change to the faecal microbiota and no improvement in diarrhoea (Costa, et al. 2021; Kinoshita, et al. 2022). Although reports of resolution of diarrhoea and changes to the microbiota are variable, the evidence suggests that FMT is safe and that there has been no evidence to suggest that it has a negative effect on outcomes(Costa, et al. 2021; Dias, et al. 2018; Kinoshita, et al. 2022; McKinney, et al. 2021; McKinney, et al. 2020a). There are currently no studies assessing the effect of FMT in foals.

In adult horses, methods of preparation and administration are yet to be optimised. The preparation method of FMT varies between studies and storage of equine FMT has rarely been assessed. Fresh manure is usually collected and combined in aerobic conditions with water or saline to result in a final FMT filtrate (Tuniyazi, et al. 2023). FMT is administered by nasogastric intubation once daily, for a period of 1-7 days (Costa, et al. 2021; McKinney, et al. 2021; McKinney, et al. 2020a). The FMT is administered fresh, within 15 minutes of

preparation (McKinney, et al. 2021). One study that used frozen thawed faeces for FMT preparation observed that the resultant FMT did not resolve dysbiosis (Kinoshita, et al. 2022). The microbiota does not significantly change between fresh manure and traditionally prepared FMT by use of an immersion blender (Loublier, et al. 2023). Storage of FMT at -20°C for more than one week resulted in a reduction in viable bacteria (Kopper, et al. 2021), however the microbiota of frozen FMT has not been assessed. The short term storage of FMT in refrigerator conditions (4°C) has also not been assessed. This information would assist equine clinicians when preparing FMT solution and allow for potential short term storage when donors are scarce.

Research objectives

The objectives of this research project were to:

Assess the clinical and microbiota response to administration of FMT in foals with diarrhoea

Evaluate the changes of the microbiota of FMT in storage conditions of 4°C and -20°C

Evaluate the changes in the microbiota associated with a single dose of anthelmintic treatment

Thesis Structure

This thesis is compiled in accordance with Charles Sturt University recommendations for a professional doctorate by publication. Chapter 2 is a literature review comprising of the important concepts that underpin the understanding of the equine gastrointestinal microbiota. This includes; anatomy, physiology, microbiota in health and disease, microbiota of adult horses and foals, effect anthelmintic treatment on the microbiota, the therapeutic use of FMT in adult horses and foals and preparation and storage of FMT.

Chapter 3 consists of a manuscript accepted and published in the *Journal of Veterinary Internal Medicine* entitled 'Effects of fecal microbiota transplantation on clinical outcomes and fecal microbiota of foals with diarrhea.'

Chapter 4 consists of a manuscript accepted and published in *The Veterinary Journal* 'Storage of equine faecal microbiota transplantation has minimal impact on major bacterial community and structure.'

Chapter 5 consists of a manuscript accepted and published in *Veterinary Parasitology* entitled 'The effect of anthelmintic treatment and efficacy on the faecal microbiota of healthy adult horses.'

Chapter 6 is an exegesis discussing the results of the research and provides comprehensive discussion of the contributions of the research to scientific literature and the veterinary profession.

Chapter 2: Literature Review

2.1 Equine gastrointestinal tract

The equine gastrointestinal tract (GIT) is an substantial organ system with a potential length and volume in the adult horse of 30 metres and 150 litres (Ericsson, et al. 2016). It contains one of the most dynamic and complex bacterial populations (microbiomes) of any known environment (Costa, et al. 2012b). Broadly, the tract is composed of the stomach and the intestines (small and large) (Figure 1). The lumen of the stomach is lined dorsally by stratified squamous epithelium and ventrally by glandular epithelium, divided by the margo plicatus (Dyce 1996). In the stomach, ingesta buffers the hydrochloric acid, there is a small amount of enzymatic digestion and there is limited fermentation, due to the presence of resident acid-tolerant bacteria (Ericsson, et al. 2016). The small intestines (duodenum, jejunum and ileum) measure approximately 25 metres long (Dyce 1996). These compartments are the primary sites for digestion of protein, soluble carbohydrate and fat, and are colonized by commensal microbial communities. The large intestine (caecum, ascending colon, transverse colon and descending colon) is the primary site for fermentation, where fibrolytic bacteria produce nutritionally beneficial short chain fatty acids (SCFAs) (Costa, et al. 2012b; Ericsson, et al. 2016). The small intestine, colon and caecum reabsorb nutrients, ions and water, and play a role in fluid shifts and balance and electrolyte homeostasis (Wj 1996).

2.2 Equine gastrointestinal microbiota in health

A properly functioning intestinal tract and microbiota is critical for maintenance and normal health, and imbalance of this community can lead to catastrophic consequences (Costa and Weese 2018; Dougal, et al. 2013a). The gastrointestinal microbiota is a diverse ecosystem of microorganisms that has a synergistic and specifically adapted relationship to digestive,

metabolic and immunologic function of the host (Stewart, et al. 2018). The intestinal microbiota has been extensively recognised for its importance in the breakdown of food particles, protection against pathogenic organisms, maintenance of the intestinal epithelium and modulation of local immune and metabolic function (Costa and Weese 2018). The bacterial community is the most extensively studied and is thought to be the most important in maintaining the homeostasis of this complex environment (Costa and Weese 2018). The role of other microorganisms including protozoa, viruses and fungi has received limited investigation in horses. In humans, studies have investigated the contribution of these microorganisms in health and disease (Dubik, et al. 2022; Guzzo, et al. 2022; Laforest-Lapointe and Arrietaa 2018). Extrapolation from the human literature would suggest that protozoa, viruses and fungi also play an important role in homeostasis of the gastrointestinal microbiota of horses. A table describing the main concepts of terms used to describe the microbiota is included in Figure 2.1.

Table 1
Definition of the main concepts used in microbial ecology

Microbiota	All microorganisms of a particular environment
Microbiome	All microorganisms along with their genetic material and their interaction with an environment
Alpha diversity	Describes characteristics of individual samples (eg, richness, evenness, and diversity)
Richness	Total number of taxa (eg, species or genera, families, phyla) present in an environment, either through direct measure (observed richness) or through calculations to estimate the true richness that would have been detected if the entire population had been studied (estimated richness) Alpha diversity indices can be described and compared between groups (eg, newborn foals have a richer microbiota than older animals)[7]
Evenness	Distribution of species (eg, prevalence or relative abundance of each population within a community)
Diversity	Mathematical equation that takes into account richness and evenness (ie, it quantifies how equal a microbial community is)
Beta-diversity	Comparisons between samples or groups assessed in a variety of ways with different indices Compares the overall composition of the microbiota, typically based on membership or community structure
Membership	Members (eg, species) that are or are not present
Structure	Broader comparison that takes into account the members that are or are not present, and their relative abundance

Figure 2.1: Table describing the main concepts of terms used to describe the microbiota. Table sourced from (Costa and Weese 2018) Used with permission, ELSEVIER LICENSE 5841691347539.

Characterization of the normal gastrointestinal microbiota in humans has been widely reported (Flint, et al. 2012; Phillips, et al. 2022; Yatsunenko, et al. 2012; Zoetendal, et al. 2006). Recently, investigations have furthered the current understanding of the equine microbiota. There is marked variation of the intestinal microbiota between healthy horses and an overabundance of contributing factors such as geographic location, ages and management systems that influence an individual's microbiota. Variation in the microbiota of healthy individual horses over time has been observed (Salem, et al. 2018). Despite the commonly observed variation between horses, one study has reported consistencies of the microbiota between healthy adult horses, particularly of the microbiota of the distal gastrointestinal tract (Costa, et al. 2015a).

The intestinal microbiota varies greatly between some compartments of the gastrointestinal tract, with less variation between neighbouring compartments (Costa, et al. 2015a). Multiple studies have demonstrated limited similarities in richness of the gastrointestinal microbial populations between small intestine, large intestine and faeces of individual horses (Costa, et al. 2015a; Ericsson, et al. 2016; Schoster, et al. 2013).

In contrast to the human gastrointestinal microbiota, the equine 'core' microbiota is not well defined. The adult equine gastric microbiota is dominated by the phyla Proteobacteria, Bacteroidetes and Firmicutes with *Lactobacillus, Sarnia* and *Streptococcus* being the main genera (Costa, et al. 2015a; Perkins, et al. 2012). Small intestinal microbiota are reportedly largely composed of *Lactobacillus* ssp*.* and *Streptococcus* spp (Costa, et al. 2015a). The caecum, ventral colon and dorsal colon have similar microbiome composition (Ericsson, et al. 2016), with Firmicutes the main bacterial phylum found in the distal intestinal tract of healthy adult horses. Second to this, Bacteroidetes and Verrucomicrobia members are highly represented (Costa and Weese 2018). These findings are not surprising given the horse diet

primarily contains fibrous plant material that requires bacterial digestion by these phyla. One study reported variation between mucosal and luminal microbiota of the proximal gastrointestinal tract (stomach, jejunum and ileum), but similar composition of the microbiota between the mucosa and lumen of the distal gastrointestinal tract of healthy horses (Ericsson, et al. 2016). Contrary to these findings (Arroyo, et al. 2020) reported similarities between the mucosal and luminal microbiota, however this study reported the small sample size as a limitation and this may be what prohibited the detection of differences.

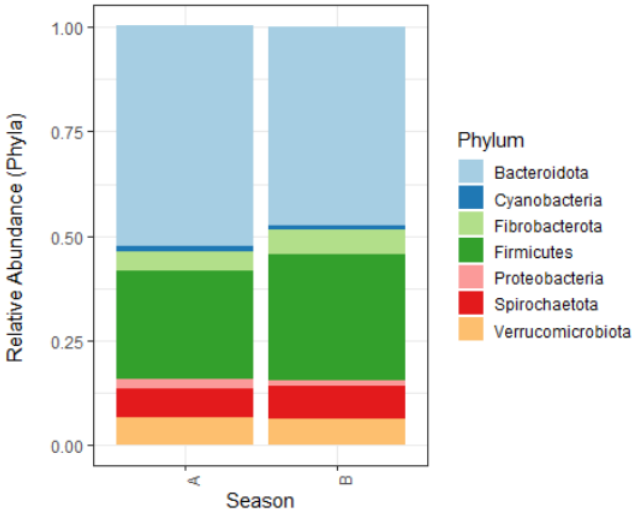

Figure 2.2: bar charts displaying the relative abundance of phyla of the faecal microbiota of healthy adult horses and ponies in The Netherlands in A) Summer and B) Winter.. Sources by (Theelen, et al. 2021). Used with permission LICENSE; https://creativecommons.org/licenses/by/4.0/.

Faecal samples have been used to as a proxy for the distal gastrointestinal tract for evaluation of the microbiota. It is broadly recognised that the faecal microbiota does not provide a reasonable profile of the luminal context of the stomach or small intestine (Ericsson, et al. 2016). Throughout the literature there are conflicting reports of the similarities and differences between the distal gastrointestinal tract segments and faecal microbiota. It has been reported that bacterial communities identified in faeces do not differ from those found in the large colon, supporting the use of faecal analysis as a representative sample for the

distal gastrointestinal tract (Costa, et al. 2015a). Conversely, there are reported differences between the microbiota of faeces and the distal gastrointestinal tract segments (Dougal, et al. 2013b; Dougal, et al. 2012). The disparities between studies highlight the complexity of this diverse ecosystem and the challenges associated with characterising the normal gastrointestinal microbiota in horses. In a clinical context, faeces serve as a proxy for the distal gastrointestinal tract microbiota segments because of the easy and non-invasive accessibility *antemortem* and capacity for longitudinal sampling.

The faecal microbiota of healthy foals has been investigated, with composition of the gastrointestinal microbiota studied from birth through to late weaning (Costa, et al. 2016b; De La Torre, et al. 2019; Husso, et al. 2020; Schoster, et al. 2017). In humans, disruption of the microbiota in early life is reported to alter metabolic signalling, inflammation and development, and ultimately leads to changes in body composition (Cox, et al. 2014). In the neonatal period, foals have an increased risk of morbidity and mortality associated with respiratory disease, gastrointestinal disease and sepsis. Understanding the origins and development of the early equine microbiota and the relationship with pathologies of the gastrointestinal tract (particularly enteritis and enterocolitis associated with bacterial, viral or mucosal injury) is essential in understanding the role of the gastrointestinal microbiota in health and disease, and for directing appropriate management and treatment strategies.

It has been postulated that the foal gastrointestinal microbiota begins to form during pregnancy, through amniotic fluid. The authors of Quericia (2019) reported that the microbiota of foal faeces is similar to the microbiota of amniotic fluid collected at parturition. Immediately after birth, small amounts of bacterial DNA can be detected within the rectum of neonates that is comparable to the core vaginal or faecal microbiota of the mare. It is reported that the foal develops an evolving gastrointestinal microbiota, which eventually

forms a microbiota with similar phyla, but different genera, to adults in the first 7 days of life (Husso, et al. 2020). Several studies have reported different compositions and proportions of certain phyla for the 24 hours after birth. It is postulated that this is due to a variety of factors, including environment and housing (De La Torre, et al. 2019; Husso, et al. 2020).

As the foal grows, synchronisation occurs between the changes in dietary needs, changes in type of food consumed and shifts in the gastrointestinal microbiota to more effectively utilize diet (De La Torre, et al. 2019). The bacterial population in normal healthy foals appears to continue to change in composition in an age dependant manner. By 28 days, the four phyla that dominate the faecal microbiota include *Firmicutes, Actinobacteria, Bateroidetes* and *Proteobacteria.* As the foal matures, the proportions of the genera and classes of these phyla changes, influenced by a number of factors including diet, region and management systems (De La Torre, et al. 2019).

2.3 Epidemiology

The effect of environmental changes on gastrointestinal microbiota has been evaluated in horses and other species including humans (Clemente, et al. 2015; Cox, et al. 2014; Metcalf, et al. 2017b). Disruption between mammals and their current microbial partners has a great impact on host health. In humans, the transition from foraging, to farming, to urban lifestyles and exposure to 'new age' medical advancements (such as antibiotics) has been associated with a less diverse gastrointestinal microbiota and a rise in immunologic and metabolic diseases. (Clemente, et al. 2015; Cox, et al. 2014; Kamada, et al. 2013; Metcalf, et al. 2017a)

The domestication of horses in this time period has played an important part in aiding humans throughout history (Massacci, et al. 2020). Horses have been bred for specific qualities such as

strength, speed, jumping and appearance, which has led to differences between equine breeds. The domestication of horses has modified feeding, housing, environment and breeding and these lifestyle factors affect the faecal microbiota composition and diversity (Metcalf, et al. 2017b).

Studies have demonstrated a reduction in diversity of the faecal microbiota in aged animals (Dougal, et al. 2014). In the human literature, it has been shown there is a similar age related reduction in faecal microbiota diversity (Biagi, et al. 2010; Woodmansey, et al. 2004). Breed has limited effect on the equine faecal microbiota (Massacci, et al. 2020), but it has been postulated that changes in weather (rainfall and ambient temperature) have an effect on the faecal microbiota of horses, due either to direct weather conditions and feed types available for horses or because of the effect on composition of environmental microbial communities (soil, grass and hay microbiota) (Salem, et al. 2018).

Bacteria of the phyla Firmicutes and Bacteroidetes consistently dominate the faecal microbiota in comparison with other phyla members, regardless of the different westernised geographical locations (Dougal, et al. 2014; O' Donnell, et al. 2013; Salem, et al. 2018).

2.4 Diet

Diet plays a key role in affecting the composition of the gastrointestinal microbiota. It has been demonstrated that changes in the diet of a horse alters the composition of the faecal microbiota (Bulmer, et al. 2019; Dougal, et al. 2014; Fernandes, et al. 2014). Differences in microbiota can be associated with differences among forage diets (pasture vs. hay), high starch diets and oil content in diets. In human medicine, there is emergence of understating of the gastrointestinal microbiota-gut-brain axis, in which there is evidence to support the role in of the gastrointestinal microbiota in regulating anxiety, mood, cognition and pain in people (Cryan and Dinan 2012). Studies in naïve ponies have demonstrated a direct effect of diet on

the faecal microbiota and behaviour (Bulmer, et al. 2019). Reports have described an increase in alertness, reactivity and increased difficulty in handling horses that are fed a high starch diet (Bulmer, et al. 2019), compared to baseline behaviour. It is hypothesised that high starch diets and the associated changes in microbiota may contribute to changes in behaviour in horses.

It has been observed that there are compositional and functional changes in the faecal microbiota of horses fed different diets. A study by. Venable et al (2017) showed that meal size and frequency has an effect on caecal microbiota composition, with changes of *Prevotella, YRC22, Lactobacillus, Streptococcus, Coprococcus,* and *Phascolarctobacterium* observed. In addition, sudden dietary changes such as the ingestion of high amounts of starch, have been associated with instability of the equine gastrointestinal microbiota (Al Jassim and Andrews 2009; Garner, et al. 1978). It has been well documented that large amounts of non-structural carbohydrates in the diet are related to a change in gastrointestinal microflora and strongly linked to colic, laminitis and increased incidence of gastric ulceration (Al Jassim and Andrews 2009; Garner, et al. 1978; Julliand and Grimm 2017). An increase and proliferation of lactic-acid producing bacteria and a decrease in cellulytic bacteria has been measured in horses receiving high-starch diets (Al Jassim and Andrews 2009). It has also been demonstrated that starch from different sources (corn, oats, wheat) has differing effects on the faecal microbiota (Harlow, et al. 2016). Horses that are fed a forage based diet have an increased diversity of faecal microbiota, with increases in abundance of *Ruminococcaceae* family and *Streptococcus* (Bulmer, et al. 2019). A study performed by Hansen et al 2015 found that a low nutrient availability diet of hay only, was associated with a higher level of both diversity and temporal stability of the caecal microbiota compared to the high nutrient availability diet of hay and whole oats. These findings suggest a low nutrient available diet has a stabilising effect on the

biological diversity and that a high nutrient availability has a destabilising effect (Hansen, et al. 2015). It is reported abrupt changes in the diet are associated with microbial disturbances in the equine gastrointestinal tract (Julliand and Grimm 2017).

2.5 Antimicrobial drug therapy

Antimicrobial drug therapy has an important role in increasing the risk of dysbiosis and diarrhoea in horses (Costa, et al. 2015b). Studies have shown that antimicrobials reduce faecal microbiota bacterial species richness (total number of taxa present in the environment), diversity (richness and eveness/distribution of species), and results in differences in population structure (Costa, et al. 2015b; Harlow, et al. 2013). Most of the common antimicrobial drugs used in equine veterinary practice, including procaine penicillin, trimethoprim sulfadimidine, gentamicin, enrofloxacin, doxycycline and ceftiofur sodium, increase the risk of development of antimicrobial-associated diarrhoea (Barr, et al. 2013; Costa, et al. 2015b; Garber, et al. 2020). With antimicrobial treatment, the normal gastrointestinal microbiota is disrupted and the intricate balance between the different bacterial species needed for normal function can be greatly altered. Changes in the microbiota in healthy horses associated with antimicrobial therapy have been related to the presence of enteropathogens such as *Clostridium difficile, Clostridium perfringens* and *Salmonella* (Barr, et al. 2013; Garber, et al. 2020; Harlow, et al. 2013). In horses, dysbiosis can cause increase in these enteropathogens is a particularly important consideration due to the associated risk of potentially life-threatening antimicrobial-associated diarrhoea and biosecurity and zoonotic risk.

2.6 Travel/transportation

Horses are frequently transported for competitions and training, pleasure rides, reproductive purposes, for medical attention and after purchasing (Garber, et al. 2020). It has been

demonstrated that transportation and fasting have an effect on the composition and structure of the microbiota of healthy horses (Faubladier, et al. 2013; Schoster, et al. 2016b). Transportation for as little as 2 hours can disturb the gastrointestinal bacterial microbiota in a manner that could increase the risk of dysbiosis of the equine large intestine (Faubladier, et al. 2013). A decreased abundance of clostridial species has been identified after transportation and fasting of 1 hour (Schoster, et al. 2016b).

2.7 Anaesthesia

Horses commonly undergo general anaesthesia for treatment of a variety of conditions. It has been demonstrated that bacterial community and structure can be altered after general anaesthesia (Schoster, et al. 2016b). The effect of general anaesthesia on the gastrointestinal microbiota has been assessed in other species. A study using neonatal rats showed a relative change in abundance of the faecal microbiota of rats that were exposed to isoflurane vs. no exposure (control group), suggesting that the anaesthetic agents commonly used in practice also have an effect on the gastrointestinal microbiota (Wang, et al. 2019). It could be extrapolated from these findings that isoflurane may also have a similar effect on horse gastrointestinal microbiota, however further investigation in this area is needed.

2.8 Non-steroidal anti-inflammatory drugs

Non-steroidal anti-inflammatory drugs (NSAIDs) are one of the most used pharmaceutical classes world-wide. In equine veterinary practice, NSAIDs are frequently used to treat a range of conditions due to the anti-inflammatory and analgesic properties of the drugs (Whitfield-Cargile, et al. 2018). NSAID administration has been associated with changes to the gastrointestinal microbiota of people and laboratory animals (MÄKivuokko, et al. 2010; Uejima, et al. 1996). It stands to reason that NSAIDs also have a similar effect on the gastrointestinal microbiota in horses. (Whitfield-Cargile, et al. 2018) reported that NSAID administration in adult horses, temporarily reduced that alpha diversity of the faecal microbiota irrespective of cyclo-oxygenase selectivity. This supported previous thoughts that the administration of NSAIDs results in a transient dysbiosis of the faecal microbiota of healthy adult horses. The changes in phyla reported in this study were primarily characterised by loss of members of *Firmicutes,* specifically the family *Lachnospiraceae.* The mechanism by which NSAID administration causes dysbiosis is still unknown (Whitfield-Cargile, et al. 2018).

2.9 Omeprazole

Omeprazole is a commonly administered, highly effective medication used in the treatment and management of equine gastric ulcer syndrome (Andrews, et al. 1999; Birkmann, et al. 2014). Omeprazole is a H+/K+ ATPase inhibitor and is a potent suppressant of hydrochloric acid production (Andrews, et al. 1999). Evaluation of this medication and the effects on microbiota of the gastric fluid, gastric mucosa and faeces has demonstrated no effect on the faecal or gastric mucosal microbiota (Cerri, et al. 2020), or the faecal microbiota of treated horses compared to a control group (Tyma, et al. 2019).

2.10 Microbiota in disease

Diseases affecting the gastrointestinal tract are one of the leading causes of morbidity and mortality in horses, and alterations of the intestinal microbiota appear are likely to contribute to disease. Disruption of the intestinal microbiota has been associated with postpartum colic, colitis and equine grass sickness (Costa and Weese 2018; Harlow, et al. 2013; Leng, et al. 2018). It has also been reported that diseases and conditions of other body systems are associated with differences in the gastrointestinal microbiota, including laminitis, asthma and metabolic syndrome (Costa and Weese 2018; Garner, et al. 1978; Leclere and Costa 2020).

2.10.1 Colic

Colic (abdominal pain) is associated with important causes of morbidity and mortality in horses. Epidemiologic studies have investigated factors associated with the likelihood of colic including management, age, breed, exercise, parasites, pasture access, stabling, transportation and vaccination (Scantlebury, et al. 2015), highlighting the multi-factorial nature of this diseases causing colic. Investigations of the faecal microbiota of horses with colic have demonstrated changes in bacterial populations observed (Stewart, et al. 2019; Stewart, et al. 2021; Weese, et al. 2015). One study reported fewer bacterial species and lower diversity of the faecal microbiota of horses presenting for abdominal discomfort compared to elective surgical procedures (Stewart, et al. 2019). In addition to reduced species richness at admission to hospital, (Stewart, et al. 2021) reported continued decrease in species richness in horses with colic >60 hours when compared with horses with colic <6 hours. In another study, changes in the faecal microbiota in post-partum mares was investigated, with significantly higher abundance of Proteobacteria observed and lower relative abundance of Firmicutes prior to episodes of abdominal discomfort (Weese, et al. 2015). These reports highlight the importance of the gastrointestinal microbiota in colic.

Further characterisation of the changes of the faecal microbiota may lead to early predication of disease and revision of current prevention and/or treatment recommendations.

2.10.2 Intestinal helminth infestation

Virtually all horses, especially those exposed to pasture, experience some level of parasitism continuously (Reinemeyer and Nielsen 2009), and hence infestation of horses in ubiquitous. Intestinal nematodes including small strongyles, *Parascaris* spp*, Strongylus vulgaris, S. edentatus, S. equinus* and cestodes are important, and have been associated with poor growth, weight loss and clinical disease (Andersen, et al. 2013). Throughout the equine GIT, internal parasites inhabit the same environment as bacteria, archaea and fungi and, as such, it is reasonable to expect that there is likely a relationship between parasites, microbiota and host immunity (Garber, et al. 2020). The interaction that these have with each other is important, but not yet completely understood. It has been observed that cyathostomin infection in horses is associated with global shifts in faecal microbial composition and diversity (Peachey, et al. 2018; Peachey, et al. 2019) and there is evidence that acute larval cyathostominosis is associated with dysbiosis (Walshe, et al. 2021). Changes in the gastrointestinal microbiota have been reported after administration of anthelmintic drugs in horses, including decreased diversity (Kunz, et al. 2019), a decrease in *Bacteroidetes* and increase in *Firmicutes* (Walshe, et al. 2019). Further, it has been speculated that individual host factors may influence the effects of anthelmintic treatments on the equine gastrointestinal microbiota (Kunz, et al. 2019). Anthelminthic administration is a common and standard equine husbandry practice, and characterising the changes to the gastrointestinal microbiota associated with endoparasites and the administration of anthelmintic drugs is important to better understand effects on the physiological and health status of horses. This information may lead to improved recommendations for anthelminthic treatment regimens

and the possibility of novel, biological treatment strategies for helminths that manipulate host-parasite-microbiota interactions.

2.10.2.1 Assessment of helminth infestation with faecal egg counts

In clinical practice, faecal eggs counts are often used for assessment of large and small strongyle burden. Antemortem, faecal egg counts are the best proxy available for assessment of helminth infestation. There is an inherit and unavoidable variability that is encountered when assessing faecal egg counts in horses. The Simple McMaster method has been described as the most accurate technique for detection of strongyle eggs (Nápravníková, et al. 2019). To perform a faecal egg count, fresh manure (which that has been voided <12 hours) is collected, and if FEC cannot be performed immediately, manure should be stored in airtight conditions at <6°C for up to 5 days (Matthews and Lester 2015). Horses are considered to have low egg shedding when counts are 0-200 epg, moderate shedding at 200-500 epg and high shedding >500 epg (Bayless 2020).

2.10.2.2 Faecal egg count reduction test

Faecal egg count reduction (FECR) tests have been used to determine anthelminthic treatment efficacy and as a tool used to monitor anthelmintic resistance (Denwood, et al. 2010). Other methods to identify AR include egg reappearance period (ERP), measurement of worm count at necropsy, larval migration inhibition test, motility based tests, larval feeding tests, egg hatch tests and molecular tests (Avramenko, et al. 2019; Coles, et al. 2006; Kaplan, et al. 2023; Taylor, et al. 2002). Due to the practicality and logistical problems associated with other methods, FECRT is the technique of choice for assessment of AR.

When considering study designs for performance of FECRT there are two broad methods undertaken 1) the use of a treatment and control group, and 2) the use of the same animals and pre and post-treatment samples. Experiments undertaken using pre and post treatment

samples have superior sensitivity compared to studies that include control groups (McKenna 2006). As a result, it is now recommended that FECRT are performed on the same animals pre and post treatment.

Put simply, the FECR is expressed as a percentage (%), and is calculated as the difference between the mean FEC at Day 0 and Day 14 (FEC % = $100(1- FEC_{d14})/FEC_{d0}$)(Armstrong, et al. 2014). The arithmetic mean is used to represent egg counts at Day 0 and Day 14 (Coles, et al. 1992). In general, helminths are considered susceptible when the lower 90% CI is greater than or equal to the lower efficacy threshold and the upper 90% is greater than or equal to the expected efficacy (Kaplan, et al. 2023). Helminths are considered resistant when the upper 90% CI is less than the expected efficacy (Kaplan, et al. 2023) and inconclusive if neither of the two criteria are met (Kaplan, et al. 2023). If the criteria for susceptibility and resistance are met simultaneously AR could be considered as 'low-resistant', however multiple authors prefer the simplified three classifications (Denwood, et al. 2023; Kaplan, et al. 2023).

The *World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.)* have recently published revised guidelines for the diagnosis of anthelmintic resistance testing. For anthelmintic resistance to be present, they state that the below conditions must all be true (Figure 2.3).

Box 1
Conditions that must all be true before one can conclude that reduced efficacy in an FECRT is consistent with a diagnosis of anthelmintic resistance.

1. Animals were treated with the proper dose (all animals received the minimum label dosage).
2. Animals were treated using a proper administration technique.
3. Anthelmintic drug was used within the expiration date and was properly stored prior to using in the FECRT.
4. The same animals were sampled both pre- and post-treatment with an interval appropriate for the anthelmintic used, and animals were correctly identified.
5. Both pre-treatment and post-treatment faecal samples were freshly collected, and labelled and stored correctly.
6. A faecal egg count method suitable for the FECRT was applied and proper laboratory techniques were used.
7. The drug being tested had been previously demonstrated to be efficacious against the target parasite species in the same host animals when administered at the dosage being tested.
8. Adequate numbers of animals were tested and adequate numbers of eggs were counted in the pre-treatment samples.
9. An appropriate statistical method was used, and the statistical results indicated sufficient confidence in the repeatability of the result (i.e. confidence intervals were calculated using an appropriate method and these were used for making the diagnosis).
10. The quality of the anthelmintic product can be assured.

Figure 2.3: World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) guidelines and conditions for conclusion of AR (Kaplan, et al. 2023). ***Permissions obtained; http://creativecommons.org/licenses/bync-nd/4.0/.***

2.10.2.3 Anthelmintic treatments

There are three main classes of anthelmintics used in equine preventative medicine: benzimidazoles (BZ), imidazothiazoles/tetrahydropyrimidines (IT) and macrocyclic lactones (ML) (Kaplan, et al. 2023). Each of these classes has been shown to differ in their efficacy in horses against susceptible small strongyle populations. The currently suggested cut-off for reduction in FEC is 95% for ML, 90% for BZ and 85% for imidazothiazoles/tetrahydropyrimidines (IT) (Bayless 2020).

2.10.3 Anthelminthic Treatment and the microbiota

Anthelminthic treatment is a common and standard equine husbandry practice and, as such, characterisation of the changes to the gastrointestinal microbiota associated with this treatment is pertinent in advancing equine preventative medicine. It has been demonstrated that administration of anthelmintics is associated with changes in the faecal microbiota of horses (Kunz, et al. 2019; Peachey, et al. 2018; Peachey, et al. 2019; Walshe, et al. 2019). Administration of fenbendazole or moxidectin has been shown to reduce alpha and beta

diversity, and results in changes to the relative abundance of taxa at phylum, family and genus levels (Walshe, et al. 2019). In another study, administration of anthelmintics moxidectin and praziquantel resulted in reduced alpha diversity and changes to relative abundances of taxa. However in contrast, administration of ivermectin has been associated with increased alpha diversity in one study (Peachey, et al. 2019).

