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Development of a Protocol with Concentrated Bacteria for Fecal Microbiota Transplantation and Impact on the Equine Fecal Microbiota After Antibiotic-Induced Dysbiosis

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Ce mémoire intitulé

Development of a Protocol with Concentrated Bacteria for Fecal Microbiota Transplantation and Impact on the Equine Fecal Microbiota After Antibiotic-Induced Dysbiosis

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Résumé

Le microbiote intestinal équin joue un rôle important dans le maintien de la santé de l'hôte. Le microbiote intestinal est composé de nombreux micro-organismes tels que les bactéries, les virus, les champignons et les archées. Cependant, la majorité de ces cellules microbiennes sont bactériennes, et par conséquent, de nombreuses études, y compris la présente, se concentrent sur l'exploration des communautés bactériennes dans l'intestin. Un déséquilibre du microbiote intestinal, appelé dysbiose, a été observé dans plusieurs conditions, telles que la colite, après l'administration d'antibiotiques ou la modification du régime alimentaire. La restauration du microbiote peut être effectuée par la transplantation de microbiote fécal (FMT). Des études utilisant les recommandations actuelles pour la FMT ont montré une récupération clinique chez les chevaux souffrant de diarrhée, mais le microbiote reste largement inchangé après la FMT et aucune étude randomisée avec contrôle placébo n'a été réalisée.

Les hypothèses de ce projet étaient que le traitement avec une FMT concentrée corrigera la dysbiose plus rapidement qu'une FMT conventionnelle et le véhicule, et que le microbiote intestinal des chevaux traités avec une FMT concentrée ressemblera au microbiote intestinal du cheval donneur. L'objectif de ce projet était de développer un protocole pour améliorer la FMT chez les chevaux, en augmentant la concentration de bactéries présentes dans les selles du donneur par centrifugation, et de le tester chez les chevaux atteints de dysbiose intestinale induite par les antibiotiques.

L'antibiotique triméthoprime sulfadiazine (TMS) a été administré à neuf chevaux pour induire une dysbiose intestinale. Les chevaux ont été séparés en trois groupes: les chevaux recevant une FMT concentrée (cFMT, n = 3); les chevaux recevant la FMT fraîche (fFMT), selon les recommandations actuelles (n = 3); et les chevaux recevant un véhicule (VEH) avec 10% de glycérol dans une solution saline à 0,9% (n=3). Des échantillons fécaux ont été prélevés avant et après l'administration du TMS, ainsi qu'avant, pendant et après la transplantation. Le séquençage a été réalisé à l'aide de la plateforme Illumina MiSeq et les données analysées à l'aide du logiciel Mothur.

Tel qu'attendu, l'antibiotique TMS a significativement diminué la richesse microbienne chez tous les chevaux. De manière inattendue, la composition des suspensions fécales des donneurs cFMT et fFMT était significativement différente de la composition de base des receveurs cFMT et fFMT, respectivement. La composition du microbiote des chevaux ayant reçu une transplantation fécale (concentrée ou non) était significativement différente après la transplantation, alors que ce n'était pas le cas chez les chevaux ayant reçu le véhicule. En outre, l'abondance relative de *Escherichia* était significativement plus élevée dans les suspensions fécales du donneur cFMT par rapport aux suspensions fécales du donneur fFMT.

Les principales limites de ce projet sont la petite taille des groupes et l'exposition des selles des donneurs à l'oxygène et à la congélation-décongélation. En outre, le modèle de dysbiose peut ne pas être optimal pour tester l'efficacité de la FMT, et des études réalisant la FMT chez les chevaux souffrant de diarrhée sont nécessaire. Cette étude a contribué à la recherche de nouvelles approches pour améliorer la FMT chez les chevaux. Le faible effet mesuré avec les deux protocoles de FMT et l'augmentation de *Escherichia* démontre que les protocoles actuels doivent être optimisés avant de pouvoir recommander la FMT pour traiter et prévenir la dysbiose chez les chevaux.

Mots-clés : Transplantation de microbiote fécal, manipulation du microbiote, dysbiose intestinale, microbiote intestinal équin, séquençage de nouvelle génération, microbiome, flore intestinale, antibiotiques.

Abstract

The equine gut microbiota plays an important role in maintaining the health of the host. The gut microbiota is composed of many microorganisms such as bacteria, viruses, fungi, and archaea. However, the majority of these microbial cells are bacterial cells, and consequently, many studies, including the present one, focus on exploring bacterial communities in the gut. An imbalance of the gut microbiota, termed dysbiosis, has been observed in several conditions such as colitis, colic, after antibiotic administration, or diet modification. Restoration of the gut to a healthy state can be performed through fecal microbiota transplantation (FMT). Studies using current recommendations for FMT have shown clinical recovery in horses with diarrhea, but the microbiota remains largely unchanged after FMT and no controlled studies have been performed.

The hypotheses of this project were that treatment with concentrated FMT will correct dysbiosis faster than conventional FMT and the vehicle, and that the gut microbiota of horses treated with concentrated FMT will resemble the gut microbiota of the donor. The objective of this project was to develop an improved protocol for FMT in horses, by increasing the concentration of bacteria found in the donor stool using centrifugation, and to test it in horses with antibiotic-induced intestinal dysbiosis.

The antibiotic trimethoprim sulfadiazine (TMS) was administered to nine horses to induce intestinal dysbiosis. Horses were separated into three groups: horses receiving concentrated FMT (cFMT) (n=3); horses receiving fresh FMT (fFMT), as per current recommendations (n=3); horses receiving a vehicle (VEH) with 10% glycerol in 0.9% saline (n=3). Fecal samples were collected before and after antibiotic administration, as well as before, during, and after transplantation. Sequencing was performed using the Illumina MiSeq platform and data analysed using the software Mothur.

As expected, the antibiotic TMS significantly decreased the richness in all horses ($P < 0.05$). Unexpectedly, the membership of the cFMT and fFMT donor fecal suspensions was significantly different from cFMT and fFMT recipients' baseline membership, respectively. The membership of the cFMT and fFMT recipient horses was significantly different after transplantation, while the

vehicle recipients were not. In addition, the *Escherichia* genus was found in significantly higher relative abundances in the cFMT donor fecal suspensions when compared to the fFMT donor fecal suspensions.

The main limitations of this study are the small sample size and exposure of cFMT donor stool to oxygen and freeze-thawing. In addition, the dysbiosis model may not be optimal to test the efficacy of FMT, and studies performing FMT in horses with diarrhea are warranted. This study contributed to the search for novel approaches to improve FMT in horses. The weak effect of both FMT protocols on the gut microbiota and the increase in *Escherichia* suggest that further clinical studies are needed before FMT can be recommended to treat and prevent dysbiosis in horses.

Keywords : Fecal microbiota transplantation, microbiota manipulation, intestinal dysbiosis, equine gut microbiota, next generation sequencing, microbiome, intestinal flora, antibiotics.

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List of Acronyms and Abbreviations

AMOVA	Analysis of Molecular Variance
ANOVA	Analysis of Variance
ASD	Autism Spectrum Disorder
cFMT	Concentrated Fecal Microbiota Transplantation
VEH	Vehicle
FDA	Food and Drug Administration
fFMT	Fresh Fecal Microbiota Transplantation
FMT	Fecal Microbiota Transplantation
FOS	Fructo-oligosaccharides
GI	Gastrointestinal
IBD	Inflammatory Bowel Disease
LDA	Linear Discriminant Analysis
LEfSe	Linear Discriminant Analysis Effect Size
LGG	*Lactobacillus rhamnosus* strain GG
NGS	Next-Generation Sequencing
OTU	Observed Taxonomic Units
PCoA	Principal Coordinate Analysis
rCDI	recurrent *Clostridioides difficile* infection
SCFA	Short-Chain Fatty Acid
SCOD	Simple Colonic Obstruction and Distension

TLR	Toll-Like Receptor
TMS	Trimethoprim/sulfadiazine

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Introduction

The gut microbiota is a collection of microorganisms playing an important role in maintaining the health of the host, such as in nutrition (1), energy metabolism (2), immune development (3), and host defense against harmful pathogens (4). Similarly, the equine gut microbiota plays a critical role in cellulose fermentation and short-chain fatty acid (SCFA) production to provide a horse with its main energy sources (5). The gut microbiota is composed of many microorganisms such as bacteria, viruses, fungi, and archaea (6). However, the majority of these microbial cells are bacterial cells (7), and consequently, our study focuses on exploring bacterial communities in the gut.

When an imbalance of the gut microbiota occurs, the host is said to have dysbiosis. In horses, dysbiosis is associated with certain events such as colitis, diet change, and antimicrobial administration (8). Importantly, diseases affecting the GI tract are the leading causes of morbidity and mortality in horses (9). Current treatments for colitis include analgesics, fluid therapy, laxatives (10, 11), antibiotic-probiotic administration, prebiotics, and diet modification (12, 13). However, the mortality rate remains elevated, therefore advances in therapeutic approaches of equine colitis are warranted (14).

There are many methods of microbiota manipulation that might be used to restore a dysbiotic environment including probiotics, prebiotics, synbiotics, postbiotics, antibiotics, and fecal microbiota transplantation (FMT). Of these methods, FMT is increasingly being explored as an alternative therapy for GI diseases due to its success in treating patients with recurrent *Clostridioides difficile* infection (rCDI) (15). FMT originated in China and involves administration of stool from a healthy donor to a patient with dysbiosis (16). FMT is being studied for its potential efficacy in treatment of several other GI tract diseases, including irritable bowel syndrome, inflammatory bowel disease (IBD), and metabolic syndrome (17). FMT has also seen success in several other species such as in puppies (18), cattle (19, 20), and pigs (21, 22). Moreover, a recent study showed diarrhea relief in 3 out of 5 geriatric horses after FMT (23), while another group reported clinical recovery in four horses with diarrhea after FMT, however, no microbiota analysis was performed (24) and a control group was absent in both studies. In addition, a study from our

group in which FMT was administered to six horses with chronic diarrhea failed to detect changes in the gut microbiota after treatment (Costa et al., under review). Therefore, well-designed studies of FMT treatment have not yet been evaluated in horses.

In humans, FMT is often given directly to the large intestine by enema (25). However, the GI tract of the horse differs substantially from that of the human. It is known that horses have a long small colon, measuring approximately three meters, thereby precluding bacteria from reaching the large colon (26). Consequently, FMT through enema is not feasible in horses as it is in humans. Another possible route of administration is through a nasogastric tube; however, this method is thought to have limitations as well since the bacteria must pass through the fermentation chamber present in the caecum, and through the small intestine where gastric acidity and enzymatic actions may decrease bacterial viability (26). The limitations of these methods strongly suggest that more research is required to determine an efficient method of FMT administration for intestinal colonization in horses.

In order to evaluate changes in bacterial composition or successful bacterial engraftment, the gut microbiota must be characterized. To date, culture-dependent methods have been used to characterize microbial communities. Although these methods have permitted the discovery of novel microorganisms and detection of antibiotic resistance, many species remain difficult to grow in culture (27). Recently, the development of culture-independent DNA-sequencing technologies, such as next generation sequencing, has made it possible for in-depth characterization of the bacterial communities present in the intestinal microbiota (28). These methods have allowed a greater understanding of the interactions between the intestinal microbiota and the host.

A previous study evaluating the effects of antibiotic administration on the intestinal microbiota in horses has shown that administration of penicillin, ceftiofur, and trimethoprim sulfadiazine (TMS) significantly alters the intestinal microbiota. Of importance, TMS had the greatest impact on species richness and diversity (8). However, although the negative and long-lasting impacts of antimicrobial administration are known (29), challenges to move away from these treatments arise due to a lack of alternative therapies.

In summary, the equine gut microbiota is essential in maintaining the health of the host (5). Microbial dysbiosis in horses is associated with several conditions such as colitis, diet modifications, and antimicrobial administration (8, 30). While FMT has shown success in treating GI diseases in other species (15, 18-22), larger randomized placebo-controlled trials are required to test the treatment efficacy in horses. Additionally, as the current FMT protocol fails to alter the gut microbiota of the recipients, the protocol must be improved before FMT can be recommended to treat horses with dysbiosis.

Chapter 1 – Literature Review

1.1 What is the gut microbiota?

The number of microbial cells in the human body was found to have an approximate ratio of 1:1 with the number of human cells, at an order of magnitude of 10^{13} (7). These microbial cells have co-evolved with the human body and have developed a mutualistic relationship. The majority of these microbial cells are found to be bacterial cells as they greatly outnumber eukaryotes and archaea (7). Consequently, many studies focus on exploring bacterial communities. Although bacteria reside in many places in the human body, they are most abundant in the gastrointestinal (GI) tract, and have been termed the gut microbiota (7). In humans, the gut microbiota is an important contributor to maintaining the health of the host, such as in nutrition (1), energy metabolism (2), immune development (3), and host defense against harmful pathogens (4). In horses, the gut microbiota plays a critical role in cellulose fermentation and SCFA production, providing them with their main energy sources (5). The bacteria most commonly present in a healthy human and equine gut microbiota belong to the Firmicutes and Bacteroidetes phyla (31, 32). In humans, Actinobacteria, Proteobacteria, Fusobacteria, and Verrucomicrobia are also highly present (6). In horses, there is also a high abundance of Verrucomicrobia (0-24%), Proteobacteria (0-14%), Spirochaetes (1-9%), Fibrobacteres (1-7%), and Actinobacteria (0-2%) (32-35).

The human gut microbiome, which consists of the set of genomes from all of the microorganisms present within the gut, has been recently studied using metagenomics, transcriptomics, and proteomics. It has been proposed that the gut microbiomes of humans generally fall into three possible clusters based on species and functional composition, known as enterotypes (31). However, these findings have been controversial due to variations in sampling, DNA purification, sequencing and analysis protocols. The first enterotype suggested by Arumugam et al. mostly consisted of *Bacteroides* and other closely related genera with saccharolytic functions and enzymes involved in the degradation of carbohydrates and proteins providing the main energy sources. The second enterotype was rich in *Prevotella* and *Desulfovibrio*, which may synergise in the mucosal layer of the gut to degrade mucin

glycoproteins. The third enterotype was the most frequently encountered in this study, and was mostly composed of *Ruminococcus* and *Akkermansia*, both of which have important functions in mucin degradation. Nevertheless, the enterotypes have yet to be refined by accounting for environmental and genetic factors, and by developing better sampling methods of the different GI compartments.

The presence of enterotypes has also been examined in certain animal species. Whereas mice housed in controlled experimental environments expressed strong clustering of enterotypes (36), stratification of the gut microbiome of animals living in the wild, such as mice (37), primates (38, 39), and pigs (40, 41), was reduced. The lack of external influences on the gut microbiome of housed mice may allow the proliferation of favoured communities, forming clear clusters. The search for equine enterotypes may be an interesting avenue to explore as these enterotypes may allow classification of gut microbiomes and may permit the diagnosis of intestinal disorders and prediction of individual responses to treatments (31).

Indeed, asthmatic horses on different diets seemed to fall into two different enterotypes (42). The majority of horses on pasture demonstrated the first enterotype, which had higher abundance of unclassified subdivision 5, unclassified Ruminococcaceae, unclassified Clostridiales, unclassified Verrucomicrobia, *Pseudobutyrivibrio*, *Oligosphaera*, unclassified Anaerollineaceae, and *Synergistes*. On the other hand, most horses eating hay demonstrated the second enterotype, with higher levels of unclassified Cytophagales, *Lactobacillus*, unclassified Lactobacillaceae, *Streptococcus*, and *Fibrobacter*. These results suggest that changes in diet strongly induce changes in the gut microbiota of horses, despite underlying conditions such as asthma.