Administration of anthelmintics with acceptable reduction in faecal egg count has been shown to effect the faecal microbiota (Peachey, et al. 2019; Walshe, et al. 2019), however it is unknown how much of this effect is due to the intrinsic nature of the drug or removal of helminths from the gastrointestinal tract. A study published by Kunz et al. 2019, reported changes in the microbiota associated with anthelmintic treatment where the parasite burden was undetectable, suggesting that administration of drug alone can affect the microbiota. The effect of anthelmintic treatment on the faecal microbiota of horses where helminth resistance is present is currently unknown. Throughout the literature, there is evidence and support for the need to further investigate the effects of alteration to the gastrointestinal microbiota by anthelminthic treatment (Kunz, et al. 2019). In particular, there is a need to further investigate the effect of anthelmintic treatment in helminth populations where there is resistance and susceptibility.

2.10.4 Diarrhoea in adults

Marked differences have been reported in the gastrointestinal microbiota between healthy horses and horses with colitis (Costa, Arroyo et al. 2012), indicating that colitis and diarrhoea is a disease process of dysbiosis, rather than simply an overgrowth a single pathogen (Costa, Arroyo et al. 2012). In one study, Firmicutes phyla predominated in the faecal microbiota of

healthy horses, whereas in horses with colitis, Bacteroidetes were the most abundant phyla (Costa, Arroyo et al. 2012). A study involving the use of faecal microbiota transplantations for diarrhoea reported that Firmicutes was the most abundant phyla, followed by Verrucomicrobia (5.8%), Bacteroidetes (5.8%), Spirochaetes (5.0%), and Proteobacteria (Costa, et al. 2021). (McKinney, et al. 2021) reported that Bacteroidetes and Firmicutes were the most abundant taxa in the investigation of the faecal microbiota in horses with colitis. Colitis/typhlocolitis in horses has been associated with a variety of bacterial pathogens including *Salmonella* spp., *Clostridium perfringens*, *Clostridium difficile* and, although exotic (not reported) to Australia, *Neorickettsia risticii* (Chapman 2009, Weese, Staempfli et al. 2010). While laboratory testing is available commercially for the detection of specific enteropathogens, evaluation of the microbiota and assessment for dysbiosis of the GIT is not performed clinically. As such, there is limited data of the microbiota of horses with GIT disease in Australian horses. Consequently, there is a need for investigation of the gastrointestinal microbiota associated with typhlocolitis in horses, to better understand the aetiology and pathophysiology of this syndrome and progress novel and targeted treatment options.

2.10.5 Diarrhoea in foals

Diarrhoea is a common problem in neonatal foals, with up to 80% of foals developing diarrhoea in the first weeks of life (John, Roediger et al. 2015, Schoster, Staempfli et al. 2015). Comparable to adult horses, the infectious bacterial agents of foals with diarrhoea reported include *Salmonella* spp., *Clostridium perfringens, Clostridium difficile, Rhodococcus* equi and *Lawsonia intracellularis* (Oliveira, et al. 2019; Slovis, et al. 2014; Wong, et al. 2009). In addition to enteropathogens, diarrhoea in foals can be due to changes of the gastrointestinal microbiota as the foal develops or in response to non-infectious causes. More recently, the gastrointestinal microbiota in foals with diarrhoea has been investigated using next

generation sequencing. In one study, foals with diarrhoea had an underrepresentation of Lachnospiraceae and Ruminococcaceae (Clostridial class) compared to healthy foals of 2 and 4 weeks of age (Schoster, et al. 2017). Similar to adult horses with GIT disease, foals experiencing diarrhoea had lower richness and diversity of the faecal microbiota. Diarrhoea caused by enteropathogens is also common in foals aged up to 6 months (Frederick, Giguère et al. 2009), suggesting dysbiosis can develop in foals of all ages.

2.11 Diagnosis

2.11.1 Culture

Historically the equine gastrointestinal microbiota has been evaluated using culture techniques, which are now known to only capture 10-20% of the actual microbiota present (Fouhy, et al. 2015). Due to the fastidious or poorly characterised atmospheric and nutrient requirements, the vast majority of gastrointestinal microbes remain largely unculturable, making studies in this area challenging (Hart, et al. 2015). Moreover, given the complexity of microbiota ecology within the gastrointestinal tract, different responses to organisms during culture processes mean that it is difficult to ensure the relative abundance of each organism after culture is representative of that *in vitro*. There are a number of pathogens associated with disease in adults and foals that can be cultured for identification including *Salmonella spp, Clostridium difficile* and *Clostridium perfringens* (Shaw and Stämpfli 2018).

2.11.2 PCR analysis and ELISA

Polymerase chain reaction technique is a common molecular technique that is utilised in the veterinary industry for diagnosis of infectious diseases. PCR is used to detect infectious causes of diarrhoea in horses including *Salmonella spp, Clostridium difficile, Clostridium perfringens, Coronavirus* and *Neorickettsia risticii* (Shaw and Stämpfli 2018) in faeces. PCR technique is very

sensitive and allows for detection and identification of gene sequences by rapid amplification of specific fragments of DNA (Garibyan and Avashia 2013). The modification of specific versions of PCR have facilitated quantitative measurement of gene expression, called real-time PCR (Garibyan and Avashia 2013). The limitations of use of PCR for investigation of the microbiota include the inability to describe the entire microbiota, as PCR targets only specific microorganisms and genes (Cason, et al. 2022).

Enzyme-linked immunosorbent assay (ELISA) is also used to aid in the diagnosis of infectious agents including *Clostridium difficile* and *Clostridium perfringens* (Shaw and Stämpfli 2018). ELISA is a technique used in clinical analysis, where most commonly, a sample with a specific antigen (for example *Clostridium difficile)* is added to and allowed to bind with a test antibody to form a 'sandwich complex' (Alhajj, et al. 2024). The amount of produce generated represents a quality of antigen or toxin within a sample (Alhajj, et al. 2024). The test allows for the identification and sometimes quantification of the amount of antigen or toxin within a sample.

2.11.3 Next Generation Sequencing (NGS)

With advancement of molecular technologies, an increase in molecular approaches to evaluate the microbiota has provided a greater accuracy than culture alone to characterise these microbial communities, increasingly feasible and affordable (Fouhy, et al. 2015; Hart, et al. 2015). Next generation sequencing (NGS) has been used in recent years to allow for more comprehensive characterization of the complex gastrointestinal microbiota of horses and other species (Costa, et al. 2016a). NGS is able to sequence a high number of DNA fragments simultaneously, which are then mapped by bioinformatic analysis with comparison to reference genomes (Behjati and Tarpey 2013).

2.11.3.1 Amplicon sequencing and shotgun metagenomic sequencing

One primary methodology of NGS is amplicon sequencing (Wensel, et al. 2022). Amplicon sequencing is a common NGS method, where amplification of a specific region of DNA is performed by PCR, with sequencing of the resulting product (Wensel, et al. 2022). Amplification of the V3-V4 region of the 16S rRNA is most commonly performed to assess the microbiota of horses (Arroyo, et al. 2020; Costa, et al. 2012b; De La Torre, et al. 2019; Schoster, et al. 2017). After amplification, amplicons are sequenced and data 'cleaned' (Wensel, et al. 2022). There are multiple sequencing platforms available including but not limited to Illumina, MiSeq and NovaSeq. Multiple stages are involved in data cleaning including adapter and primer sequence trimming, chimeric sequences, removal of poor-quality bases and sequences, elimination of sequences that match control libraries, human contaminants and other contaminants (Wensel, et al. 2022). Analysis of sequence data results in operational taxonomic units (OTUs) and then taxonomic identification in inferred with references 16srRNA data bases (Wensel, et al. 2022). An alternative to OTUs are amplicon sequence variants (ASVs), that rely on exact nucleotide matching to obtain ASVs. Both OTUs and ASVs are approaches to estimate taxonomy and downstream analysis is the performed using these results (Wensel, et al. 2022).

Shotgun metagenomic sequencing differs from amplicon sequencing in that all DNA and RNA in a sample is analysed (Wensel, et al. 2022). After DNA extraction, DNA is randomly fragmented, with barcodes and adapters ligated at the ends of each segment for sample identification and sequencing (Wensel, et al. 2022). The reads are aligned with a reference database for taxa identification and functional prediction (Wensel, et al. 2022).

2.11.3.2 Bioinformatic analysis

There are several publicly available (Wensel, et al. 2022)platforms for the undertaking of bioinformatics and analysis including QIIME(Knight, et al. 2010), QIIME2 (Bolyen, et al.

2019)and mothur (Schloss, et al. 2009). These platforms have been utilised in studies investigating the equine microbiota (Arroyo, et al. 2020; De La Torre, et al. 2019; Glatter, et al. 2019; McKinney, et al. 2021; Schoster, et al. 2017). Initial results of bioinformatic analysis include OTU or ASV analysis, alpha diversity analysis and beta diversity analysis. Thereafter, statistical analysis of species and community variance can be performed, as well as advanced analysis including environment and correlation analysis and functional prediction. Functional prediction cannot be directly obtained from 16S rRNA sequencing and as such methods including PICRUSt and PICRUSt2 are instituted in an attempt to predict the functional genes of bacteria (Wensel, et al. 2022).

2.11.3.3 Considerations of 16s rRNA sequencing

The benefits of 16s rRNA NGS in the investigation of the microbiota include a reduced risk of contamination of samples, as the genes sequenced are specific to bacteria and a lower risk of false positives due to extensive reference databases and error correction tools (Wensel, et al. 2022). However, there is an increased bias due to the primer-dependant PCR amplification and differences between variable regions (Wensel, et al. 2022).

2.11.4 Sampling

The proposed question “What samples should we obtain to evaluate the gastrointestinal microbiota” has been of debate. Multiple different conclusions have been drawn between studies for the use of faeces as a proxy for the large intestine microbiota. Multiple reports (Costa, et al. 2015a; Grimm, et al. 2017) have demonstrated that faecal samples and their bacterial analyses, can be used to represent the changes that occur in the microbiota of the distal compartments of the gastrointestinal tract. Conversely, reports from (Schoster, et al. 2013) stated that faecal samples were not entirely representative of the distal tract. In a clinical setting where obtainment of ingesta from the multiple different compartments is

contraindicated (due to invasiveness, expense and impracticality of these procedures), faecal samples prove to be the best proxy available in representing the distal gastrointestinal tract microbiota. It has been shown that immediate (<6 hours) sampling of faeces is required to obtain accurate representation of the distal gut microbiota, after this time the microbial community changes (Beckers, et al. 2017) and it is best to analyse the centre of the faecal ball(Stewart, et al. 2018). In cases where collection of manure is also unachievable (for example diarrhoea in foals), swabs of manure have been obtained, with adequate DNA concentrations and quality achieved in previous reports (Bordin, et al. 2013).

2.11.5 Sample storage

Throughout the literature, authors have stored faecal microbiota samples at freezing temperatures of -20°C (Bai, et al. 2012; De La Torre, et al. 2019) and -80°C prior to DNA extraction (Costa, et al. 2012b; Ericsson, et al. 2016; Stewart, et al. 2018). Human studies have reported little impact of storage on the microbiota at these temperatures over a 6-month period (Bai, et al. 2012) and the freezing of faecal samples for microbiota analysis has been shown to have no significant effects on processing or results (Fouhy, Deane et al. 2015). It is important for samples to be stored at freezing temperatures, as the microbial community changes after 6 hours of ambient temperature (Beckers, et al. 2017).

2.11.6 DNA extraction

DNA extraction of faecal samples has been performed using commercial kits. The type of kit and extraction method varies depending on sample type, and numerous kits have been used to perform the procedure. Commercially available kits have been used to extract DNA for assessment of equine faecal microbiota and include kits from companies such as QIAGEN (Álvarez-Narváez, et al. 2020), Zymo Research (Bordin, et al. 2013; De La Torre, et al. 2019;

Husso, et al. 2020), Omega Bio-Tek (Costa, et al. 2016a; Schoster, et al. 2016b; Schoster, et al. 2017), and Ivitek Diagnostics (Stewart, et al. 2018). Depending on what sample was obtained, specimens of either faeces (Costa, et al. 2016b; De La Torre, et al. 2019; Stewart, et al. 2018) or swabs (Álvarez-Narváez, et al. 2020; Bordin, et al. 2013; Husso, et al. 2020; Schoster, et al. 2016b; Schoster, et al. 2017) have been processed for DNA extraction.

The Quick-DNA Fecal/Soil Microbe Miniprep Kit® by Zmyo Research has been utilised in equine faecal microbiota studies and extraction from manure samples and swabs have been performed with the commercial kit (Bordin, et al. 2013; De La Torre, et al. 2019; Husso, et al. 2020). Assessment of the concentration and quality of the extractions is satisfied by NanoDrop UV spectrophotometer, which is utilised commonly to assess DNA extraction quality prior to gene sequencing (Costa, et al. 2012b; De La Torre, et al. 2019; Schoster, et al. 2016a).

2.12 Treatment

As it has been demonstrated that alterations in the microbiota can be associated with gastrointestinal disease, it stands to reason that restoration of the normal microbiota is a rational goal for prevention and treatment. The treatment of diarrhoea in adult horses and foals is dependent on the severity of disease and presenting condition. Treatment is mostly supportive, and specific and tailored treatment relies on identifying an inciting cause, which is not always possible.

2.12.1 Probiotics

Probiotic treatment in adult horses has been widely used as a nutritional supplement, although no clear benefits have been identified for improvement of diarrhoea. Initial studies reported that treatment of adult horses with human strains of probiotics did not induce any adverse effects (Weese, et al. 2003). A trial involving 24 colic patients, (Kim, et al. 2001)

reported no difference in diarrhoea incidence of treated horses and control horses. This was similar to earlier studies of the effect of probiotic administration on diarrhoea in post operative colic patients, where no improvement of diarrhoea was observed (Parraga, et al. 1997). Seemingly, the current area of interest for administration of probiotics to adult horses at this time is for performance enhancement. (Laghi, et al. 2018) reported reduced post exercise lactate in training Standardbred horses after multi-strain probiotic administration, suggesting a positive impact on performance. (Zavistanaviciute, et al. 2019) reported that probiotic supplementation reduced blood lactate concentrations in endurance horses that are undergoing athletic activities. The current literature suggests that although there is no clear benefit in administration of probiotics for diarrhoea in adult horses, there may be other advantageous effects of the supplement in performance athletes.

Probiotic treatment in foals has been evaluated in multiple studies, with variable results. Initial research trials reported no adverse effects of treatment (Weese, et al. 2004; Weese, et al. 2003). Two subsequent studies reported decreased diarrhoea incidence and increased growth rate in thoroughbred foals treated with multi-strain probiotic (Tanabe, et al. 2014b; Yuyama, et al. 2004). Contrary to these initial reports, however, further evaluation of the administration probiotics showed increased severity of diarrhoea in foals and treated foals displayed a reduced growth rate (Ströbel, et al. 2018; Weese and Rousseau 2005). In a study involving 153 foals, (Weese and Rousseau 2005) observed development of diarrhoea associated with probiotic administration, which was severe enough to warrant veterinary intervention and treatment in some foals. Another large study involving 72 healthy foals also concluded that administration of probiotics showed no benefit to the enrolled foals and that administration of probiotic increased the likelihood of development of diarrhoea (Schoster, et al. 2015). (Urubschurov, et al. 2019) and (Schoster, et al. 2016a) observed no change to the

faecal microbiota of foals administered probiotic stains of Lactobacillus, Enterococcus and Bifidobacterium. In addition to this, (Swarthout, et al. 2017) reported no effect of probiotic administration on the severity of diarrhoea in neonatal foals and (John, et al. 2015) reported no effect on diarrhoea and clinical status of foals supplemented with probiotic. In summary, there's not been any robust evidence in the literature to state that probiotic treatment prevents diarrhoea or assists in the treatment of diarrhoea in a clinical setting in foals.

2.12.2 Faecal microbiota transplantation (FMT)

The concept of establishing a more normal gastrointestinal microbiota has also been evaluated with faecal microbiota transplantation (FMT). Although not novel, this treatment has recently received increasing attention both in the human medicine and veterinary medicine. The approach has been historically used with anecdotal success by equine practitioners, predominantly through the administration of faecal slurries by nasogastric intubation to horses with diarrhoea. Interest in the veterinary industry has swelled, likely due to the recent advancements in human medicine, where FMT has been shown to have high cure rates in humans with *Clostridium difficile* infection, particularly due to antibiotic-associated diarrhoea (Brandt, et al. 2012; Lee, et al. 2016; Shahinas, et al. 2012) and ulcerative colitis (2015; Kunde, et al. 2013) . In dogs, initial data suggests that FMT is an effective treatment for diarrhoea caused by canine parvovirus and decreases the duration of disease (Pereira, et al. 2018).

Recently, three studies have assessed the clinical and faecal microbiota response to treatment with FMT in adult horses with diarrhoea. McKinney et al. (2020) published a study of FMT treatment of five geriatric horses presented for diarrhoea. In this study, three of five responded to treatment (McKinney, et al. 2020), specifically, the FMT treatment increased the alpha-diversity of the recipient microbiota. A key limitation of this study was the lack of a

control group. Subsequent to this, McKinney (2021) published a study of 22 horses with colitis, where horses receiving once daily treatment with FMT showed a greater overall reduction in diarrhoea and greater normalization of the microbiota score than control horses. However, this study was performed in two hospital locations, with treated horses and untreated horses housed in different environments, hence location may have influenced these results. Costa et al. (2021) reported no significant changes to the microbiota of diarrhoeic horses treated with FMT in a study involving 6 client owned horses with chronic and acute diarrhoea. However only faecal samples were obtained, and perhaps changes in the microbiota were present in the more proximal aspects of the intestinal tract, where samples cannot be obtained in the live animal. In this study, authors reported improvement of diarrhoea in 4/6 horses. To the authors' knowledge, there are no descriptions of the use of FMT for the treatment of diarrhoea in foals.

Administration of FMT to horses as a prophylactic approach in preventing dysbiosis in animals receiving antimicrobial therapy has also been investigated (Kinoshita, et al. 2022). Faecal transplantation prior to metronidazole administration did not ameliorate the induced dysbiosis, which was assessed by clinical signs of the horses and microbiota analysis of manure (Kinoshita, et al. 2022). FMT has also been trialled in the treatment of free faecal water syndrome (Laustsen, et al. 2021b). This publication reported that FMT reduced the severity of disease, although some animals only had temporary reduction in clinical signs and there was no difference of the faecal microbiota of treated horses (Laustsen, et al. 2021a).

2.12.3 Faecal microbiota transplantation collection, preparation and storage

There is no current validated protocol for preparation and administration of FMT in horses, but reported protocols are relatively similar throughout the literature. It has been recommended that faeces are collected from donor horses that are healthy, have not recently

been administered antimicrobials and screened for infectious diseases including *Salmonella* sp, gastrointestinal parasites and equine coronavirus (Mullen, et al. 2018). Ideally donor horses are housed outdoors and fed a forage based diet, and are also from the same herd or facility as the recipient (Mullen, et al. 2018). Faeces are collected per rectum or freshly passed (<6 hours old) (Costa, et al. 2021; Dias, et al. 2018; Kinoshita, et al. 2022; Laustsen, et al. 2021b; McKinney, et al. 2020a). Preparation methods vary slightly from use of 0.1kg – 0.5kg of manure per 1 litre of warm water, with frequency of administration varying from 1 – 3 days consecutively via nasogastric intubation (Costa, et al. 2021; Dias, et al. 2018; Kinoshita, et al. 2022; Laustsen, et al. 2021a; McKinney, et al. 2021; McKinney, et al. 2020a). The volume administered is reported to be 2-3 litres for an average sized (500kg) adult horse and recommendations of 200mL in foals (Mullen, et al. 2018).

The ability to store faecal microbiota transplantations for use as required would be of great benefit in clinical practice. Preparation of transplantations can be time consuming, particularly in settings where transplantations may be performed daily for a number of days. Changes to the microbial population over time and the viability of the bacterial community in different storage conditions is important to consider. Studies assessing the change in equine faecal microbiota transplant communities in different storage conditions has rarely been investigated. As previously stated, the microbial composition in fresh faeces is stable until approximately 6 hours, and after that time the population changes (Beckers, et al. 2017). There are currently no studies that have utilised next generation sequencing to report changes in the microbiota of transplantations over time in horses. (Kopper, et al. 2021) evaluated the viability of microbial populations of FMT in storage conditions -20°C over time. They reported that viability significantly decreased after more than 1 week of storage, with gram negative bacteria most significantly affected.

Frozen FMT material is widely used in human medicine, and studies have shown that the use of frozen-thawed material is non-inferior to fresh faeces and does not result in differences in clinical outcomes (Lee, et al. 2016). In the human literature it has been reported that long term freezing has limited effect on the diversity and composition of the microbial community (Tap, et al. 2019). Cryoprotectants such as glycerol are often used in human medicine to protect microbial communities during storage. (Youngster, et al. 2014) demonstrated that use of frozen stools resuspended with 10% pharmaceutical grade glycerol and stored at -80°C was effective in treating CDI, and other studies have replicated this protocol with reported success (Cheminet, et al. 2018). One equine study performed by (Kinoshita, et al. 2022) used frozen thawed stools to perform transplantations on horses that were simultaneously being administered metronidazole. The study reported that once daily FMT did not prevent metronidazole induced GIT dysbiosis, but did not investigate the effects of storage on the transplantations prior to administration. There is need for further investigation in this area.

Study Objectives

This project will aim to:

- Determine the clinical efficacy of FMT in foals with diarrhoea and assess the responses of the faecal microbiota to FMT
- Determine the stability of the microbiota of FMT in short term shortage conditions of 4°C and -20°C
- Assess the impact of administration of two anthelmintics that the cyathostomin population is known to be susceptible to and resistant to and determine the effects on the faecal microbiota

The expected outcomes of this thesis include improved clinical outcomes in foals with diarrhoea, including resolution of clinical signs and changes to the faecal microbiota. It was

expected that administration of anthelmintics would result in changes to the faecal microbiota and that removal of larger cyathostomin burdens would result in more dramatic changes. It was hypothesized that short term storage of FMT at 4°C would result in significant change to the microbiota and that storage at -20°C for up to 4 weeks would result in minimal changes to the microbiota.

Chapter 3: Effects of faecal microbiota transplantation on clinical outcomes and faecal microbiota of foals with diarrhoea.

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Effects of faecal microbiota transplantation on clinical outcomes and faecal microbiota of foals with diarrhoea.

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3.1 Abstract

Diarrhoea in foals can be associated with disruption of the intestinal microbiota (dysbiosis). Effective management of intestinal dysbiosis in foals has not been demonstrated. The hypothesis of the study was that faecal microbiota transplantation (FMT) in foals with diarrhoea influences the intestinal microbiota and improves clinical and clinicopathological outcomes.

The study included twenty five foals <6 months of age with diarrhoea at 3 veterinary hospitals. This was a prospective randomised placebo-controlled cohort study. Foals in the FMT group (*n* = 19) or control group (*n* = 9) received FMT or electrolyte solution once daily for 3 days. Faecal samples were obtained on Day 0 (D0), D1, D2, D3 and D7. Within group and between group data analyses were performed for clinical, clinicopathological and microbiota variables.

Treatment had no effect on survival (FMT 79%; control 100%, *P* = .3) or resolution of diarrhoea (FMT 68%; control 55%, *P* = .4). On D3, the white blood cell count of the FMT group was lower than the control group (D3 FMT group median 6.4g/L [5-8.3g/L]; D3 control group median 14.3g/L [6.7-18.9g/L] *P* = .04). Heart rate (HR) reduced over time in the FMT group (D0 median 80bpm [60-150bpm]; D2 median 70bpm [52-110bpm] (*P* = .005); D3 median 64, [54-102bpm] (*P* < .001). Phylum Verrucomicrobiota, genus *Akkermansia* and family *Prevotellaceae* were enriched in the FMT group on D1 (LDA >4). In foals with diarrhoea, FMT appears safe and can be associated with some clinical and microbiota changes suggestive of beneficial effect.

3.2 Introduction

Diarrhoea affects up to 80% of foals in the first 6 months of life,(Frederick, et al. 2009; Urquhart 1981) and can be associated with non-infectious and infectious causes. Infectious causes of diarrhoea in foals include equine rotavirus,(Frederick, et al. 2009) equine coronavirus,(Frederick, et al. 2009) *Clostridioides difficile,(Frederick, et al. 2009) Clostridium perfringens,*(Frederick, et al. 2009) *Lawsonia intracellularis,*(Wong, et al. 2009) *Rhodococcus equi,*(Slovis, et al. 2014) *Salmonella* spp., *Cryptosporidium* spp.(Frederick, et al. 2009) and *Strongyloides westeri.*(Frederick, et al. 2009; Slovis, et al. 2014) Disruption of intestinal microbial communities (dysbiosis) can occur in diarrhoea in both adult horses and foals. Differences in the relative abundance of bacteria, reduced alpha-diversity and increased beta-diversity are described in adult horses with colitis.(Arnold, et al. 2021; Costa, et al. 2012; Li, et al. 2022; McKinney, et al. 2021; McKinney, et al. 2020b) Similarly, diarrhoea in foals is associated with reduced bacterial richness and diversity, and changes in faecal microbiota composition.(De La Torre, et al. 2019; Schoster, et al. 2017) Dysbiosis can disrupt intestinal function, energy metabolism and mucosal health, predispose to inflammation, impair immunity and reduce resistance to colonisation by enteric pathogens. Consequently, restoration of the microbiota is an important objective of management of horses and foals with diarrhoea. Optimal methods for manipulation of intestinal microbiota are yet to be established.

Treatment of diarrhoea in foals is usually non-specific and supportive, including administration of fluids intravenously and enterally, intra-luminal toxin binding agents, and nutritional support.(Oliver-Espinosa 2018) However, these treatments do not address dysbiosis and there is limited information on microbiota manipulation in foals. Administration of antimicrobial drugs is common in the treatment of diarrhoea in foals, but is associated with disturbances of

the microbiota in adult horses,(Arnold, et al. 2020; Costa, et al. 2015b; Liepman, et al. 2022) and these changes likely occur in foals as well. Early studies evaluating probiotic administration to foals report no adverse effects; (Weese, et al. 2004; Weese, et al. 2003) however, subsequent studies demonstrate that the administration of probiotic to foals with diarrhoea increases the severity of disease,(Schoster, et al. 2015) reduces growth rate(Ströbel, et al. 2018; Weese and Rousseau 2005) and, in healthy foals, is associated with the development of diarrhoea requiring veterinary treatment.(Schoster, et al. 2015; Weese and Rousseau 2005)

Faecal microbiota transplantation (FMT) in adult horses reduces diarrhoea,(McKinney, et al. 2021; McKinney, et al. 2020a) increases alpha-diversity of the faecal microbiota(McKinney, et al. 2020a) and results in greater phylogenetic normalisation of microbiota.(McKinney, et al. 2021) Conversely, retrospective evaluation of the use of FMT in adult horses reported no improvement to hospitalisation duration, faecal consistency or clinical and clinicopathological variables with treatment.(Quattrini, et al. 2023) In addition, FMT administration did not alter the faecal microbiota of horses with diarrhoea in another study(Costa, et al. 2021) and administration of FMT to horses undergoing treatment with metronidazole failed to prevent or inhibit dysbiosis.(Kinoshita, et al. 2022) The administration of FMT to adult horses with free faecal water syndrome resulted in no change to the faecal microbiota.(Laustsen, et al. 2021b) The use of FMT in foals with diarrhoea is not reported and it unknown if this method for manipulation of the intestinal microbiota is safe and effective.

The objective of this study was to investigate the clinical and clinicopathological outcomes and changes in the faecal microbiota associated with administration of FMT in foals with diarrhoea. Our hypothesis was that FMT in foals with diarrhoea is associated with improved

clinical and clinicopathological outcomes, resolution of diarrhoea and restoration of faecal microbiota.

3.3 Materials and methods

3.3.1 Animals and study design

A prospective randomised placebo-controlled cohort study was conducted between 2019-2022, using foals <6 months of age presented for treatment of diarrhoea 3 veterinary hospitals [Veterinary Clinical Centre Charles Sturt University, Scone Equine Hospital, South Eastern Equine Hospital]. Foals were excluded if there was evidence of abdominal pain, ileus or gastric reflux. Foals were randomly allocated by ballot into a control or treatment (FMT) group. Foals in the FMT group were administered 200-400 mL (4-6 mL/kg) of freshly prepared FMT by nasogastric intubation once daily for 3 days. Foals in the control group received 200-400 mL of isotonic electrolyte solution once daily for 3 days. All foals were administered omeprazole (non-enteric coated 4mg/kg or enteric coated 2mg/kg) per os at least 60 minutes before FMT or electrolyte administration.

Faeces or faecal swabs were collected 3-5 minutes before treatment on Day 0 (D0) and on D1, D2, D3 and D7. Once collected, samples were stored at -20°C until DNA extraction. Samples were analysed for infectious causes of diarrhoea: PCR was used to test for *Salmonella* spp., *C. difficile* toxins A and B genes, *C. perfringens* toxin A, CPE, cpb2 and netF genes, *Cryptosporidium* spp, equine coronavirus and equine rotavirus A (Vetnostics, ASAP Laboratory)and enrichment and selective culture methods were used to test for *Salmonella* spp. (Veterinary Diagnostics Laboratory Charles Sturt University, ASAP Laboratory, Scone

Equine Hospital Laboratory). Faecal smears were also used to confirm *Cryptosporidium* spp. (Scone Equine Hospital Laboratory).

Animal age, sex, breed, current co-morbidities and duration of diarrhoea prior to enrolment were recorded. Clinical data including demeanour, faecal consistency, heart rate, respiratory rate, rectal temperature, mucous membrane colour and capillary refill time were recorded at admission and the results of daily clinical examinations were recorded. Results of haematological and serum biochemical examinations (including serum amyloid A and fibrinogen) were recorded on D0, D1, D2, D3 and D7. Outcome data consisted of time to resolution of diarrhoea and survival to discharge from hospital.

Throughout the study, foals received treatment for diarrhoea discretion of the attending veterinarians and according to veterinary hospital protocols. This research was approved by the Animal Care and Ethics Committee of Charles Sturt University (Protocol number A19299). Owner consent was obtained before enrolment in the study.

3.3.2 Donor horses

One donor horse was recruited at each veterinary hospital. Donor horses were determined to be healthy based on physical examination and underwent screening yearly for infectious agents including *C. difficile*, *C. perfringens*, equine coronavirus, *L. intracellularis* and *Salmonella* spp. by PCR analysis (Vetnostics, Australia).Cultured donor faeces were assessed for extended spectrum B lactamase (ESBL) producing *E. coli* isolates using the Cefpodoxime Combination Kit (Thermofisher Scientific, Australia). Organisms were interpreted as containing an ESBL if zone size was increased ≥5mm between the combination disc compared to cephalosporin alone (Veterinary Diagnostic Laboratory, Charles Sturt University).

3.3.3 FMT preparation

Fresh manure was collected from donor horses by rectal evacuation, and FMT solution was prepared 15 minutes before treatment each day. Approximately 300 grams of manure was combined with one litre of warm chlorinated tap water (approximately 95°F) and macerated using an immersion blender for 30-60 seconds to facilitate release of bacteria from faecal particulate matter. The preparation was then strained through a wire strainer or 4 cm x 4 cm gauze swabs, and collected into a clean container.

3.3.4 DNA Extraction and Next-Generation Sequencing

Each faecal sample was thawed for 10 minutes in a warm water bath (approximately 30°C/86°F) and DNA was extracted using Quick-DNA™ Fecal/Soil Microbe Miniprep Kit (Zymo Research, CA). Cotton tips of rectal swabs were placed into a bead beating tube with lysis buffer and bead beating was performed at 2800 rpm for 5 minutes using the Digital Disruptor Genie (Scientific Industries, New York, USA). Poor DNA yield and quality were identified at the initiation of DNA extraction of particular batches of samples and one hundred microliters of sterile poly-buffered saline were added to the cotton tip of some swabs prior to bead beating due to poor DNA yield and quality. The remainder of the extraction was performed as per the manufacturer's instructions. Concentration and purity were assessed by NanoDrop 2000 Spectrophotometer (Thermo Scientific, Australia). DNA was stored at -20°C for 8 weeks prior to submission for sequencing.

For each sample, the extracted DNA underwent 16S rRNA sequencing at Novogene sequencing facility (Singapore). Samples were filtered, buffered, washed and eluted as per product protocol. Prior to sequencing all samples underwent quality control (QC). PCR amplification was performed on the V3-V4 regions with primer sequence

CCTAYGGGRBGCASCAG-GGACTACNNGGGTATCTAAT. To select PCR products of appropriate size, 2% agarose gel electrophoresis was used. The products were then pooled, end-repaired, A-tailed and ligated with Illumina adapters. Library sequencing was undertaken on a paired-end Illumina platform to generate 250 bp paired-end raw reads.

3.3.5 Analysis of sequence data

Raw data were spliced and filtered to acquire clean data. Sequences from the clean data with abundances <5 were filtered out using DADA2 (Callahan, et al. 2016) to obtain the final amplicon sequence variants (ASV). The Quantitative Insights Into Microbial Ecology (QIIME2) algorithm was used for species annotation of the ASV.(Bokulich, et al. 2018; Bolyen, et al. 2019) The representative sequence of each ASV was annotated using QIIME2 software. Relative abundance of phylum, class, order, family and genus taxonomic levels were determined for the FMT, control and donor groups. For each group, alpha diversity measures of richness (Chao1, Observed_otus), evenness (Shannon's index) and diversity (Simpson's index of diversity) were generated in QIIME2.(Li, et al. 2013) Beta diversity was assessed with weighted and unweighted UniFrac distance metric,(Lozupone, et al. 2007; Lozupone and Knight 2005; Lozupone, et al. 2011) and visualised using principal coordinate analysis (PCoA) plots generated in QIIME2.

3.3.6 Statistical analysis

Continuous clinical and clinicopathological data were assessed for normality by the Shapiro-Wilk test.(Shapiro and Wilk 1965) Non-normally distributed data were log transformed. Comparisons between treatment and control groups were performed using independent t tests and Mann-Whitney tests for normally distributed and non-normally distributed variables, respectively. Proportions were compared between groups using Fisher's exact or Chi-squared

test. Repeated measures for continuous variables from Day 0-3 inclusive were analysed by repeated measures ANOVA with post-hoc testing by Tukey test or, for non-parametric data, Friedman's test and post-hoc analysis with Dunn's tests were used. Analyses were performed using commercial software (Prism version 9.5.0, GraphPad Software Inc, San Diego, CA, USA). For all analyses, significance was set at $P \leq .05$.

To compare the relative abundances of ASV, independent t tests were performed using R Software (version 3.5.3). Multiple comparison testing of t test results was performed using Benjamin and Hochberg false discovery rate to provide adjusted p-values.(Benjamini and Hochberg 1995) Taxa enriched in the faecal samples of each foal group were determined using linear discriminant analysis effect size (LEfSe),(Segata, et al. 2011) based on $P < .05$ and a linear discriminate analysis (LDA) score > 4. For LEfSe analysis, LEfSe software (version 1.0) was used. Alpha diversity was assessed using the ASV annotated by QIIME2 to result in Observed features, Chao1, Shannon and Simpson indices and Kruskal-Wallis(Kruskal and Wallis 1953) and Tukey tests were used to compare between groups and time points. Beta diversity was calculated based on weighted and unweighted Unifrac distances.(Lozupone and Knight 2005; Lozupone, et al. 2011) Principal coordinate analysis (PCoA) was performed to visualise differences of samples in complex multi-dimensional data. To assess differences of community structure between groups, analysis of similarity test (ANOSIM) was performed by the ANOSIM function in QIIME2 software.(Maslen, et al. 2023)

3.4 Results

3.4.1 Clinical data

Twenty five foals were enrolled into the study (Veterinary Clinical Centre(13), South Eastern Equine Hospital (3) and Scone Equine Hospital (9)), including Thoroughbred (13), Standardbred (4), pony (2), Warmblood (2), Quarter horse (2), Australian stockhorse (1) and Arabian (1)

foals. There were 16 colts and 9 fillies. The median age of the foals was 45 days (range 3 - 122). The duration of diarrhoea prior to presentation ranged from 0-22 days. Treatment that foals had received before enrolment included intravenously and enterally administered fluids, anti-inflammatory medication, intestinal adsorbents, gastrointestinal protectants, nutritional support and antimicrobial drugs. Twelve and 8 foals were administered antimicrobial drugs and non-steroidal anti-inflammatory drugs, respectively, before enrolment into the study. Treatments that foals in the FMT and Control groups received during the study period included antimicrobial drugs (*n* = 24), butorphanol (*n* = 3), di-tri-octahedral smectite (Bio-Sponge®) (*n* =16), bio-absorbent clay (Bioclay®) (*n* = 1), sucralfate (*n* = 11), polyionic crystalloid fluids administered intravenously (*n* = 21), plasma (*n* = 9), polyionic crystalloid fluids administered enterally (*n* = 5), electrolyte paste administered enterically (*n* = 9), lactase (*n* = 10), hypertonic saline (*n* = 1), meloxicam (*n* = 4), flunixin meglumine (*n* = 11), phenylbutazone (*n* = 1), yoghurt (*n* = 1), misoprostol (*n* = 2), parental nutrition (*n* = 4), natural colloidal volcanic minerals (*n* = 2), lignocaine continuous rate infusion (*n* = 1) and paracetamol (*n* = 1). Antimicrobial drugs that were used include gentamicin (*n* = 13), metronidazole (*n* = 7), cefazolin (*n* = 7), benzyl penicillin (*n* = 5), ceftiofur (*n* = 5), procaine penicillin (*n* = 4), oxytetracycline (*n* = 3), sulfadiazine-trimethoprim combination (*n* = 2), ceftriaxone (*n* = 2), clarithromycin (*n* = 2), rifampin (*n* = 2), doxycycline (*n* = 2) and azithromycin (*n* = 1). Sixteen foals in the FMT group and 8 foals in the Control group received antimicrobial drugs during the study period. Duration of antimicrobial drug treatment ranged from 2-7 days and the number of antimicrobial drugs that a single animal received during hospitalization ranged from 0-4. There was no difference between the FMT and control groups for age (*P* = .5), sex (*P* = .9), duration of diarrh0ea (*P* = .1), antimicrobial administration (*P* = .9) or veterinary hospital (*P* = .8).

Sixteen foals were allocated to the FMT group and 9 foals were allocated to the Control group. Three foals initially enrolled into the Control group were subsequently enrolled into the FMT group due to persistence of diarrhoea. Persistence of diarrhoea was defined as presence of diarrhoea for 4 days or longer after initial enrolment into the Control group.

Co-morbidities were present in 11 foals (44%) at the time of presentation: pneumonia (*n* = 4), prior abdominal surgery for intestinal disease (*n* = 2), corneal ulceration (*n* = 1), neonatal isoerythrolysis (*n* = 1), dorsal displacement of the soft palate (*n* = 1), osteomyelitis (*n* = 1) and uroperitoneum (*n* = 1). Enteric pathogens were identified in 16/25 (77%) of foals: *Salmonella* spp. (*n* = 9); *rotavirus* A (*n* = 9); C*ryptosporidium* spp. (*n* = 7) and *C. difficile* (*n* = 1) (Figure 3. 1). Seven foals had multiple pathogens identified. No enteropathogens were detected in 9 foals.

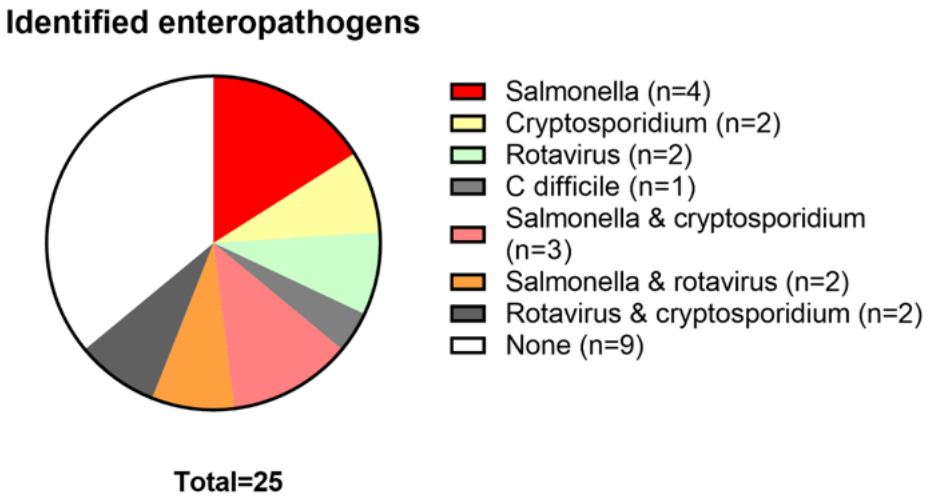

***Figure 3.1**: Pie chart displaying the identified enteropathogens from foals with diarrhoea.*

On D0, there were no differences in clinical, haematological or blood biochemical variables between groups, with the exception of mildly increased respiratory rate in the Control group.

There was a reduction in heart rate over time in the FMT group (*P* = .002), with differences between D0 and D2 (*P* = .005) and D0 and D3 (*P* < .001). By contrast, no reduction in heart rate was evident in the Control group (*P* = .07). Over time, there was no difference in respiratory rate in the FMT group (*P* = .46), while there was a reduction in respiratory rate in the control foals (*P* = .03), but differences between time points were not significant. There was no difference in rectal temperature between groups. Clinical and clinicopathological data are presented in Appendix 1.

On D3, the white blood cell count of the FMT group was lower than that of the Control group (*P* = .04) (Figure 3.2). The PCV of the FMT group was lower than that of the Control group on D3; however, this finding was not significantly different (*P* = .09) (Figure 3.2). In the FMT group, the PCV reduced over time and was lower on D3 compared to D0 (*P* = .04). A corresponding decrease in PCV was not observed in Control group foals (*P* = .4).

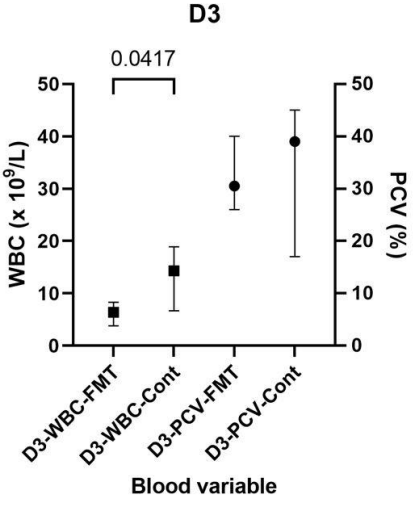

***Figure 3.2**: WBC and PCV of the FMT group and Control group on D3.*

The concentration of chloride in serum increased over time in the FMT group (*P* = .05), with a mean difference between D0 and D1 of 4.5 mmol/L (95% CI 0.5 – 8.5, *P* = .03). A change in serum chloride concentration was not observed in the Control group.

Resolution of diarrhoea within 7 days of initiation of treatment was more often observed in the FMT group (13/19, 68%) than in the Control group (4/9, 55%) (*P* = .4); however, differences were not significant (Figure 1.3). There was no difference in survival to discharge between the FMT (15/19, 79%) and Control groups (9/9, 100%) (*P* = .3).

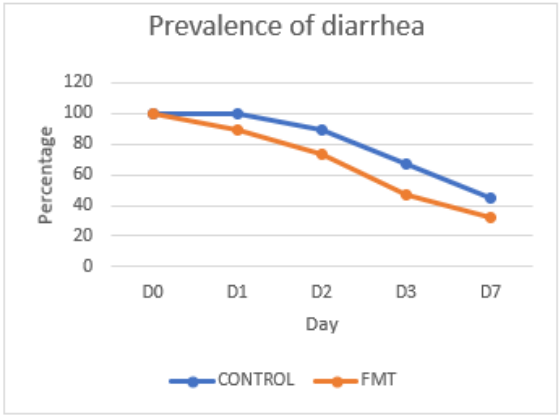

***Figure 3.3**: Bar graph displaying the prevalence of diarrhoea within the FMT group and the control group during the study period.*

3.4.2 Sequence analysis

Faeces from two of the three donor horses were obtained for microbiota analysis. The 106 samples from control foals (*n* = 34), FMT foals (*n* = 70) and donors (*n* = 2) yielded 3 992 044, 9 099 602 and 262 199 raw reads respectively, with a total yield of 13 353 765. Quality filtering and primer sequence removal resulted in 11 253 352 qualified reads that were used for analysis.

3.4.3 Relative abundance

The ASVs were clustered and allocated to levels of taxonomic classification including 31 phyla, 126 orders, 59 classes, 220 families and 443 genera. The 5 most abundant phyla contributed 95.9% of the identifiable ASVs in the foals and included Firmicutes (54.4%), Bacteroidota (22%), Proteobacteria (12.6%), Fusobacteroita (3.6%) and Actinobacteroita (3%). The 5 most abundant phyla in the donor horses contributed 95.8% of the identified ASVs and included Firmicutes (69.4%), Bacteroidota (18.8%), Spirochaetota (4.5%), Fibrobacter (2%) and Verrucomicrobiota (1.1%). On D0, the 10 most abundant genus in the foals included *Bacteroides* (12.7%), *Escherichia-Shigella* (7.4%), *Lactobacillus* (5.8%), *Ligilactobacillus* (3.8%), *Enterococcus* (3.2%), *Akkermansia* (3.2%), *Corynebacterium* (1.9%), *Klebsiella* (1.7%), *Clostridium_sensu_stricto_1* (1.3%) and *Actinobacillus* (1.1%) (Figure 3.4). In the donor horses at D0, the 10 most abundant genus were *Christensenellaceae_R-7_group* (10.3%), *NK4A214_group* (6.1%), *Treponema* (4.5%), *UCG-005* (3.5%), *Lachnospiraceae_XPB1014_group* (3.4%), *Rikenellaceae_RC9_gut_group* (3%), *Fibrobacter* (2%), *UCG-002* (2%), [*Eubacterium*]*_hallii_group* (1.8%) and *Blautia* (1.6%).

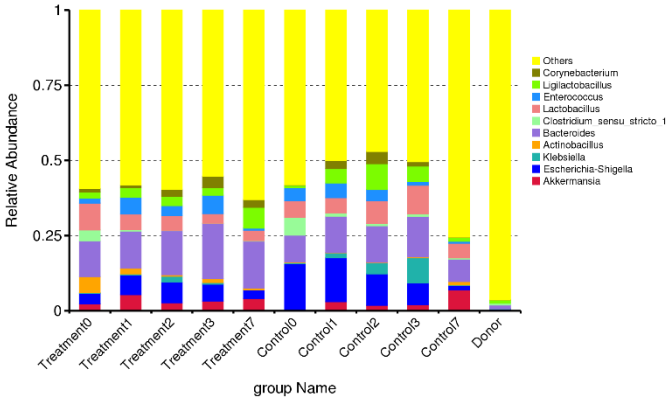

Figure 3.4*: The faecal microbiota relative abundance at genus level. The bar chart shows the relative abundance of the donor horses, and each foal group at each sampling time point.*

There were no differences in the relative abundance at the phylum or genus taxonomic level between FMT and control foals at each sampling time point, or within groups over time. There were differences in class Negativicutes (*P* = .05) and families *Enterobacteriaceae* (*P* = .05), *Veillonellaceae* (*P* = .05), *Burkholderiaceae* (*P* = .05) and *Streptococcaceae* (*P* = .05) between the FMT and Control group at D0. In the FMT group over time, there was an increase in order Rhizobiales (*P* = .05) at D0 compared to D3. Differences in relative abundances between and within groups are presented in Appendix 2.

LEfSe analysis revealed differences in enrichment of orders, families and genera at D0 between the Control and FMT group prior to treatment (Figure 3.5). On D1, a number of taxa were enriched in the FMT group compared to the Control group, including Phylum Verrucomicrobiota, genus *Akkermansia* and family *Prevotellaceae* (Figure 3.6).

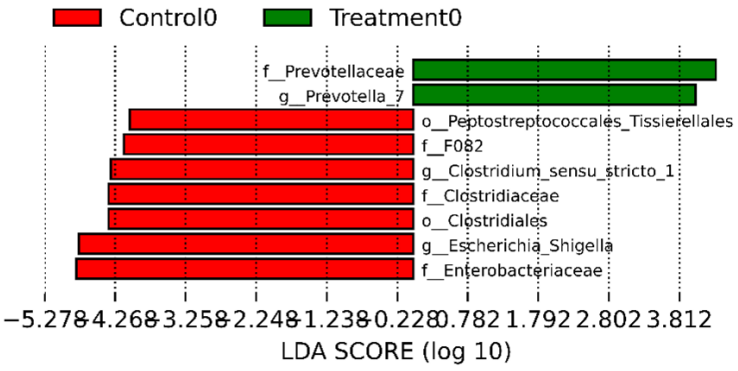

***Figure 3.5**: The enriched faecal microbiota displayed by LEfSe histogram, which shows a number of differences in taxa at D0*

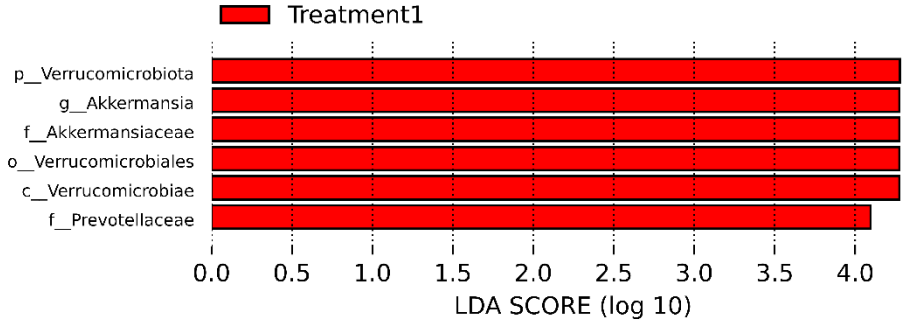

Figure 3.6: *The enriched faecal microbiota displayed by a LEfSe histogram, which shows enriched features in the FMT group at Day 1 compared to the Control group on Day 1*

3.4.4 Alpha diversity measures

There were no significant differences in alpha diversity between the FMT and Control group at D0, D1, D2, D3 or D7. There was increased richness in donor horses compared to foals (Figure 3.7).

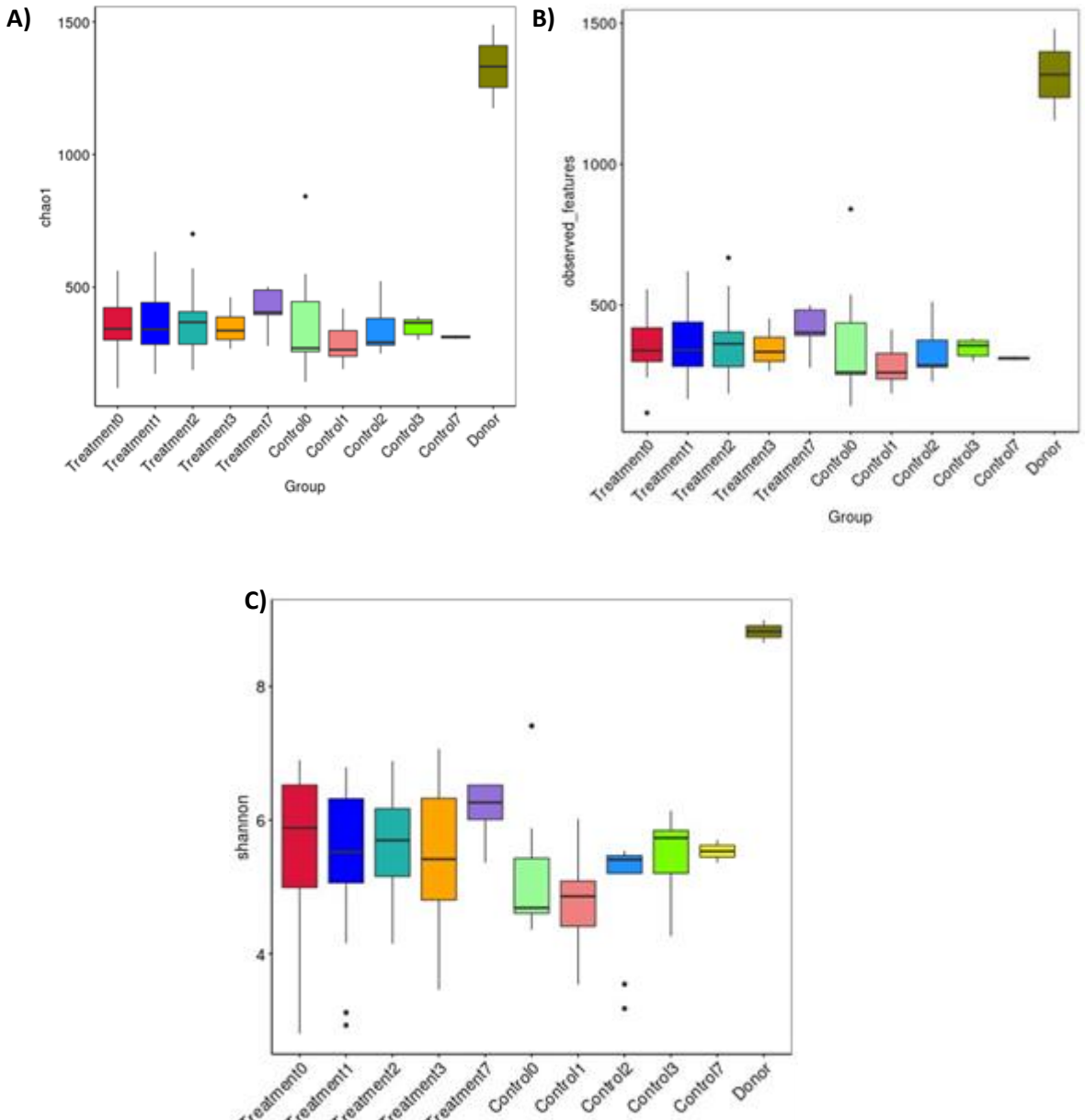

***Figure 3.7**: Choa 1 (A) and Shannon (B) box plot displaying differences in alpha diversity between groups. The donor horses had significantly increased richness and diversity compared to all foals (C).*

3.4.5 Beta diversity measures

Principal coordinate analysis analyses of unweighted and weighted UniFrac distances revealed considerable overlap between FMT and Control groups over all time points (Figure 3.8) indicating similarities in bacterial community membership and structure (ANOSIM, *P* = .1-1).

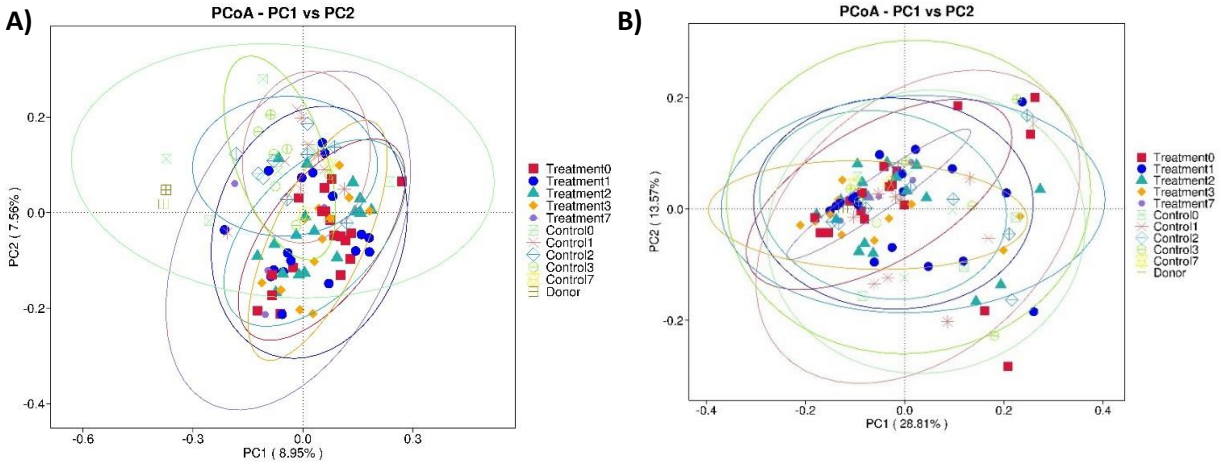

***Figure 3.8:** Weighted (A) and unweighted (B) Principal Coordinate Analysis of the faecal microbiota of foals with diarrhoea and adult donor horses. Each shape represents each group at each time point during the study period*

3.5 Discussion

In this study, the effect of FMT in foals with diarrhoea was assessed. Administration of FMT was associated with improvement of some clinical and clinicopathological variables. There were no differences in the relative abundance of phylum or genus between the FMT and Control group; however, there was enrichment of phylum Verrucomicrobiota and genus

Akkermansia in the FMT group at D1. There were no differences in alpha diversity within or between groups over time. Although the resolution of diarrhoea and survival to discharge were not significantly different between groups, a beneficial effect of FMT treatment cannot be discounted. The concurrent administration of other medications, most importantly antimicrobials, could have precipitated persistent dysbiosis and had an effect on outcome variables.

Foals receiving FMT evidenced reduced heart rate, WBC and PCV, and increased serum chloride concentrations, relative to pre-treatment values or corresponding results from control foals. Reductions in PCV and heart rate could be associated with improved hydration and improved haemodynamic status in treated animals. Increased chloride concentrations at D1 compared to D0 could reflect decreased mucosal secretion and improved absorptive capacity associated with resolving intestinal damage. This could have resulted from improvement of the integrity and function of the intestinal mucosa by re-establishment of the intestinal microbiota. The decreased WBC count in the FMT group, compared to control foals at D3, suggest reduced inflammation in treated foals. The gastrointestinal microbiota has an important role in metabolic and immune function in the host,(Costa and Weese 2018; Stewart, et al. 2018) and it is possible that the administration of FMT assisted in restoration of local immune responses in those foals.

In this study, there was no difference in resolution of diarrhoea or survival between the two groups. There was no evidence that FMT exacerbated diarrhoea or had a negative effect on outcome. Of the three foals initially enrolled into the Control group and then re-enrolled into the FMT group, two had resolution of diarrhoea after FMT. This could be due to continued supportive care and time; however, this finding could reflect a beneficial effect of FMT on the

intestinal microbiota and intestinal function. Clinical indicators of response to FMT in adult horses include improved faecal consistency(Costa, et al. 2021; McKinney, et al. 2021; McKinney, et al. 2020b) and resolution of diarrhoea;(Dias, et al. 2018) however, studies of adult horses with diarrhoea have demonstrated conflicting responses to FMT. Normalisation of faecal microbiota has been associated with FMT in three of five geriatric horses with diarrhoea.[20] In a study of four adult horses that developed diarrhoea post-laparotomy, FMT was associated with resolution of pyrexia and diarrhoea within 24 hours of the first treatment.(Dias, et al. 2018) However, that study did not report on the faecal microbiota of recipients[40] and neither study included a control group. Other studies assessing the response of FMT treatment in adult horses report no improvement in diarrhoea,(Costa, et al. 2021; Kinoshita, et al. 2022) resolution of free faecal water syndrome,(Laustsen, et al. 2021b) or differences in clinical and clinicopathological findings in comparison control horses.(McKinney, et al. 2021)

In the current study, a major effect of FMT on relative abundance of taxa in the faecal microbiota was not observed. The microbiota of horses is affected by environmental factors including season, feed types, weather conditions(Salem, et al. 2018) and geographic location. (McKinney, et al. 2021) Importantly, the faecal microbiota of foals changes dramatically within the first 6 months of life.(De La Torre, et al. 2019) The microbiota of foals is different to adult horses and gradually becomes similar to that of mares at the time of weaning.(Costa, et al. 2016a; De La Torre, et al. 2019) Phyla including Firmicutes, Bacteroidetes and Verrucomicrobia are more enriched at 2 months of age compared to 1 day of age, reflective of a higher forage based diet in older foals.(Salem, et al. 2018) In the current study, it was not possible to control for age, and this might have contributed to differences in taxa observed at D0 in FMT and control foals, although there was no significant difference in foal age between groups.

In the current study, FMT was associated with enrichment of phylum Verrucomicrobiota, genus *Akkermansia* and family *Prevotellaceae*. Genus *Akkermansia* is associated with maintenance of the integrity of the mucin layer of the intestinal tract and reportedly decreases bowel inflammation in people.(Everard, et al. 2013) In addition, members of the *Prevotellaceae* family have anti-inflammatory and modulating effects on the intestines, and have a role in maintaining intestinal health.(Bedarf, et al. 2017) The enrichment of this family supports the possible establishment of a more normalized gastrointestinal microbiota and assistance in immune modulation. The gastrointestinal microbiota has an important role in metabolic and immune function in the host,(Costa and Weese 2018; Stewart, et al. 2018) and it is possible that the administration of FMT assisted in restoration of local immune responses in those foals. The significant differences of enrichment and relative abundance detected at D0 likely reflect individual variation of the microbiota and introduces difficulty in interpretation of the changes observed over time.

In our study, alpha diversity of foals was less than donors. This finding is consistent with the findings of previous studies where alpha diversity of the adult horse faecal microbiota was greater than foals, and increasing alpha diversity in foals was correlated with age.(De La Torre, et al. 2019; Liu, et al. 2021) Foals with diarrhoea have a lower richness compared to healthy age matched peers, represented by lower Chao index (Schoster, et al. 2017) and decreased alpha diversity has been associated with gastrointestinal disease in adult horses.(Stewart, et al. 2021) Studies assessing FMT in adult horses with diarrhoea have reported increased alpha diversity(McKinney, et al. 2021; McKinney, et al. 2020b) and normalization of microbiota scores.(McKinney, et al. 2021) In contrast, other studies reported no change in alpha diversity after administration of FMT to horses with diarrhoea of free faecal water syndrome.(Costa, et

al. 2021),(Laustsen, et al. 2021b) In the current study, FMT administration was not associated with changes in alpha diversity.