Another study examining changes in the gut microbiota of foals before and after weaning reported substantial clustering of the gut microbiota in three community types, at 3 days post-weaning (43). Community type 1 was found in the majority of suckling foals and demonstrated a higher abundance of *Acinetobacter*, *Adlercreutzia*, *Bacillus*, *Fibrobacter*, *Rikenella*, and *Treponema*, and lower abundance of *Eubacterium*, *Anaerovibrio*, *Blautia*, *Clostridium* XI, *Coprococcus*, *Lachnospiracea incertae sedis*, and *Prevotella*, whereas community type 2 was

present after weaning when foals were on a cereal-based diet, and displayed an opposite abundance pattern from community type 1. Community type 3 seemed to be present in foals during the transition to weaning, showing a gradient of dominant taxa between community type 1 and type 2, such as *Acinetobacter*, *Adlercreutzia*, *Fibrobacter*, *Bacteroides*, *Rikenella*, and *Clostridium IV*. Moreover, community type 2 expressed lower cortisol levels, higher telomere length, increased production of the SCFA N-butyrate, and was associated with increased average daily gain, when compared to community type 3. The authors suggest that higher cortisol levels may induce an increase in the abundance of bacteria found in community type 3, possibly priming ROS production by the gut, which in turn may lead to DNA damage and telomere length shortening (43). Contrarily, N-butyrate production in community type 2 may have a protective effect on telomere length and may play a positive role in average daily weight gain. Therefore, it is possible that individuals with a community type 2 may adapt better to weaning (43).

Several studies have also observed an increased richness and diversity to be associated with a healthy gut microbiota (44). Richness is a measure of the number of different taxa in a sample, while diversity measures both richness and evenness, which considers the distribution of those taxa in each sample. For instance, foals that experienced less stress during weaning had a higher diversity in their gut microbiota when compared to foals experiencing increased stress (43). In addition, a significant reduction in richness and diversity was observed in dogs with acute diarrhea when compared to healthy individuals (45).

1.2 Development of new technologies for microbiota characterisation

Traditionally, culture-based methods have been used to characterize bacterial communities in the intestinal tract. These methods have allowed detection of antibiotic resistance, as well as detection of specific bacteria using selective media. Additionally, the development of culturomics has allowed the growth and discovery of numerous bacteria present in the human intestine (46, 47). Culturomics involves a high scale culture technique aimed to grow bacteria that are unculturable using traditional media due to the complex microbial ecosystems they inhabit (such as the intestinal tract) (48). Although culturing has important advantages and has led to significant discoveries, many bacterial species remain challenging to culture in traditional media (27). This

may lead to underestimating the number of species present in a community (richness), as well as overestimating the abundance, and thus importance, of certain species due to advantageous growth in certain media (49). Furthermore, these methods have made it challenging to identify many other microbial cells, such as archaea, fungi, viruses, and eukaryotes (50).

Recently, culture-independent methods have been developed that have made it possible for in-depth characterization of the bacterial communities present in the intestinal microbiota (28). In turn, interactions between the intestinal microbiota and the host are better understood. Such methods include molecular fingerprinting, fluorescent in situ hybridization, microarrays, and quantitative PCR. Many of these molecular methods can quantify the bacteria present in a sample or target the amplification of specific taxa. However, a significant limitation is the relatively low number of species that can be sequenced in parallel, which in turn may underestimate the richness of a community (50). Moreover, these techniques are time consuming and cost ineffective. On the other hand, NGS is the only method that can sequence thousands of samples simultaneously, identifying millions of bacteria at an affordable cost in a reasonable time frame (50). Still, certain limitations arise with this method as well. For instance, due to the limited size of DNA fragments that can be sequenced, it is challenging to classify organisms at lower taxonomic levels (e.g. species) (50). However, a recently developed technology by Pacific Biosciences enables accurate sequencing of long reads, such as the full-length 16S rRNA gene, enabling organism classification at the species level (51). Another limitation to NGS is that it can only provide the relative abundance of bacteria present, as opposed to an absolute quantification. Nevertheless, this method has recently gained popularity in characterizing bacterial communities present in the gut microbiota.

1.3 Indices used to evaluate the gut microbiota

One of the ways in which a microbial community is characterized is through alpha diversity. This measure involves looking at the richness, evenness, and diversity of a single community. Richness is the number of different taxa present in a community, and evenness describes how each taxa is distributed within that community (relative abundance), whereas diversity is a mathematical index that accounts for both richness and evenness (50). Furthermore,

the beta diversity analysis is used to compare two or more communities. This analysis involves a measure of membership, which evaluates the taxa that are shared or different between communities. Structure is also assessed, which is a measure of both membership and evenness. In other words, it compares the shared and distinct taxa between communities, while also accounting for their relative abundance (50).

One of the plots used to describe beta diversity is a relative abundance plot (Figure 1). This plot can be used to visualize the different taxa present between communities at any taxonomic level, in relation to the total number of taxa in each community. However, when dealing with low taxonomic levels (such as genus and species, compared to phylum and order), the large amount of data may lead to a misrepresentation of community analyses. Hence, statistical analyses are applied to correct over-interpretation, and to appropriately evaluate significant differences of results. Figure 1 shows an example of a relative abundance plot of two groups with different microbiota profiles.

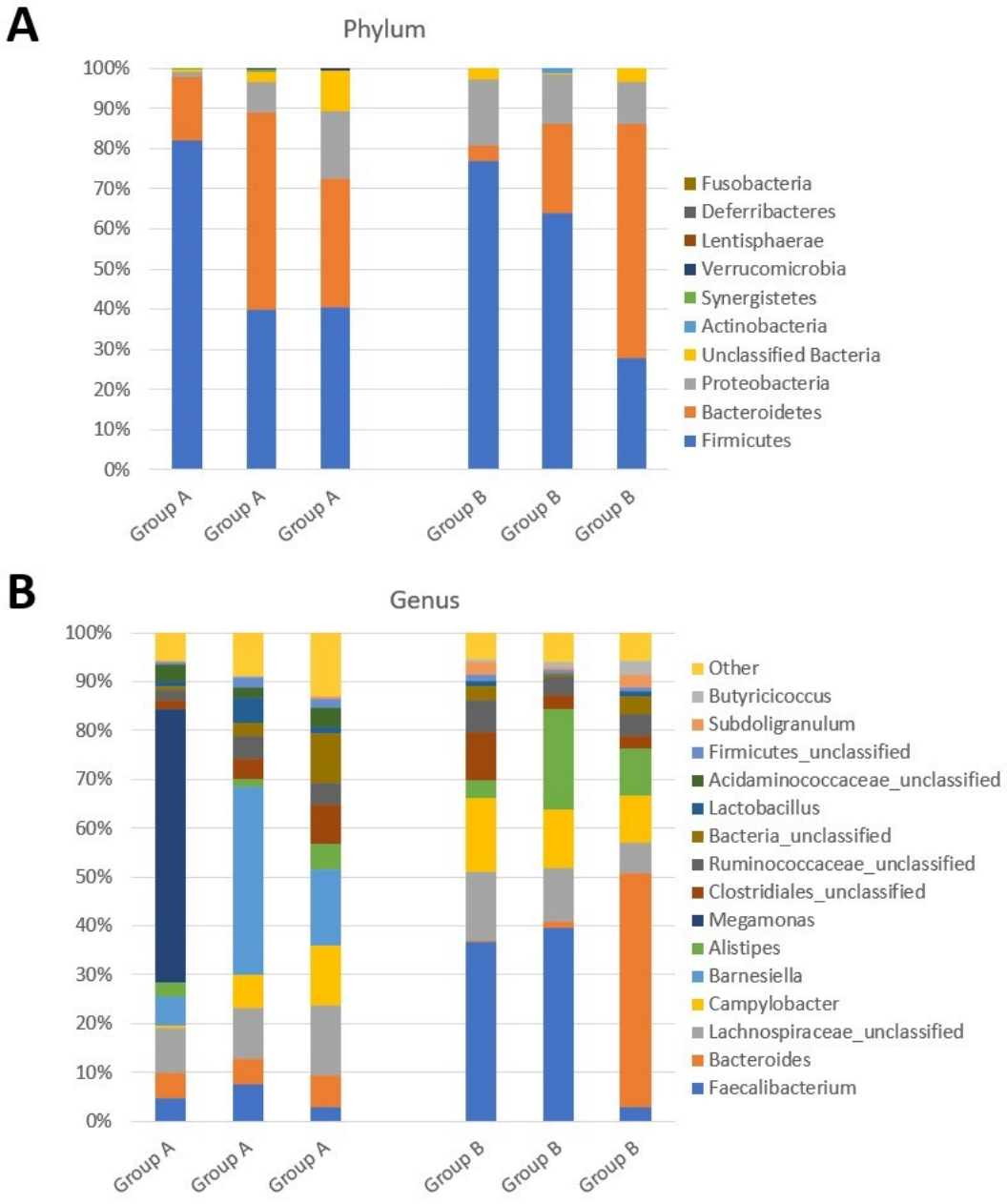

Figure 1. – Relative abundace of predominant bacteria at the phylum (A) and genus (B) levels. Group A and Group B are represented. Only the 10 most common phyla and 16 most common genera are represented.

Another plot used to describe beta diversity is principal coordinate analysis (PCoA) (Figure 2). This representation is based on a distance matrix that calculates the number of times a taxon is present in a community. The closer two dots are on the graph, the more similar their communities

are. Contrarily, the further two dots are from each other, the greater the difference in their communities (50). This plot can be made in two or three dimensions, depending on which will best demonstrate the variation of each community. Similarly, statistical analyses are used to determine significant differences.

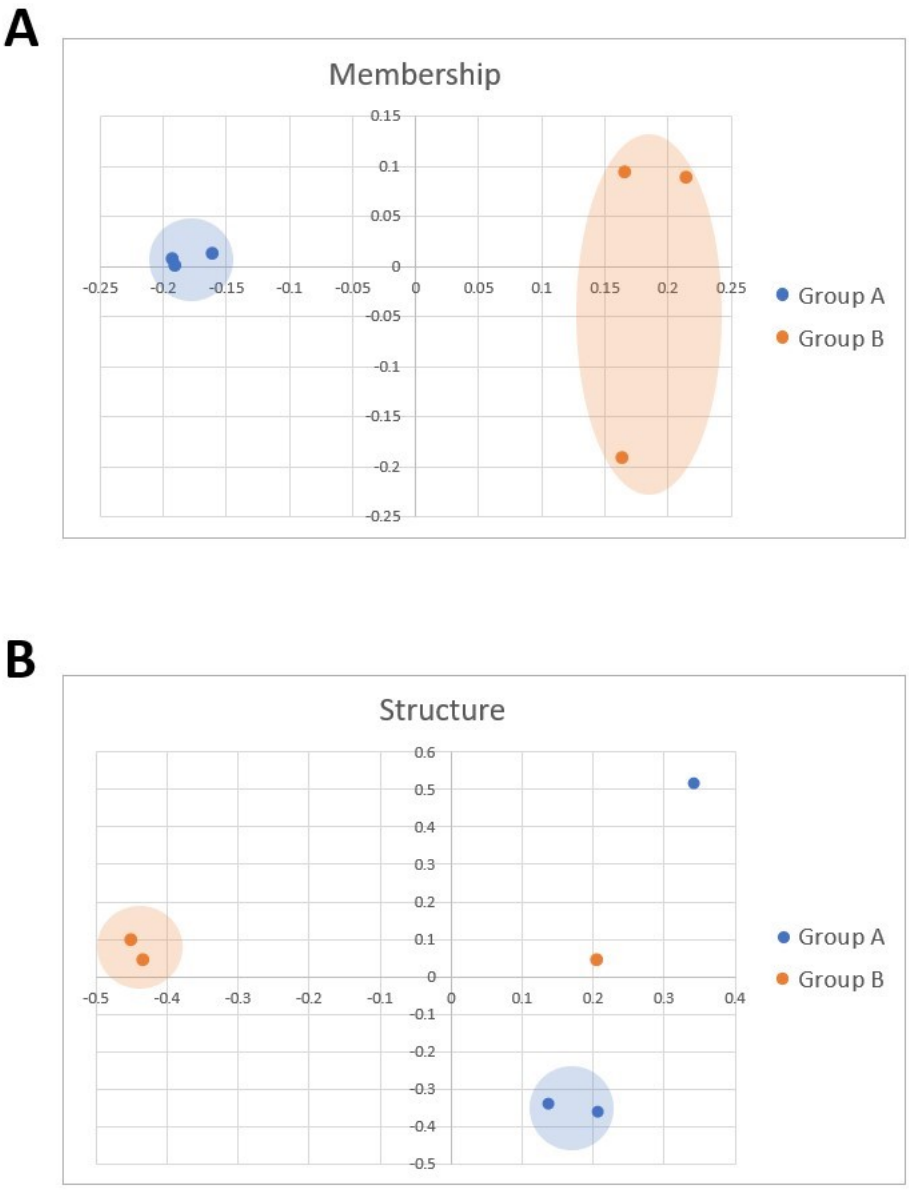

Figure 2. – Principal coordinate analysis (PCoA) of bacterial communities present in Group A and Group B. Bidimensional representation of the principal coordinate analysis of bacterial communities' membership addressed by the Classic Jaccard analysis (A) and structure

addressed by the Yue and Clayton analysis (B). Most samples cluster together in membership and structure with their respective groups.

Moreover, a dendrogram is another useful way to visualize differences and similarities between communities (Figure 3). A dendrogram is comprised of a tree diagram that assembles samples with similar communities on the same branch (50).

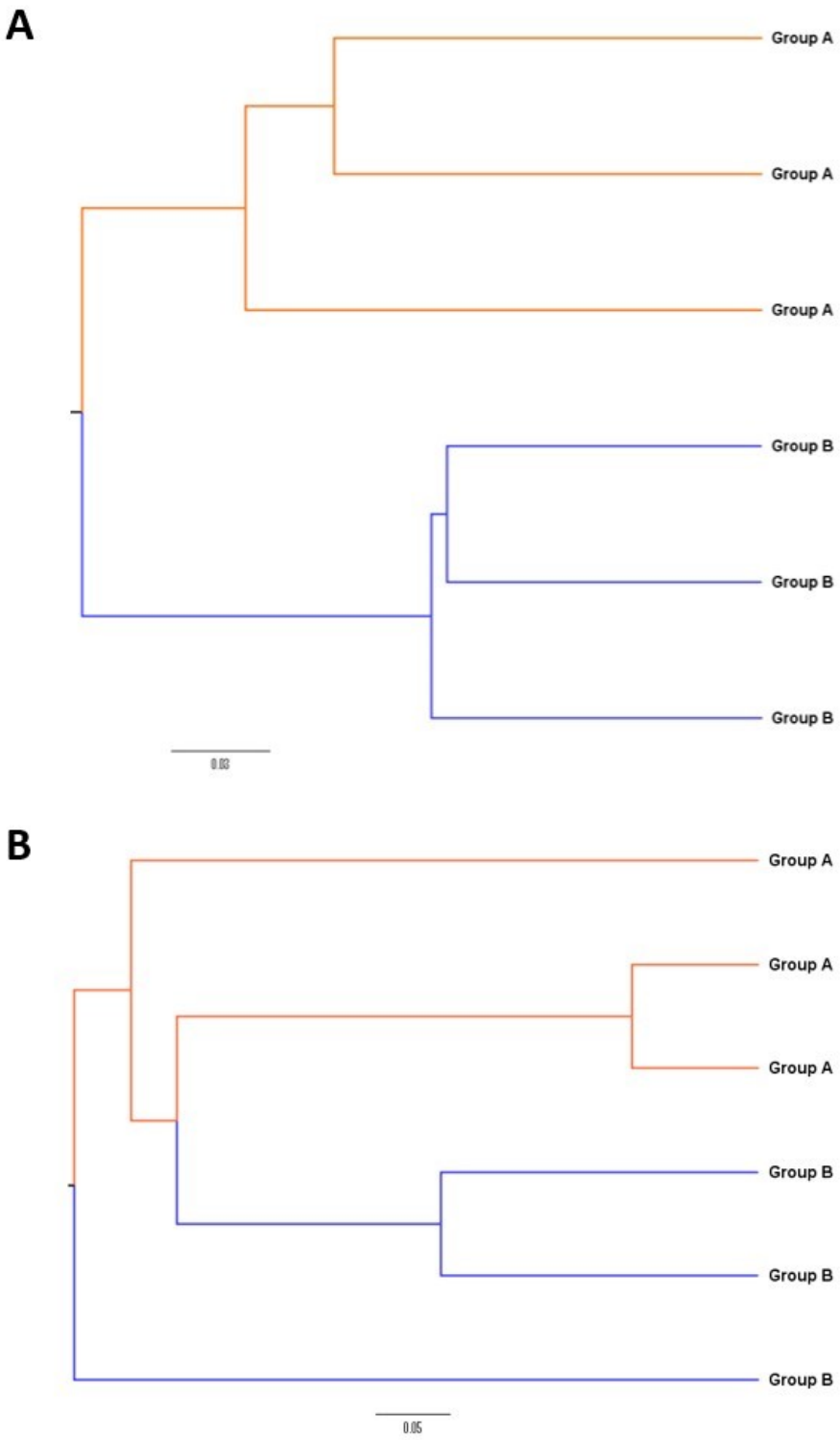

Figure 3. – Dendrograms representing similarities of bacterial communities present in Group A and Group B. Phylogenetic representation of bacterial communities' membership addressed by the Classic Jaccard analysis (A) and structure addressed by the Yue and Clayton analysis (B).

1.4 Role of the gut microbiota

The gut microbiota has many important roles within the body, many of which have been clearly demonstrated in germ-free mice (complete removal of the microbiota) and antibiotics-treated mice (depletion of select members of the microbiota). Germ-free mice were shown to have deteriorated Peyer's patches with fewer germinal centers, immature mesenteric lymph nodes, insufficient lymphoid follicles, low levels of antimicrobial peptides and immunoglobulin A (IgA), and thus reduced numbers of B, T and dendritic cells (52). Hence, the microbiota plays a crucial role in immune function.

1.4.1 Immune function

One of the ways the immune system and the gut microbiota interact is through the expression of pattern recognition receptors, such as toll-like receptors (TLRs). TLRs allow differentiation between commensals and pathogens, and are involved in immunological tolerance by inhibiting an inflammatory response against non-harmful microbiota components (53). In fact, TLR-9 depleted mice showed increased production of regulatory T-cells in the small intestine (54). Additionally, there was significantly decreased production of IFN-γ-producing $CD4^+$ T cells in the lamina propria compared to wildtype mice. The dysregulated production of these cells may lead to an impaired immune response to infection. Indeed, when the same mice were orally infected with *Encephalitozoon cuniculi*, a bacterium that normally produces a robust IFN-γ response required for protection, IFN-γ concentrations were significantly reduced.

Interactions between the immune system and the gut microbiota have been further demonstrated through immune challenges. Some of the mechanisms by which commensals of the gut inhibit pathogen invasion are through the production of bacteriocins and metabolites, and nutrient competition. If the microbiota is depleted or removed, these functions may be impaired, thus allowing pathogen colonization. For instance, a study performed by Oh et al. showed that antibiotic-treated mice had an impaired antiviral immune response to vaginal Herpes Simplex Virus 2, suggesting its role in antiviral immunity (55). Moreover, the role of the gut microbiota in pathogen defense was shown in microbiota-depleted mice which were more susceptible to bacterial pathogens. Antibiotic-treated mice infected with *Salmonella typhimurium*

exhibited a correlation between higher bacterial loads and higher IFN-γ production in the mesentery lymph node, suggesting once again an impaired immune response to infection (56). Additionally, mice treated with antibiotics showed decreased production of secondary bile acids, resulting in spore germination and overgrowth of *C. difficile* infection in the large intestine (57).

1.4.2 Energy metabolism

The gut microbiota also plays a crucial role in providing the host with energy. The commensal bacteria do this by turning primary bile acids into secondary bile acids, and by breaking down complex carbohydrates otherwise indigestible by the host and producing SCFAs for energy (58). This energy uptake can be illustrated through colonizing germ-free mice with a known set of bacteria. For instance, colonized germ-free mice showed increased body weight and adiposity despite lower food ingestion and higher energy expenditure, when compared to germ-free mice (59). Furthermore, the presence of the gut microbiota is required for diet-induced obesity, as germ-free mice are resistant to obesity when given a Western diet high in fat and sugar (60). Indeed, germ-free mice that were given cecal content from obese donor mice had increased weight gain compared to mice receiving cecal content from lean donors (61). Thus, while the gut microbiota is essential for providing a host with energy, it may also cause harm through excessive energy harvesting.

1.4.3 Gut-brain axis

The gut microbiota has also been shown to be involved in the gut-brain axis through neural, hormonal, and immunological signalling (62, 63). It is described as a bidirectional relationship where the gut can influence behavior and emotion, while the brain can influence GI and immune functions (63). Most of the studies investigating this relationship have been conducted in animal models receiving a fecal transplant or a single strain of bacteria. For instance, germ-free BALB/c mice of naturally low exploratory behavior receiving a fecal transplant from highly explorative NIH Swiss mice had increased exploratory behavior (64). Contrarily, germ-free NIH Swiss mice receiving a fecal transplant from BALB/c mice showed decreased exploratory behavior.

The gut microbiota has also been shown to modulate stress and depressive-like behaviors. Germ-free mice exposed to restraint stress showed significantly higher stress levels than controls

(65). When the same mice were given *Bifodobacterium infantis*, the stress response was reversed. Moreover, microbiota-depleted rats receiving a fecal transplant from depressed human patients developed depressive characteristics (66). Interestingly, patients with depression, anxiety, and GI disease receiving a fecal transplant revealed improved sleep score, and a lower depression and anxiety score on the Hamilton Rating Scale, which correlated with increased gut microbiota diversity (67). However, as this study lacked a control group, further studies are required to confirm these results.

The gut microbiota is also thought to play a role in certain neurodevelopmental disorders, such as autism spectrum disorder (ASD), which is associated with maternal obesity during pregnancy (68). A study done by Buffington et al. showed that the addition of a single bacterial species, *Lactobacillus reuteri*, to the offspring of mice fed a Maternal-High-Fat-Diet reverted deficient social behaviors in the offspring (69). Similarly, several fecal transplants were performed in 18 children aged 7-17 years old that had both autism spectrum disorders and GI symptoms (70). Remarkably, the treatment revealed an 80% reduction in GI symptoms (abdominal pain, reflux, indigestion, diarrhea, and constipation), improved symptoms of ASD behavior, and partial colonization of *Bifidobacterium*, *Prevotella*, and *Desulfovibrio* 8 weeks after treatment. As this study did have untreated age- and gender-matched control patients with neurotypical behavior lacking GI disorders, the lack of a placebo-controlled group precludes confirmation of the beneficial effects of fecal transplants on GI and ASD symptoms. Therefore, as these results are only correlative, larger randomized and placebo-controlled studies are needed.

1.5 Disruption of the gut microbiota

Since the gut microbiota is important for maintaining the health of the host in many ways, it is not surprising that various diseases were found to be associated with dysbiosis, a term encompassing the imbalance of microbial populations. In humans, conditions such as IBD, diabetes, allergies, obesity, rectal cancer, and behavior changes were found to be associated with dysbiosis (28). Similarly in horses, several events such as colitis, colic (30), diet change, antimicrobial administration (8), and stressful conditions such as exercise and transport (26) were

all found to be associated with dysbiosis. Of importance, diseases affecting the GI tract are the leading causes of morbidity and mortality in horses (9).

1.5.1 Colitis

Many cases of colitis in horses remain without a clear etiology, although the causes can usually be classified as non-infectious or infectious. Non-infectious etiologies include antibiotic associated diarrhea, sand impaction, dietary imbalances, or NSAIDs toxicity (71). Additionally, colitis has been shown to be associated with certain pathogens such as *Clostridioides difficile*, *Clostridium perfringens*, *Salmonella* spp., *Neorickettsia risticii*, and equine coronavirus (72, 73). Furthermore, in a study by Costa et al., *Fusobacterium necrophorum* and *Fusobacterium nucleatum* were found in a significantly higher abundance in horses with colitis when compared to healthy controls (74). Interestingly, *Fusobacterium* spp. has been found to be associated with several gastrointestinal diseases in humans, such as Crohn's disease (75, 76), colorectal cancer (77, 78), and appendicitis (79). On the other hand, the *Lachnospiraceae* family is suggested to play an important role in maintaining health of the gut microbiota, as they have the capacity to ferment nutrients into SCFAs, which aid in reducing intestinal inflammation, maintaining intestinal barrier function, and controlling gut motility (80).

1.5.2 Colic

Etiologies for colic (abdominal pain) in horses include sand impaction (81), and parasite infections, (82). Studies have also shown a change in microbiota composition in horses with colic (83, 84). Pregnant mares developing colic were observed to have significantly lower relative abundances of *Ruminococcus* (phylum Firmicutes), unclassified Sphingobacteriales (phylum Bacteroidetes), unclassified Bacteroidales (phylum Bacteroidetes), and *Acetivibrio* (phylum Firmicutes), and a higher relative abundance of *Rhodopseudomonas*, unclassified Enterobacteriaceae, and *Enhydrobacter* (all of which belong to the phylum Proteobacteria), when compared to pregnant mares without colic (84). On the contrary, a study by Venable et al. showed an increase in Bacteroidetes in horses with colic (85).

1.5.3 Dietary imbalance

Dietary imbalance is also an important factor associated with intestinal dysbiosis. In humans, bacteria in the gut help supply essential nutrients, produce vitamin K, and aid in cellulose digestion (86). Similarly, in horses, diet and the gut microbiota have an important relationship due to the ingested plant fiber that is undigestible by the host enzymes. Hence, the structural carbohydrates in the fiber (such as cellulose, hemicellulose, and lignin) reach the hindgut where breakdown will occur through microbial fermentation. These components are broken down into volatile fatty acids, providing the horse with 50-70% of its energy requirements (5). Additionally, horses have evolutionally adapted to continuously graze low amounts of a fiber-rich diet over the course of a day. However, with increasing high energy demands in performance horses, a diet rich in readily fermentable carbohydrates fed only a few times a day is imposed. Overconsumption of this alternative diet may lead to lower microbiota diversity and decreased production of SCFAs and secondary bile acids (87, 88). For instance, the gut microbiota of ponies fed a high-starch diet tended to have lower richness and diversity when compared to those fed a high-fiber diet (89). Similarly, overweight dogs showed a significant decrease in alpha diversity (90).

Several studies have shown a correlation between dietary changes and the development of intestinal diseases that involve changes in the gut microbiota. For instance, a study done by Daly et al. found a significant increase in Lachnospiraceae, *Bacteroidetes* assemblage and the lactic-acid producing group (*Bacillus-Lactobacillus-Streptococcus*) in horses fed a concentrate diet and in horses fed the concentrate diet with simple colonic obstruction and distension (SCOD), when compared to horses fed a grass-only diet (91). Of interest, the significant increase in colonic lactic acid concentrations and the contrasting constant population size of lactate utilizing bacteria (Veillonellaceae) in the concentrate diet group and the SCOD group suggests an inability to respond to the increased lactic acid, thereby leading to a decrease in colonic pH and thus to a vulnerable environment for the development of intestinal disease (91).

Like animals, humans from various parts of the world have evolved with different diets, allowing the relationship between diet and gut microbiota to be compared. A study comparing the diets and intestinal microbiota of children in Europe (whose diet is high in animal protein and

fat) and rural Africa (whose diet is high in fiber content) found a greater abundance of SCFA-producing bacteria in children from rural Africa, suggesting the ability to provide the host with a higher supply of energy, and the possibility of preventing pathogenic microbes from colonizing the gut (92). Furthermore, an increased microbial richness and diversity was found in children from rural Africa, suggesting further prevention of pathogenic establishment in the gut (92). Perhaps this is an explanation for lower incidences of intestinal diseases observed in populations consuming a traditional fiber-rich diet, first investigated by Burkitt in 1973 (93).

Moreover, the initial diet of a newborn may also play a role in the observed differences in initial gut microbial colonization between individuals. The gut microbiota of newborns who were breast-fed was found to be dominated (up to 90%) by bifidobacteria and lactobacilli (94). In contrast, newborns who were formula-fed were found to have 40-60% of their gut microbiota dominated by bifidobacteria and lactobacilli, whereas the rest was composed of Enterobacteriaceae and Bacteroides (94). Bifidobacteria were found to have several beneficial effects on host health, such as pathogen defense, barrier function, and modulation of immune and inflammatory processes (95).

1.5.4 Antimicrobial administration

For several decades, antimicrobial therapy has been the first line of treatment and prevention for many infectious diseases (96). However, with the recent advances of high throughput sequencing, the negative impacts of antimicrobials on the intestinal microbiota have been discovered. A study in which five healthy volunteers were treated with clindamycin for one week found lasting changes of up to 2 years on bacterial composition in the gut microbiota, as well as an increase in antibiotic-resistant strains of the genus *Bacteroides* and in antibiotic resistance genes (97). Comparably, a study in which mice were treated with clindamycin found long-lasting changes in bacterial composition and decreased bacterial diversity for up to 4 weeks (98). In horses, antimicrobials can have profound effects on the intestinal microbiota, potentially leading to life-threatening colitis (8). One previous study demonstrated significant changes in population structure and community membership in horses treated with procaine penicillin, ceftiofur sodium, and TMS, up to 25 days post-treatment, with differences still remaining (8). Another

study in which metronidazole was administered to horses directly in the caecum observed a decrease in diversity and reduced abundances of Lentispaerae and Spirochaetes (99). In addition, a study evaluating the effects of rifampin on the fecal microbiota of foals with subclinical pneumonia found decreased fecal microbiota diversity and increased antimicrobial resistance genes in feces compared to untreated controls (100).