In our study, ANOSIM analysis revealed no differences in beta diversity. The lack of difference could be due to the small group size, the absence of change in the distal intestinal tract, or limitations in using faecal microbiota to characterise the microbial population within the gastrointestinal tract. In one study, administration of FMT to adult horses with diarrhoea was associated with changes in beta diversity,(McKinney, et al. 2021) while in other studies, there were no changes in beta diversity associated with treatment with FMT.(Costa, et al. 2021; Laustsen, et al. 2021b)

Foals in this study were administered isotonic polyionic fluids and plasma intravenously, antimicrobial drugs, anti-inflammatory drugs, intestinal adsorbents and nutritional care prior to study enrolment. The administration of FMT could have facilitated improved haemodynamic status by promoting the re-establishment of the intestinal microbiota and restoration of the integrity and function of the intestinal mucosa. However, the likely negative effect of concurrent antimicrobial drug administration on the efficacy of FMT cannot be ignored. Administration of antimicrobial drugs causes changes to the faecal microbiota(Costa, et al. 2015b; Liepman, et al. 2022; Theelen, et al. 2023) and can lead to dysbiosis and diarrhoea.(Barr, et al. 2013; Kinoshita, et al. 2022) Antimicrobial drugs commonly administered to horses, including metronidazole,(Arnold, et al. 2020; Arnold, et al. 2021; Kinoshita, et al. 2022)penicillin,(Arnold, et al. 2021; Theelen, et al. 2023) gentamicin,(Arnold, et al. 2021) ceftiofur,(Arnold, et al. 2021; Costa, et al. 2015b) trimethoprim-sulfadiazine combination,(Costa, et al. 2015b) doxycycline(Chapuis, et al. 2023) and enrofloxacin(Liepman, et al. 2022) affect the faecal microbiota in adult horses. Significant changes in taxa including phyla Spirochaetes, Lentisphaerae, Fibrobacteres and Verrucomicrobia and family

Lachnospiraceae, and significant reductions in alpha diversity(Costa, et al. 2015b; Gomez, et al. 2023; Theelen, et al. 2023) and changes in beta diversity(Kinoshita, et al. 2022; Theelen, et al. 2023) are associated with antimicrobial administration.(Arnold, et al. 2020; Chapuis, et al. 2022; Gomez, et al. 2023; Theelen, et al. 2023) In the current study, the limited changes to the faecal microbiota and similar clinical results in the FMT and control groups could have been influenced by concurrent administration of antimicrobial drugs. In foals with diarrhoea, it is possible that FMT did not address the underlying antimicrobial-associated dysbiosis,[26] or the continued administration of antimicrobials adversely impacted on FMT efficacy.

The current study was a multi-centre, prospective randomised clinical trial. The number of animals enrolled is similar to or greater than reports in adult horses;(Costa, et al. 2021; Kinoshita, et al. 2022; McKinney, et al. 2021; McKinney, et al. 2020a) albeit modest. As expected in prospective clinical studies, it was not possible to standardise concurrent veterinary treatments required for animal care, and these treatments could have influenced outcomes in these foals. Some clinical and microbiota data were not available, influenced by individual animal needs and owner decisions, resolution of diarrhoea and discharge from hospital. Inclusion of animals in three veterinary hospitals in differing locations could have confounded the results, as it has been demonstrated that geography also influences the gastrointestinal microbiota.(Arnold, et al. 2021) Donor horses were used over a 3 year period and changes in the microbiota of these animals may have occurred during this time.

3.6 Conclusion

In conclusion, this study demonstrated that FMT in foals is safe and did not exacerbate the occurrence of diarrhoea or clinical disease in treated foals. Although survival and resolution of diarrhoea were similar in both groups, there was evidence to suggest that foals treated with FMT had improvement in some clinical and clinicopathological findings, and a beneficial effect

of FMT on the gastrointestinal microbiota cannot be discounted. Further studies assessing the outcomes associated with FMT in foals and preparation protocol would further refine treatment recommendations in the future.

3.7 Off-label antimicrobial use

Off-label antimicrobials were administered to foals in this study including cefazolin, gentamicin and ceftiofur.

3.8 Funding

This research was funded by Agrifutures Australia.

3.9 Conflicts of interest

K J Hughes serves as an Associate Editor for the Journal of Veterinary Internal Medicine. He was not involved in the review of the manuscript.

3.10 Acknowledgements

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3.11 Supplementary information

A copy of the clinical and clinicopathological results is included in Appendix 1.

A copy of the results of differences of relative abundance between and within groups is included in Appendix 2.

Chapter 4: Storage of equine faecal microbiota transplantation solution has minimal impact on major bacterial communities and structure.

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Storage of equine faecal microbiota transplantation solution has minimal impact on major bacterial communities and structure.

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4.1 Abstract

Management of diarrhoea in horses is usually non-specific and supportive. Faecal microbiota transplantations (FMT) are used to manage dysbiosis in horses with diarrhoea. There are few studies investigating the effects of storage on prepared FMT solutions. This study was an in vitro non-randomised controlled experiment that investigated the effects of FMT solution preparation and storage on the faecal microbiota. Fresh faeces were collected from five healthy adult horses and used for DNA extraction and preparation of FMT. From each FMT, seven aliquots were collected and DNA was extracted immediately after FMT preparation (0hr), after storage at 4oC for 24, 48 or 72 hours, and after storage at -20°C for 7 days, 14 days or 28 days. The extracted DNA was used for 16S rRNA gene sequencing.

The relative abundance, alpha diversity and beta diversity between fresh faeces and FMT 0hr showed no differences ($P \geq 0.05$). There were minimal changes in the microbiota of FMT stored at 4°C for up to 72 hours and -20°C for up to 28 days. The results of this study indicate that preparation of equine FMT solution has minimal effect on the microbiota in comparison to fresh faeces. FMT solution can be stored at 4°C for up to 3 days and -20°C for 28 days without major change in microbiota.

Keywords: horse; freezing; dysbiosis; refrigeration; diarrhoea.

4.2 Introduction

Faecal microbiota transplantation (FMT) is used to treat various gastrointestinal diseases in several species. In humans, FMT is associated with resolution of Clostidioides difficile infection (Brandt, et al. 2012; Shahinas, et al. 2012) and ulcerative colitis (Kunde, et al. 2013). In dogs, FMT reduces the duration of parvoviral-associated diarrhoea (Pereira, et al. 2018). In horses, FMT has been used to treat diarrhoea for several decades, although, to date, few studies have critically investigated the efficacy of FMT in this species. Some studies report that FMT in adult horses is associated with increased alpha-diversity, normalization of microbiota score and reduction in diarrhoea severity (McKinney, et al. 2021; McKinney, et al. 2020a), while others reported limited effect on the faecal microbiota in horses with diarrhoea (Costa, et al. 2021).

Preparation and storage of FMT solution in humans has been well characterised (Burz, et al. 2019). In contrast, there is no standardised FMT preparation method for horses and proposed guidelines are based on clinician experience rather than scientific literature (Mullen, et al. 2018). Reported methods used faeces that were collected per rectum or immediately after defecation, with considerable variation in the amount of faeces used (0.1-0.5kg per 1 litre of warm water) (Costa, et al. 2021; Dias, et al. 2018; Kinoshita, et al. 2022; Laustsen, et al. 2021; McKinney, et al. 2020b). The faeces and water are blended and the resulting mixture is then strained to result in the final FMT filtrate (Loublier, et al. 2023; Mullen, et al. 2018).

In humans, the clinical response of patients with recurrent C. difficile infection is reportedly non-inferior when FMT is prepared from frozen faeces compared to fresh faeces (Lee, et al. 2016). In addition, long term storage of human faeces had limited effect on microbial diversity and composition (Tap, et al. 2019). In horses, the bacterial community of faeces at ambient temperature is reported to change within 6 hours (Beckers, et al. 2017) and 12 hours (de

Bustamante, et al. 2021) after defecation. Further, there is minimal information on the effect of storage on the microbiota of equine FMT, on subsequent outcomes of recipients (Kinoshita, et al. 2022) or comparisons between fresh and frozen FMT and the effects on the microbiota. In one study, the process of preparation of FMT from horse faeces had no impact on bacterial viability or composition (Loublier, et al. 2023). In another study, the viability of Gram-negative bacteria decreased over time with storage of FMT at -20°C (Kopper, et al. 2021). The objective of the current study was to compare the microbiota of fresh faeces, freshly prepared FMT solution and FMT solution stored at 4°C or -20°C. It was hypothesised that FMT storage at 4°C would result in changes in the microbiota composition and storage in conditions of -20°C would result in minimal changes in the microbiota composition.

4.3 Materials and methods

4.3.1 Ethical Statement

This study was approved by the Animal Ethics Committee of Charles Sturt University (Protocol number A23461, approval date 21th January 2023).

4.3.2 Animals

Five mares of various breeds and ages from the Charles Sturt University teaching herd were used for manure collection. The horses were housed on pasture. Within the previous 6 weeks, no horse had any illness, dietary or management change or had been administered anthelmintic, non-steroidal anti-inflammatory or antimicrobial medications.

4.3.3 Manure collection

Approximately 300 grams of faeces was collected from each horse immediately after defecation or per rectum. Approximately 3 grams of fresh faeces was collected from the centre of each pile for DNA extraction.

4.3.4 Faecal microbiota transplantation solution preparation

All equipment used for FMT solution preparation was cleaned and disinfected before use and between animals. Disposable gloves were worn during FMT preparation. Faeces (150 g) was weighed by an electronic scale (NUWEIGH, Digital Bench Scale) and added to a bucket containing 500mL of warm tap water (approximately 35°C). The combination of faeces and water was blended using an immersion blender for approximately 30-60 seconds. The preparation was sieved to result in the final FMT filtrate. Each preparation was divided into seven 3 mL aliquots. The FMT solutions were prepared immediately after collection of faeces and preparation time took approximately 5 minutes.

4.3.5 Storage conditions

Fresh faeces and FMT0hr were stored at 4°C, and DNA was extracted within 6 hours of collection and preparation. FMT solution aliquots were stored at 4°C or -20°C and analysed at 24 hours (FMT24hr), 48 hours (FMT48hr) and 72 hours (FMT72hr) and 7 days (FMT7d), 14 days (FMT14d) and 28 days (FMT28d) after preparation, respectively. One sample per horse, per time point was obtained (5 horses per time point/group).

4.3.6 DNA Extraction

A volume of 100 µL was used for FMT solution and ≤150 mg was used for extractions from faeces. Genomic DNA was extracted with Quick-DNA™ Fecal/Soil Microbe Miniprep Kit (Zymo Research, CA) following the manufacturer's instructions. NanoDrop 2000 Spectrophotometer (Thermo Scientific, Australia) was used to assess concentration and purity.

4.3.7 16s rRNA Next Generation Sequencing, sequencing data and statistical analysis

The extracted DNA underwent 16s rRNA sequencing. All samples underwent quality control prior to sequencing and passed. Polymerase chain reaction amplification was performed on the V3-V4 regions with primer sequence CCTAYGGGRBGCASCAG-GGACTACNNGGGTATCTAAT. Paired-end Illumina platform (NovaSeq 6000 PE250) was used for library sequencing to

generate 250bp paired-end raw reads and then merged (Mago and Salzberg 2011) and pre-treated to obtain clean tags. The reagents used in the Illumina platform were V1.5 kits. The chimeric sequences of the clean tags were detected and removed resulting in the effective tags (Edgar, et al. 2011).

To improve accuracy of the bioinformatic analysis, raw data were spliced and filtered to acquire clean data. DADA2 was used to reduce noise from the clean data (Callahan, et al. 2016; Li, et al. 2020), to obtain the final amplicon sequence variants (ASVs). Quantitative Insights Into Microbial Ecology (QIIME2) (Bokulich, et al. 2018; Rideout, et al. 2019) algorithm was used for species annotation of each ASV (annotation database Silva 138.1). Relative abundance of phylum, class, order, family and genus was undertaken using QIIME2. Differences in relative abundance was evaluated by T-test using R Software (Version 3.5.3), with significance set at $P \leq 0.05$. Multiple comparison testing of t test results was performed using the storey method to provide adjusted p values (Storey 2002). Linear discriminant analysis (LDA) in combination with linear discriminant analysis effect size (LEfSe) was used to detect enriched taxa (at different taxonomic levels) between groups and the results were composed of histograms containing LDA scores. Chao1, Dominance, Goods Coverage, Pielou, Shannon and Simpson indices were used to assess alpha diversity. Tukey and Kruskal-Wallis tests were performed to assess for differences of alpha diversity between groups. Rarefaction curves were created to assess sequencing depth. Beta diversity was calculated based on weighted and unweighted Unifrac distances. Beta diversity intergroup difference analysis was undertaken using principal coordinate analysis (PCoA). PCoA undertaken using unweighted and weighted UniFrac distances was used to graphically represent multidimensional data. Analysis of similarities (ANOSIM) was used to detected significant variation between groups.

4.4 Results

Forty aliquots were analysed and yielded 4,269,650 raw reads. Filtering and primer sequence removal resulted in 4,134,661 qualified reads that were analysed. The ASVs were clustered and allocated to the respective level of classification, including 25 phyla and 210 genera.

The predominant 10 phyla of all samples contributed to 99.1% of ASVs: Firmicutes (43.1%), Bacteroidota (24.6%), Fibrobacterota (9.8%), Halobacterota (6.3%), Spirochaetota (6.1%), Verrucomicrobiota (5.3%), Euryarchaeota (2.2%), Actinobacteriota (0.8%), Proteobacteria (0.3%) and Thermoplasmatota (0.1%). The top ten genus contributed to 38.2% of ASVs and included Fibrobacter (9.8%), Methanocorpusculum (6.3%), Treponema (5.5%), Christensenellaceae_R-7_group (3.7%), NK4A214_group (3.2%), Methanobrevibacter (2.3%), Akkermansia (2.2%), Prevotellaceae_UCG-001 (2.1%), Lachnospiraceae_XPB1014_group (1.6%) and Alloprevotella (1.2%) (Figure 4.1).

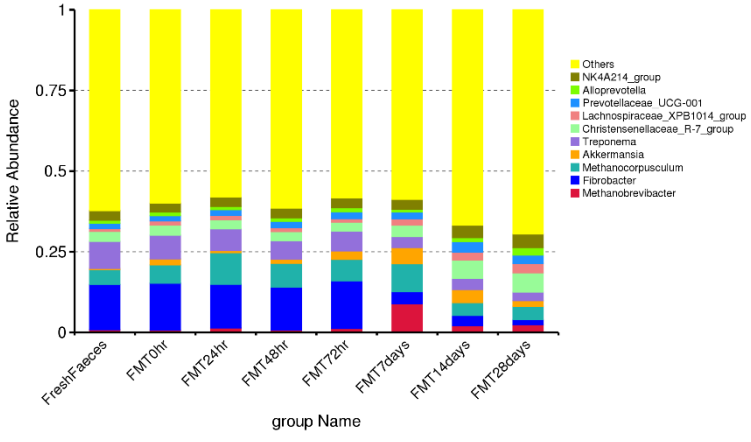

***Figure 4.1**: The relative abundance of the predominant 10 genus of fresh faeces or FMT solution for each storage time and temperature*

4.4.1 Impact of FMT solution preparation on the faecal microbiota

There were no enriched taxa and no differences in relative abundances at phyla, class, order family, or genus taxonomic levels between fresh faeces and FMT0hr. There was no difference in alpha or beta diversity indices.

4.4.2 Impact of short term storage of FMT solution at 4°C

There was no difference in relative abundances between FMT0hr, FMT24hr, FMT48hr and FMT72 hr at the level of phylum, class, order, family or genus. There were no enriched taxa between FMT0hr and FMT24hr, FMT48hr or FMT72hr. Comparison of FMT24hr, FMT48hr and FMT72 hr showed enrichment of order WCHB1_41 and class Kiritimatiellae at FMT48hr. There were no differences in alpha diversity indices between FMT0hr, FMT24hr, FMT48hr and FMT72hr (Figure 4.2A & 2B). Using PCoA, there was no evidence of clustering associated with storage at 4°C. There were no differences in community structure between FMT0hr, FMT24hr, FMT48hr and FMT72hr (ANOSIM, P = 0.82-0.95).

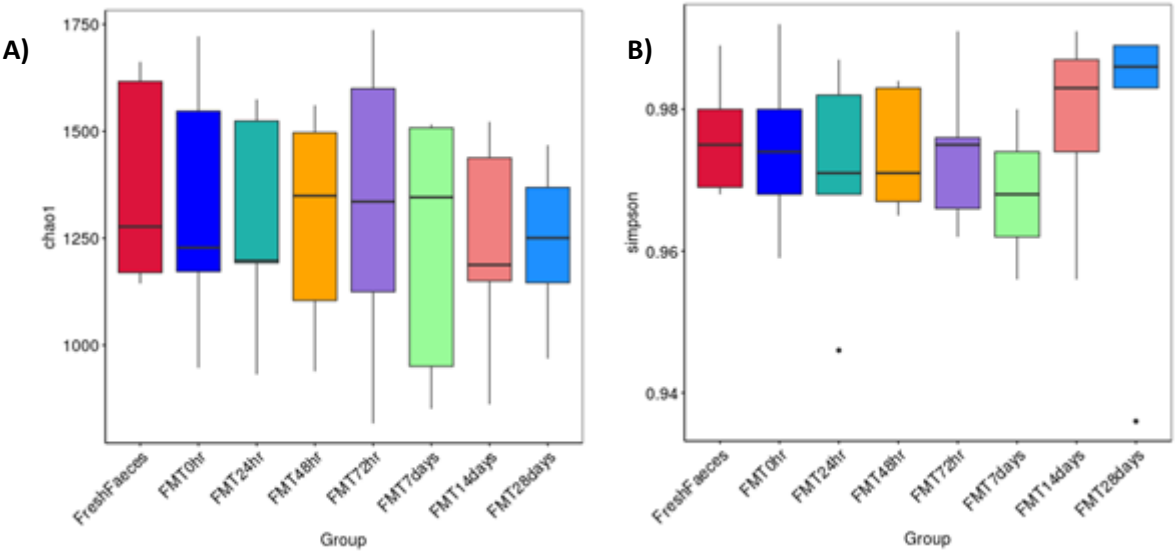

Figure 4.2: *boxplot displaying the Chao1 (A) and Simpson (B) indices in fresh faeces and FMT solution for each storage time and temperature*

4.4.3 Impact of storage of FMT at -20°C

There was no difference in the relative abundance of taxa between FMT0hr compared to FMT7d, FMT14d and FMT28d. Storage of FMT solution at -20°C was associated with enrichment of family Erysipelatoclostridaceae and genus UCG_004 at FMT0hr compared to FMT7d, FMT14d and FMT28d. Comparison of FMT7days, FMT14 days and FMT28 days showed no enriched taxa. There were no differences in alpha diversity indices between FMT0hr, FMT7d, FMT14d and FMT28d. Assessment of beta diversity by PCoA showed no evidence of clustering associated with storage at -20°C(Figure 4.3). There was no differences in community structure between FMT0hr, FMT7d, FMT14d and FMT28d (ANOSIM, P = 0.35-0.63).

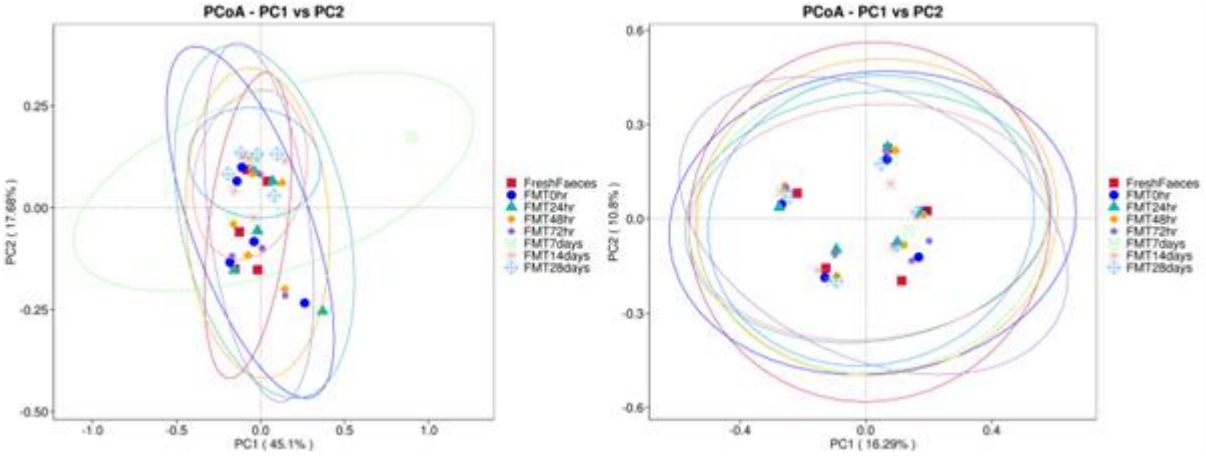

***Figure 4.3**: Principal coordinate analysis of the microbiota of fresh faeces, FMT0hr, FMT24hr, FMT48hr, FMT72hr, FMT7days, FMT14days and FMT28days. The unweighed Unifrac distances by storage condition displays the beta diversity of each group. Plots that appear closer in position are more similar. The axes indicate percent variation.*

4.4.4 Beta diversity of individual animals

There was clustering of samples of individual animals within the weighted PCoA plot, however no clustering of samples at any particular time point (Figure 3).

4.5 Discussion

The findings of our study demonstrated that the preparation methodology of FMT solution in this study did not affect the microbiota community or structure and short term storage of FMT at 4oC up to 72 hours and -20°C up to 28 days resulted in minimal changes to the microbiota.

In our study, there were no differences in relative abundance or enrichment of taxa between fresh faeces and FMT0hr. Similarly, there were limited differences in bacterial populations between faeces and FMT filtrate in a recent study of FMT preparation (Loublier, et al. 2023). These findings indicate that FMT solution likely reflects the microbiota of fresh faeces, supporting the use of FMT solution in horses.

Storage of FMT solution at 4°C for up to 72 hours resulted in minimal differences in the relative abundance of taxa (phylum, class, order and genus). The enrichment of order WCHB1-41 and class Kiritimatiellae at FMT48hr indicates an increase in taxa belonging to phylum Kiritimatiellaeota. Taxa of phylum Kiritimatiellaeota, in particular order WCHB1-41, have been shown to be increased in healthy donor horses compared to horses with colitis and diarrhoea (McKinney, et al. 2021; McKinney, et al. 2020b) and an increased relative abundance of order WCHB1-41 is strongly associated with an increased alpha diversity (McKinney, et al. 2021). As such, the changes associated with short-term refrigeration of FMT solution are unlikely to have a negative effect of clinical efficacy of equine FMT and could assist in restoration of the microbiota. Similar to the findings of the current study, storage of canine and feline faeces at 4°C for 14 days had minimal impact on the microbiota, including no differences in richness, diversity, evenness, membership or structure and few differences of relative abundance (Weese and Jalali 2014). In some studies, storage of human faeces at 4°C for up to 14 days had no effect of structure and diversity of communities (Carroll, et al. 2012; Lauber, et al. 2010a;

Tedjo, et al. 2015) and short term storage of faeces at 4°C was safe for transplantation (Burz, et al. 2019).

Storage of FMT at -20°C resulted in no difference in the relative abundance of taxa by t test and minimal differences with LEfSe analysis. The enrichment of family Erysipelatoclostridaceae, and genus UCG_004, at FMT0hr compared to FMT7d, FMT14d and FMT28d suggests that the relative abundance of these taxa decrease with freezing and storage. Family Erysipelatoclostridaceae and genus UCG_004 have been associated with increased concentrations of total branched-chain fatty acids in equine faeces (Weinert-Nelson, et al. 2023); however, the roles of these taxa within the equine microbiota are not defined. These taxa belong to phylum Firmicutes, which is one of the predominating phyla of the equine hindgut (Kauter, et al. 2019). Storage of human faeces at -20°C for 14 days has no major effect on the microbiota (Carroll, et al. 2012; Lauber, et al. 2010b; Tedjo, et al. 2015). The lack of change in the relative abundance of taxa with storage of -20°C in the current study suggests that FMT could be stored in these conditions for up to 28 days prior to use.

The lack of differences in alpha diversity indices between fresh faeces and FMT0hr is consistent with those of a previous study (Loublier, et al. 2023), where there were no differences in diversity, richness or evenness with FMT preparation. Conversely, another recent study demonstrated marked changes of the microbiota of donor faeces with FMT preparation (Di Pietro, et al. 2023). However in the latter study, the method of mixing of faeces and water was not detailed and FMT was also concentrated after filtration (Di Pietro, et al. 2023). In our study, an immersion blender was used to prepare the FMT solution in an effort to facilitate the availability of bacteria closely attached to fibrous material. However, this method increases the oxygen exposure of the FMT and might be detrimental to anaerobic bacteria, including members of the Firmicutes phylum (Chu, et al. 2017; Loublier, et al. 2023).

In humans, preparation of FMT in aerobic conditions has been associated with degradation of bacterial communities (Chu, et al. 2017). However, preparation of FMT solution in our study had no effect on community structure or alpha and beta diversity and differences between studies could be due to the volume of faeces used and mixing method (Loublier, et al. 2023). The lack of differences of alpha diversity between FMT0hr and FMT solution suggests that storage at 4°C or -20°C has minimal effect on the community distribution.

There was no difference in the beta diversity of the microbiota between faeces and FMT0hr, which is consistent with a previous study (Loublier, et al. 2023) where no differences in beta diversity between FMT solution preparation and fresh faeces were observed. In our study, there was clustering between the 5 animals used, and greater difference in beta diversity between individual animals than between FMT preparations and storage conditions.

The current study did not explore the impact of storage on bacterial viability. A recent study of equine FMT preparation that used a similar method to the current study, demonstrated no differences in bacterial viability between faeces and fresh FMT solution (Loublier, et al. 2023). Storage of equine FMT solution at -20°C has been associated with no viable Gram negative organisms and a 98-fold reduction of total enteric bacteria after introduction to simulation of the proximal gastrointestinal tract (Kopper, et al. 2021). In humans, frozen FMT solution is reported to have fewer viable bacteria than fresh faeces, although this does not impact clinical results with repeated administration (Costello, et al. 2015; Gangwani, et al. 2023). However, freezing of human faeces without cryoprotectants substantially alters the microbiota (Bilinski, et al. 2022). The viability of bacteria of equine FMT solution with storage 4°C and -20°C and the impact of administration on the microbiota of recipient horses requires further investigation.

The results of our study indicate that FMT solution storage at 4°C for up to 72 hours and -20°C for up to 28 days is not associated with substantial changes in microbial community and structure in comparison to fresh faeces or freshly prepared solution. The faecal microbiota of horses with colitis and diarrhoea has marked differences in community structure compared to healthy adults (Costa, et al. 2012; McKinney, et al. 2020a) and to stored FMT solution in the current study (Costa, et al. 2012), where horses with colitis have a reduced relative abundance of phylum Firmicutes and an increased relative abundance of Bacteroidetes and Proteobacteria (Costa, et al. 2012).

Limitations of this study were the use of a small number of horses and that the effects of storage on bacterial viability, the effects of cryoprotectants of FMT solution and the effects of administration of FMT solution on the faecal microbiota of recipient horses were not assessed. Further investigations of the effects of storage of FMT solution on bacterial viability and efficacy in clinical cases are required.

4.6 Conclusions

Storage of FMT at 4° for up to 72 hours and -20°C for up to 28 days has minimal effect on the microbiota community, structure or diversity indices. The modest change in microbiota of FMT solution stored in these conditions suggest that preparations can be stored for up to 72 hours at 4°C and up to 28 days at -20°C prior to use in horses.

4.7 Conflicts of interest

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

4.8 Funding

This study was funded by the Faculty of Science and Health, Charles Sturt University.

4.9 Acknowledgements

The authors thank that staff of the CSU Equine Centre for their assistance.

4.10 Supplementary Items

Results of differences in relative abundance between and within groups of FMT solution in different storage conditions are located in Appendix 3.

Chapter 5: The effect of anthelmintic treatment and efficacy on the faecal microbiota of healthy adult horses.

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The effect of anthelmintic treatment and efficacy on the faecal microbiota of healthy adult horses.

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5.1 Abstract

Changes to the faecal microbiota of horses associated with administration of anthelmintic drugs is poorly defined. This study included horses with cyathostomin infection where susceptibility and resistance to different anthelmintics was known. This study assessed the changes to the faecal microbiota associated with administration of two different anthelmintics in this population. Twenty-four adult horses were included. Faecal egg counts were performed on all horses prior to random allocation into abamectin (n=8), oxfendazole (n=8) or control groups (n=8) and at Day 14 post treatment. Faecal samples were collected for microbiota analysis prior to anthelmintic administration and on Day 3 and Day 14. From each faecal sample, DNA was extracted prior to PCR amplification, next generation sequencing and analysis using QIIME2. Anthelmintic treatment was associated with changes in alpha diversity (p <0.05), with increased evenness and diversity at Day 14 and increased richness at Day 3 within the abamectin group. Differences in relative abundance of bacteria at the phyla, family and genus taxonomic levels occurred after treatment; indicating that the microbiota was altered with anthelmintic administration.

The results support that anthelmintic administration and removal of cyathostomins from the large intestine of horses is associated with changes in the faecal microbiota. The results suggest that removal of cyathostomins is associated with greater differences in microbiota, compared to anthelmintic drug administration that is ineffective in reducing cyathostomin infection. Cyathostomin removal was supported by adequate reduction of faecal egg counts, determined by faecal egg count reduction testing.

Keywords: equine; microbiota; faecal; anthelmintic; cyathostomin

5.2 Introduction

Infections with cyathostomins are ubiquitous in grazing horses, due to continued exposure to infectious stages on pasture and often incomplete immunity (Collobert-Laugier et al., 2002; Morariu et al., 2016). Cyathostomins are an important intestinal nematode of horses and infection can be associated with colic (Reinemeyer and Nielsen, 2009), diarrhoea, peripheral oedema and weight loss (Lawson et al., 2023). Acute larval cyathostominosis (ALC) is an important disease in horses and can be characterised by diarrhoea, weight loss and death (Bodecek et al., 2010) and is associated with the sudden emergence of encysted larvae from the mucosa of the large intestine (Reid et al., 1995). Further, recent anthelmintic administration has been associated with the development of ALC (Reid et al., 1995), suggesting disruption to host-parasite relationships, including immune responses. Strategies for the control of cyathostomins and other helminths in horses often involves the administration of anthelmintics. However, frequent use has predisposed to emergence of anthelmintic resistance, including in cyathostomins (Abbas et al., 2021; Kuzmina and Kharchenko, 2008; Traversa et al., 2009).

There are complex and poorly understood relationships between the host, gastrointestinal parasites and the microbiota. In mice, infiltration of IL-4 expressing memory CD4+ T cells and macrophages have been observed with helminth infection (Anthony et al., 2006). In humans, an increase in Th2 type cytokines including IL-5, IL-4 and IL-13 are observed in infected individuals compared to non-infected individuals (Loke et al., 2022). The role of cyathostomins in the homeostasis of the gastrointestinal tract in horses is poorly understood (Walshe et al., 2019), however, cyathostomins likely have a role in immunoregulation (McKay, 2009) and this relationship modulates responses to infectious and immunologic stimuli (Davidson et al., 2005; Nielsen et al., 2015b; Pickles et al., 2010). It has been demonstrated that cyathostomin larvae burden is associated with increased concentrations of IL-4 and IL-10 in the large intestine of horses (Davidson et al., 2005). In addition, mast cells (du Toit et al., 2007; Pickles

et al., 2010), mast cell proteinases (Pickles et al., 2010) and 5-LOX enzyme (Giacominelli-Stuffler et al., 2014) have been demonstrated to be increased in cyathostomin infected horses (du Toit et al., 2007). The removal of cyathostomins from the gastrointestinal tract of horses has resulted in systemic inflammatory responses in some studies (Betancourt et al., 2015; Walshe et al., 2019), while in another study, there was little inflammatory reaction (Nielsen et al., 2015a). Cyathostomin infections also influence the faecal microbiota of horses, including reduction in alpha diversity (Peachey et al., 2019; Peachey et al., 2018; Walshe et al., 2021) and decrease of relative abundance of particular taxa including phyla Bacteroidetes and *Ruminococcus* species (Peachey et al., 2019; Walshe et al., 2021). Acute larval cyathostominosis has been associated with intestinal dysbiosis, evidenced by reduced alpha diversity and changes of relative abundance of specific taxa, including increased relative abundance of genus *Streptococcus*, class Bacilli, order *Lactobacillales* and family *Streptococcaceae* (Walshe et al., 2021).