1.5.5 Stressful conditions

Stress is increasingly being recognized for its impact on the gut microbiota and may contribute to the development of dysbiosis. Stressors may be psychological, physical, or environmental and may include factors such as anxiety, fear, extreme climate, noise, demanding exercise, and sleep deprivation, all of which were evaluated in military personnel (101, 102). Soldiers exposed to a multiple-stressor military training environment developed an increase in intestinal permeability, associated with a post-stress modulation of 23% of metabolites in stool (103). The increase in intestinal permeability may allow the entry of pathogenic compounds, potentially leading to barrier damage, inflammation, and ultimately, intestinal disease. The biological stress response induces activation of the hypothalamus-pituitary-adrenal axis and sympathetic nervous system, thereby stimulating the release of glucocorticoids, catecholamines, and other hormones. In turn, these hormones may modulate the immune system and GI function (104).

As mentioned previously, stress may also be involved in changes in the gut microbiota. A study comparing the gut microbiota of mice exposed to either voluntary wheel running or forced treadmill running showed increased richness in the cecal contents of the voluntary exercise group (105). Additionally, the voluntary exercise group had a higher diversity than the forced exercise group. Lastly, Proteobacteria and Tenericutes phyla were elevated in the forced exercise group. The majority of Proteobacteria were from a single class and genus, namely Epsilonproteobacteria and *Nautilia* spp., respectively. This genus is closely related to *Helicobacter* spp. and *Campylobacter* spp. which are known to be pathogenic in mammalian GI tracts. Moreover, all the Tenericutes were represented by a single family, namely Mollicutes, which has been associated with ulcerative colitis in humans. The authors thus suggest that these bacteria may negatively impact the gut microbiota in mice during forced exercise (105).

Similarly, in horses, stressful conditions such as excessive exercise, transport, fasting, anaesthesia, and weaning have been associated with dysbiosis of the gut microbiota (43, 106). A study evaluating the gut microbiota of 8 horses at baseline, during transport, fasting, and post-anaesthesia found significant differences in community membership and structure 24h and 48h after anaesthesia, respectively (106). Additionally, a study comparing abrupt weaning and progressive weaning of foals found increased salivary cortisol in the abrupt group at time of weaning, suggesting higher stress levels (43). However, *Prevotella* (saccharolytic)*, Paraprevotella* (saccharolytic), and *Ruminococcus* (fibrolytic) were less abundant in the progressive group compared to the abrupt group before weaning, suggesting that the constant separation between foal and mare in the progressive group may have triggered a constant release of stress hormones, inhibiting growth of these genera (43).

Many studies could only prove an associative relationship between intestinal dysbiosis and disease, rather than a causal one. Therefore, it is not yet known whether the previously mentioned events are causes for the onset of dysbiosis, or rather consequences of it. Nevertheless, as intestinal diseases are the leading cause of morbidity and mortality in horses, further studies are required to deepen our understanding of these complex relationships.

1.5.6 Age, genetics, and environment

Other factors that may be involved in disruption of the gut microbiota are age, genetics, and environment. The development of a diverse gut microbiota begins at birth and may differ greatly between a neonate born by a natural birth and a neonate born through a C-section. It has been shown that a woman's vaginal canal is a highly colonized environment that is dominated by *Lactobacillus* and *Prevotella* spp. (107). Consequently, the majority of the bacteria found on the skin of vaginally delivered newborns are similar to those of the mother's vaginal canal. In contrast, the bacteria found on the skin of newborns born through C-section resemble those found on the skin surface of the mother, such as *Staphylococcus*, *Corynebacterium*, and *Propionibacterium* spp. (107). The effects of the different delivery modes on the child's health have yet to be elucidated.

Additionally, it has been shown that the gut microbiota changes with age. As intestinal motility decreases, the rate of intestinal transit and thus defecation decreases as well, possibly leading to

constipation, thereby altering the gut fermentation processes, and ultimately negatively affecting the homeostatic gut environment (108). A group studying the fecal microbiota of young adults (20-40 years old), elders (60-80 years old), and centenarians (over 100 years old) found the microbiota of the centenarians to be enriched with facultative anaerobes, most being Proteobacteria and Bacilli (108). On the other hand, the fecal microbiota of young adults and elders was enriched by *Clostridium* cluster XIVa. Interestingly, this study found no significant differences in structure and diversity of the gut microbiota of young adults compared to elders. The authors suggest that the gut microbiota may remain stable for longer than initially anticipated, proposing to increase the threshold for an aged microbiota to 75-80 years old.

Moreover, genetic impacts on the gut microbiota have been evaluated through twin studies. For instance, a study found greater similarity between monozygotic twins than unrelated persons (109). However, this study used denaturing gradient gel electrophoresis to compare bacterial communities, a method that lacks specificity as it prohibits taxonomic classification, and that may underestimate bacterial richness as a result of overlapping bands (50). On the other hand, another study performing NGS to analyse the fecal samples of mono- and dizygotic twins found no significant differences in gut microbiota between the two groups (110). However, this may be because the sampling was done on individuals 25-32 years old, precluding assessment of the gut microbiota at younger ages. Therefore, whereas twin studies may be beneficial to study the genetic effects on the gut microbiota, larger-scale studies are needed to confirm these results.

Furthermore, environmental influences on gut microbiota changes and establishment have also been an interesting field of study. In western societies such as Italy and the US, the diversity of the gut microbiota of healthy adults is decreasing when compared to those of rural societies such as Papua New Guinea (111), Malawi, and Amerindians (112), and hunter-gatherers from Tanzania (113). Likewise, the diversity of the gut microbiota of children (1-5 years old) in rural societies is greater than that in western societies (92). This western lifestyle seems to be associated with certain chronic inflammatory diseases on the rise, such as IBD, diabetes, asthma, and allergies (114). This may be because of changes in certain practices, such as increased sanitation, antibiotic use, indoor isolation (115), and reduced care for our land, decreasing the diversity of microbes in the soil (116).

In horses, it has been shown that elderly horses (19-28 years old) have a less diverse gut microbiota than younger horses (5-12 years old) (117). However, changes in bacterial structure were lacking. Moreover, in horses, environmental changes such as variations in temperature, geography, and social interactions seem to have an impact on the diversity of the gut microbiota. A study evaluating the fecal microbiota of horses over 12 months found alterations in the gut microbiota of horses associated with seasonal changes (83). In addition, another study evaluating the effects of spatial structure and social interactions reported that close interactions between mares and their offspring, and between stallions and mares, resulted in significantly similar gut microbiota (118). Furthermore, foals showed a significantly different gut microbiota compared to adult horses, where changes in diet may play an important role.

1.6 Treatments for intestinal dysbiosis

Many of the predisposing conditions associated with dysbiosis involve the development of chronic diarrhea. Various treatments have been tried to manipulate the equine gut microbiota in order to correct chronic diarrhea, such as prebiotics, probiotics, antibiotics, and diet modification. However, conflicting results have been obtained on the safety and efficacy of probiotics. Moreover, the effects of many of these treatments are transient and do not lead to a cure. Hence, fecal microbiota transplantation has been increasingly explored to treat horses with chronic diarrhea, as it has shown success in several other species (119-122).

1.6.1 Prebiotics

Prebiotics, first described in 1995 by Glenn Gibson and Marcel Roberfroid, are non-digestible dietary substances degraded by the gut microbiota to selectively feed the beneficial microbes present, leading to favorable proliferation and ultimately, gastrointestinal health (123). In order to be classified as a prebiotic, a compound must be resistant to the acidic pH of the stomach, must not be hydrolyzed by mammalian enzymes, and should not be absorbed in the GI tract. Additionally, it must be fermentable by the intestinal microbiota, and lastly, it must be able to stimulate selective growth and/or activity of the gut microbiota to improve the host's health (123).

Prebiotics are mostly comprised of oligosaccharide carbohydrates, fructo-oligosaccharides (FOS), galacto-oligosaccharides, starch, and glucose-derived oligosaccharides. When they are fermented by the gut, SCFAs are produced, including lactic acid, butyrate, and propionate, which are known to have beneficial effects on host health (124). These compounds can naturally be found in various food products such as asparagus, sugar, beet, garlic, chicory, onion, artichoke, wheat, honey, peas, beans, and more. However, since the concentration of these prebiotics in the diet are minimal, they are mass-produced to increased concentrations, and thus, increased efficacy.

An increase of fecal IgA has been observed in mice fed FOS in their diet, suggesting crucial roles for IgA as a toxin and pathogen neutralizer (125). Additionally, patients with moderately active Crohn's disease receiving supplementary FOS showed increased fecal and mucosal bifidobacteria, and decreased disease activity (126). However, as this study did not include a control group, the therapeutic efficacy of the prebiotic cannot be confirmed.

In horses, diet supplementation with FOS and inulin resulted in increased diversity in all parts of the GI tract when compared to the control group, suggesting better resilience of the GI microbiota (127). Moreover, administration of FOS to horses following induction of digestive stress through diet change resulted in decreased concentrations of lactate-utilizing bacteria and lactobacilli in the colon 29 hours after stress induction, suggesting a preventative role for FOS as it may inhibit potential lactate accumulation (128).

Although these results are promising, large randomized placebo-controlled trials are needed to confirm the various beneficial effects of prebiotics in horses. Additionally, there have been no reported studies on the use of prebiotics to prevent or treat colitis in horses.

1.6.2 Probiotics

Probiotics are microorganisms thought to have beneficial effects on the host health, most of which are already naturally present in the host. In order to be considered a probiotic, the microorganism must survive gastric environments, contain antimicrobial properties, adhere to mucus and epithelial cells, and endure constant production (129). The most common probiotics are *Lactobacillus*, *Bifidobacterium*, and *Saccharomyces*, all of which have been widely investigated

for their beneficial roles in GI diseases (130). In fact, probiotics have been found to be associated with decreased risk of antibiotic-associated diarrhea in children and adults (131-133), as well as increased remission rates in adults with ulcerative colitis (134). Nevertheless, severe side effects such as infections and sepsis have been reported, the majority being in immunocompromised patients due to a weakened intestinal barrier function and immune system, and an impaired microbial clearance (135). Additionally, probiotics have been shown to transfer resistance genes to opportunistic pathogens (136-138). Therefore, the bacteria chosen must be rigorously evaluated. Whereas this regulation is enforced in Europe (139), North America has yet to implement it.

Probiotics may be in the form of food or dietary supplements. Since probiotics can be classified as a drug, preapproval from Health Canada (Canada), the Food and Drug Administration (FDA, United States), or the European Food Safety Authority (EFSA, Europe), is required. Currently, there are no FDA-approved probiotics for horses, however the EFSA approved four commercial products, three of which contain *Saccharomyces cerevisiae* marketed to improve fiber digestion, and one of which includes *Escherichia coli* claiming to improve fecal consistency and odor (140). A study testing the potential of *Lactobacillus rhamnosus* strain GG (LGG) as a probiotic in horses and foals found that LGG persistence in feces was limited to 48 hours in adult horses and reached a maximum of 9 days in foals (13). The low levels reported in adult horses may be due to passive movement through the GI tract rather than intestinal colonization, thereby making LGG less attractive as a probiotic in adult horses. However, the increased persistence in foals may be due to their immature GI microbiota, thus enabling intestinal colonization (13). Similarly, a study in which *Saccharomyces boulardii* was administered to 7 horses with acute enterocolitis found persistence in feces at day 5 post-treatment but not at day 10 (141).

These studies raise the question of whether the beneficial effects of probiotics can be acquired quickly or whether a long-term repeated treatment is necessary. A study in which a combination of probiotics was administered to mice with dextran sodium sulfate-induced chronic colitis found increased colon length, increased body weight, decreased mucosal lesions, and decreased proinflammatory cytokines (142). Accordingly, it is possible that a probiotic mixture might increase its persistence and efficiency of intestinal colonization in horses. Administration

of a probiotic mix typically designed for humans to 5 horses after 21 days of exercise resulted in a reduced concentration of post-exercise blood lactate, suggesting less accumulation of lactate in the muscles and thus a reduced onset of fatigue (143).

Although probiotics may be safe and effective in some studies, other studies have demonstrated conflicting results. For instance, a study in which the probiotic *Lactobacillus pentosus* WE7 was administered once daily for 7 days to 70 foals revealed associations between probiotic administration and the development of colic signs and increased diarrhea persistence, compared to 83 placebo-controlled horses (144). In addition, administration of *Saccharomyces boulardii* to 12 horses with antimicrobial-associated diarrhea found no significant effects on fecal consistency, resolution of watery diarrhea, attitude and appetite improvement, and survival at discharge, compared to 9 placebo-controlled horses (145). Similarly, a study in which one of two commercially available probiotics (gel, *Lactobacillus plantarum, L. casei, L. acidophilus, Streptococcus faecium*; paste, *L. acidophilus, S. faecium, Bifidohacterium thermophilum, B. longum*) was administered to horses for 7 days post-surgery for colic did not find significant effects on *Salmonella* shedding, diarrhea prevalence, or duration of antimicrobial therapy and hospitalization (146).

Although probiotics have the potential to treat gastrointestinal diseases with a low probability of adverse effects, research remains to be conducted in order to confirm their effectiveness in horses.

1.6.3 Synbiotics

The generally accepted definition for a synbiotic is: a mixture of probiotics and prebiotics that act synergistically, aimed to benefit the host by improving survival and implementing microbial dietary supplements in the GI tract of the host (147). Although the concept is interesting, there has been little evidence of success in the use of synbiotics to treat cases involving dysbiosis. It is possible that the amount of prebiotic or probiotic is too low to exert beneficial effects from a given product. For example, de Vrese and Schrezenmeir mention that several products on the German market contain an estimated 2.5 g inulin or oligofructose, which

may be insufficient to prevent gastrointestinal discomfort (148). However, the quantity of the ingredients was not specified, therefore this statement could not be verified.

A study in which prebiotics (inulin and oligofructose), probiotics (*Lactobacillus rhamnosus* and *Bifidobacterium lactic*), or synbiotics (a combination of the two) were administered to rats with experimentally induced colon cancer found a significantly decreased number of tumours and increased SCFAs in the cecum in the rats given prebiotics and synbiotics (149). The synbiotics were able to further reduce the number of tumours, although not significantly, suggesting that this synbiotic may have an additive effect rather than a synergistic one. Nevertheless, no other significant differences were found between the prebiotic and synbiotic treatments, suggesting that the prebiotic alone is sufficient to decrease carcinogenesis. In addition, colonic proliferation was lower in the prebiotic group, further favoring the use of prebiotics over synbiotics in this study. The insignificant effect of probiotics could be due to exposure time, as the authors mention that probiotics may only be effective when given before carcinogen introduction. The results could also be explained by the diet composition, as the carbohydrates given (sucrose and maltodextrins) seem to be poor substrates for bacterial fermentation, as suggested by the observed low SCFA production in the cecum (149).

Furthermore, a study evaluated changes in the fecal microbiota of mice administered synbiotics (comprising 9 bacterial strains) after antibiotic-induced dysbiosis (150). When synbiotics were given before and during antibiotic treatment, Lactobacillales, Verrucomicrobiales and Bifidobacteriales orders remained stable, whereas Bacteroidales decreased and Enterobacteriales increased (150). When synbiotics were continued after antibiotic treatment, Verrucomicrobiales was preserved whereas Lactobacillales and Bifidobacteriales levels decreased. The results suggest that continuous synbiotic administration after antibiotic treatment is more efficient at re-establishing homeostasis in the gut microbiota, compared to solely administering synbiotics before and during antibiotic treatment. While these results are intriguing, it would have been interesting to describe the bacterial composition at the genus and/or species level, as this would allow a clearer understanding of the effects of the synbiotic on specific bacterial populations.

Although the concept of synbiotics may revolutionize treatment efficiency, studies regarding the synergistic mechanisms of action and the effects of diet are still needed.

1.6.4 Postbiotics

Postbiotics are metabolic products secreted by live bacteria in the gut or released after bacterial lysis, including metabolites, SCFAs, microbial cell fractions, functional proteins, extracellular polysaccharides, cell lysates, teichoic acid and pili-type structures (151). Interestingly, fractions and extracts of *Lactobacillus* spp. were found to have significant tumor-suppressing effects *in vitro* (152). In addition, *Lactobacillus paracasei* culture supernatant showed anti-inflammatory effects on *ex vivo* mucosal tissues infected with *Salmonella* (153).

To the author's knowledge, the effect of postbiotics on the equine microbiota have not been evaluated. However, postbiotics may be a great alternative to administering live microorganisms in the form of probiotics or FMT as its shelf-life would be increased, and packaging and transport would be simplified (151). In addition, postbiotics may be safer than live microorganisms for immunocompromised patients and young children whose gut microbiota is not fully developed to combat pathogenic microorganisms (151).

1.6.5 Antimicrobials

It is without a doubt that antimicrobials are vital weapons for fighting infectious diseases. Prior to 1928, infectious diseases caused high morbidity and mortality around the world, restricting the average life expectancy to 47 years (154). However, with the invention of penicillin by Sir Alexander Fleming, infectious disease rates plummeted and average life expectancy increased to 78.8 years (154). Since then, antimicrobials have been the first line of treatment for many bacterial, fungal, and viral infections in humans and animals. Antimicrobials are also widely used in farm animals to prevent the onset and spread of disease, as well as to enhance animal growth (155). For instance, although only 16% of all lactating dairy cows in the U.S. receive antimicrobials for clinical mastitis every year, almost all dairy cows receive prophylactic antimicrobials to prevent the onset of mastitis (156). Moreover, 88% of swine in the U.S. are given antimicrobials in their feed for disease prevention and growth promotion (156). In horses,

antimicrobials are given to treat diseases such as colitis and colic (157). However, information regarding worldwide antimicrobial use and resistance in horses is lacking.

While antimicrobials have revolutionized treatment of infectious diseases, they can be thought of as a double-edged sword. For instance, the same antibiotics that are given to humans with *C. difficile* infection may generate antibiotic resistance, thus leading to recurrence of infection, and possibly death of the host (158, 159). In addition, certain antimicrobials added to animal feed are also used to treat human infections, which may spread antibiotic resistance to humans when animal-based products are consumed (156). Additionally, antimicrobials have been shown to cause long-lasting changes in function and composition of the intestinal microbiota of the host, as mentioned previously (8, 97, 98).

1.6.6 Diet

Diet plays a major role in the gut microbiota composition, diversity, and richness. An extreme example of how diet impacts the microbes present in the gut is the comparison between herbivores, carnivores and omnivores, where their gut microbiota differs greatly as each diet requires different microbes to digest the food consumed and to produce energy for the host (160). Diet also has an impact on the gut microbiota from birth. In fact, human milk is known to contain nutrients, maternal antibodies and commensal maternal bacteria such as bifidobacteria and lactobacilli that may be transferred to the newborn if breastfed (161). Accordingly, newborns who were breastfed had lower levels of *Atopobium* and increased levels of *Bifidobacterium* compared to newborns who were formula-fed (162). Differences were also seen in gut microbiota composition when comparing Western diets that are either high in fiber and carbohydrates, or high in protein and fat (163). Dietary fibers and SCFAs stimulate mucus production and secretion, which is important to prevent pathogen invasion (164). If this mucus barrier is disrupted (due to dietary deficiency, for example), the host becomes increasingly susceptible to infections and chronic inflammatory diseases (164). For instance, mice fed a fiber-free diet were increasingly susceptible to infection by *Citrobacter rodentium*, a pathogen that must cross the mucus layer to cause colitis (164).

Dietary management has also been attempted in horses to treat intestinal diseases such as IBD. However, a study in which 49 horses with IBD were treated by dietary management (laxative, high-protein, high-fat, high-fiber, pasture turn out, probiotics) did not find an association between dietary changes and a positive outcome (165).

1.6.7 Fecal microbiota transplantation (FMT)

Fecal microbiota transplantation is a process that involves taking feces from a healthy host (containing millions of microbes) and transferring it to a patient with intestinal dysbiosis. This idea originated in 4th century China to treat cases of severe food poisoning and diarrhea in humans (16). Currently, FMT is commonly used to treat humans with recurrent *Clostridioides difficile* infection as a last-resource therapy, and has reached a success rate of 90% (15). The most common routes of administration in humans include the upper GI tract by capsule ingestion or by nasogastric, nasoduodenal, or nasojejunal tube and the lower GI tract via colonoscopy or enema (166). The use of FMT has also been studied for several other diseases such as irritable bowel syndrome (167), IBD (which includes ulcerative colitis and Crohn's disease) (168-171), slow transit constipation (172), hepatic encephalopathy (173), and metabolic syndrome (174, 175). However, the complexity of these diseases makes it challenging to reach a high success rate.

1.6.7.1 FMT in other species

FMT is believed to have first been used in veterinary medicine in the 17th century by the Italian anatomist Fabricius Aquapendente (176). FMT has shown success in several other species such as mice, cows, dogs, and poultry. For instance, administration of FMT to mice with colitis alleviated intestinal inflammation, resulting in increased colon length, decreased weight loss, increased expression of anti-inflammatory factors IL-10 and TGF-β, and decreased expression of inflammatory factors TNF-α and IL-1β (119, 177). In addition, rumen transfaunation, which involves transferring rumen fluid of a healthy donor to a sick cow, has seen success in improving rumen microbiota activity, dry matter intake and total VFA production in cows with indigestion (120, 178-180). Furthermore, FMT has been effective in the treatment of dogs with diarrhea associated with parvovirus infection (121), as well as in a dog with *C. difficile*-associated diarrhea (181). Moreover, in poultry, FMT from highly feed-efficient donors succeeded in altering bacterial

composition and microbe-host signaling pathways of recipient chickens (122). However, this study was unsuccessful in transferring high feed-efficient phenotypes to the recipients (122).

1.6.7.2 FMT in horses

As the horse is a hindgut fermenter, microbes are crucial in providing its energy demands. Many of the studies evaluating changes in bacterial composition of the equine gut microbiota use fecal matter for analysis due to its easy acquisition and low level of invasiveness (34). Fecal samples have been shown to appropriately represent the microbiota of the large colon, but may not be suitable for evaluating the proximal intestinal tract (34, 182). Accordingly, significant changes in the fecal microbiota have been identified in horses with colitis, laminitis, and after antimicrobial administration (8, 74, 183).

It is known that foals practice coprophagia with feces from their dams between 2 to 5 weeks of age, which suggests this is an evolutionary process to inoculate the GI tract (184). Between days 2 and 30 of age, the foal's fecal microbiota is subjected to changes in bacterial diversity and relative abundance. However, by day 30, foals seem to develop a stable fecal microbiota for the first year of life (184). An imbalance of these microbes may lead to intestinal diseases, which are the leading causes of morbidity and mortality in horses (9). Although some intestinal diseases in horses are self-limiting, others may be life-threatening. Thus, a suggested protocol for FMT in a foal is to administer fresh feces from its respective dam (26).

As adult horses usually don't practice coprophagia, administration of FMT may help the host intestinal microbiota return to a homeostatic state (26). McKinney et al. (2020) reported diarrhea relief in 3 out of 5 geriatric horses after FMT (23), while another group reported clinical recovery in four horses with diarrhea after FMT, however, no microbiota analysis was performed (24) and a control group was absent in both studies.

It has been speculated that the presence of a core microbiota in all healthy members of a species may provide a guideline to disease prevention, diagnosis, and treatment (32, 74, 110). A study done by Dougal et al. (2013) found the core microbiota to differ between proximal and distal regions of the horse's large intestine. The proximal large intestine, including the cecum, right ventral colon, and left ventral colon, was largely dominated by an unclassified family of the

Bacteroidales order, followed by the *Lachnospiraceae* family (*Firmicutes* phyla), *Prevotellaceae* (*Bacteroidetes* phyla), *Erysipelotrichaceae* (*Firmicutes* phyla), *Ruminococcaceae* (*Firmicutes* phyla) and *Fibrobacteraceae* (*Fibrobacteres* phyla) (32). Contrastingly, the right dorsal colon, small colon, and feces of the distal region were dominated by *Prevotellaceae, Fibrobacteraceae, Lachnospiraceae*, unclassified family (*Bacteroidetes* phyla) and *Clostridiaceae 1* (Firmicutes phyla), while the left dorsal colon was mostly comprised of *Lachnospiraceae, Clostridiaceae 1*, and an unclassified family belonging to the order *Bacteroidales* and *Erysipelotrichaceae*. In comparison, Costa and colleagues compared the fecal microbiota of four healthy horses and found *Roseburia* spp. and four unclassified bacteria, all members of the *Lachnospiraceae* family, to be the most abundant among individuals (74).

Of importance, the largest member in any region of the large intestine in the study by Dougal et al. accounted for only 2% of all members of each region, suggesting the core of the horse hindgut contains many members at very low abundances, possibly explaining the increased vulnerability and severity to intestinal disease in this species (32). In contrast, the microbiota of the rumen of cows has been shown to have highly abundant members that make up the core microbiota (185). A study done in human twins revealed that, although differences were observed in terms of structure and membership (high beta-diversity), there were large similarities in metabolic profiles, suggesting that the core microbiota may be present on a functional basis (core 'microbiome') rather than a taxonomical one (110). Nonetheless, this has yet to be investigated in the equine species.

A standard protocol for FMT has not yet been developed for horses, as is the case for humans. While equine veterinarians have often reported administering FMT to horses with GI disease, placebo-controlled peer-reviewed studies are lacking.

1.6.7.3 Challenges of equine FMT

Although there have been reports of FMT administration in horses, little success has been observed. This may be due to the complex horse anatomy that differs greatly from that of humans, ruminants, and the dog or to the lack of studies evaluating the effects of storage

temperature and time on bacterial viability in the FMT preparations, as well as the precise mechanisms of action of FMT.

1.6.7.3.1 Route of administration

Although the standard methods for FMT administration in humans have been used in horses, the anatomy of the horse GI tract differs greatly from that of the human, possibly hindering FMT efficacy. In humans, FMT is most effective when delivered rectally (186, 187). However in the horse, the lengthy small colon (measuring up to 3.5 metres) precludes the bacteria from colonizing the intestine, therefore excluding the use of this route (26). On the other hand, FMT may also be given orally to humans via nasogastric tube, but this was shown to be less effective (188, 189). In horses, the presence of the fermenting chamber in the caecum, and of the gastric acid and enzymes in the small intestine may decrease bacterial viability, further inhibiting intestinal colonization (26). Therefore, research is needed to determine an efficient method of FMT administration for intestinal colonization in horses.

1.6.7.3.2 Bacterial viability

The processing and storage of FMT has been standardized by Hamilton et al. for human patients with rCDI. The method includes collecting 50 g of fecal material from a healthy donor and placing it in a blender for homogenization in 250 ml of sterile, non-bacteriostatic normal saline. In an anaerobic chamber, the slurry is passed through a 0.25-mm sieve to remove large particles, centrifuged, resuspended in half the original volume, and either administered to the patient immediately or stored in 10% glycerol at -80°C. The frozen suspension is thawed in an ice bath 2-4h before the following FMT procedure.

While the method for FMT processing may be standardized, few studies have evaluated the impact of oxygen exposure and storage time on bacterial viability of the fecal suspension. A study by Papanicolas et al. compared the bacterial composition of stools processed under strict anaerobic conditions, in ambient air, and freeze-thawed, and found that the viability decreased to 50%, 19% and 23%, respectively (190). However, freeze-thawing was not found to significantly modify viable bacterial composition. In contrast, exposure to ambient air significantly reduced the abundance of *Faecalibacterium prausnitzii*, *Subdoligranulum variable*, and *Eubacterium hallii*,

all of which are important butyrate producers in a healthy gut microbiota. Additionally, extended periods of oxygen exposure may not only decrease the abundance of anaerobic bacteria but may also encourage the growth of opportunistic bacteria such as *E. coli* (190). Nevertheless, processing delays may be inevitable in most clinical settings, thus studies evaluating the impact of processing time for FMT in horses are also needed.

Although the processing method mentioned above is efficient in humans, several challenges may arise when dealing with equine fecal preparations. Firstly, horses are anatomically larger than humans, requiring a larger amount of feces resuspended in a larger volume. Secondly, studies evaluating the changes in bacterial viability in equine FMT suspensions after storage are lacking, thus indications of maximum storage time or suggested storage temperature are warranted. Lastly, the need to prepare FMT suspensions in an anaerobic chamber limits the feasibility of the procedure in the field.

Nevertheless, studies in horses comparing FMT suspensions under aerobic and anaerobic conditions are needed, as well as studies evaluating the effects of bacterial viability at different temperatures and storage periods.

1.6.7.3.3 Donor screening and selection

Although FMT has the potential to treat life-threatening intestinal diseases, it is important to acknowledge the risks associated with this technique. Before donor selection, important screening protocols must be performed to prevent transmission of multi-drug resistant organisms or development of bacteraemia. In June of 2019, the FDA issued a safety alert concerning these risks, in response to serious invasive infections in two immunocompromised patients who received extended-spectrum beta-lactamase-producing *E. coli* (191). In October of 2019, OpenBiome, the largest public stool bank in the United States, published a standardized donor screening program (192). The first stage evaluates the candidate's general health and risk of infectious disease. The second stage screens for transmissible diseases and microbiome-mediated conditions such as gastrointestinal, autoimmune, atopic, allergic, metabolic, neurologic, and psychiatric conditions. The third stage involves screening the stool and nasal cavities of each candidate. The fourth and final stage is to perform serological screening. After the four screening

stages, only 3% of 15,317 candidates qualified as healthy stool donors (192). This indicates the importance of donor screening and the subsequent immense challenge of donor selection.

FMT use in animals has yet to establish a standardized screening and selection protocol for the donor candidates. In the study done by Pereira et al. in which puppies with canine parvovirus infection were treated with FMT, each donor was screened for vaccine history, antimicrobial use history, vomiting, diarrhea, serum biochemical analysis, blood count, fecal parasites, and viruses (121). In a case report by Sugita et al., the donor screening protocol included a blood count, serum biochemical analysis, radiography, abdominal ultrasound, fecal examination, and pathogen detection through real-time PCR (181). Nevertheless, both studies lacked specificity and standardization of donor screening. Of importance, a standardized donor screening protocol for horses has not been developed, which may potentially affect the outcome of current and future FMT studies.

1.7 Hypotheses and Objective

Hypothesis 1: Treatment with concentrated FMT will correct dysbiosis faster than conventional FMT and the vehicle.

Hypothesis 2: The gut microbiota of horses treated with concentrated FMT will resemble the donor's microbiota.

General Objective : To compare an FMT protocol with concentrated bacteria to current recommendations of FMT treatment for horses with dysbiosis.

Specific objective 1: To develop a protocol to increase the concentration of bacteria from horse feces by centrifugation.

Specific objective 2: To test a protocol using concentrated FMT to correct antibiotic-induced dysbiosis in horses.