Anthelmintics are commonly administered to aid in the management of cyathostomin infection in horses, and administration of anthelmintics has been associated with changes to the faecal microbiota (Kunz et al., 2019; Peachey et al., 2019; Peachey et al., 2018; Walshe et al., 2019). It remains unknown whether changes to the faecal microbiota associated with anthelmintic treatment occur due to removal of intestinal parasites or intrinsic effects of the anthelmintic drugs. It has been shown that administration of fenbendazole or moxidectin resulting in an acceptable reduction in strongyle faecal egg count (FEC) (94-99%), is associated with changes in the faecal microbiota including reduction in alpha and beta diversity, as well as changes in relative abundance at phylum, family and genus levels (Walshe et al., 2019). In another study, administration of ivermectin with marked reduction in strongyle FEC (100%), was associated with increased alpha diversity and changes in the relative abundance of taxa (Peachey et al., 2019). In addition, administration of moxidectin and praziquantel in horses with no nematode eggs detected on FEC, has been shown to result in reduced alpha diversity

and changes in taxanomic abundances (Kunz et al., 2019), suggesting that anthelmintic drugs alone can have minor effects on the microbiota. The influence of anthelmintic administration in cyathostomin populations with established resistance to the administered drug is unknown.

The objectives of this study were to assess the effect of administration of anthelmintic drugs, with known susceptibility (abamectin) and resistance (oxfendazole) in a population of cyathostomins, on the faecal microbiota of healthy adult horses. The first hypothesis was that anthelmintic drug administration would be associated with change to the faecal microbiota. The second hypothesis was there would be a greater change in microbiota in the abamectin group compared to the oxfendazole group.

5.3 Materials and methods

5.3.1 Ethical Statement

This study was approved by the Animal Care and Ethics Committee of Charles Sturt University (Protocol number A22052).

5.3.2 Animals

Twenty four adult geldings and mares, of mixed breeds and ages from the Charles Sturt University teaching herd were used. All horses were housed on pasture in the same conditions in, two separate paddocks (*n* = 10 and *n*= 14). Horses had not received any anthelmintic treatment for at least 16 weeks prior to the onset of the study. No horse had been administered antimicrobial drugs within 6 months of commencement of the study.

5.3.3 Administration of anthelmintic medication

Two anthelmintic drugs were used in this study; abamectin (MecWorma®), at the recommended label dose 0.2mg/kg per os and oxfendazole (Equinox®), at the recommended label dose 10mg/kg per os. Horse bodyweight was estimated by use of a weight tape and anthelmintic dose was calculated based on the nearest 50kg above the tape measurement.

5.3.4 Study design

This study was conducted as a prospective experimental study. At the commencement of the study (Day -3), a faecal sample was collected from each horse by rectal palpation for faecal worm egg count (FEC). Horses were ranked by FEC and using a blocked design, horses were randomly allocated into abamectin (ABA, *n*=8), oxfendazole (OXF, *n*=8) or control (*n*=8) groups.

5.3.5 Sampling

On Day 1 (D1), faeces was collected from each horse and the allocated anthelmintic was administered to horses in the ABA and OXF groups. From each horse, faecal samples were collected 3 days (D3) and 14 days (D14) post-treatment. Microbiota analysis was performed on faecal samples from D1, D3 and D14. Faecal worm egg counts (FEC) were performed on D14 samples and results from -3 and D14 were used for calculation of the FEC reduction (FECR) to assess the efficacy of the anthelmintic drugs administered. Samples collected for FEC were stored at 4°C and analysed within 3 days of collection. Samples collected for microbiota analysis were stored within 2 hours of collection at -20°C DNA extraction was performed.

5.3.6 Faecal Egg Counts and Faecal Egg Count Reduction Testing

Faecal egg counts were performed using a modified McMaster method with an egg detection limit of 10 eggs per gram (EPG)(Mines, 1977). Due to the inhomogeneity of the density of nematode egg shedding, the count was performed three times and the mean FEC was calculated (Wilkes et al., 2016).

For each horse, the FECR (%) was calculated as $100(1- FEC_{D14})/FEC_{D0}$. The efficacy of each anthelmintic drug was determined by calculation of FECRs and lower 95% confidence limits (LCL) according to 3 methods: arithmetic mean (AM) of individual FECRs (Method 1), AMs of arcsin-transformed individual FECRs (Method 2) (Pook et al., 2002) and correction of the AM of the treatment group FECR by changes in the AM of control group FECR (Method 3) (Dash et al., 1988). To define the presence of susceptibility, a FECR(%) of > 95% for abamectin and >

90% for oxfendazole was used. Additionally, 95% lower confidence limits (LCLs) of 90% and 80% were selected for classifying resistance to abamectin and oxfendazole, respectively. A FECR% of <95% and/or a LCL of <90% for abamectin and a FECR of <90% and/or a LCL of <80% for oxfendazole were used to define anthelmintic resistance (Abbas et al., 2021).

5.3.7 Larval culture

Faeces from four horses randomly selected were submitted, to confirm that FEC were representative of cyathostomins. Proportions of faeces were mixed with vermiculite and incubated at approximately 28°C for 10 days at controlled humidity (Henriksen and Korsholm, 1983) before third stage larvae were harvested and identified by the number and shape of intestinal cells (Russell, 1948).

5.3.8 DNA Extraction

Genomic DNA was extracted using Quick-DNA™ Fecal/Soil Microbe Miniprep Kit (Zymo Research, CA) as per the manufacturer's instructions. Concentration and purity were assessed by NanoDrop 2000 Spectrophotometer (Thermo Scientific, Australia).

5.3.9 16s rRNA Amplification and Next-Generation Sequencing

The extracted DNA samples underwent 16s rRNA sequencing. Prior to sequencing, all samples underwent quality control (QC): one sample initially failed QC, but subsequently passed after one round of purification. PCR amplification was performed on the V3-V4 regions with primer sequence CCTAYGGGRBGCASCAG-GGACTACNNGGGTATCTAAT. Electrophoresis on 2% agarose gel was used to selected the PCR products of appropriate size. The products were then pooled, end-repaired, A-tailed and ligated with Illumina adapters. Library sequencing was undertaken on a paired-end Illumina platform to generate 250 bp paired-end raw reads.

5.3.10 Analysis of sequence data

Raw data was spliced and filtered to acquire clean data. Sequences from the clean data with abundances <5 were filtered using DADA2 (Callahan et al., 2016) to obtain the final amplicon sequence variants (ASV). The Quantitative Insights Into Microbial Ecology (QIIME2) algorithm

was used for species annotation of the ASV. The representative sequence of each ASV was annotated using QIIME2 software. Relative abundance of phylum, class, order, family and genus taxonomic levels were determined for the abamectin, oxfendazole and control groups. For each group, alpha diversity measures of richness (Chao1, Observed_otus), evenness (Shannon's index) and diversity (Simpson's index of diversity) were generated in QIIME2. Beta diversity was assessed with weighted and unweighted UniFrac distances, principal component analysis (PCA) and principal coordinate analysis (PoCA) generated in QIIME2.

5.3.11 Statistical analysis

To compare the relative abundances of ASV, t tests were performed and the Benjamini Hochberg method applied for false discovery rate adjustments using R Software (Version 3.5.3). Taxa enriched in the faecal samples of each group were determined using linear discriminant analysis effect size (LEfSe), based on $p < .05$ and a linear discriminate analysis (LDA) score > 4. LEfSe software (Version 1.0) was used to perform LEfSe analysis. Alpha diversity was assessed using the ASV annotated by QIIME2 to result in Observed_otus, Chao1, Shannon, Simpson and Dominance indices. Kruskal-Wallis tests were used to analyse differences between groups and time points. Beta diversity was calculated based on weighted and unweighted Unifrac distances. Cluster analysis was performed with principal component analysis (PCA). Principal coordinate analysis (PoCA) was performed to visualise differences of samples in complex multi-dimensional data. To assess clustering between groups and assess changes in community structure as part of beta diversity analysis, ANOSIM performed.

5.4 Results

5.4.1 Subjects

Ten geldings and fourteen mares were enrolled in the study. Breeds included Thoroughbred (*n* = 13), Standardbred (*n* = 9), Quarter horse (*n* = 1) and Brumby (*n* = 1). Age of horses enrolled ranged from 6 to 23 years of age with a median of 12 years.

5.4.2 Larval culture

Equine larval culture from 4 randomly chosen horses enrolled in the study showed culture results of 100% cyathostomins.

5.4.3 Faecal egg count reduction testing

The results of the FECRT for each group are provided in Table 5.1. Faecal egg count reduction testing results were consistent with resistance to oxfendazole and susceptibility to abamectin.

***Table 5.1**: Faecal egg count reduction test (FECRT) results for oxfendazole and abamectin in horses with cyathostomin infection. Results of arithmetic mean (AM) of individual faecal egg count reduction (FECR), AM of arcsin-transformed individual FECR and AM of treatment group corrected for control group FECR, each with 95% lower confidence limit (LCL) are included.*

Group	AM of individual FECR (95% LCL)	AM of arcsin-transformed individual FECR (95% LCL)	AM of treatment group corrected for control group FECR
Control	-59 (-211)		
Abamectin	95 (91)	96 (92)	97
Oxfendazole	29 (-34)	44(13)	50

5.4.4 Next generation sequencing and bioinformatic analysis

5.4.4.1 Sequence analysis

Seventy one samples from ABA (*n* = 23), OXF (*n* = 24) and Control groups (*n* = 24) were available for analysis and yielded 2,754,082, 3,241,973 and 3,164,134 sequences, respectively with a total yield of 9,160,189 raw reads. One sample from the ABA group was unable to be obtained on D1 due to lack of manure at time of faecal collection. Primer sequence removal and quality filtering resulted in a total number of qualified reads of 2,649,134 (abamectin), 3,103,635 (oxfendazole) and 3,041,564 (control) that were subsequently analysed.

5.4.4.2 Relative abundance and LEfSe analysis

The ASVs were clustered and allocated to the respective level of classification including 38 phyla and 650 genera. The top 10 phyla of all horses contributed to 98.9% of the identifiable ASVs and included Firmicutes (56.3%), Bacteroidota (28.6%), Verrucomicrobiota (3%), Spirochaetota (4%), Fibrobacterota (2%), Proteobacteria (1.6%), Actinobacteroita (1.2%), Euryarchaeota (0.6%). Halobacterota (0.5%) and Fusobacteriota (0.2%) (Figure 5.1). The relative abundance of Firmicutes increased over time in both treatment groups, but decreased in the Control group. Bacteroidota increased in the Control and OXF groups, but decreased in the ABA group after treatment.

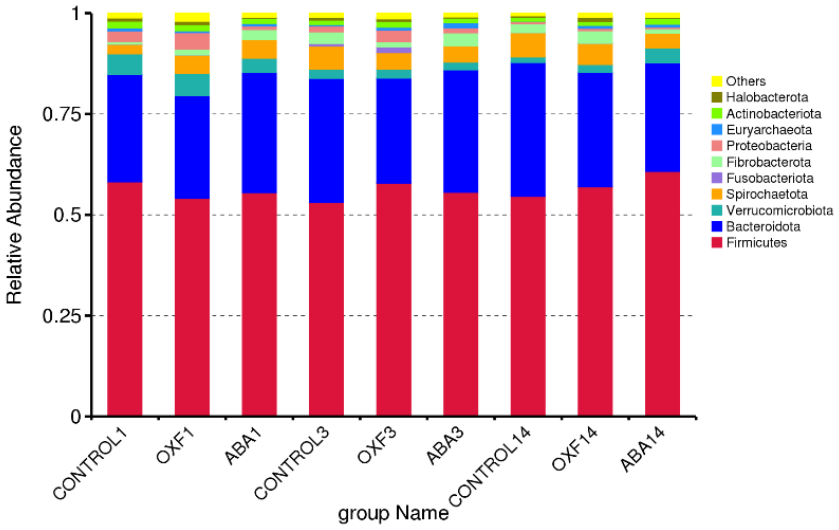

Figure 5.1: *the faecal microbiota relative abundance at phylum level. The bar chart shows the relative abundance of the top 10 phyla for each group (Control, Abamectin [ABA] and Oxfendazole [OXF]) on days 1, 3 and 14.*

Differences in relative abundance of phyla, class, order, family and genus are presented in Table 5.2. Compared to the Control group, the ABA group had reduced relative abundance of phyla Halobacterota ($p = 0.03$) on D3. On D14, there was an increased relative abundance of phyla Firmicutes ($p = 0.04$) and Cyanobacteria ($p = 0.03$) and decreased relative abundance of Bacteroidota ($p = 0.02$) in the ABA group compared to the Control group. There were 4

differences in relative abundance at the class taxonomic level on D14 between the ABA and Control groups (Table 5.2).

Taxon (P value)					
Oxfendazole v control	*Phyla*	*Class*	*Order*	*Family*	*Genus*
D1	-	-	-	-	-
D3	-	Bacilli(↑)(0.04), Nagativicutes(↑)(0.04), Desulfovibrionia(↑) (0.01)	-	-	-
D14	Halobacterota(↑) (<0.01), Cyanobacteria(↑) (<0.01), Thermoplasmatota(↑) (0.05)	Methanomicrobioa(↑) (0.01), Vampirivibrionia(↑) (0.01), Alphaproteobacteria(↑) (0.03)	Gastranaerophilales(↑) (0.05)	Methanocorpusculaceae(↑) (0.05), Gastranaerophilales(↑) (0.05), Sutterellaceae(↑) (0.05)	Gastranaerophilales(↑) (0.03), Anaerovorax(↑) (0.01), Lachnospiraceae_NK4B4_group(↑) (0.03 Sediminispirochaeta(↑) (0.01)
Abamectin v control	*Phyla*	*Class*	*Order*	*Family*	*Genus*
D1	-	-	Lachnospirales(↓) (0.02), Firbobacterales(↑) (0.02), Clostridia(↑) (0.02), Xanthomonodales(↓) (0.02), Campylobacterales(↓) (0.02), Chloroplast(↓) (0.02), Pseudonocardiales(↑) (0.02)	-	-
D3	Halobacterota(↓) (0.03)	-	-	-	-
D14	Firmicutes(↑) (0.04), Bacteroidota(↓) (0.02), Cyanobacteria(↑) (0.03), Themoplasmatota(↑) (0.03)	Bacteroidia(↓)(0.04), Bacilli(↑)(0.04), Vampirivibrionia(↑) (0.03), Desulfovibrionia(↑) (0.04), Thermoplasmata(↑) (0.04)	-	-	-
Abamectin v oxfendazole	*Phyla*	*Class*	*Order*	*Family*	*Genus*
D1	-	-	-	-	-
D3	-	-	-	-	-
D14	Halobacterota(↓) (<0.01)	Bacilli(↑)(<0.01), Methanomicrobia(↓) (<0.01)	Methanomicrobiales(↓) (<0.02)	Methanocorpusculaceae(↓) (0.03)	-

***Table 5.2**: Significant differences of relative abundance of phyla, class, order, family and genus within and between groups. In comparisons between treatment groups and the control group, the relative abundance is compared to the treatment group. In comparisons between the abamectin and oxfendazole groups, the relative abundance is the abamectin group compared to the oxfendazole group.*

Differences in relative abundance were observed between the OXF and Control groups after treatment (Table 5.2). There was increased relative abundance of class Bacilli (p = 0.04), Nagativicutes (p = 0.04) and Desulfovibrionia (p = 0.01) in the OXF group on D3. On D14 there was increased relative abundance of phyla Halobacterota (p =<.01), Cyanobacteria (*p* =<.01) and Thermoplasmatota (*p* = 0.03) in the OXF group. There was an increased relative abundance in genus *Gastranaerophilales* (p = 0.03), *Anaerovorax* (p = 0.01),

Lachnospiraceae_NK4B4_group (p = 0.03) and *Sediminispirochaeta* (p = 0.01) in the OXF group at Day 14.

Differences in relative abundance of taxa were observed between the ABA group at the OXF after treatment (Table 5.2). At Day 14, there was a decrease in phyla Halobacterota in the ABA group (p <0.01).

LEfSe results are presented in Appendix 5. Within the OXF group, phyla Proteobacteria and Verrucomicrobiota and genus *Akkermansia* were enriched on D1 compared to D3 and D14 (Figure 5.2a). Within the ABA group, family Lachnospiraceae was enriched on D14, compared to D1 and D3 (Figure 5.2b). Comparison of the Control, ABA and OXF group on D3 showed an increase in genus Rikenellaceae_RC9_gut_group in the ABA group and an increase of class Bacteroidia in the Control group (Figure 5.2c). On D14, family *Prevotellaceae* and genus *Prevotellaceae*_UCG_001 where enriched in the Control group compared to the OXF and ABA group (Figure 5.2d).

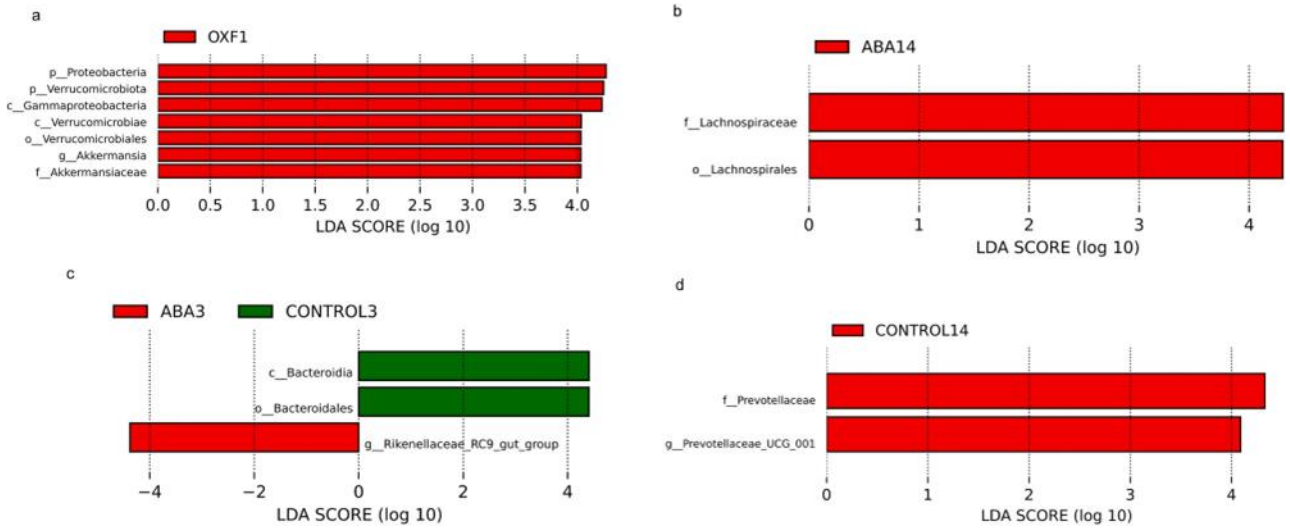

***Figure 2.2**: . LEfSe histogram displaying significant differences of relative abundance including: in the OXF group, enrichment of phyla Proteobacteria and Verrucomicrobiota and genus Akkermansia on D1 compared to D3 and D14 (Fig. a). Within the ABA group, family Lachnospiraceae was enriched on D14, compared to D1 and D3 (Fig. b). Comparison of the Control, ABA and OXF group on D3 showed an increase in genus Rikenellaceae_RC9_gut_group in the ABA group and an increase of class Bacteroidia in the Control group (Fig. c). On D14, family Prevotellaceae and genus Prevotellaceae_UCG_001 were enriched in the Control group compared to the OXF and ABA group (Fig.2d).*

Differences of relative abundance of taxa were observed between groups prior to anthelmintic treatment and within the Control group over time (Appendix 5).

5.4.4.3 Alpha diversity

Comparisons within each treatment group (ABA, OXF and control) at D1, D3 and D14 were performed. Comparisons between treatments groups (ABA, OXF, control) at D1, D3 and D14 were also performed. There were no differences within or between groups in Chao1 index. Shannon indices showed greater evenness at D14 compared to D3 in the ABA group (P=<.01) (Figure 5.3). Simpson indices showed greater diversity at D14 compared to D3 in the ABA group (P=<.01).

Differences of richness were observed within the ABA group between D3 and D14 (P=<.01) using the Dominance index (Figure 5.3).

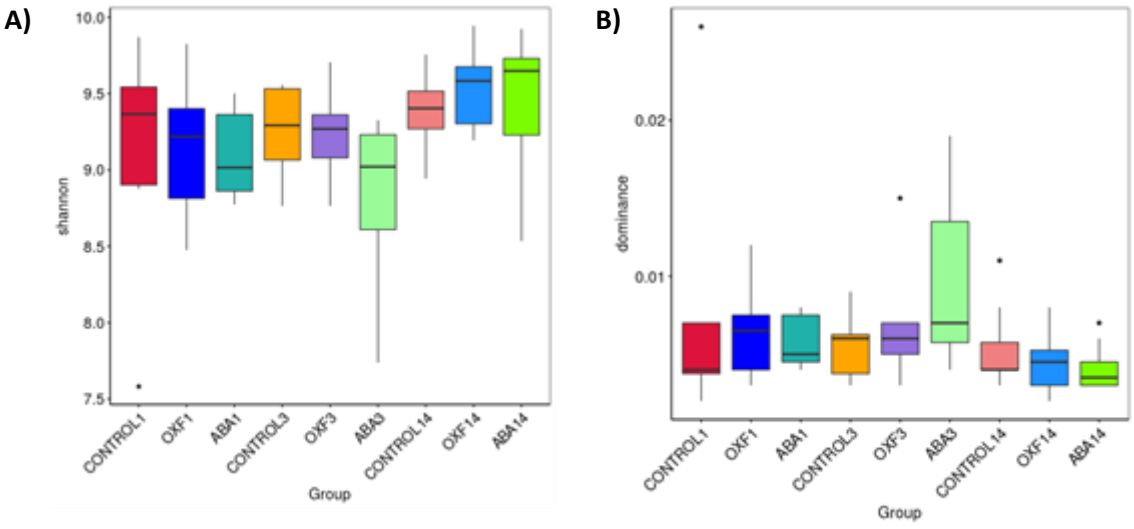

***Figure 5.3**: Boxplot displaying Shannon indices of all groups and time points (A), and boxplot of Dominance indices showing changes in richness of the Abamectin group (B).*

5..4.4.4 Beta diversity

Comparisons of the ABA, OXF and control group at D1, D3 and D14 time was performed. Unweighted PCoA analysis showed clustering of the OXF and ABA groups at D3 and D14 and clustering of the Control group at D14 (Figure 5.4). Weighted PCoA did not reveal clustering of any groups. Unweighted NMDS plot analysis showed clustering of the OXF group on D14 and weighted NMDS plot showed no clustering of any groups.

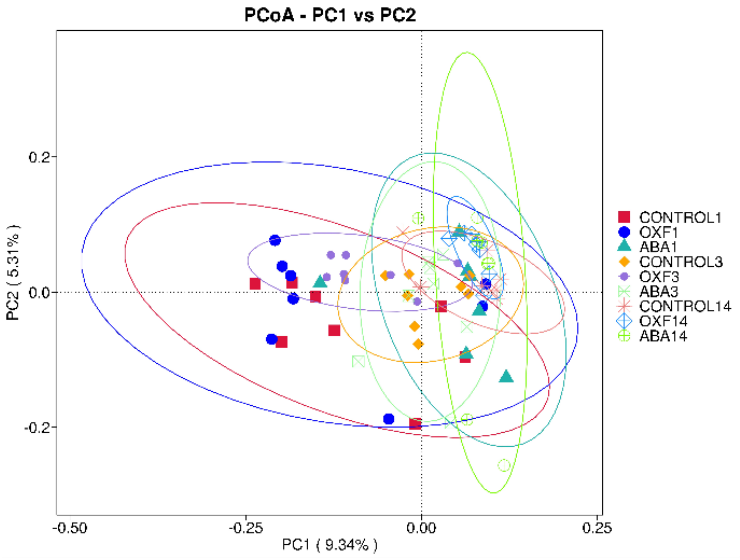

***Figure 5.4**: Unweighed principal coordinate analysis of the faecal microbiota of horses treated with oxfendazole (OXF) or abamectin (ABA) and control group horses on D1, D3 and D14. The Unifrac distances show the beta diversity of each group. Each shape represents a different group and time point. Shapes that are visually closer together are more similar*

Results of analysis of similarity (ANOSIM) are provided in Table S3. There was no difference in the bacterial community structure between groups on D1. There were differences within the Control group between D1 and D14 and between D3 and D14 (p =.01) and differences in the OXF group between D1 and D14 and between D3 and D14 (p =<.01). There were no differences within the ABA group. Comparison of groups showed differences between the Control group, and both the OXF group (p =<.01) and ABA group (p =<.01) at Day 14. There was a significant difference in diversity between the Control group and ABA group at Day 3 (p

=.04). There were no differences between the ABA group and the OXF group at any time point.

5.5 Discussion

In this study, the effects of anthelmintic drug administration on the faecal microbiota of horses infected with cyathostomins that were resistant to oxfendazole and susceptible to abamectin were assessed. Despite the ubiquity of intestinal parasites in horses and the common use of anthelmintic drugs, only a few studies have assessed the response of the faecal microbiota to treatment of cyathostomin populations with known susceptibility to the administered drug (Kunz et al., 2019; Walshe et al., 2019). Overall, there were limited changes to the relative abundance of predominant microbiota, although significant differences of phyla, class, order, family and genus between and within groups after treatment were observed. Administration of abamectin was associated with differences in alpha diversity and administration of both abamectin and oxfendazole was associated with changes in beta diversity. In this study, administration of an anthelmintic with determined efficacy against cyathostomins was associated with changes to the microbiota and may reflect changes in the host-parasite-microbiota relationship, which is important in health and disease.

In the current study, differences in the relative abundance of faecal bacteria at various taxonomic levels occurred with administration of oxfendazole and abamectin, in comparison to the Control group. These findings are similar to previous reports that administration of anthelmintics alters the faecal microbiota (Kunz et al., 2019; Peachey et al., 2018; Walshe et al., 2019). The enrichment of *Prevotellaceae* (family) on D14 in the Control group in comparison to the ABA and OXF groups, could suggest that the animals who did not receive an anthelmintic had a healthier microbial population. *Prevotellaceae* are short-chain fatty acid producing bacteria and have anti-inflammatory and modulating effects on the intestinal mucosa and have a role in maintaining gut health (Bedarf, et al. 2017). The increase of phyla Firmicutes and enrichment of family *Lachnospiraceae* (phylum Firmicutes, class Clostridia) in

the ABA group on D14 could reflect normalisation of the microbiota (Venable et al., 2016) and improvement in intestinal health. The increased relative abundance of class Bacteroidia in the control group compared to the ABA group at D14, could reflect higher cyathostomin burdens in untreated horses. Bacteroidetes members have been associated with nematode infection in mice (Rausch et al., 2013) and in horses with strongyle infection (Peachey et al., 2018; Walshe et al., 2019). In addition to this, effective anthelmintic treatment in horses has been associated with decreased phylum Bacteroidetes (Walshe et al., 2019).

Changes of the relative abundance of taxa was different within treatment groups. In the OXF group, reductions in phylum Proteobacteria, phylum Verrucomicrobiota and genus *Akkermansia* were present on D3 and D14 after treatment. Phylum Verrucomicrobiota, dominates the equine hindgut and genus *Akkermansia* is reported to maintain the integrity of the mucin layer and decreases bowel inflammation in people (Everard, et al. 2013). In addition, increases in the relative abundance of family *Akkermansiaceae* has been associated with disease stabilisation in human chemotherapy patients (Grenda et al., 2022). Within the ABA group, there was enrichment of family Lachnospiraceae on D14. In humans, members of this taxa have been correlated with strengthening of the intestinal barrier, anti-inflammatory, immune modulation and production of short-chain fatty acids (Vacca et al., 2020). The increases in family *Lachnospiraceae* at D14 could suggest that stabilisation of the microbial community occurs approximately 2 weeks after anthelmintic administration and removal of cyathostomins. Increased abundances of family *Lachnospiraceae* have been associated with the faecal microbiota of healthy horses in comparison to horses with colitis (Costa et al., 2012) and colic (Weese et al., 2015), supporting a role of this taxon in the health and functional integrity of the intestinal tract. Further, increased abundance of family *Lachnospiraceae* has been found in horses with smaller strongyle burdens (Clark et al., 2018).

Differences in relative abundance between the ABA group and the OXF group on D14 could reflect different effects of the two anthelmintic drugs. The greater relative abundance of phylum Halobacterota in the OXF group compared to the ABA group on D14, suggest different types of change to the microbiota associated with susceptibility and resistance to particular anthelmintic drugs. Alternatively, these differences could reflect direct impact of administration of two different drugs on the intestinal microbiota. In the current study, increases in the relative abundance of class Methanomicrobia, order Methanomicrobiales and family *Methanocorpusculaceae* in the OXF group at D14 were observed, suggesting that methanogens may be associated with cyathostomin infection in horses. In contrast, another study reported that the relative abundances of methanogens were greater in horses with low FEC (Peachey et al., 2018), suggesting that there was a negative associated between methanogen abundance and FEC. The results of the current study and previous studies identify a potential association between methanogen bacteria and cyathostomin infection, indicating that more investigation is required to further elucidate the relationship between parasite, host immunity and the microbiota.

In the current study, the increased evenness and diversity at D14 compared to D3 in the ABA group highlight the effect of anthelmintic drug administration on alpha diversity. Similarly, decreases in alpha diversity after anthelmintic drug treatment on Day 7 and subsequent increase on D14 were reported in one study (Walshe et al., 2019), and decreased alpha diversity 2 days after treatment was found in another study (Kunz et al., 2019). In contrast, other studies have reported no differences in alpha diversity (Peachey et al., 2018) or increases in alpha diversity (Peachey et al., 2019) following treatment with ivermectin. Richness refers to the total number of taxa, evenness refers to the relative abundance of those taxa and diversity is the result of interpretation of the richness and evenness together. The increased evenness on D14 compared to D3 in the abamectin group supports changes of the microbiota after treatment and is consistent with findings of a previous study (Peachey et

al., 2019). A study performed by (Kunz, et al. 2019) reported only mild decrease in alpha diversity in horses that had a low FEC after anthelmintic treatment, and this supports the results of the current study; in horses where the parasite burden remains similar or unchanged, the effect on the microbiota is less, compared to large changes in helminth population.

In the current study, administration of anthelmintic treatment altered beta diversity. Unweighted PCoA analysis revealed clustering of treatment groups over time, with oxfendazole and abamectin clustering at different points in the PCoA analysis. The clustering of each treatment group at different time points could be due to a difference in mode of action of the anthelmintic drugs, or changes in cyathostomin populations in the ABA group. The results of ANOSIM analysis indicated there were no differences in beta diversity between groups prior to treatment. The differences between the Control group and OXF group on D14 and ABA group on D3 and D14 indicate that anthelmintic drug administration resulted in changes to beta diversity. In addition, there were differences within the OXF group between D3 and D14 and D1 and D14. In previous studies, anthelmintic drug treatment was associated with no change (Kunz et al., 2019; Peachey et al., 2018) or differences in beta diversity (Walshe et al., 2019). The low R values in the current study highlight there is overlap and similarity between groups. Other studies have reported similar observations (Kunz et al., 2019), where the differences between individual animals were more pronounced than between treatment groups. The lack of differences between the ABA and OXF groups suggest horses receiving anthelmintic drug treatment have similar beta diversity and may suggest that differences in susceptibility of the population do not have an adverse effect on beta diversity.

The differences in beta diversity in the Control group over time suggests that some of the differences observed within the oxfendazole and abamectin groups were due to other factors other than drug administration. In addition, the differences in relative abundance present on

D1 make interpretation of the importance of changes post-treatment difficult. Differences prior to treatment could be due to small group sizes and individual variation of the microbiota between horses (Costa et al., 2012; Dougal et al., 2014; Kunz et al., 2019; Theelen et al., 2021). Although some of the differences between groups could be due to variability, the results overall support the likelihood that anthelmintic drug administration resulted in changes to the faecal microbiota, as has been reported previously (Kunz et al., 2019; Walshe et al., 2019).

Changes in the faecal microbiota associated with administration of oxfendazole or abamectin in the current study are supportive of interactions between cyathostomins and the microbiota, that could include involvement of mucosal and systemic host immunity. Previous studies have highlighted that host factors likely contribute to the changes in microbiota after treatment, (Kunz et al., 2019; Peachey et al., 2018) emphasizing the important but not yet completely understood relationship between the microbiota, host immune responses and helminths. The gastrointestinal microbiota is likely affected by changes in parasite populations, as helminths occupy the same environment as bacteria, archaea and fungi as part of the gastrointestinal ecosystem (Garber et al., 2020). Modulation of inflammatory responses associated with vaccination and concurrent anthelmintic administration have been observed, characterised by reduced concentrations of haptoglobin, fibrinogen and IL-4 in horses with reduced helminth burden (Nielsen et al., 2015b). In another study, changes in microbiota and inflammatory responses, including increased concentrations of serum fibrinogen and faecal albumin, coincided with acceptable reduction in FEC after anthelmintic administration (Walshe et al., 2019). Inflammatory responses following anthelmintic administration could be either indirectly or directly associated with manipulation of the microbiota (Walshe et al., 2019). In addition, inflammation and tissue damage has been reported after anthelmintic treatment (Steinbach et al., 2006), which was hypothesised to be due to reactions to encysted fourth stage cyathostomin larvae and may mimic synchronous mass emergence of fourth stage larvae in larval cyathostominosis. In the current study, abamectin was associated with

some changes in metabolic and gene processing function compared to the control group and this supports that administration of an anthelmintic effective against cyathostomins in horses is likely associated with changes in host responses and immune function. The lack of differences in function prediction of genes between the control group and OXF group support that with minimal change to FEC (and presumably lack of change in cyathostomin population) local immune function is minimally affected.

The limitations of this study include small group sizes, similar to those previously reported in the study of anthelmintics in horses (Kunz et al., 2019; Walshe et al., 2019). Individual animal variability precluded the identification of convincing changes in relative abundance or determination of the magnitude of change to beta diversity due to anthelmintic drug treatment. However, the use of a control group assisted in the detection of variability in beta diversity and microbiota community structure. The use of faeces for sampling of microbiota analysis may not entirely reflect the large intestinal microbiota, however is the best proxy available antemortem. The use of two different anthelmintic drugs with different nematocidal profiles could introduce variability that influenced the microbiota.

5.5 Conclusions

In conclusion, the findings of the current study indicate that administration of anthelmintic drugs, specifically oxfendazole and abamectin, has effects on the faecal microbiota of horses. Further, these findings suggest that administration of an anthelmintic that the cyathostomin population is susceptible to, has a greater impact on the gastrointestinal microbiota than the anthelmintic drug alone. Further studies investigating the host-parasite-microbiota relationship could lead to refined parasite control plans and recommendations.

5.6 Author Contributions

JB doctorate student and KH doctorate supervisor were involved in the conceptualisation, methodology, sample collection, data analysis and manuscript preparation. SR doctorate co-supervisor was involved in manuscript preparation and review.

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5.9 Supplementary Information

Results of LEfSe analysis of differences within and between groups in included in Appendix 4.

Results of ANOSIM analysis is included in Appendix 5.

A table of the raw faecal egg count data in included in Appendix 6.

Chapter 6: Exegesis

The gastrointestinal microbiota plays an important role in the overall health of horses (Garber, et al. 2020; Venable, et al. 2016). The microbiota assists in digestion, protection against pathogens, development of the intestinal epithelium and modulation of local and systemic immune systems and metabolic functions (Costa and Weese 2018). Disruption of the GI microbiota is associated with disease including colitis (Costa, et al. 2012b), laminitis (Milinovich, et al. 2008), diarrhoea (Li, et al. 2022), asthma (Leclere and Costa 2020), equine metabolic syndrome (Elzinga, et al. 2016) and equine grass sickness (Leng, et al. 2018).Given these associations, the identification and refinement of targeted management and treatment options to preserve and re-establish a balanced microbiota might represent an effective tool in the prevention and management of disease. Consideration of the effects of common husbandry practices including anthelmintic treatment is important in reconsidering current regimens. There is currently no targeted treatment of the GI microbiota in foals with diarrhoea and the implementation of FMT treatment is a reasonable therapeutic consideration. There is little investigation of the side effects of storage on equine and the identification of safe storage options will assist veterinarians in the future.

This thesis assessed several aspects of the equine GI microbiota from health to disease and targeted treatment. The thesis has assessed the effects of commonly used anthelmintic treatments on the faecal microbiota, the effects of FMT administration to foals with diarrhoea and the effects of storage on the microbiota of FMT.

6.1 FMT as a treatment of diarrhoea in foals

6.1.2 Diarrhoea in foals

Up to 80% of foals experience one or more instances of diarrhoea within the first 6 months of life (Frederick, et al. 2009; Oliver-Espinosa 2018). Clinical signs associated with diarrhoea in foals may include a quiet mentation, reduced nursing, abdominal discomfort and weight loss

(Oliver-Espinosa 2018). Non-infectious causes of diarrhoea include foal heat diarrhoea, dietary intolerance, neonatal maladjustment syndrome associated diarrhoea and sand ingestion (Frederick, et al. 2009). In Chapter 3, 36% of foals (n = 9) had no identifiable infectious cause of diarrhoea.

A number of infectious agents can cause diarrhoea including *Salmonella* (Frederick, et al. 2009), *Clostridium perfringens* (Frederick, et al. 2009)*, Clostridium difficile* (Frederick, et al. 2009), *Lawsonia intracellularis* (Wong, et al. 2009), *Rhodococcus equi* (Slovis, et al. 2014), equine rotavirus (Frederick, et al. 2009) and equine coronavirus (Frederick, et al. 2009). In a previous study by Netherwood et al. (1996), *Clostridium perfringens,* rotavirus, *Cryptosporidium* spp. and *Strongyloides westeri* were associated with foal diarrhoea. In Chapter 3, the prevalence of pathogens detected in foals presenting with diarrhoea was 77% (n = 16). The enteropathogens detected included *Salmonella* spp. (n = 9), *rotavirus* (n = 4), Cryptosporidium spp. (n = 7) and *Clostridium difficile* (n =1). *Clostridium perfringens*, *Salmonella* spp and *Cryptosporidium* spp. have been previously associated with fatal illness (Netherwood, et al. 1996). This was in part observed in Chapter 3, where two foals with enteric salmonellosis developed marked clinical and clinicopathological derangements and were subsequently subjected to euthanasia.

The prevalence of enteropathogens detected in foals in Chapter 3 is similar to previous reports. A study investigating the prevalence of infectious agents in fifty-one foals with diarrhoea has reported a prevalence of 63.2% of enteropathogens (Slovis, et al. 2014) and another study authors reported a prevalence of infectious causes of diarrhoea 55% percent of foals (Frederick, et al. 2009). These and other studies have been undertaken internationally; however, Chapter 3 provides one of the first studies presenting the prevalence of different infectious causes of diarrhoea in foals in Australia. The findings of Chapter 3 suggest that the

prevalence of infectious agents in foals with diarrhoea are similar in Australia to other parts of the world.

6.1.3 Microbiota of healthy and diarrheic foals

The faecal microbiota of healthy foals and foals with diarrhoea has been previously investigated, although investigation of the microbiota of foals with diarrhoea is limited (Schoster, et al. 2017). The faecal microbiota of healthy foals changes from birth through to weaning and becomes more similar to the dam as the foal ages (De La Torre, et al. 2019). The changes to the microbial community as foals age is reflected in changes of beta diversity (De La Torre, et al. 2019), alpha diversity (Costa, et al. 2016a; De La Torre, et al. 2019) and relative abundance of taxa (Costa, et al. 2016a; De La Torre, et al. 2019). In Chapter 3, foals were aged between 0-6 months of age, which likely influenced the results of the microbiota analysis. The absence of significant changes or differences within and between the FMT and control groups might reflect the wide age range of the study population. Grouping of foals into closer age groups within the 6 month period was not possible, due to the small number of animals eligible for enrolment. This is one of the limitations of Chapter 3. If more animals were available and eligible for enrolment in the study, foals could have been grouped into closer ages within the 6 month age requirement.

The faecal microbiota of foals with diarrhoea has been investigated (Schoster, et al. 2017). In this study, authors reported that there were 117 species enriched in healthy foals compared to diarrheic foals, with 48% of those species being members of *Lachnospiraceae* or *Ruminococcaceae* families (Schoster, et al. 2017). Foals with diarrhoea were reported to have a lower richness than non-diarrheic foals, and authors observed that diarrhoea had an inconsistent effect of microbial community structure (Schoster, et al. 2017). The most abundant phyla identified in diarrheic foals included Firmicutes, Verrucomicrobia and Proteobacteria, and the most abundant families included Ruminococcaceae, Lachnospiraceae and Verrucomicrobiota (Schoster, et al. 2017). In Chapter 3, the most abundant phyla were

Verrucomicrobia, Bacteroidota and Proteobacteria and most abundant families were Lachnospiraceae, Lactobacillaceae and Bacteroidaceae. The study performed by Schoster et al 2017 evaluated the microbiota of foals of 2 and 4 weeks of age and as such, the microbiota of these foals would be different to the to the microbiota of the foals in Chapter 3 due to differences of age. Although direct comparison of taxa cannot be performed due to age differences, it could be assumed that reduced richness would be present in the foals of Chapter 3 as well, as decreased richness is also associated with diarrhoea in adult horses (Costa, et al. 2012b; McKinney, et al. 2021; McKinney, et al. 2020a; Rodriguez, et al. 2015).

6.1.4 Changes of the microbiota associated with FMT

Although FMT has been used by equine clinicians for decades with anecdotal success, studies assessing the response of the microbiota to FMT have only recently been undertaken (Costa, et al. 2021; Dias, et al. 2018; Kinoshita, et al. 2022; Laustsen, et al. 2021b; McKinney, et al. 2021; McKinney, et al. 2020b; Quattrini, et al. 2023) and changes of the faecal microbiota in response to treatment are inconsistent. In a study of 6 horses with diarrhoea, authors reported no change to the faecal microbiota associated with FMT (Costa, et al. 2021) and a study of FMT use in free faecal water syndrome reported no changes to the faecal microbiota with treatment (Laustsen, et al. 2021b). In another study, FMT has also been shown to have no effect on preventing antimicrobial associated dysbiosis when administered simultaneously with antimicrobial drugs (Kinoshita, et al. 2022); however, the continued administration antimicrobials could have contributed to the persistence of dysbiosis. In contrast, other studies in adult horses have reported some changes to the faecal microbiota subsequent to FMT. The frequent concurrent use of antimicrobial drugs in foals of Chapter 3, could also have contributed to the absence of an obvious effect of FMT administration on the microbiota. It has been reported that adult recipients responding favourably to FMT had a higher alpha diversity and taxonomic groupings which were more similar to donor manure (McKinney, et al. 2020a). Following this, another study assessing FMT in adult horses, including a control group,

was published. Here the authors reported normalization of the microbiota of twelve FMT recipients, compared to untreated control horses (McKinney, et al. 2021).

In Chapter 3, evidence of an obvious effect of FMT on the relative abundance of taxa was not observed and convincing differences were not detected between the Control group and the FMT group over time. The enrichment of some taxa at D1 in the FMT group could suggest that FMT administration had a positive effect on the microbiota, however these differences were not evident throughout the remainder of the study period. In addition to this, taxa were enriched between the Control group and FMT group at D0, introducing difficulty in interpreting the significance of enrichment of taxa observed at D1. The lack of convincing change in microbiota associated with FMT in recipient foals in Chapter 3 is in agreement with a study of adult horses, where FMT administration was not associated with changes in the microbiota (Costa, et al. 2021).

The presence of differences of taxa between groups at Day 0 and within the control group over time highlight the variability between animals. As previously explored, the microbiota of foals changes dramatically over the first 6 months of life (De La Torre, et al. 2019) and this may have had an impact on the microbiota analysis of groups. It is also known that other factors including geographical location (Arnold, et al. 2021), antimicrobial administration (Gomez, et al. 2023) and diet (Leng, et al. 2021) can have an effect on the microbiota and these variables could not be controlled in foals studied in Chapter 3. In addition to this, analytical methods to reduce the impact of these variables is not available [and would necessitate a much larger study population.

In contrast to the reports in adult horses (McKinney, et al. 2021; McKinney, et al. 2020b), there was no increase in alpha diversity or normalisation of the microbiota associated with FMT in Chapter 3. The results of Chapter 3 were consistent with one study of FMT in adult horses, where there was no change in beta diversity associated with FMT administration

(Costa, et al. 2021), but contrasted another study that observed a reduction of beta diversity between recipients and donors associated with FMT (McKinney, et al. 2021).

6.1.5 Clinical variables and FMT treatment

Clinical variables of animals provide invaluable insight into the course of disease and response to treatment. The clinical variables of adult horses receiving FMT have been described infrequently. In a retrospective study of FMT in adult horses, authors reported no significant differences between respiratory rate, heart rate or body temperature when comparing FMT recipients to control horses (Quattrini, et al. 2023). In another study of FMT for treatment of diarrhoea in adult horses, authors also reported no differences between control and FMT groups (McKinney, et al. 2021). Other studies that were performed without a control group didn't report on analysis of clinical variables over time (Costa, et al. 2021; Dias, et al. 2018; McKinney, et al. 2020b). In Chapter 3, the median heart rate of foals receiving FMT treatment was significantly decreased between D0 and D2 and D0 and D3. In a study examining prognostic indicators of horses with acute diarrhoea, a lower heart rate was associated with survival (Kovač, et al. 2017). This decrease in heart rate over time could have reflected improved haemodynamic status and a positive response to treatment, and this could have occurred in the FMT group foals in Chapter 3.

6.1.6 Haematologic and blood biochemistry variables associated with FMT treatment

In studies investigating the effect of FMT in adult horses, authors have not reported evidence of clinically relevant changes to haematologic or blood biochemistry variables over time between or within groups (Costa, et al. 2021; Dias, et al. 2018; McKinney, et al. 2021; McKinney, et al. 2020a; Quattrini, et al. 2023). Prognostic indicators of survival in adult horses with acute diarrhoea include the packed cell volume (PCV) or haematocrit, with horses that have a lower haematocrit having increased chances of survival (Kovač, et al. 2017).

Complementary to this, in horses undergoing small intestinal surgery, an increasing PCV has been associated with non-survival (Proudman, et al. 2005). As PCV is influenced by fluid balances and endotoxaemia, a lowered PCV reflects positive response to treatment and restoration of hydration and resolution of systemic inflammation. The ability of FMT to positively influence hydration may be due to re-establishment of the intestinal microbiota and normalisation of the intestinal epithelium and local immune responses (Costa and Weese 2018; Stewart, et al. 2018). This would then lead to improved intestinal integrity and function, resulting in reduced intestinal losses and improved absorptive capacity of the intestine.

The microbiota has been recognised to play a role in both the local and systemic immune responses (Costa and Weese 2018; Garber, et al. 2020). Re-establishment of a healthy gastrointestinal microbiota is expected to act as a barrier against pathogenic organisms and assist in the local immunity to counteract infection (McKinney, et al. 2020b; Tlaskalová-Hogenová, et al. 2011). Mechanisms by which the microbiota assists in protecting the host includes inhibition of pathogen colonisation, competition for nutrients and other mechanisms including direct killing and enhancement of immune responses (Pickard, et al. 2017). The ability of The microbiota is also thought to be associated with inflammatory and anti-inflammatory mediators within the intestinal tract (McKinney, et al. 2020b; Tlaskalová-Hogenová, et al. 2011). In humans, it has been hypothesised that dysbiosis is associated with immune mediated diseases (Lerner, et al. 2016) and related to failure of the localised immune systems of the gastrointestinal tract (Chang and Choi 2023).

In Chapter 3, the WBC count of the FMT group at Day 3 was significantly lower than the control group, showing normalisation of the inflammatory responses. This normalisation may have been a consequence of restoration of the microbiota, resolution of disease at the level of the intestines, and reduced local inflammation. Improvement in intestinal function would reduce the degree of endotoxin absorption into the bloodstream, lowering the systemic

inflammatory response. The WBC count is increased in certain disease processes, including neonatal illness (Yue, et al. 2022) and markedly decreased in septicaemia in foals (Gayle, et al. 1998). The WBC count of foals within the treatment group was within normal range at Day 3,supporting improvement of disease. .

6.1.7 Outcomes associated with FMT treatment

Faecal microbiota transplantation (FMT) is used in humans to treat recurrent *Clostridium difficile* infection (Jiang, et al. 2018), ulcerative colitis (Ianiro, et al. 2019) and inflammatory bowel disease (Mazzawi, et al. 2018). The use of FMT to treat recurrent *Clostridium difficile* in humans is reported to have very high clinical efficacy (Gangwani, et al. 2023). The efficacy of FMT in adult horses with diarrhoea and colitis has been previously investigated (Costa, et al. 2021; Dias, et al. 2018; McKinney, et al. 2021; McKinney, et al. 2020b). The first clinical trial of FMT in adult horses was published relatively recently, in 2018 (Dias, et al. 2018). In this study, the authors reported resolution of diarrhoea within 24 hours of FMT (Dias, et al. 2018). Other studies have reported FMT recipients have improved faecal consistency (Costa, et al. 2021) and greater day-to-day improvement of diarrhoea (McKinney, et al. 2021). Contrary to these reports, Quattrini et al (2023) reported that horses treated with FMT did not have normalization of faeces compared to control horses. However this study has several limitations due to its retrospective nature including FMT protocols and assessment, treatments administered to patients varied, most horses received antibiotics at the time of FMT, and the FMT protocol was inconsistent (Quattrini, et al. 2023). In Chapter 3, resolution of diarrhoea was not significantly different between the FMT group and the control group. However, more foals in the FMT group (68%) had resolution of diarrhoea than foals in the control group (55%).

In Chapter 3, survival to discharge was not significantly different between groups, with 79% of FMT foals surviving and 100% of control group foals surviving. In one study of FMT administration in adult horses, authors reported a 100% survival rate of the FMT recipients

and a 80% survival rate of control horses (McKinney, et al. 2021). In another study, there was no significant differences in survival to discharge in a retrospective evaluation of outcomes in adult horses receiving FMT (75%) compared to control horses (75%) (Quattrini, et al. 2023). In the remaining studies of FMT in adult horses with diarrhoea, FMT recipients were reported to have a survival rate of 75% (Dias, et al. 2018), 66.7 % (Costa, et al. 2021) and 60% (McKinney, et al. 2020a). The results of Chapter 3 show that the outcomes associated with FMT in foals are similar to those reported in adult horses

6.1.8 Limitations

The limitations of Chapter 3 include the small number of animals enrolled, which increase the occurrence of Type 1 statistical errors. The number of animals included is similar or greater to those included in studies investigating FMT administration in adult horses (Costa, et al. 2021; Kinoshita, et al. 2022; McKinney, et al. 2021; McKinney, et al. 2020a). Another limitation includes the inability to standardise treatment regimens of foals, as each animal required individualised care and treatment. The inability to standardise these treatments arguably had a major impact on the microbiota of foals and clinical outcomes. The inclusion of animals from three different locations is also expected to influence the results in Chapter 3. With these limitations in mind, Chapter 3 was undertaken in a clinical setting, where these variables will always present and as such, the outcomes observed accurately reflect the outcomes associated with treatment within true veterinary context.

6.2 Contribution of the research to scientific literature

Chapter 3 is the first study investigating the clinical and microbiota outcomes associated with FMT administration in foals. Diarrhoea is a major cause of morbidity in foals in the first 6 months of life (Frederick, et al. 2009; Urquhart 1981) and with limited targeted treatment options for dysbiosis available (Oliver-Espinosa 2018), the search for improved treatments is important. Reduced antimicrobial usage is a treatment imperative to preserve clinical efficacy

of these drugs, and likely to facilitate restoration of the normal GIT microbiome. Formulation of efficacious targeted treatment options would reduce duration of disease, reduce duration of hospitalisation and reduce cost associated with treatment. This chapter has extrapolated on previous equine FMT preparation and administration studies (Costa, et al. 2021; Dias, et al. 2018; McKinney, et al. 2021; McKinney, et al. 2020b) in adult horses to provide a scaffold for FMT treatment of foals. FMT preparation is quick and cost effective, and administration of the transplantation can be performed by veterinarians in a variety of settings. This chapter also supports that FMT is tolerated well by recipient foals.

The microbiota results show that treatment with FMT was not associated with convincing changes in recipient foals. Chapter 3 also contributed insight into the clinicopathological and clinical changes observed during the study period and support improved haemodynamic status and reduction of inflammation in foals receiving FMT. Resolution of diarrhoea and survival outcomes were not different between groups.

This research provides a good foundation for use of FMT in the treatment of foals with diarrhoea. The findings suggest that administration of FMT may be associated with improvements in haemodynamic status. Although the faecal microbiota and clinical outcomes were not different between groups, a positive impact of FMT may still have occurred. Further investigation into the FMT preparation and administration protocols may refine use of FMT in foals in the future and provide better outcomes of resolution of diarrhoea, survival associated with FMT and changes to the microbiota of recipients. Further investigation into the FMT preparation and administration protocols may refine use of FMT in foals in the future and provide better outcomes of resolution of diarrhoea and survival associated with FMT.

6.3 Preparation and storage of equine FMT

6.3.1 Preparation of FMT

The preparation methods used in both Chapter 3 and 4 were the same and the method was extrapolated from previously described methods (Loublier, et al. 2023; Mullen, et al. 2018). A

number of varied FMT preparation methods have been described (Costa, et al. 2021; Di Pietro, et al. 2023; Kinoshita, et al. 2022; Loublier, et al. 2023; McKinney, et al. 2021; McKinney, et al. 2020a). The majority of methods prepare FMT using manure and warm water (Costa, et al. 2021; Kinoshita, et al. 2022; Loublier, et al. 2023; McKinney, et al. 2021; McKinney, et al. 2020b), however some authors have reported inclusion of saline (Laustsen, et al. 2021b), bicarbonate (Dias, et al. 2018), bismuth subsalicylate (Quattrini, et al. 2023), feed supplements (Quattrini, et al. 2023) and di-tri-octahedral smectite (Quattrini, et al. 2023) in transplantation preparation. In Chapter 3 and 4, the use of a protocol utilising only manure and warm water consistent with the majority of the existing literature adopting this approach. The risk of addition of other substances to FMT could include a negative effect on bacterial viability and changes to the microbiota composition, although these effects are unknown. It has been shown in Chapter 4 that combination of lukewarm water and manure has no effect on the faecal microbiota and this also supported by (Loublier, et al. 2023), where authors observed that there was no effect on bacterial viability.

The ratio of manure to water varies greatly throughout the literature, with a range of 0.1-0.5kg of manure per 1 litre of water previously described (Costa, et al. 2021; Di Pietro, et al. 2023; Dias, et al. 2018; Kinoshita, et al. 2022; Laustsen, et al. 2021b; McKinney, et al. 2021; McKinney, et al. 2020a). In Chapter 3 and 4, the use of 0.3kg of manure per 1 litre of water was based on this existing literature. Following the results of Chapter 3, it would be reasonable to consider an increased ratio of manure to water (0.5kg per 1 litre of water) in future studies. This could facilitate transplantation of a higher concentration of microbiota, and increase the rate of re-establishment of a more normalised gastrointestinal flora.

The processing of water and manure to result in a final FMT filtrate also varies in previous studies (Costa, et al. 2021; Di Pietro, et al. 2023; Kinoshita, et al. 2022; Loublier, et al. 2023; McKinney, et al. 2021; McKinney, et al. 2020b; Mullen, et al. 2018). In Chapters 3 and 4,

manure and warm water were blended with an immersion blender to result in a homogenous mixture. Blending is thought to assist in detaching bacteria closely associated with fibrous material and allow these taxa to be present in the final filtrate in proportions similar to manure (Loublier, et al. 2023). The findings of Chapter 4 supports this theory, where the relative abundance of taxa was the same between fresh faeces and final FMT filtrate, and this is similar to the findings of Loublier et al. 2023. Considering the horse's diet is largely composed of fibrous material, the ability to maintain the bacteria associated with cellulose degradation and hindgut metabolism in the final FMT filtrate would be expected to increase efficacy. Other methods of FMT preparation include mixing manure and water freely (Costa, et al. 2021; Di Pietro, et al. 2023; Laustsen, et al. 2021b; McKinney, et al. 2021) or soaking within a bouffant cap (McKinney, et al. 2021) or muslin cloth (Quattrini, et al. 2023), followed by straining. The effect of these methods on bacterial viability and community and structure of FMT have not been evaluated.

6.3.2 Short term storage of FMT

There are currently no studies assessing the effect of short term storage on the microbiota of equine FMT. The effects of storage of equine faeces at ambient temperatures has shown that storage in these conditions for >6 hours resulted in changes to the faecal microbiota (de Bustamante, et al. 2021), including changes in alpha diversity, beta diversity and relative abundance of taxa (de Bustamante, et al. 2021). In Chapter 4, fresh FMT underwent DNA extraction <6 hours after preparation and consistent with the previously mentioned study, there was no difference in the microbiota of fresh faeces and fresh FMT.

Short term storage of faeces for microbiota analysis has been studied sparingly in veterinary species. Storage of canine and feline faeces at 4°C for 14 days had a limited effect on community membership and structure (Weese and Jalali 2014). Specifically, storage of canine faeces at 4°C for 14 days resulted in significant increases in Actinobacteria and Proteobacteria (Weese and Jalali 2014). In cat faeces, Firmicutes increased by Day 7 and members of the

phyla Proteobacteria became enriched at 14 days of storage at 4°C (Weese and Jalali 2014). In Chapter 4, storage of FMT at 4°C for up to 72 hours had limited to no effect on the relative abundance of individual taxa. The lack of change with short term storage demonstrates the stability of the bacterial community and structure in these conditions.

Storage of faeces at 4°C for up to two weeks had no effect on diversity, evenness or richness in cats and dogs (Weese and Jalali 2014). This is consistent with the results of Chapter 4, where storage was not associated with changes in beta and alpha diversity. In Chapter 4, samples maintained their individual microbial profiles and samples became no more similar or different over time. There is no current existing literature containing information or recommendations for short term storage of equine FMT. The findings of Chapter 4 suggest that short term storage at 4°C for up to 3 days may be a viable option.

6.3.3 Storage of FMT at -20°C

The findings of Chapter 4 showed that there were minimal differences in the relative abundance of taxa associated with storage at -20°C for up to 28 days. The enrichment of family *Erysipelatoclostridaceae* and genus *UCG_004* at FMT0hr suggests that freezing has a negative effect on the abundance of these taxa. However, the role of these taxa within the equine gastrointestinal tract are not currently defined, and it is unknown what effect this may have on clinical efficacy or safety. In addition to this, there was no effect on alpha diversity or beta diversity associated with storage of -20°C through the 4 week study period. The results of Chapter 4 support that there is minimal change of the microbiota of FMT solution with storage at -20°C for 28 days. This finding is of particular interest as there could be scope to store and have FMT solution readily available in a clinical veterinary setting. Although FMT are routinely frozen for use in humans (Gangwani, et al. 2023), the clinical use of frozen FMT in horses has not yet been investigated. In humans, some changes of the microbiota of FMT have been reported with freezing and prolonged storage, however microbial profiles remain

relatively stable (Dorsaz, et al. 2020). Despite the evidence to suggest change in the microbiota associated with storage, the use of frozen stool for faecal microbiota transplantations has high clinical efficacy in humans (Gangwani, et al. 2023). In one study of horses, authors reported that use of frozen equine faeces in FMT preparation was ineffective in preventing antibiotic associated dysbiosis (Kinoshita, et al. 2022). However in that study, horses continued to receive antimicrobials concurrently with FMT, which may have reduced the efficacy of transplantation.

The effect of storage at -20°C on bacterial viability of FMT is another important consideration. In Chapter 4 of this thesis, bacterial viability was not assessed. It is understood that, for FMT to be clinically effective, viable microorganisms need to be present (Kopper, et al. 2021). In one study of horses, storage of FMT at -20°C for up to 4 weeks reduced viability of Gramn negative bacterial viability after 1 week of storage (Kopper, et al. 2021). The impact of storage of FMT solution at 4°C and -20°C on bacterial viability requires further investigation.

6.4 Contribution of the research to scientific literature

Chapter 4 of this thesis details a standardised preparation protocol of equine FMT that can be easily performed by equine clinicians. Although this preparation protocol has been extrapolated from existing literature, this is only the second study published describing the microbiota between fresh manure and FMT preparation in horses. In this thesis, there were no differences in relative abundance, alpha diversity or beta diversity associated with preparation. This provides valuable information supporting the use of the described preparation method in clinical practice. Although oxygen exposure has previously been a concern with aerobic preparation (Loublier, et al. 2023) this did not affect the microbiota in Chapter 4.

Chapter 4 is the first study investigating the effects of storage of equine FMT solution at 4°C and -20°C on the microbiota composition. Chapter 4 of the thesis showed that storage of FMT at 4°C for up to 72 hours and -20°C up to 28 days has minimal effect on the microbiota. There was negligible effect on the relative abundance of taxa and no effect on alpha or beta diversity. These findings support short term storage of FMT in these conditions. Although Chapter 4 details the microbiota profile, the effect of storage on bacterial viability requires further investigation as this is also likely to affect clinical efficacy.

6.5 The use of anthelmintic treatment in horses and effects on the microbiota

Chapter 5 investigated the effects of administration of anthelmintics on the faecal microbiota. The differences in relative abundance between the treatment groups and control group supports previous literature, that anthelmintic administration results in change to the faecal microbiota (Kunz, et al. 2019; Peachey, et al. 2018; Peachey, et al. 2019; Walshe, et al. 2019). A number of the taxa that were reduced with anthelmintic treatment have been associated with intestinal health, and this finding suggests that anthelmintics may have a negative effect of the balance of the microbiota, albeit small. In line with previous studies, the results of Chapter 5 show that overall, there were limited changes to the predominant taxa of the faecal microbiota. Considering the common and wide-spread use of anthelmintics in horses, these are important findings when contemplating revised anthelmintic control programs.