Chapter 2 – Scientific Manuscript

Development of a Protocol with Concentrated Bacteria for Fecal Microbiota Transplantation and Impact on the Equine Fecal Microbiota After Antibiotic-Induced Dysbiosis

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Abstract

The equine gut microbiota plays an important in maintaining the health of the host. An imbalance of the gut microbiota, termed dysbiosis, has been observed in equine GI diseases. Restoration of the gut to a healthy state can be performed through methods of microbiota manipulation, such as fecal microbiota transplantation (FMT). Studies using current commendations for FMT have shown clinical recovery in horses with diarrhea, but the microbiota remains largely unchanged after FMT and no controlled studies have been performed. The objective of this study was to develop a new protocol to improve FMT in horses, by increasing the concentration of bacteria found in the donor stool using centrifugation, and to test it in horses with antibiotic-induced intestinal dysbiosis. The antibiotic trimethoprim sulfadiazine (TMS) was given to nine horses for five consecutive days to induce intestinal dysbiosis. Horses received either the concentrated FMT (cFMT, n=3), fresh FMT (fFMT, n=3), or 10% glycerol in 0.9% saline (vehicle, VEH, n=3). Fecal samples were collected, and DNA extraction was performed. Sequencing was performed using the Illumina MiSeq platform and analysed using the software Mothur. The membership of the cFMT and fFMT donor fecal suspensions was significantly different from the membership of cFMT and fFMT recipients at baseline, respectively. The membership of the cFMT and fFMT recipient horses was significantly different after transplantation, while the membership of the vehicle recipients was not. In addition, the *Escherichia* genus was found in significantly higher relative abundances in the cFMT donor fecal suspensions when compared to the fFMT donor fecal suspensions. Further clinical studies are needed before FMT can be recommended to treat dysbiosis in horses.

Introduction

The gut microbiota is an important contributor to maintaining the health of the host, such as in nutrition (1), energy metabolism (2), immune development (3), and host defense against harmful pathogens (4). Similarly, the equine gut microbiota plays a critical role in cellulose fermentation and short-chain fatty acid (SCFA) production to provide a horse with an important source of energy (5). The gut microbiota is composed of many microorganisms such as bacteria,

viruses, fungi, and archaea (6). However, the majority of these microbial cells are bacterial cells (7), and consequently, our study focuses on exploring bacterial communities in the gut.

When an imbalance of the microbiota occurs, the host is said to have dysbiosis. In horses, dysbiosis is associated with diet change and antimicrobial administration (8), as well as with diseases affecting the gastrointestinal (GI) tract such as colitis and colic (9). Importantly, GI diseases are the leading causes of morbidity and mortality in horses (10). Current treatments for colic include analgesics, fluid therapy, and laxatives (11, 12), while colitis may also be treated by antibiotic-probiotic administration, prebiotics, and diet modification (13, 14). However, many of these treatments are transient and recurrence of the disease or animal death is often the outcome. Therefore, advances in therapeutic approaches of equine colitis are warranted (15).

There are many methods of microbiota manipulation that might be used to restore a dysbiotic environment including probiotics (16), prebiotics (17), synbiotics (18), postbiotics (19), and fecal microbiota transplantation (FMT) (20). Of these methods, FMT is increasingly being explored as an alternative therapy to GI diseases due to its success in treating human patients with recurrent *Clostridioides difficile* infection (rCDI) (21). FMT involves administration of stool from a healthy donor to a patient with dysbiosis (22). FMT is being studied for its potential efficacy in treatment of several other GI diseases in humans, including irritable bowel syndrome, inflammatory bowel disease (IBD), and metabolic syndrome (23). FMT has also seen success in several other species such as dogs (24), cattle (25, 26), and pigs (27, 28). Moreover, a recent study showed diarrhea relief in 3 out of 5 geriatric horses after FMT (29), while another study reported clinical recovery in 4 horses with diarrhea after FMT, however, no microbiota analysis was performed (30) and a control group was absent in both studies. In addition, a study from our group in which FMT was administered to six horses with chronic diarrhea failed to detect changes in the gut microbiota after treatment (Costa et al., under review). Therefore, controlled studies of FMT treatment have not yet been evaluated in horses.

In humans, FMT is often given directly to the large intestine by enema (31). However, the GI tract of the horse differs substantially from that of the human. It is known that horses have a long small colon, measuring approximately three meters, thereby precluding bacteria from reaching

the large colon (32). Consequently, FMT through enema is not feasible in horses as it is in humans. Another possible route of administration is through a nasogastric tube; however, this method is thought to have limitations as well since the bacteria must pass through the gastric acidity, enzymatic actions of small intestine and fermentation present in the cecum, decreasing bacterial viability (32). The limitations of these methods strongly suggest that more research is required to determine an efficient method of FMT administration for intestinal colonization in horses.

In order to evaluate changes in bacterial composition or successful bacterial engraftment, the gut microbiota must be characterized. The development of culture-independent DNA-sequencing technologies, such as next generation sequencing, has made it possible for in-depth characterization of the bacterial communities present in the intestinal microbiota (33). These methods have allowed a greater understanding of the interactions between the intestinal microbiota and the host.

A previous study evaluating the effects of antibiotic administration on the intestinal microbiota in horses has shown that administration of penicillin, ceftiofur, and trimethoprim sulfadiazine (TMS) significantly alter the intestinal microbiota. Of importance, TMS had the greatest impact on species richness and diversity (8). Therefore, oral administration of TMS could be used as a model for equine dysbiosis.

Based on what has been said, we are conducting the present work to test a protocol for FMT in horses with dysbiosis by concentrating the bacteria in the donor fecal suspension. The hypothesis is that treatment with a concentrated FMT solution will correct dysbiosis faster than conventional FMT. We also hypothesize that the gut microbiota of horses treated with concentrated FMT will resemble the donor's microbiota.

Materials and Methods

Ethics statement

Experimental procedures were performed in accordance with the Canadian Council for Animal Care guidelines and were approved by the Animal Care Committee of the Université de Montréal (#19Rech2025).

Animal selection

One healthy 11-year old female Standardbred horse (teaching animal housed at the institution) of 490 kg was used as a fecal donor (DON) with no history of gastrointestinal disease and did not receive antimicrobials or other medications during the 3 months prior to the study. The donor horse was fed hay for the duration of the study, received regular mineral supplements and a salt block, and had daily access to a paddock. Feces from the donor horse were tested negative for the presence of *Salmonella enterica*, *Clostridium perfringens*, *Clostridioides difficile* and parasitic eggs, as previously recommended (32). The microbiological tests were performed by the Diagnostic Service at the Faculté de médecine vétérinaire.

Nine adult horses belonging to a research herd of asthmatic horses of the Faculté de médecine vétérinaire, Université de Montréal were enrolled and were housed in a different research facility from the donor horse. All horses were in remission and had no history of gastrointestinal diseases or antimicrobial administration during the previous 3 months. 4 months prior to the study, horses received a dewormer (Eqvalan), methylprednisolone, and a Vetera Gold vaccine. The animals were kept on pasture and were fed grass and silage and had access to a salt block. Table 1 summarizes the studied population including previous treatments received.

Protocol for cFMT by bacterial centrifugation

Feces from the donor horse were collected using a fecal collector. The fecal collector was installed on the donor and kept overnight to obtain approximately 10 kg of feces. The cFMT was made by adding 2 L of water to 1 kg of feces, mixing thoroughly to break the fecal balls, and then strained with a cheese cloth to remove large particles. The strained feces were then put into 500 mL centrifuge bottles and centrifuged at 24 470 x *g* for 30 minutes. The supernatant from each centrifuge bottle was discarded, and the pellet was resuspended in 400 mL of 10% glycerol in 0.9% saline. The cFMT was transferred into plastic bags and stored at -80 °C until use. Figure 1 shows a bacterial culture of the cFMT (A) and fFMT (B) fecal suspensions.

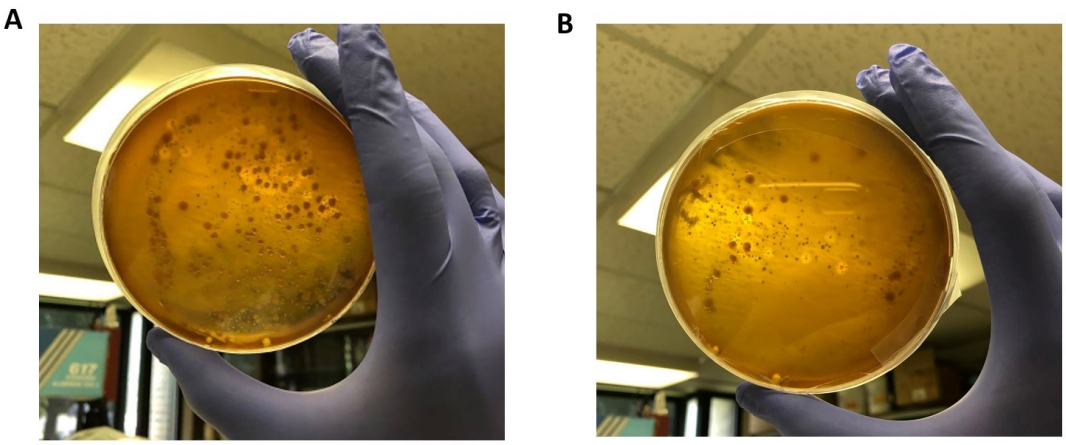


Figure 1. – Bacterial culture of concentrated and fresh fecal suspensions on MacConkey agar. **(A)** After centrifugation of the donor fecal suspension and resuspension in 10% glycerol and 0.9% saline, the suspension was plated on MacConkey agar. **(B)** Before centrifugation, the fresh fecal suspension was plated on MacConkey agar. Both plates were incubated at 37 °C for 24 hours in ambient air.

Protocol for fFMT

The fresh FMT (fFMT) was made by installing the fecal collector on the donor horse the evening before each treatment day (days 6, 7, and 8). On the day of the transplants, 3.2 L of water were added to 1.6 kg of feces, and after mixing thoroughly, the mixture was strained with a cheese cloth to remove large particles, as previously recommended (32). This procedure was repeated until enough fFMT was made to treat the three horses (3.2 L x 3 horses = 9.6 L).

Study design

All nine horses received trimethoprim sulfadiazine (TMS) (30mg/kg) twice a day for 5 days (D0 to D4). After two days of rest, the nine horses were then randomly assigned to each of two treatment groups or a vehicle group and received transplants for three consecutive days (D7 to D9) by nasogastric tube. The first group received 3.2 L of concentrated FMT (cFMT) twice a day. 3.2 L of cFMT was obtained by thawing 8 plastic bags each containing 400 mL of cFMT. The second group received 3.2 L of fresh FMT (fFMT) as per current recommendations, once a day. 3.2 L of fFMT was obtained by straining 1.6 kg of fresh feces in 3.2 L of water. The vehicle group (VEH)

received 3.2 L of 10% glycerol in 0.9% saline once a day. All horses received 500 mL of 0.1 molar solution of sodium bicarbonate before treatment administration to increase the pH of the stomach (193). A detailed experimental timeline can be found in Figure 2.

Recipient characteristics, diet, housing type, and previous treatments received.

Recipient	Age	Sex	Breed	Weight (kg)	Group	Diet	Housing type	Previous treatments
1	15	Mare	Crossed Quarter Horse	530	cFMT	Pasture and silage	Turnout with shelter	Methylprednisolone, dewormer (Eqvalan), Vetera Gold vaccine
2	14	Gelding	Quarter Horse	483	cFMT	Pasture and silage	Turnout with shelter	Methylprednisolone, dewormer (Eqvalan), Vetera Gold vaccine
3	14	Gelding	Crossed Quarter Horse	496	cFMT	Pasture and silage	Turnout with shelter	Methylprednisolone, dewormer (Eqvalan), Vetera Gold vaccine
4	19	Mare	Paint Horse	588	fFMT	Pasture and silage	Turnout with shelter	Methylprednisolone, dewormer (Eqvalan), Vetera Gold vaccine
5	16	Mare	Crossed Paint Horse	540	fFMT	Pasture and silage	Turnout with shelter	Methylprednisolone, dewormer (Eqvalan), Vetera Gold vaccine
6	14	Mare	Thoroughbred	514	fFMT	Pasture and silage	Turnout with shelter	Methylprednisolone, dewormer (Eqvalan), Vetera Gold vaccine
7	15	Mare	Crossed Quarter Horse	510	VEH	Pasture and silage	Turnout with shelter	Methylprednisolone, dewormer (Eqvalan), Vetera Gold vaccine
8	12	Gelding	Canadian Horse	622	VEH	Pasture and silage	Turnout with shelter	Methylprednisolone, dewormer (Eqvalan), Vetera Gold vaccine
9	10	Gelding	Quarter Horse	556	VEH	Pasture and silage	Turnout with shelter	Methylprednisolone, dewormer (Eqvalan), Vetera Gold vaccine

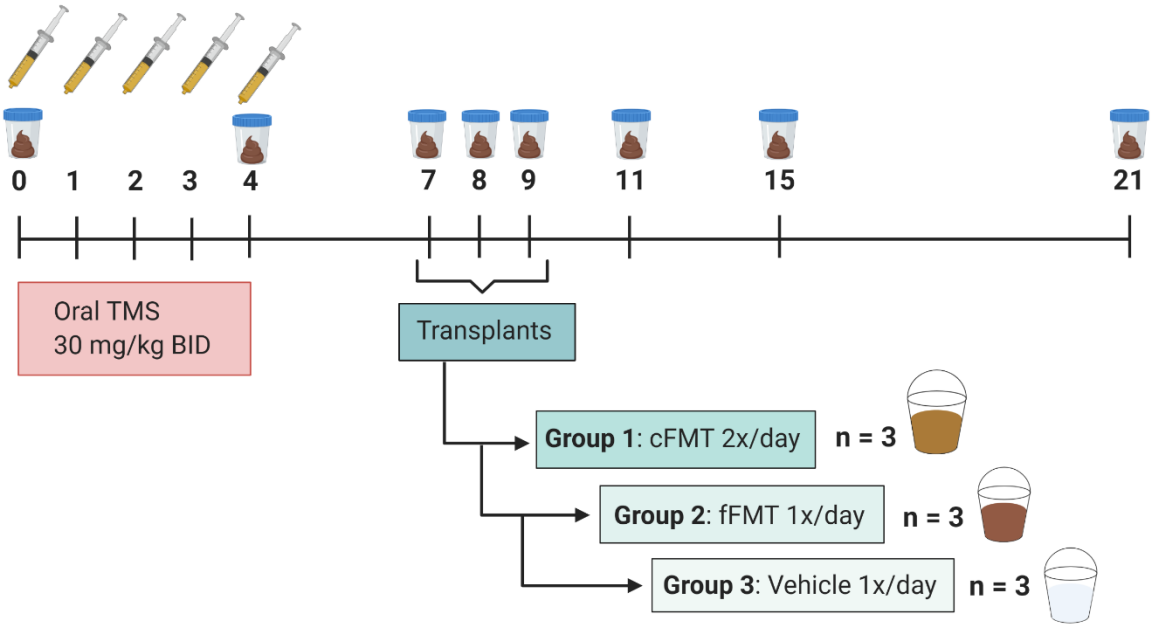


Figure 2. – Experimental timeline. Trimethoprim/sulfadiazine (TMS) was administered for 5 consecutive days (day 0 to day 4). Transplants were performed by nasogastric tube for three consecutive days (day 7, 8, 9). Group 1 contained three horses who received the cFMT twice a day for three consecutive days. Group 2 also contained three horses and received the fFMT once a day for three consecutive days. Group 3, the VEH group, contained three horses, and received 10% glycerol in normal saline once a day for three consecutive days. Fecal samples were collected on days 0, 4, 7, 8, 9, 11, 15, and 21.

Fecal samples in the form of a fecal ball were collected by rectal palpation using one glove per animal. Samples were collected before and after antibiotic administration (days 0 and 4, respectively), before and during the transplants (days 7 and 8), as well as after the transplants (days 9, 11, 15, and 21) (Figure 2). A fecal sample directly from the rectum of the donor horse was also collected. Fecal samples were stored in plastic bags and frozen at -80 °C within 3 hours after collection until DNA extraction.

Microbiota analysis

Total DNA was extracted using a commercial kit (DNeasy PowerSoil Kit, QIAGEN) following the manufacturer's instructions. PCR amplification of the V4 region of the 16S rRNA gene was performed using the primers 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) as previously recommended (35). Sequencing was performed using an Illumina MiSeq platform for 250 cycles from each end at the Génome Québec Innovation Centre. Sequences will be made available at the NCBI Sequence Read Archive.

Sequence analysis and statistical analysis

Bioinformatic analysis was performed using the software Mothur (36) following the Standard Operating Procedure previously described (37). Sequencing reads were aligned with the SILVA reference database, clustered at 97% similarity and classified using the Ribosomal Databank Project (RDP). Sequences classified as the same genus (94% similarity) were clustered together for further analyses (Phylotypes).

The Chao richness estimator, Simpson's diversity index, and Shannon index were calculated for characterization of richness (number of different genera present in a community) and diversity (number of genera present and their relative abundance). Those indices were compared between donor and recipients, and between recipients at the different time points using a paired Student's two-tailed t-test and one-way analysis of variance (ANOVA). Beta diversity (comparison of taxonomic composition between each sample) was characterized by the Jaccard index and the Yue and Clayton index to evaluate community membership and structure, respectively. It is important to note that membership analysis considers only the presence or absence of a bacterial taxa while the structure also considers how often that bacteria appeared in the analysis (relative abundance). A 2-dimensional Principal Coordinate Analysis (PCoA) plot was generated to visualize the similarity between samples. Analysis of molecular variance (AMOVA) was used to determine significance of clustering between recipients in different treatment groups at different time points.

The most abundant bacteria (>1%) were visualized by generating bar charts representing the relative abundance of the main phyla and genera found in each horse. The linear discriminant

analysis effect size (LEfSe), which uses a non-parametric factorial Kruskal-Wallis with a subsequent Unpaired Wilcoxon test, was used to detect significant differences in relative abundances with respect to each group of interest (recipients before and after antibiotic administration, and after treatment), followed by a linear discriminant analysis (LDA) to estimate the effect size of each differentially abundant group (38).

Results

Horses

The same donor was used for all horses receiving fFMT and cFMT. Three horses received 3.2 L of cFMT twice a day for three consecutive days, while another three horses received 3.2 L of fFMT once a day for three consecutive days. Lastly, three horses received 3.2 L on 10% glycerol in 0.9% saline once a day for three consecutive days. All horses were closely monitored by physical examination including changes in behavior, appetite, temperature, respiratory and cardiac frequency, GI motility, and stool consistency. No side effects such as discomfort or diarrhea were recorded. All horses completed the experiment, and no severe side effects were observed.

Microbiota analysis

A total of 11,084,884 reads were obtained from 78 samples of which 6,543,737 passed all quality filters and were assigned into OTUs. To normalize the number of reads across all samples and decrease bias of non-uniform sizes, a subsample of 12,144 reads per sample was used for analysis.

Alpha diversity

As expected, a significant decrease in richness (Chao richness estimator) was observed after antibiotic administration ($P < 0.01$, paired Student's t-test of D0 fecal samples vs. D4 fecal samples, Figure 3A). No significant difference in richness was observed after microbiota transplant in neither fFMT nor cFMT groups (Figure 3B). Diversity (Simpson's and Shannon indices) were not significantly different after antibiotic administration, nor when comparing

samples after antibiotic administration to those after the microbiota transplant (Figure 3C, D, E, F).

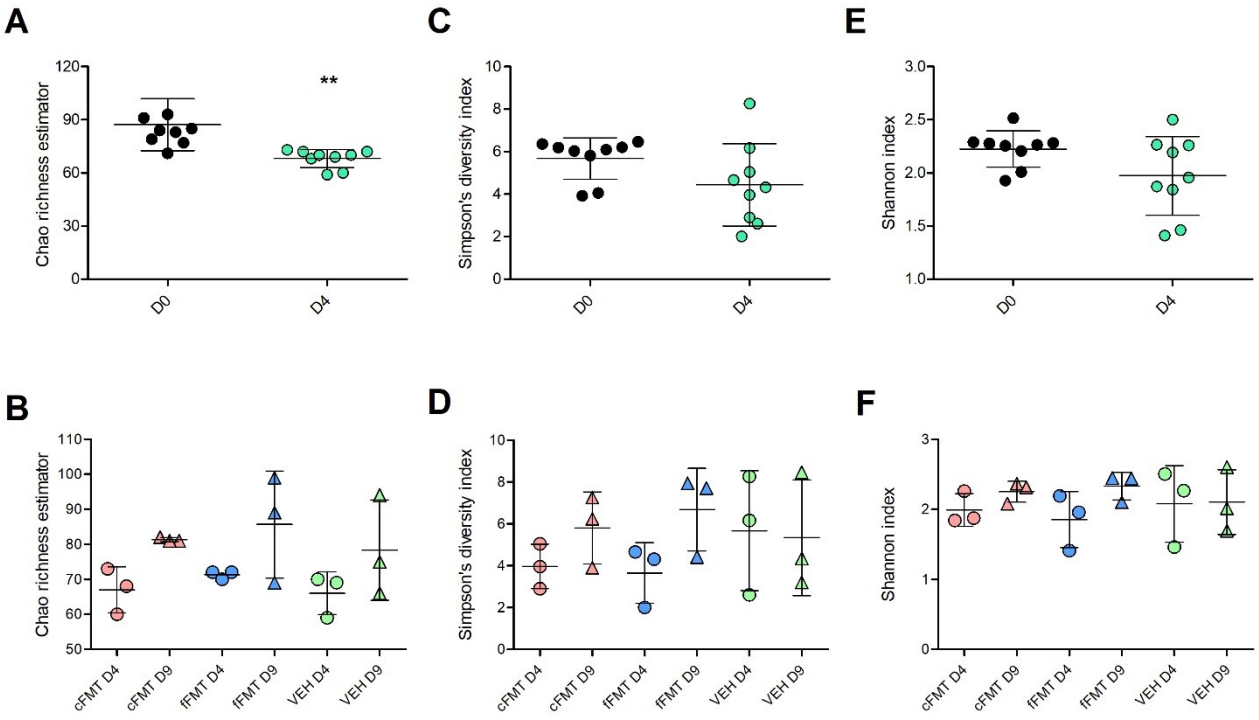
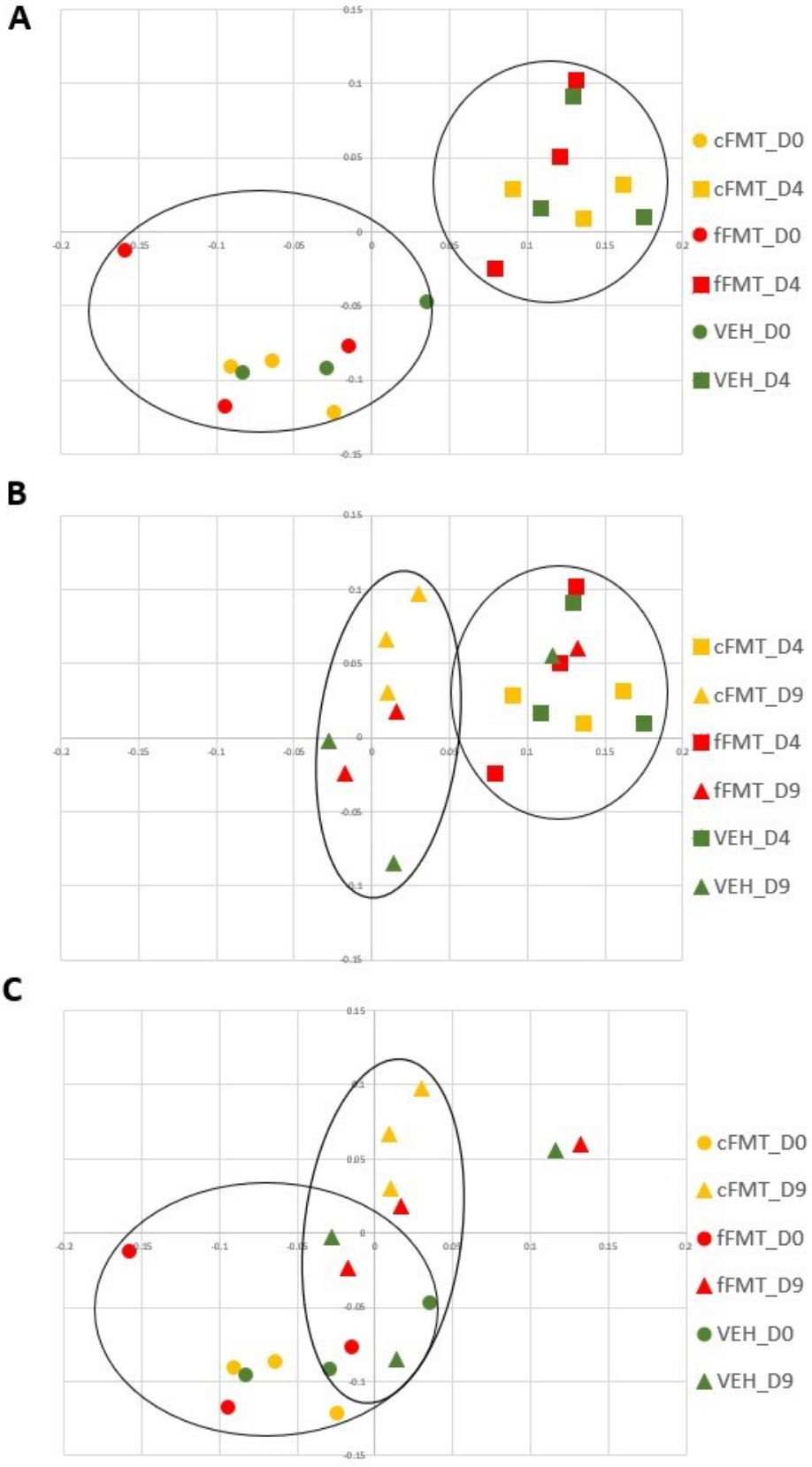


Figure 3. – Alpha diversity indices. Chao richness estimator of all horses before (D0) and after (D4) antibiotic administration **(A)** and before (D7) and after (D9) transplantation **(B)**. Simpson's diversity index of all horses before (D0) and after (D4) antibiotic administration **(C)** and before (D7) and after (D9) transplantation **(D)**. Shannon index of all horses before (D0) and after (D4) antibiotic administration **(E)** and before (D7) and after (D9) transplantation **(F)**. cFMT represents horses receiving the concentrated FMT, while fFMT represents the horses receiving the fresh FMT. VEH represents the horses receiving 10% glycerol in 0.9% saline. Statistical analysis was performed using paired Student's t tests (A, C, E) and one-way ANOVA (B, D, F). Bars represent mean and SD. $^{**}P \leq 0.01$.

Beta diversity

A complete list of *P*-values for beta-diversity obtained from AMOVA test can be found in Table 2. A significant difference was observed in beta diversity membership after antibiotic administration when compared to baseline values ($P < 0.001$, D0 vs. D4, Figure 4A) confirming the