6.5.1 Anthelmintic administration without change to parasite population

Chapter 5 is the first studying investigating the effects of anthelmintic administration in a cyathostomin population where anthelmintic resistance was demonstrated. Chapter 5 demonstrated that the administration of anthelmintics, without subsequent change in parasite burden, causes change to the faecal microbiota. This is similar to the findings of a previous study, where anthelmintic administration affected the faecal microbiota of horses without an observed helminth infection (Kunz, et al. 2019). Oxfendazole was administered in Chapter 5 was used as it was known that the population of cyathostomins included in the

study were resistant to this particular anthelmintic and hence there would be little or no change in cyathostomin infestation in treated horses. It is known that there is variability and inhomogeneity in the faecal egg counts of horses (Wilkes, et al. 2016) and, as such, relying on FEC to determine significant infection can result in both false negatives and false positives. In previous studies, the assumption that low FEC equated to low helminth infection may not have been representative and administration of anthelmintic may still have changed the parasite population in that study (Kunz, et al. 2019). In Chapter 5, the use of an anthelmintic that the population is known to have resistance to ensured that the cyathostomin population within the intestinal tract remained unchanged. FECRT demonstrated resistance to oxfendazole and supported that the cyathostomin population remained unchanged.

6.5.2 Anthelmintic administration with change in parasite population

Chapter 5 provided further understanding of the effects of anthelmintic administration on the faecal microbiota with changes to the helminth population. In Chapter 5, abamectin was used as the cyathostomin population was known to be susceptible to this compound, and this was confirmed on FECR testing. Administration of abamectin and removal of helminths in Chapter 5 resulted in changes in alpha diversity, beta diversity and relative abundance. The results are Chapter 5 are consistent with previous studies, where anthelmintic treatment has resulted in changes in alpha diversity, beta diversity (Peachey, et al. 2018; Walshe, et al. 2019) and relative abundance (Peachey, et al. 2018). The findings suggest that removal of helminths from the intestinal tract has a greater effect on the microbiota than administration of anthelmintic compound alone.

This leads to the important consideration of effect of removal of helminths and the interplay between the gastrointestinal microbiota and host immunity. In horses, the strength and nature of the relationship between host, helminth and the gastrointestinal microbiota is poorly characterised (Boisseau, et al. 2023). It is understood that interactions between the host, gastrointestinal parasites and microbiota is complex and involves mediation of both

direct and indirect interactions through the host's immune system (Boisseau, et al. 2023; Leung, et al. 2018; Rynkiewicz, et al. 2015). Shifts in systemic and local immune responses have been reported with fluctuations in helminth infestations (Bancroft, et al. 2012; Fricke, et al. 2015; Gause and Maizels 2016), however much of the understanding of these interactions is extrapolated from rodent and humans models (Fricke, et al. 2015; Lee, et al. 2021; Peachey, et al. 2018; Walk, et al. 2010). Investigation into the effect of helminth infestation and anthelmintic administration on immune responses was outside the scope of Chapter 5. However, it is important to consider the interplay of helminth, microbiota and host immunity when extrapolating the results in a broader clinical context.

6.5.3 Individual variation and the faecal microbiota

The inclusion of a control group in Chapter 5 allowed for a greater understanding of individual variation. The differences in relative abundances between each group detected prior to anthelmintic treatment, as well as within the control group over time, could be attributed to individual variation or variation in environmental factors. In Chapter 5, adult horses of various breeds and ages were used and, although all horses received the same diet, they were housed in different pastures. It is understood breed can also have an effect on the relative abundance of taxa (Massacci, et al. 2020) as well as seasonal, spatial and social interactions (Garber, et al. 2020). The results of Chapter 5 highlight the significant individual variability that can be present between horses with minimal external influencing factors. Possible approaches that would reduce this variability would include larger group sizes or investigation into the use of other methodologies that can reduce the influence of these variables.

6.6 Contribution of the research to scientific literature

Chapter 5 is the first study of horses investigating the effect of anthelmintics on the microbiota including use of a compound the population is resistant to, as well as inclusion of a control group. The changes in relative abundance and beta diversity in oxfendazole recipients support that administration of anthelmintics without changes in parasite population exerts an

effect on the microbiota. The differences in relative abundance, beta diversity and alpha diversity in horses receiving abamectin showed that removal of helminths has a greater effect on microbial community structure. These findings are consistent with previous studies. The results highlight that, while administration of anthelmintics exerts an effect on the microbiota, removal of helminths leads to more substantial changes.

The methodology of Chapter 5 allowed for further understanding of individual variation of the faecal microbiota and the effect on interpreting results. It highlights the importance of inclusion of a control group, to allow for identification of true differences between groups. This research supports the need for larger group sizes and investigation into bioinformatic technologies that allow for individual animal variation. The findings of this study provide valuable insight into the effect of anthelmintic administration on the relationship between host, helminth and the microbiota.

6.7 Conclusion

The research presented in this thesis provides important contributions to the scientific literature and to the veterinary and equine professions. The results of Chapter 3 pioneer the use of faecal microbiota transplantations in foals.. Outcomes of resolution of diarrhoea and discharge from hospital were not different between FMT recipients and control group foals, however the clinical and clinicopathological results were suggestive of a positive response to FMT administration. Although convincing changes in the relative abundance of taxa were not observed with FMT, a positive effect on the microbiota cannot be discounted. . Chapter 3 provides a protocol of preparation and administration of FMT, including transplant volume and frequency of administration. The results of Chapter 3 also support that administration of FMT to foals <6 months of age is safe.

Chapter 4 provided valuable information on the effects of preparation and storage of equine FMT in different conditions. The results Chapter 4 show that preparation of FMT in aerobic

conditions with an immersion blender had negligible effect on the microbiota. These findings show that preparation of FMT using the described methodology preserves the microbiota of donor horse manure, which is optimal for transplantation. Chapter 4 provided important contribution to the literature by showing that storage of FMT at 4°C for up to 72 hours and -20°C for up to 28 days results in minimal changes to the microbiota. . The lack of significant differences in alpha diversity and beta diversity of FMT stored in either condition suggest that major changes in bacterial community and composition do not occur. Chapter 4 did not investigate the effect of FMT preparation and storage on viability, which would affect clinical efficacy of FMT. The effect of storage on bacterial viability requires further investigation.

Chapter 5 provided further evidence that the administration of anthelmintics affects the faecal microbiota of horses. Chapter 5 is the first study investigating the effect of anthelmintic treatment in a population of cyathostomins where resistance and susceptibility to particular anthelmintics was known. The administration of anthelmintics that the helminth population is resistant to resulted in changes in relative abundance of taxa and beta diversity. The results show that the administration of compound alone affects the microbial community. Chapter 5 showed that administration of anthelmintics and removal of helminths from the gastrointestinal tract resulted in changes in alpha diversity, beta diversity and relative abundance of taxa. The changes to microbiota associated with removal of helminths after anthelmintic treatment were greater than treatment without changes in parasite population. These findings support the recommendation against the indiscriminate use of anthelmintics, and investigation into revised anthelmintic control programs. The results provide a scaffold for understanding of the host, helminth and microbiota relationship, however investigation into the role of host immune responses requires further exploration. Chapter 5 showed that the inclusion of a control group is important when assessing the response of the microbiota to different interventions, to improve determination of true changes in microbial community.

References

Al Jassim, Rafat A. M., and Frank M. Andrews

2009 The Bacterial Community of the Horse Gastrointestinal Tract and Its Relation to Fermentative Acidosis, Laminitis, Colic, and Stomach Ulcers. Veterinary Clinics of North America: Equine Practice 25(2):199-215.

Alhajj, M., M. Zubair, and A. Farhana

2024 Enzyme Linked Immunosorbent Assay. *In* StatPearls. Treasure Island (FL): StatPearls Publishing

Álvarez-Narváez, S., et al.

2020 A Common Practice of Widespread Antimicrobial Use in Horse Production Promotes Multi-Drug Resistance. Scientific reports 10(1):911-911.

Andersen, U. V., et al.

2013 Recent advances in diagnosing pathogenic equine gastrointestinal helminths: The challenge of prepatent detection. Veterinary parasitology 192(1-3):1-9.

Andrews, F. M., et al.

1999 Efficacy of omeprazole paste in the treatment and prevention of gastric ulcers in horses. Equine veterinary journal 31(S29):81-86.

Armstrong, E. A., et al.

2022 Poor body condition is associated with lower hippocampal plasticity and higher gut methanogen abundance in adult laying hens from two housing systems. Sci Rep 12(1):15505.

Armstrong, S. K., et al.

2014 The efficacy of ivermectin, pyrantel and fenbendazole against Parascaris equorum infection in foals on farms in Australia. Veterinary parasitology 205(3-4):575-580.

Arnold, Carolyn E., et al.

2020 The cecal and fecal microbiomes and metabolomes of horses before and after metronidazole administration. PloS one 15(5):e0232905.

Arnold, Carolyn E., et al.

2021 The effects of signalment, diet, geographic location, season, and colitis associated with antimicrobial use or Salmonella infection on the fecal microbiome of horses. Journal of veterinary internal medicine 35(5):2437-2448.

Arroyo, Luis G., et al.

2020 Luminal and Mucosal Microbiota of the Cecum and Large Colon of Healthy and Diarrheic Horses. Animals (Basel) 10(8):1403.

Bai, Guoyun, et al.

2012 Comparison of storage conditions for human vaginal microbiome studies. PloS one 7(5):e36934-e36934.

Bancroft, Allison J., Kelly S. Hayes, and Richard K. Grencis

2012 Life on the edge: the balance between macrofauna, microflora and host immunity. Trends in parasitology 28(3):93-98.

Barr, B. S., et al.

2013 Antimicrobial-associated diarrhoea in three equine referral practices. Equine Veterinary Journal 45(2):154-158.

Bayless, Rosemary

2020 Update: AAEP parasite control guidelines. DVM360 51(6):20-22.

Beckers, Kalie F., Christopher J. Schulz, and Gary W. Childers

2017 Rapid regrowth and detection of microbial contaminants in equine fecal microbiome samples.(Research Article)(Report). PLoS ONE 12(11):e0187044.

Bedarf, J. R., et al.

2017 Functional implications of microbial and viral gut metagenome changes in early stage L-DOPA-naïve Parkinson's disease patients. Genome medicine 9(1):39-39.

Behjati, S., and P. S. Tarpey

2013 What is next generation sequencing? Arch Dis Child Educ Pract Ed 98(6):236-8.

Betancourt, A., E. T. Lyons, and D. W. Horohov

2015 Characterisation of the inflammatory cytokine response to anthelmintic treatment in ponies. Equine veterinary journal 47(2):240-244.

Biagi, Elena, et al.

2010 Through Ageing, and Beyond: Gut Microbiota and Inflammatory Status in Seniors and Centenarians. PloS one 5(5):e10667.

Bilinski, Jaroslaw, et al.

2022 Fresh Versus Frozen Stool for Fecal Microbiota Transplantation—Assessment by Multimethod Approach Combining Culturing, Flow Cytometry, and Next-Generation Sequencing. Frontiers in microbiology 13:872735-872735.

Birkmann, K., et al.

2014 Efficacy of Omeprazole Powder Paste or Enteric-Coated Formulation in Healing of Gastric Ulcers in Horses. Journal of veterinary internal medicine 28(3):925-933.

Boisseau, Michel, et al.

2023 Species interactions, stability, and resilience of the gut microbiota - Helminth assemblage in horses. iScience 26(2):106044-106044.

Bokulich, Nicholas A., et al.

2018 Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. Microbiome 6(1):90-90.

Bolyen, Evan, et al.

2019 Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nature biotechnology 37(8):852-857.

Bordin, Angela I., et al.

2013 Effects of Administration of Live or Inactivated Virulent Rhodococccus equi and Age on the Fecal Microbiome of Neonatal Foals.(Research Article). PLoS ONE 8(6):e66640.

Brandt, Lawrence J., et al.

2012 Long-Term Follow-Up of Colonoscopic Fecal Microbiota Transplant for Recurrent Clostridium difficile Infection. The American journal of gastroenterology 107(7):1079-1087.
Bulmer, Louise S., et al.

2019 High-starch diets alter equine faecal microbiota and increase behavioural reactivity. Scientific reports 9(1):18621-18621.
Burz, Sebastian D., et al.

2019 A Guide for Ex Vivo Handling and Storage of Stool Samples Intended for Fecal Microbiota Transplantation. Scientific reports 9(1):8897-16.
Cain, Jennifer L., et al.

2020 Diagnostic performance of McMaster, Wisconsin, and automated egg counting techniques for enumeration of equine strongyle eggs in fecal samples. Veterinary parasitology 284:109199-109199.
Callahan, Benjamin J., et al.

2016 DADA2: High-resolution sample inference from Illumina amplicon data. Nature methods 13(7):581-583.
Cardona, Silvia, et al.

2012 Storage conditions of intestinal microbiota matter in metagenomic analysis. BMC microbiology 12(1):158.
Carroll, Ian M., et al.

2012 Characterization of the Fecal Microbiota Using High-Throughput Sequencing Reveals a Stable Microbial Community during Storage (Stability of the Fecal Microbiota during Storage). 7(10):e46953.
Cason, Carolina, et al.

2022 Next-generation sequencing and PCR technologies in monitoring the hospital microbiome and its drug resistance. Frontiers in microbiology 13:969863-969863.
Cerri, Simona, et al.

2020 Effect of oral administration of omeprazole on the microbiota of the gastric glandular mucosa and feces of healthy horses. Journal of veterinary internal medicine 34(6):2727-2737.
Chang, Sung-Ho, and Youngnim Choi

2023 Gut dysbiosis in autoimmune diseases: Association with mortality. Frontiers in cellular and infection microbiology 13:1157918-1157918.
Chapuis, Ronan J. J., et al.

2022 Characterisation of faecal microbiota in horses medicated with oral doxycycline hyclate. Equine veterinary journal.
—

2023 Characterisation of faecal microbiota in horses medicated with oral doxycycline hyclate. Equine veterinary journal 55(1):129-141.
Cheminet, G., et al.

2018 Faecal microbiota transplantation with frozen capsules for relapsing Clostridium difficile infections: the first experience from 15 consecutive patients in France. The Journal of hospital infection 100(2):148-151.
Chu, Nathaniel D., et al.

2017 Profiling living bacteria informs preparation of fecal microbiota transplantations. PloS one 12(1):e0170922-e0170922.
Clemente, Jose C., et al.

2015 The microbiome of uncontacted Amerindians. Science advances 1(3):e1500183.
Coles, G. C., et al.

1992 World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) methods for the detection of anthelmintic resistance in nematodes of veterinary importance. Veterinary parasitology 44(1):35-44.
Collobert-Laugier, C., et al.

2002 Prevalence, abundance and site distribution of equine small strongyles in Normandy, France. Veterinary parasitology 110(1):77-83.
Costa, M. C., et al.

2015a Characterization and comparison of the bacterial microbiota in different gastrointestinal tract compartments in horses. The Veterinary Journal 205(1):74-80.
Costa, M. C., et al.

2016a Development of the faecal microbiota in foals. Equine veterinary journal 48(6):681-688.
Costa, M. C., et al.

2016b Development of the faecal microbiota in foals. Equine Veterinary Journal 48(6):681-688.
Costa, M. C., et al.

2015b Changes in the equine fecal microbiota associated with the use of systemic antimicrobial drugs. Bmc Veterinary Research 11(1).
Costa, Marcio C., et al.

2012a Comparison of the fecal microbiota of healthy horses and horses with colitis by high throughput sequencing of the V3-V5 region of the 16s rRNA gene. PloS one 7(7):e41484-e41484.
—

2012b Comparison of the Fecal Microbiota of Healthy Horses and Horses with Colitis by High Throughput Sequencing of the V3-V5 Region of the 16S rRNA Gene (Equine Fecal Microbiota). 7(7):e41484.
Costa, Marcio Carvalho, and Jeffrey Scott Weese

2018 Understanding the Intestinal Microbiome in Health and Disease. Veterinary Clinics of North America: Equine Practice 34(1):1-12.
Costa, Marcio, et al.

2021 Evaluation of changes in microbiota after fecal microbiota transplantation in 6 diarrheic horses. Canadian veterinary journal 62(10):1123-1130.
Costello, S. P., et al.

2015 Faecal microbiota transplant for recurrent Clostridium difficile infection using long-term frozen stool is effective: clinical efficacy and bacterial viability data. Alimentary pharmacology & therapeutics 42(8):1011-1018.
Costello, S. P., et al.

2017 Systematic review with meta-analysis: faecal microbiota transplantation for the induction of remission for active ulcerative colitis. Alimentary pharmacology & therapeutics 46(3):213-224.

Cox, Laura M, et al.

2014 Altering the Intestinal Microbiota during a Critical Developmental Window Has Lasting Metabolic Consequences. Cell (Cambridge) 158(4):705-721.

Craven, J., et al.

1998 Survey of anthelmintic resistance on Danish horse farms, using 5 different methods of calculating faecal egg count reduction. Equine veterinary journal 30(4):289-293.

Cringoli, Giuseppe, et al.

2010 FLOTAC: new multivalent techniques for qualitative and quantitative copromicroscopic diagnosis of parasites in animals and humans. Nature protocols 5(3):503-515.

Cryan, John F., and Timothy G. Dinan

2012 Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. Nature reviews. Neuroscience 13(10):701-712.

Daniels, Simon

2019 Friend or foe? Intestinal parasites of horses and sustainable worm control mechanisms. Veterinary Nursing Journal 34(3):72-77.

Dash, K. M., E. Hall, and I. A. Barger

1988 The role of arithmetic and geometric mean worm egg counts in faecal egg count reduction tests and in monitoring strategic drenching programs in sheep. Australian veterinary journal 65(2):66-8.

de Bustamante, Michelle Martin, et al.

2021 Impact of ambient temperature sample storage on the equine fecal microbiota. Animals (Basel) 11(3):1-10.

De La Torre, Ubaldo, et al.

2019 Utilizing the fecal microbiota to understand foal gut transitions from birth to weaning.(Research Article). PLoS ONE 14(4):e0216211.

Denwood, M. J., et al.

2010 Comparison of three alternative methods for analysis of equine Faecal Egg Count Reduction Test data. Preventive veterinary medicine 93(4):316-323.

Denwood, Matthew J., et al.

2023 A statistical framework for calculating prospective sample sizes and classifying efficacy results for faecal egg count reduction tests in ruminants, horses and swine. Veterinary parasitology 314:109867-109867.

Di Pietro, Rebecca, et al.

2023 Effects of concentrated fecal microbiota transplant on the equine fecal microbiota after antibiotic-induced dysbiosis. Canadian journal of veterinary research 87(2):85-96.

Dias, Deborah P. M., et al.

2018 Efficacy of faecal microbiota transplantation for treating acute colitis in horses undergoing colic surgery. Pesquisa veterinária brasileira 38(8):1564-1569.

Dorsaz, Stéphane, et al.

2020 Changes in Microbiota Profiles After Prolonged Frozen Storage of Stool Suspensions. Frontiers in cellular and infection microbiology 10:77-77.

Dougal, Kirsty, et al.

2014 Characterisation of the Faecal Bacterial Community in Adult and Elderly Horses Fed a High Fibre, High Oil or High Starch Diet Using 454 Pyrosequencing.(Research Article). PLoS ONE 9(2):e87424.

Dougal, Kirsty, et al.

2013a Identification of a core bacterial community within the large intestine of the horse. PloS one 8(10):e77660.

—

2013b Identification of a Core Bacterial Community within the Large Intestine of the Horse.(Research Article). PLoS ONE 8(10):e77660.

Dougal, Kirsty, et al.

2012 A comparison of the microbiome and the metabolome of different regions of the equine hindgut. FEMS Microbiology Ecology 82(3):642-652.

Dubik, Magdalena, Bartosz Pilecki, and Jesper Bonnet Moeller

2022 Commensal Intestinal Protozoa—Underestimated Members of the Gut Microbial Community. Biology (Basel, Switzerland) 11(12):1742.

Dyce, K. M.

1996 Textbook of veterinary anatomy. Philadelphia: Saunders.

Elzinga, Sarah E., J. Scott Weese, and Amanda A. Adams

2016 Comparison of the Fecal Microbiota in Horses With Equine Metabolic Syndrome and Metabolically Normal Controls Fed a Similar All-Forage Diet. Journal of equine veterinary science 44:9-16.

Ericsson, Aaron C., et al.

2016 A Microbiological Map of the Healthy Equine Gastrointestinal Tract.(Research Article). PLoS ONE 11(11):e0166523.

Everard, Amandine, et al.

2013 Cross-talk between Akkermansia muciniphila and intestinal epithelium controls diet-induced obesity. Proceedings of the National Academy of Sciences - PNAS 110(22):9066-9071.

Faubladier, C., et al.

2013 Effect of transportation on fecal bacterial communities and fermentative activities in horses: impact of Saccharomyces cerevisiae CNCM I-1077 supplementation. Journal of animal science 91(4):1736-1744.

Fernandes, Karlette A., et al.

2014 Faecal Microbiota of Forage-Fed Horses in New Zealand and the Population Dynamics of Microbial Communities following Dietary Change (Faecal Microbiota of Forage-Fed Horses). 9(11):e112846.

Flint, Harry J., et al.

2012 The role of the gut microbiota in nutrition and health. Nature reviews. Gastroenterology & hepatology 9(10):577-589.

Fouhy, Fiona, et al.

2015 The Effects of Freezing on Faecal Microbiota as Determined Using MiSeq Sequencing and Culture-Based Investigations. PloS one 10(3):e0119355.
Frederick, J., S. Giguère, and L. C. Sanchez

2009 Infectious Agents Detected in the Feces of Diarrheic Foals: A Retrospective Study of 233 Cases (2003–2008. Journal of Veterinary Internal Medicine 23(6):1254-1260.
Fricke, W. Florian, et al.

2015 Type 2 immunity-dependent reduction of segmented filamentous bacteria in mice infected with the helminthic parasite Nippostrongylus brasiliensis. Microbiome 3(1):40-40.
Gangwani, Manesh Kumar, et al.

2023 Fresh Versus Frozen Versus Lyophilized Fecal Microbiota Transplant for Recurrent Clostridium Difficile Infection: A Systematic Review and Network Meta-analysis. Journal of clinical gastroenterology 57(3):239-245.
Garber, Anna, Peter Hastie, and Jo-Anne Murray

2020 Factors Influencing Equine Gut Microbiota: Current Knowledge. Journal of Equine Veterinary Science 88.
Garibyan, Lilit, and Nidhi Avashia

2013 Research Techniques Made Simple: Polymerase Chain Reaction (PCR). Journal of investigative dermatology 133(3):e6-e6.
Garner, H. E., et al.

1978 Changes in the Caecal Flora associated with the Onset of Laminitis. Equine Veterinary Journal 10(4):249-252.
Gause, William C., and Rick M. Maizels

2016 Macrobiota — helminths as active participants and partners of the microbiota in host intestinal homeostasis. Current opinion in microbiology 32:14-18.
Gayle, J. M., N. D. Cohen, and M. K. Chaffin

1998 Factors associated with survival in septicemic foals: 65 cases (1988-1995). Journal of veterinary internal medicine 12(3):140-146.
Glatter, M., et al.

2019 Modification of the equine gastrointestinal microbiota by Jerusalem artichoke meal supplementation. PloS one 14(8):e0220553-e0220553.
Gomez, Diego, et al.

2023 Longitudinal effects of oral administration of antimicrobial drugs on fecal microbiota of horses. Journal of veterinary internal medicine.
Grimm, P., C. Philippeau, and V. Julliand

2017 Faecal parameters as biomarkers of the equine hindgut microbial ecosystem under dietary change. 11(7):1136-1145.
Grinberg, A., et al.

2009 A study of neonatal cryptosporidiosis of foals in New Zealand. New Zealand veterinary journal 57(5):284-289.
Guzzo, Gina L., Jane M. Andrews, and Laura S. Weyrich

2022 The Neglected Gut Microbiome: Fungi, Protozoa, and Bacteriophages in Inflammatory Bowel Disease. Inflammatory bowel diseases 28(7):1112-1122.

Hansen, Naja C. K., et al.

2015 High nutrient availability reduces the diversity and stability of the equine caecal microbiota. Microbial Ecology in Health and Disease 26(1).

Harlow, Brittany E., Laurie M. Lawrence, and Michael D. Flythe

2013 Diarrhea-associated pathogens, lactobacilli and cellulolytic bacteria in equine feces: Responses to antibiotic challenge. Veterinary Microbiology 166(1-2):225-232.

Harlow, Brittany E., et al.

2016 Effect of Dietary Starch Source and Concentration on Equine Fecal Microbiota.(Report). PLoS ONE 11(4).

Hart, Marcia L., et al.

2015 Comparative Evaluation of DNA Extraction Methods from Feces of Multiple Host Species for Downstream Next-Generation Sequencing.(Report). 10(11).

Hu, Dini, et al.

2021 Metagenomic Analysis of Fecal Archaea, Bacteria, Eukaryota, and Virus in Przewalski's Horses Following Anthelmintic Treatment. Frontiers in veterinary science 8:708512-708512.

Husso, A., et al.

2020 The composition of the perinatal intestinal microbiota in horse. Scientific reports 10(1):441-441.

Ianiro, Gianluca, et al.

2019 Systematic review with meta-analysis: efficacy of faecal microbiota transplantation for the treatment of irritable bowel syndrome. Alimentary pharmacology & therapeutics 50(3):240-248.

Jiang, Zhi-Dong, et al.

2018 Safety and preliminary efficacy of orally administered lyophilized fecal microbiota product compared with frozen product given by enema for recurrent Clostridium difficile infection: A randomized clinical trial.(Research Article). PLoS ONE 13(11):e0205064.

John, Jenny, et al.

2015 Development of intestinal microflora and occurrence of diarrhoea in sucking foals: effects of Bacillus cereus var. toyoi supplementation. BMC Veterinary Research 11(1).

Julliand, Veronique, and Pauline Grimm

2017 The Impact of Diet on the Hindgut Microbiome. Journal of equine veterinary science 52:23-28.

Kamada, Nobuhiko, et al.

2013 Role of the gut microbiota in immunity and inflammatory disease. Nature reviews. Immunology 13(5):321-335.

Kanehisa, Minoru, et al.

2023 KEGG for taxonomy-based analysis of pathways and genomes. Nucleic acids research 51(1):D587-D592.

Kaplan, R. M., and M. K. Nielsen

2010 An evidence-based approach to equine parasite control: It ain't the 60s anymore. Equine veterinary education 22(6):306-316.

Kaplan, Ray M., et al.

2023 World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) guideline for diagnosing anthelmintic resistance using the faecal egg count reduction test in ruminants, horses and swine. Veterinary parasitology 318:109936-109936.

Kim, Lisa'Marie, et al.

2001 Factors associated with Salmonella shedding among equine colic patients at a veterinary teaching hospital. Journal of the American Veterinary Medical Association 218(5):740-748.

Kinoshita, Yuta, et al.

2022 Simultaneous Daily Fecal Microbiota Transplantation Fails to Prevent Metronidazole-Induced Dysbiosis of Equine Gut Microbiota. Journal of equine veterinary science 114:104004-104004.

Knight, Rob, et al.

2010 QIIME allows analysis of high-throughput community sequencing data. Nature Methods 7(5):335-336.

Kopper, Jamie J., et al.

2021 In Vitro Evaluation of the Effect of Storage at −20°C and Proximal Gastrointestinal Conditions on Viability of Equine Fecal Microbiota Transplant. Journal of equine veterinary science 98:103360-103360.

Kovač, Milomir, et al.

2017 Survival and Evaluation of Clinical and Laboratory Variables as Prognostic Indicators in Horses Hospitilized with Acute Diarrhea: 342 Cases (1995-2015). Acta veterinaria (Beograd) 67(3):356-365.

Kunde, Sachin, et al.

2013 Safety, Tolerability, and Clinical Response After Fecal Transplantation in Children and Young Adults With Ulcerative Colitis. Journal of Pediatric Gastroenterology and Nutrition 56(6):597-601.

Kunz, Isabelle G. Z., et al.

2019 Equine Fecal Microbiota Changes Associated With Anthelmintic Administration. Journal of Equine Veterinary Science 77:98-106.

Laforest-Lapointe, Isabelle, and Marie-Claire Arrietaa

2018 Microbial eukaryotes: A missing link in gut microbiome studies. mSystems 3(2).

Laghi, Luca, et al.

2018 Probiotic supplementation in trained trotter horses: Effect on blood clinical pathology data and urine metabolomic assessed in field. Journal of applied physiology (1985) 125(2):654-660.

Langille, Morgan G. I., et al.

2013 Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nature biotechnology 31(9):814-821.

Lauber, Christian L., et al.

2010 Effect of storage conditions on the assessment of bacterial community structure in soil and human-associated samples. FEMS Microbiology Letters 307(1):80-86.

Laustsen, Louise, et al.

2021a Free faecal water: Analysis of horse faecal microbiota and the impact of faecal microbial transplantation on symptom severity. Animals (Basel) 11(10):2776.

Laustsen, Louise, et al.

2021b Free Faecal Water: Analysis of Horse Faecal Microbiota and the Impact of Faecal Microbial Transplantation on Symptom Severity. Animals (Basel) 11(10):2776.

Lawson, April L., Fernando Malalana, and Tim S. Mair

2023 Larval cyathostominosis: Clinicopathological data and treatment outcomes of 38 hospitalised horses (2009–2020). Equine veterinary education 35(8):424-435.

Leclere, Mathilde, and Marcio C. Costa

2020 Fecal microbiota in horses with asthma. Journal of Veterinary Internal Medicine 34(2):996-1006.

Lee, Christine H., et al.

2016 Frozen vs Fresh Fecal Microbiota Transplantation and Clinical Resolution of Diarrhea in Patients With Recurrent Clostridium difficile Infection: A Randomized Clinical Trial. JAMA : the journal of the American Medical Association 315(2):142-149.

Lee, Soo Ching, et al.

2021 Correction to: Helminth colonization is associated with increased diversity of the gut microbiota (PLoS Neglected Tropical Diseases). PLoS neglected tropical diseases 8(5):e0009325-e0009325.

Leng, Joy, et al.

2021 Hay vs. haylage; forage type influences the equine urinary metabonome and faecal microbiota. Equine veterinary journal.

Leng, Joy, et al.

2018 Exploration of the Fecal Microbiota and Biomarker Discovery in Equine Grass Sickness. Journal of proteome research 17(3):1120-1128.

Lerner, Aaron, Rustam Aminov, and Torsten Matthias

2016 Dysbiosis may trigger autoimmune diseases via inappropriate post-translational modification of host proteins. Frontiers in microbiology 7:84-84.

Leung, Jacqueline M., Andrea L. Graham, and Sarah C. L. Knowles

2018 Parasite-microbiota interactions with the vertebrate gut: Synthesis through an ecological lens. Frontiers in microbiology 9(MAY):843-843.

Li, Minjuan, et al.

2020 Signatures within esophageal microbiota with progression of esophageal squamous cell carcinoma. Chinese journal of cancer research 32(6):755-767.

Li, Yaonan, et al.

2022 Comparative Analysis of Gut Microbiota Between Healthy and Diarrheic Horses. Frontiers in veterinary science 9:882423-882423.

Liepman, Rachel S., et al.

2022 Effects of Intravenous Antimicrobial Drugs on the Equine Fecal Microbiome. Animals (Basel) 12(8):1013.

Liu, Yuhong, et al.

2021 Faecal microbiota and antimicrobial resistance gene profiles of healthy foals. Equine veterinary journal 53(4):806-816.

Loublier, Clémence, et al.

2023 Evaluation of Bacterial Composition and Viability of Equine Feces after Processing for Transplantation. Microorganisms (Basel) 11(2):231.

Lurie-Weinberger, Mor N., and Uri Gophna

2015 Archaea in and on the Human Body: Health Implications and Future Directions. PLoS pathogens 11(6):e1004833-e1004833.

MÄKivuokko, Harri, et al.

2010 The effect of age and non-steroidal anti-inflammatory drugs on human intestinal microbiota composition. British journal of nutrition 103(2):227-234.

Martinez-Fernandez, Gonzalo, et al.

2020 Seasonal and nutrient supplement responses in rumen microbiota structure and metabolites of tropical rangeland cattle. Microorganisms (Basel) 8(10):1-20.

Massacci, Francesca Romana, et al.

2020 Inter-breed diversity and temporal dynamics of the faecal microbiota in healthy horses. Journal of Animal Breeding and Genetics 137(1):103-120.

Matthews, Jacqui, and Hannah Lester

2015 Control of equine nematodes: making the most of faecal egg counts. In practice (London 1979) 37(10):540-544.

Mazzawi, Tarek, et al.

2018 The kinetics of gut microbial community composition in patients with irritable bowel syndrome following fecal microbiota transplantation. PloS one 13(11):e0194904-e0194904.

McKay, Derek M.

2009 The therapeutic helminth? Trends in parasitology 25(3):109-114.

McKenna, P. B.

2006 A comparison of faecal egg count reduction test procedures. New Zealand veterinary journal 54(4):202-203.

McKinney, Caroline A., et al.

2021 Assessment of clinical and microbiota responses to fecal microbial transplantation in adult horses with diarrhea. PloS one 16(1):e0244381-e0244381.

McKinney, Caroline A., et al.

2020a The fecal microbiota of healthy donor horses and geriatric recipients undergoing fecal microbial transplantation for the treatment of diarrhea. PloS one 15(3):e0230148-e0230148.

—

2020b The fecal microbiota of healthy donor horses and geriatric recipients undergoing fecal microbial transplantation for the treatment of diarrhea. PloS one 15(3):e0230148.

Metcalf, Jessica L., et al.

2017a Evaluating the impact of domestication and captivity on the horse gut microbiome. Scientific reports 7(1):15497-9.

Metcalf, Jessica, et al.

2017b Evaluating the impact of domestication and captivity on the horse gut microbiome. Sci Rep 7(1):15497-15497.

Milinovich, Gabriel J., et al.

2008 Microbial ecology of the equine hindgut during oligofructose-induced laminitis. The ISME Journal 2(11):1089-1100.

Mines, J. J.

1977 MODIFICATIONS OF THE McMASTER WORM EGG COUNTING METHOD. Australian veterinary journal 53(7):342-343.

Morariu, Sorin, et al.

2016 The prevalence, abundance and distribution of cyathostomins (small stongyles) in horses from Western Romania. Veterinary parasitology 223:205-209.

Mshelia, Esther Solomon, et al.

2018 The association between gut microbiome, sex, age and body condition scores of horses in Maiduguri and its environs. Microbial Pathogenesis 118:81-86.

Mullen, K. R., et al.

2018 Equine faecal microbiota transplant: Current knowledge, proposed guidelines and future directions. Equine Veterinary Education 30(3):151-160.

Nápravníková, J., et al.

2019 Reliability of three common fecal egg counting techniques for detecting strongylid and ascarid infections in horses. Veterinary parasitology 272:53-57.

Netherwood, T., et al.

1996 Foal diarrhoea between 1991 and 1994 in the United Kingdom associated with Clostridium perfringens, rotavirus, Strongyloides westeri and Cryptosporidium spp. Epidemiol Infect 117(2):375-83.

Nielsen, M. K.

2022 Parasite faecal egg counts in equine veterinary practice. Equine veterinary education 34(11):584-591.

Nielsen, M. K., et al.

2015a Local and systemic inflammatory and immunologic reactions to cyathostomin larvicidal therapy in horses. Veterinary immunology and immunopathology 168(3-4):203-210.

Nielsen, M. K., et al.

2015b Interaction between anthelmintic treatment and vaccine responses in ponies naturally infected with cyathostomins. Veterinary immunology and immunopathology 164(3-4):110-117.

O' Donnell, M. M., et al.

2013 The core faecal bacterial microbiome of I rish T horoughbred racehorses. Letters in Applied Microbiology 57(6):492-501.

Oliveira, Luiz G. S., et al.

2019 Clinical and pathological findings of Rhodococcus equi infection in foals. Pesquisa veterinária brasileira 39(11):849-857.

Oliver-Espinosa, Olimpo

2018 Foal Diarrhea: Established and Postulated Causes, Prevention, Diagnostics, and Treatments. The Veterinary clinics of North America. Equine practice 34(1):55-68.

Parraga, M. E., et al.

1997 A clinical trial of probiotic administration for prevention of Salmonella shedding in the postoperative period in horses with colic. Journal of veterinary internal medicine 11(1):36-41.

Peachey, L. E., et al.

2018 The relationships between faecal egg counts and gut microbial composition in UK Thoroughbreds infected by cyathostomins. International Journal for Parasitology 48(6):403-412.

Peachey, Laura E., et al.

2019 Dysbiosis associated with acute helminth infections in herbivorous youngstock - observations and implications. Scientific reports 9(1):11121-16.

Pereira, Giorgio Q., et al.

2018 Fecal microbiota transplantation in puppies with canine parvovirus infection. Journal of veterinary internal medicine 32(2):707-711.

Perkins, G. A., et al.

2012 Equine Stomachs Harbor an Abundant and Diverse Mucosal Microbiota. Applied and Environmental Microbiology 78(8):2522.

Perry, Erin, et al.

2018 Effect of Road Transport on the Equine Cecal Microbiota. Journal of Equine Veterinary Science 68:12-20.

Phillips, Sarah, et al.

2022 A protocol paper for the MOTION Study—A longitudinal study in a cohort aged 60 years and older to obtain mechanistic knowledge of the role of the gut microbiome during normal healthy ageing in order to develop strategies that will improve lifelong health and wellbeing. PloS one 17(11):e0276118-e0276118.

Pickard, Joseph M., et al.

2017 Gut microbiota: Role in pathogen colonization, immune responses, and inflammatory disease. Immunological reviews 279(1):70-89.

Pook, J. F., et al.

2002 Evaluation of tests for anthelmintic resistance in cyathostomes. Veterinary parasitology 106(4):331-43.

Presland, S. L., E. R. Morgan, and G. C. Coles

2005 Counting nematode eggs in equine faecal samples. Veterinary record 156(7):208-210.

Proudman, C. J., et al.

2005 Factors affecting long-term survival of horses recovering from surgery of the small intestine. Equine veterinary journal 37(4):360-365.

Quattrini, Camilla, et al.

2023 Fecal microbiota transplant for treatment of diarrhea in adult hospitalized horses—111 cases (2013–2018). Open veterinary journal (Tripoli, Libya) 13(9):1135-1140.

Reinemeyer, C. R., and M. K. Nielsen

2014 Review of the biology and control of Oxyuris equi. Equine veterinary education 26(11):584-591.

Reinemeyer, Craig R., and Martin Krarup Nielsen

2009 Parasitism and Colic. The Veterinary clinics of North America. Equine practice 25(2):233-245.

Ren, Qingmiao, et al.

2020 Bacterial communities in the solid, liquid, dorsal, and ventral epithelium fractions of yak (Bos grunniens) rumen. MicrobiologyOpen (Weinheim) 9(2):e963-n/a.

Rideout, Jai Ram, et al.

2019 Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nature biotechnology 37(8):852-857.

Rodriguez, Cristina, et al.

2015 Faecal microbiota characterisation of horses using 16 rDNA barcoded pyrosequencing, and carriage rate of clostridium difficile at hospital admission. BMC microbiology 15(1):181-181.

Roesch, Luiz F. W., et al.

2009 Influence of fecal sample storage on bacterial community diversity. The open microbiology journal 3(1):40-46.

Rynkiewicz, Evelyn C., Amy B. Pedersen, and Andy Fenton

2015 An ecosystem approach to understanding and managing within-host parasite community dynamics. Trends in parasitology 31(5):212-221.

Salem, Shebl E., et al.

2018 Variation in faecal microbiota in a group of horses managed at pasture over a 12-month period. Scientific reports 8(1):8510-8510.

Sangster, N. C.

1999 Pharmacology of anthelmintic resistance in cyathostomes: will it occur with the avermectin/milbemycins? Veterinary parasitology 85(2):189-204.

Scantlebury, C. E., et al.

2015 Management and horse-level risk factors for recurrent colic in the UK general equine practice population. Equine veterinary journal 47(2):202-206.

Schloss, Patrick D., et al.

2009 Introducing mothur: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing Microbial Communities. Applied and Environmental Microbiology 75(23):7537-7541.

Schoster, A., et al.

2016a The longitudinal effect of a multi-strain probiotic on the intestinal bacterial microbiota of neonatal foals. Equine Veterinary Journal 48(6):689-696.

Schoster, A., et al.

2016b Effects of transport, fasting and anaesthesia on the faecal microbiota of healthy adult horses. Equine Veterinary Journal 48(5):595-602.

Schoster, A., et al.

2015 Effect of a Probiotic on Prevention of Diarrhea and Clostridium difficile and Clostridium perfringens Shedding in Foals. Journal of Veterinary Internal Medicine 29(3):925-931.

Schoster, A., et al.

2017 Comparison of the fecal bacterial microbiota of healthy and diarrheic foals at two and four weeks of life. BMC veterinary research 13(1):144-144.

Schoster, Angelika, et al.

2013 Comparison of microbial populations in the small intestine, large intestine and feces of healthy horses using terminal restriction fragment length polymorphism.(Report). BMC Research Notes 6(1).

Shahinas, Dea, et al.

2012 Toward an Understanding of Changes in Diversity Associated with Fecal Microbiome Transplantation Based on 16S rRNA Gene Deep Sequencing. mBio 3(5).

Shaw, Sarah D., and Henry Stämpfli

2018 Diagnosis and Treatment of Undifferentiated and Infectious Acute Diarrhea in the Adult Horse. The Veterinary clinics of North America. Equine practice 34(1):39-53.

Singh, Tanveer, et al.

2022 Fecal Microbiota Transplantation and Medical Therapy for Clostridium difficile Infection: Meta-analysis of Randomized Controlled Trials. Journal of clinical gastroenterology 56(10):881-888.

Slovis, N. M., et al.

2014 Infectious agents associated with diarrhoea in neonatal foals in central K entucky: A comprehensive molecular study. Equine Veterinary Journal 46(3):311-316.

Slusarewicz, Paul, et al.

2016 Automated parasite faecal egg counting using fluorescence labelling, smartphone image capture and computational image analysis. International journal for parasitology 46(8):485-493.

Steinbach, Tanja, et al.

2006 Small strongyle infection: Consequences of larvicidal treatment of horses with fenbendazole and moxidectin. Veterinary parasitology 139(1):115-131.

Stewart, H. L., et al.

2018 Characterization of the fecal microbiota of healthy horses. American Journal of Veterinary Research 79(8):811-819.

Stewart, H. L., et al.

2019 Differences in the equine faecal microbiota between horses presenting to a tertiary referral hospital for colic compared with an elective surgical procedure. Equine Veterinary Journal 51(3):336-342.

Stewart, Holly L., et al.

2021 Changes in the faecal bacterial microbiota during hospitalisation of horses with colic and the effect of different causes of colic. Equine veterinary journal 53(6):1119-1131.

Stoll, Norman R.

1930 On Methods of counting Nematode Ova in Sheep Dung. Parasitology 22(1):116-136.

Ströbel, Christina, et al.

2018 Effects of oral supplementation of probiotic strains of Lactobacillus rhamnosus and Enterococcus faecium on diarrhoea events of foals in their first weeks of life. Journal of Animal Physiology and Animal Nutrition 102(5):1357-1365.

Swarthout, H., et al.

2017 Effect of probiotic administration on diarrhea incidence, severity, and the fecal microbiome in neonatal foals. Journal of Equine Veterinary Science 52:83-83.

Tanabe, Soichi, et al.

2014a Anti-inflammatory and Intestinal Barrier–protective Activities of Commensal Lactobacilli and Bifidobacteria in Thoroughbreds: Role of Probiotics in Diarrhea Prevention in Neonatal Thoroughbreds. Journal of equine science 25(2):37-43.

Tanabe, Soichi, et al.

2014b Anti-inflammatory and Intestinal Barrier-protective Activities of Commensal Lactobacilli and Bifidobacteria in Thoroughbreds. Journal of Equine Science 25(2):37.

Tap, Julien, et al.

2019 Effects of the long-term storage of human fecal microbiota samples collected in RNAlater. Scientific reports 9(1):601-601.

Tedjo, Danyta I., et al.

2015 The effect of sampling and storage on the fecal microbiota composition in healthy and diseased subjects. PloS one 10(5):e0126685-e0126685.

Theelen, Mathijs J. P., et al.

2023 Longitudinal study of the short- and long-term effects of hospitalisation and oral trimethoprim-sulfadiazine administration on the equine faecal microbiome and resistome. Microbiome 11(1):33-33.

Theelen, Mathijs J. P., et al.

2021 The Equine Faecal Microbiota of Healthy Horses and Ponies in The Netherlands: Impact of Host and Environmental Factors. Animals (Basel) 11(6):1762.

Tlaskalová-Hogenová, Helena, et al.

2011 The role of gut microbiota (commensal bacteria) and the mucosal barrier in the pathogenesis of inflammatory and autoimmune diseases and cancer: contribution of germ-free and gnotobiotic animal models of human diseases. Cellular & Molecular Immunology 8(2):110-120.

Torgerson, Paul R., Michaela Paul, and Fraser I. Lewis

2012 The contribution of simple random sampling to observed variations in faecal egg counts. Veterinary Parasitology 188(3):397-401.

Tuniyazi, Maimaiti, Wenqing Wang, and Naisheng Zhang

2023 A Systematic Review of Current Applications of Fecal Microbiota Transplantation in Horses. Veterinary sciences 10(4):290.

Tyma, Jesse F., et al.

2019 Investigation of effects of omeprazole on the fecal and gastric microbiota of healthy adult horses. American journal of veterinary research 80(1):79-86.

Uejima, Motoo, et al.

1996 Role of Intestinal Bacteria in Ileal Ulcer Formation in Rats Treated with a Nonsteroidal Antiinflammatory Drug. Microbiology and immunology 40(8):553-560.

Urquhart, Ken

1981 Diarrhoea in foals. In practice (London 1979) 3(1):22-29.

Urubschurov, Vladimir, et al.

2019 Effect of oral supplementation of probiotic strains of Lactobacillus rhamnosus and Enterococcus faecium on the composition of the faecal microbiota of foals. Journal of Animal Physiology and Animal Nutrition 103(3):915.

Vacca, Mirco, et al.

2020 The controversial role of human gut lachnospiraceae. Microorganisms (Basel) 8(4):573.

Van Weyenberg, S., J. Sales, and Gpj Janssens

2006 Passage rate of digesta through the equine gastrointestinal tract: A review. Livestock Science 99(1):3-12.

Venable, Erin B., et al.

2016 Role of the gut microbiota in equine health and disease. Animal frontiers 6(3):43-49.

Walk, Seth T., et al.

2010 Alteration of the murine gut microbiota during infection with the parasitic helminth Heligmosomoides polygyrus. Inflammatory Bowel Diseases 16(11):1841-1849.

Walshe, Nicola, et al.

2019 Removal of adult cyathostomins alters faecal microbiota and promotes an inflammatory phenotype in horses. International Journal for Parasitology 49(6):489-500.

Walshe, Nicola, et al.

2021 Outbreak of acute larval cyathostominosis – A “perfect storm” of inflammation and dysbiosis. Equine veterinary journal 53(4):727-739.

Wang, Likuan, Xudong Yang, and Haiyin Wu

2019 Juvenile Rats Show Altered Gut Microbiota After Exposure to Isoflurane as Neonates. Neurochemical research 44(4):776-786.

Weese, J. S., et al.

2004 Screening of the equine intestinal microflora for potential probiotic organisms. Equine veterinary journal 36(4):351-355.

Weese, J. S., et al.

2015 Changes in the faecal microbiota of mares precede the development of post partum colic. Equine Veterinary Journal 47(6):641-649.

Weese, J. Scott, et al.

2003 Preliminary investigation of the probiotic potential of Lactobacillus rhamnosus strain GG in horses: Fecal recovery following oral administration and safety. Canadian veterinary journal 44(4):299-302.

Weese, J. Scott, and Mohammad Jalali

2014 Evaluation of the impact of refrigeration on next generation sequencing-based assessment of the canine and feline fecal microbiota. BMC veterinary research 10(1):230-230.

Weese, J. Scott, and Joyce Rousseau

2005 Evaluation of Lactobacillus pentosus WE7 for prevention of diarrhea in neonatal foals. Journal of the American Veterinary Medical Association 226(12):2031-2034.

Wensel, Caroline R., et al.

2022 Next-generation sequencing: insights to advance clinical investigations of the microbiome. The Journal of clinical investigation 132(7).

Whitfield-Cargile, Canaan M., et al.

2018 Differential effects of selective and non-selective cyclooxygenase inhibitors on fecal microbiota in adult horses.(Research Article). PLoS ONE 13(8):e0202527.

Whitlock, H. V.

1950 A. Technique for Counting Trematode Eggs in Sheep Faeces. Journal of helminthology 24(1-2):47-52.

Wilkes, E. J. A., et al.

2016 Inhomogeneity of the density of Parascaris spp. eggs in faeces of individual foals and the use of hypothesis testing for treatment decision making. Veterinary parasitology 229:131-138.

Wj

1996 Second Edition K.M. Dyce Textbook of Veterinary Anatomy 1996 W.B. Saunders 869 published by pages; 1810 illustrations; US$82.00. Journal of equine veterinary science 16(3):124-124.

Wong, D. M., et al.

2009 Impaired Intestinal Absorption of Glucose in 4 Foals with Lawsonia intracellularis Infection. Journal of veterinary internal medicine 23(4):940-944.

Woodmansey, E. J., et al.

2004 Comparison of Compositions and Metabolic Activities of Fecal Microbiotas in Young Adults and in Antibiotic-Treated and Non-Antibiotic-Treated Elderly Subjects. Applied and environmental microbiology 70(10):6113-6122.

Yap, George S., and William C. Gause

2018 Helminth infections induce tissue tolerance mitigating immunopathology but enhancing microbial pathogen susceptibility. Frontiers in immunology 9:2135-2135.

Yatsunenko, Tanya, et al.

2012 Human gut microbiome viewed across age and geography. Nature (London) 486(7402):222-227.

Youngster, Ilan, et al.

2014 Fecal Microbiota Transplant for Relapsing Clostridium difficile Infection Using a Frozen Inoculum From Unrelated Donors: A Randomized, Open-Label, Controlled Pilot Study. Clinical infectious diseases 58(11):1515-1522.

Yue, Yuanxi, et al.

2022 Comparative analysis of haematological, biochemical and nutritional parameters of Dezhou donkey with healthy and weak foals (Equus asinus). Italian journal of animal science 21(1):1140-1150.

Yuyama, Teruhiko, et al.

2004 Evaluation of a host-specific Lactobacillus probiotic in neonatal foals. Journal of Applied Research in Veterinary Medicine 2(1):26.

Zavistanaviciute, P., et al.

2019 The influence of the newly isolated Lactobacillus plantarum LUHS135 and Lactobacillus paracasei LUHS244 strains on blood and faeces parameters in endurance horses. Polish journal of veterinary sciences 22(3):513-521.

Zoetendal, E. G., E. E. Vaughan, and W. M. de Vos

2006 A microbial world within us. Molecular microbiology 59(6):1639-1650.

Appendix 1

Clinical and clinicopathological results of foals in the FMT or control group.

The results obtained throughout the study period.

Comparison of clinical and clinicopathological findings between Control and faecal microbiota transplantation (FMT) groups throughout the study period. Results are presented as median (range) or mean ± standard deviation.

Variable	D0		D1		D2		D3		D7	
	Control (*n*=9)	FMT (*n*=19)	Control (*n*=9)	FMT (*n*=19)	Control (*n*=9)	FMT (*n*=19)	Control (*n*=9)	FMT (*n*=19)	Control (*n*=6)	FMT (*n*=9)
Heart Rate (bpm)	88 (76-124)	80 (60-150)	68 (52-100)	74 (56-120)	88 (50-156)	70 (52-110)	68 (44-104)	64 (54-102)	60 (48-100)	60 (60-80)
Respiratory Rate (brpm)	28* (16-52)	20 (12-44)	20 (12-40)	23 (12-40)	24 (12-32)	20 (12-40)	20 (16-28)	20.5 (16-44)	24 (16-40)	28 (12-40)
Temperature (°F)	100.9 (100.5-103.6)	101.5 (99.9-104.5)	100.2 (99.5-101.1)	100.9 (99-102.2)	100.8 (99.1-101.8)	100.8 (99-102.6)	100.8 (99.1-101.5)	100.2 (98.8-103.5)	101.1 (100.4-103.5)	100.6 (99.5-101.8)
White blood cells (g/L)	7.2 (2.7-13.5)	8.2 (1.9-21.1)	8.7 (8.7-8.7)	11.2 (10.6-11.8)	7 (7-7)	13.1 (2.7-15.6)	14.3* (6.7-18.9)	6.4 (5.0-8.3)	NA	NA
Neutrophil (g/L)	3.6 (0.2-11.2)	5.3 (0.7-14.5)	NA	NA	NA	NA	7.6 (4.9-16.6)	4.4 (3.7-6.1)	NA	NA
PCV (%)	36 (20-46)	35 (29-55)	36 (17-37)	34 (26-48)	34.5 (24-46)	32 (28-41)	39 (17-41)	30.5 (29-40)	38.5 (36-41)	31 (28-34)
TPP (g/dL)	5.71 ± 0.76	5.84 ± 1.34	5.12 ± 0.58	5.3 ± 1.34	5.35 ± 0.7	52.0 ±15.2	5.23 ± 0.55	4.67 ± 1.55	5.97 ± 0.65	5.1 ± 0.65
SAA (mg/L)	1216 (0.73-2719)	503 (3-2096)	NA	NA	NA	NA	NA	NA	NA	NA
Sodium (mEq/L)	130 (123-137)	131 (112-137)	130 (129-135)	131.5 (119-146)	129.5 (115-137)	134 (127-145)	133 (124-140)	132.5 (125-135)	NA	NA

Chloride (mEq/L)	96.1 ±6.3	95.6 ± 6.5	96.8 ± 2.3	100 ±6.4	95.5 ± 4.4	102.6 ± 5.8	99 ± 4.8	97.8 ± 5.8	NA	NA
Potassium (mEql/L)	3.3 ± 0.8	3.4± 0.9	2.9 ± 0.6	3.4 ± 0.8	3.5 ± 1.1	3.3 ± 0.8	4.3 ±0.7	3.6 ± 0.6	NA	NA
Lactate (mmol/L)	1.6 (1.1-2.2)	1.5 (0.6 – 10.5)	0.9 (0.7-1)	0.9 (0.5- 1.9)	1 (0.7-2.5)	1.1 (0.7 -1.1)	0.9 (0.9- 0.9)	1.1 (0.4 – 2.9)	NA	NA
Glucose (mg/dL)	118.9 (90– 189.2)	122.5 (104.5- 182)	NA	NA	NA	NA	127.9 (118.9- 136.9)	129.7 (108 -140.5)	NA	NA
Creatinine (mg/dL)	1.18 (0.93 – 1.64)	1.23 (0.7-2.8)	NA	NA	NA	NA	1.0 (0.99-1.1)	1.27 (0.76-1.54)	NA	NA

Appendix 2

Results of differences of relative abundance of taxa between and within groups of foals in the FMT or control group

The results obtained throughout the study period.

Significant differences of relative abundance of phyla, class, order, family, genus and species within and between groups determined by T-test. For comparisons between groups, the relative abundance of the FMT is compared to the control group. Within groups, the relative abundance is for D1, D2 and D3 compared to D0.

FMT v Control	*Phyla*	*Class*	*Order*	*Family*	*Genus*
D0	-	Negativicutes (↑) (.05)	-	Enterobacteriaceae(↓)(.05) Veillonellaceae(↑) (.05) Streptococcaceae(↑)(.05) Burkholderiaceae (↓) (.05)	-
D1	-	-	-	-	-
D2	-		-	-	-
D3	-	-	-	-	-
FMT group					
D0-D1	-	-	-	-	-
D0-D2	-	-	-	-	-
D0-D3	-	-	Rhizobiales(↑)(.05)	-	-
Control group					
D0-D1	-	-	-	-	-
D0-D2	-	-	-	-	-
D0-D3	-	-	-	-	-

Appendix 3

Results of differences in relative abundance between and within groups of FMT solution in different storage conditions.

Significant differences (P = <0.05) of relative abundance of phyla, class, order, family and genus between storage conditions by t test. The relative abundance of samples stored at 4°C is compared to the fresh faeces. The relative abundance of samples stored at -20°C is compared to the FMT0hr.

	Phyla	Class	Order	Family	Genus
Fresh-FMT0hr	-	-	-	-	-
Fresh-FMT24hr	-	-	-	-	-
Fresh-FMT48hr	-	-	-	-	-
Fresh-FMT72hr	-	-	-	-	-
Fresh-FMT7d	-	-	-	-	-
Fresh-FMT14d	-	-	-	-	-
Fresh-FMT28d	-	-	-	-	-
FMT0hr-FMT24hr	-	-	-	-	-
FMT0hr-FMT48hr	-	-	-	-	-
FMT0hr-FMT72hr	-	-	-	-	-
FMT0hr-FMT7d	-	-	-	-	-
FMT0hr-FMT14d	-	-	-	-	-
FMT0hr-FMT28d	-	-	-	-	-

Appendix 4

Results of LEfSe analysis displaying differences in the relative abundance of taxa before and after anthelmintic treatment.

The results show differences of relative abundance of phyla, class, order, family and genus within and between groups. Arrows indicate an increase or decrease in relative abundance.

LEfSe results (LDA score)					
Control	*Phyla*	*Class*	*Order*	*Family*	*Genus*
D1-D3-D14	Verrucomicrobiota(↑) D1(>4), Proteobacteria(↑) D1 (>4), Fibrobacterota(↑)D3 (4), Spirochaetota(↑)D14 (>4),	Gammaproteobacteria(↑) D1 (>4), Kiritimatiellae(↑) D1 (4), Spriochaetia(↑) D3 (>4), Fibrobacteria(↑)D3 (4)	WCHB1_41(↑) D1 (4), Spirochaetales(↑) D3 (>4), Fibrobacterals(↑)D3 (4),	WHCB1_41(↑)D1 (4), Spirochaetaceae(↑)D3 (>4), Fibrobacteraceae(↑)D3 (4), p_251_o5(↑)D14 (>4),	WCHB1_41(↑)D1 (4), Treponema(↑)D3 (>4), Fibrobacter(↑)D3 (4), p_251_o5(↑) D14 (>4)
Oxfendazole	*Phyla*	*Class*	*Order*	*Family*	*Genus*
D1-D3-D14	Proteobacteria(↑)D1 (>4), Verrucomicrobiota(↑)D1 (>4)	Gammaproteobacteria(↑)D1 (>4)	Verrucomicrobiales(↑) D1 (4)	Akkermansiaceae(↑)D1 (4)	Akkermansia(↑)D1 (4)
Abamectin	*Phyla*	*Class*	*Order*	*Family*	*Genus*
D1-D3-D14	-	-	Lachnospirales(↑)D14 (>4)	Lachnospiraceae(↑)D14 (>4)	-
Control-oxfendazole-abamectin	*Phyla*	*Class*	*Order*	*Family*	*Genus*
D1	Proteobacteria(↑)OXF (>4)	Gammaproteobacteria(↑)OXF (>4)	-	-	-
Control-oxfendazole-abamectin	*Phyla*	*Class*	*Order*	*Family*	*Genus*
D3	-	Bacteroidia(↑)CON (>4)	Bacteroidales(↑)CON (>4)	-	Rikenellaceae_RC9_gut_group(↑)ABA (>4)
Control-oxfendazole-abamectin	*Phyla*	*Class*	*Order*	*Family*	*Genus*
D14	-	-	-	Prevotellaceae(↑)CON(>4)	Prevotellaceae_UCG_001(↑)CON (>4)

Appendix 5

ANOSIM results displaying degree of similarities between and within groups before and after anthelmintic administration.

ANOSIM results		
Oxfendazole v control	*P-value*	*R value*
D1	0.43	-<0.01
D3	0.1	0.08
D14	<0.01	0.23
Abamectin v control	*P-Value*	*R value*
D1	0.05	0.14
D3	0.04	0.13
D14	<0.01	0.14
Abamectin v oxfendazole	*P-Value*	*R value*
D1	0.15	0.08
D3	0.5	-0.01
D14	0.32	0.02
Control group	*P-Value*	*R value*
D1-D3	0.34	0.02
D3-14	0.01	0.2
D1-D14	<0.01	0.23
Oxfendazole group	*P-Value*	*R value*
D1-D3	0.62	-0.03
D3-14	<0.01	0.22
D1-D14	<0.01	0.2
Abamectin	*P-Value*	*R value*
D1-D3	0.74	-0.05
D3-14	0.08	0.09
D1-D14	0.1	0.09

Appendix 6

Faecal egg count data.

A copy of the raw faecal egg count data of horses receiving anthelmintic treatment and horses in the control group in Chapter 5.

Table 1: Faecal egg counts of horses in each group on Day -3

Animal	Group	FEC 1 (epg)	FEC 2 (epg)	FEC 3 (epg)	Average
1	OXF	530	750	1150	810
2	CONTROL	470	300	470	413.3333
3	ABA	450	500	430	460
4	OXF	360	230	620	403.3333
5	CONTROL	310	170	360	280
6	ABA	250	560	480	430
7	CONTROL	250	760	500	503.3333
8	ABA	210	230	250	230
9	OXF	210	250	190	216.6667
10	ABA	210	130	210	183.3333
11	CONTROL	180	210	120	170
12	OXF	140	290	390	273.3333
13	CONTROL	140	180	140	153.3333
14	ABA	110	230	180	173.3333
15	OXF	100	170	210	160
16	ABA	90	90	110	96.66667
17	CONTROL	80	130	90	100
18	OXF	70	70	80	73.33333
19	ABA	70	190	160	140
20	OXF	40	10	40	30
21	CONTROL	40	20	60	40
22	OXF	30	80	90	66.66667
23	CONTROL	30	80	70	60
24	ABA	30	30	30	30

Table 2: Faecal egg counts of horses in each group on Day 14

Animal	Group	FEC 1 (epg)	FEC 2 (epg)	FEC 3 (epg)	Average
1	OXF	550	580	650	593.333333
2	CONTROL	250	180	140	190
3	ABA	0	10	0	3.33333333
4	OXF	0	20	30	16.6666667
5	CONTROL	270	390	170	276.666667
6	ABA	50	30	60	46.6666667
7	CONTROL	680	780	780	746.666667
8	ABA	0	30	30	20
9	OXF	570	550	380	500
10	ABA	0	0	10	3.33333333
11	CONTROL	940	990	980	970
12	OXF	420	310	330	353.333333
13	CONTROL	290	350	510	383.333333
14	ABA	0	0	0	0
15	OXF	60	80	110	83.3333333
16	ABA	0	20	0	6.66666667
17	CONTROL	60	130	90	93.3333333
18	OXF	10	10	20	13.3333333

19	ABA	0	10	0	3.33333333
20	OXF	10	0	10	6.66666667
21	CONTROL	0	0	0	0
22	OXF	20	50	10	26.6666667
23	CONTROL	50	40	20	36.6666667
24	ABA	10	0	0	3.33333333