potential of TMS to induce compositional changes in the distal gut microbiota. Membership of cFMT and fFMT recipients after transplantation was significantly different than after antibiotic administration, whereas vehicle recipients were not (P = 0.004, cFMT_D4 vs. cFMT_D9; P = 0.04, fFMT_D4 vs. fFMT_D9; P = 0.26, VEH_D4 vs. VEH_D9; Figure 4B). When compared to baseline values, the membership of cFMT and fFMT recipients after transplantation was significantly different, but so was the membership of the vehicle recipients (P = 0.004, cFMT_D0 vs. cFMT_D9; P = 0.004, fFMT_D0 vs. fFMT_D9; P = 0.02, VEH_D0 vs. VEH_D9; Figure 4C). The membership from the cFMT and fFMT donor's fecal suspensions were significantly different from the cFMT and fFMT recipients' baseline values, respectively (P = 0.004, cFMT_D0 vs. DON_cFMT; P = 0.003, fFMT_D0 vs. DON_fFMT; Figure 4F), as well as different from the donor fecal microbiota, indicating that the composition of the microbiota present in the fecal suspension used for transplantation was different from the one found in healthy horses. The fecal microbiota membership of the donor clustered together with the baseline membership of all recipients (Figure 4F).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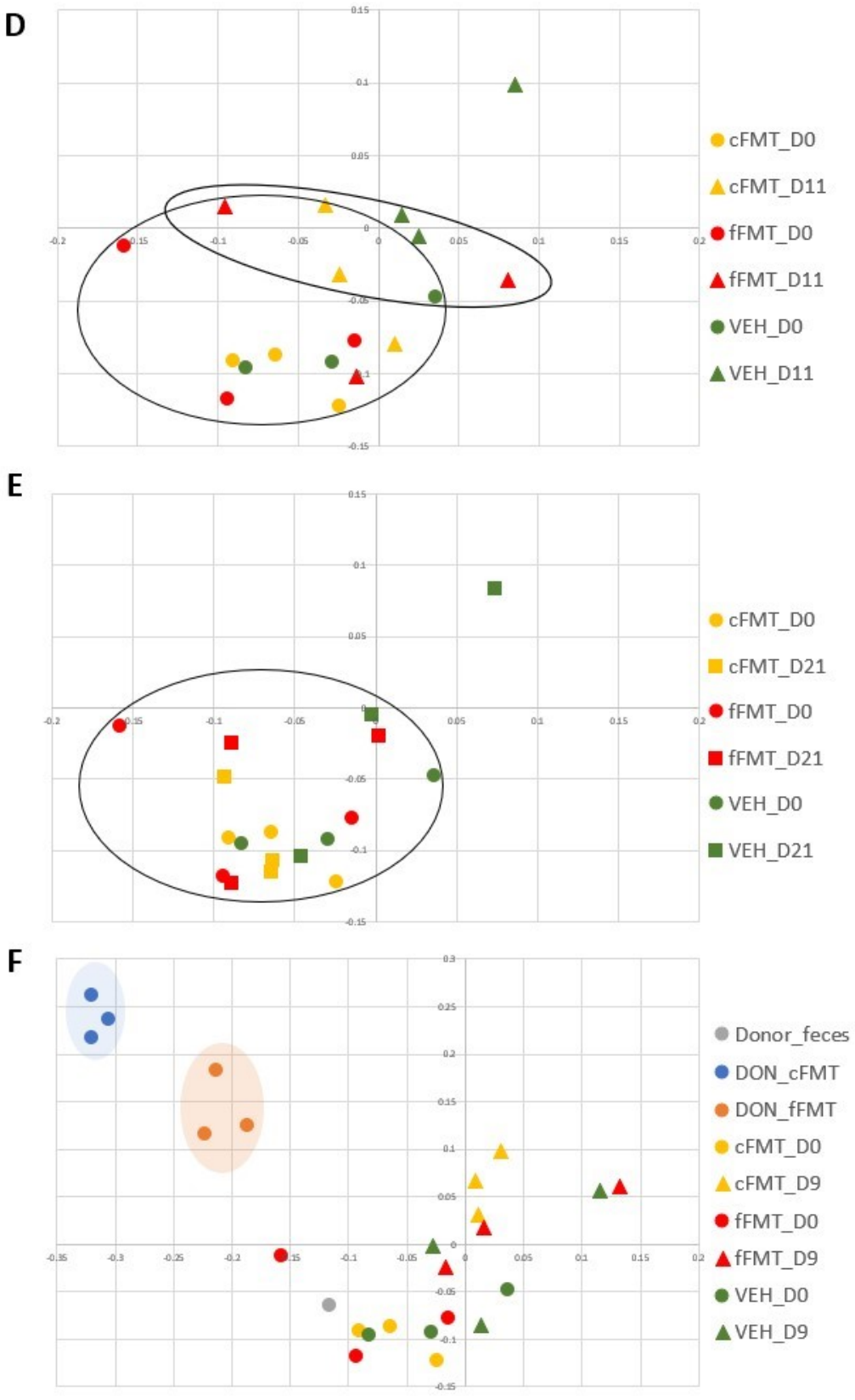
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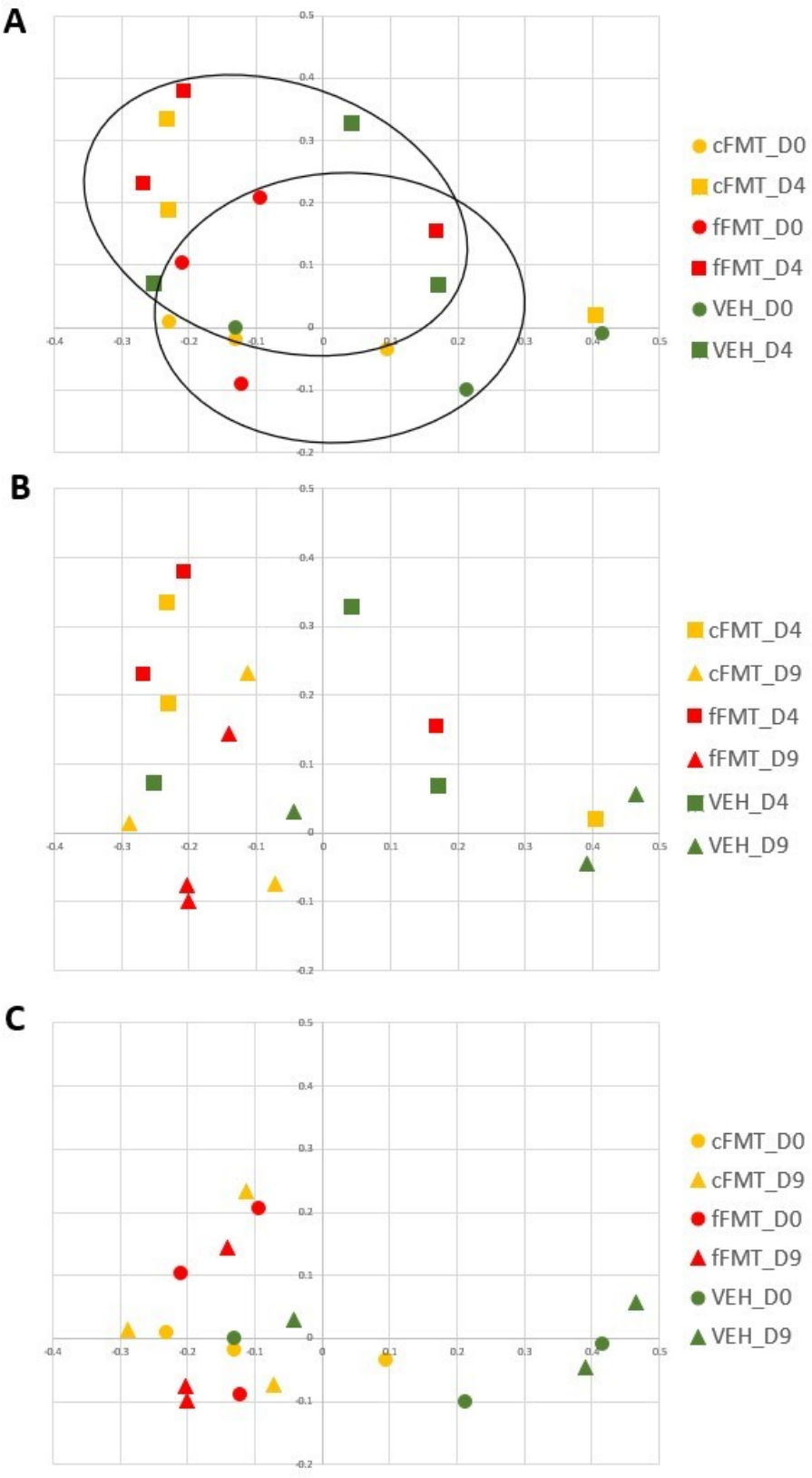
VEH_D9

Figure 4. – Principal coordinate analysis (PCoA) of bacterial communities' membership present in the feces of healthy donor horses, FMT recipients, and vehicle recipients. Bidimensional representation of the principal coordinate analysis of bacterial communities' membership addressed by the Classic Jaccard analysis. (A) Membership before antibiotic administration (D0) and after antibiotic administration (D4) of recipients receiving the concentrated FMT (cFMT), recipients receiving the fresh FMT (fFMT) and recipients receiving the vehicle (VEH). (B) Membership after antibiotic administration (D4) and after transplantation (D9). (C) Membership before antibiotic administration (D0) and after transplantation (D9). (D) Membership before antibiotic administration (D0) and 6 days after transplantation (D15). (E) Membership before antibiotic administration (D0) and 12 days after transplantation (D21). (F) Membership of the donor's fecal suspensions (DON_cFMT, DON_fFMT), and of the recipients before antibiotic administration (D0), and after transplantation (D9). Circles were used to highlight the major clustering.

No significant difference was observed in community structure after antibiotic administration when compared to baseline values of all recipients (P = 0.11, all recipients on D0 vs. D4, Figure 5A), indicating that TMS affects the rare populations of a community, but not the most abundant. The structure was not significantly different when comparing values after transplantation to values after antibiotic administration (P = 0.44, cFMT_D4 vs. cFMT_D9; P = 0.22, fFMT_D4 vs. fFMT_D9; P = 0.11, VEH_D4 vs. VEH_D9; Figure 5B), indicating no impact of treatment. Similarly, no significant difference was observed when comparing the structure of baseline values to after transplantation (P = 0.38, cFMT_D0 vs. cFMT_D9; P = 0.27, fFMT_D0 vs. fFMT_D9; P = 0.06, VEH_D0 vs. VEH_D9; Figure 5C). The fecal microbiota structure of the donor clustered together with the baseline structure of all recipients (Figure 5F). The structure from the cFMT and fFMT donor's fecal suspensions were significantly different from the cFMT and fFMT recipients' baseline structure, respectively (P = 0.003, cFMT_D0 vs. DON_cFMT; P = 0.005, fFMT_D0 vs. DON_fFMT; Figure 5F), as well as from the donor fecal microbiota (Figure 5F).


A
cFMT_D0
cFMT_D4
fFMT_D0
fFMT_D4
VEH_D0
VEH_D4
B
cFMT_D4
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fFMT_D4
fFMT_D9
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fFMT_D0
fFMT_D9
VEH_D0
VEH_D9

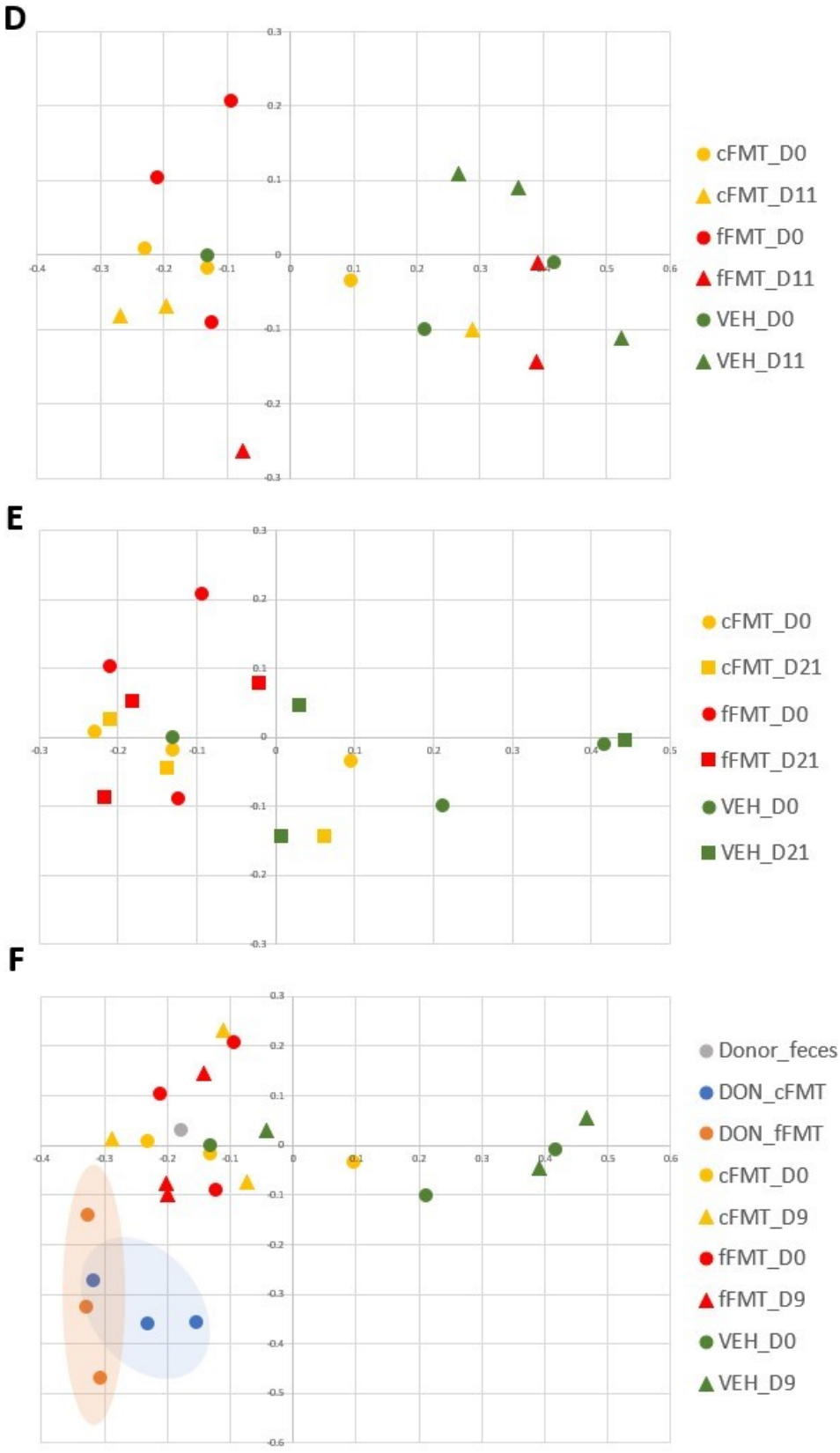
D
cFMT_D0
cFMT_D11
fFMT_D0
fFMT_D11
VEH_D0
VEH_D11
E
cFMT_D0
cFMT_D21
fFMT_D0
fFMT_D21
VEH_D0
VEH_D21
F
Donor_feces
DON_cFMT
DON_fFMT
cFMT_D0
cFMT_D9
fFMT_D0
fFMT_D9
VEH_D0
VEH_D9

Figure 5. – Principal coordinate analysis (PCoA) of bacterial communities' structure present in the feces of healthy donor horses, FMT recipients, and vehicle recipients. Bidimensional representation of the principal coordinate analysis of bacterial communities' structure addressed by the Yue and Clayton analysis. **(A)** Structure before antibiotic administration (D0) and after antibiotic administration (D4) of recipients receiving the concentrated FMT (cFMT), recipients receiving the fresh FMT (fFMT) and recipients receiving the vehicle (VEH). **(B)** Structure of recipients after antibiotic administration (D4) and after transplantation (D9). **(C)** Structure before antibiotic administration (D0) and after transplantation (D9). **(D)** Structure before antibiotic administration (D0) and 6 days after transplantation (D15). **(E)** Structure before antibiotic administration (D0) and 12 days after transplantation (D21). **(F)** Structure of the donor's fecal suspensions (DON_cFMT, DON_fFMT), and of the recipients before antibiotic administration (D0), and after transplantation (D9). Circles were used to highlight the major clustering.

P-values obtained from the AMOVA test for structure (Yue & Clayton index) and membership (Jaccard index) comparing groups at different sampling times. Samples in bold represent significant *P*-values of < 0.05.

Group comparisons	Structure	Membership
D0 vs. D4	0.11	**< 0.001**
cFMT_D4 vs. cFMT_D9	0.44	**0.004**
fFMT_D4 vs. fFMT_D9	0.22	**0.04**
VEH_D4 vs. VEH_D9	0.11	0.26
cFMT_D0 vs. cFMT_D9	0.38	**0.004**
fFMT_D0 vs. fFMT_D9	0.27	**0.004**
VEH_D0 vs. VEH_D9	0.06	**0.02**
cFMT_D9 vs. VEH_D9	**0.03**	0.37
fFMT_D9 vs. VEH_D9	**0.02**	0.89
cFMT_D9 vs. fFMT_D9	0.71	0.16
D4. vs. cFMT_D11	0.13	**0.008**
D4 vs. fFMT_D11	**0.01**	**<0.001**
D0 vs. cFMT_D11	0.68	**0.008**
D0 vs. fFMT_D11	0.08	0.13
cFMT_D11 vs. fFMT_D11	0.17	0.7
cFMT_D0 vs. DON_cFMT	**0.003**	**0.004**
fFMT_D0 vs. DON_fFMT	**0.005**	**0.003**
cFMT_D9 vs. DON_cFMT	0.09	0.09
fFMT_D9 vs. DON_fFMT	0.11	0.11
DON_cFMT vs. DON_fFMT	0.1	0.1

Relative abundances

The relative abundances at the phylum and genus levels found in each group at the various sampling times are shown in Figure 6. Bacteroidetes was the most abundant phylum among recipient horses (45%), followed by Fibrobacteres (19%), Firmicutes (15%), unclassified bacteria (9%), Spirochaetes (7%), and Verrucomicrobia (5%) (Figure 6A). The most abundant taxa classified at lower taxonomic levels included unclassified Bacteroidetes, *Fibrobacter*, unclassified bacteria, unclassified Bacteroidales, *Treponema*, unclassified subdivision 5, unclassified *Lachnospiraceae*, unclassified *Ruminococcaceae*, unclassified Clostridiales, *Prevotella*, *Phascolarctobacterium*, unclassified *Prevotellaceae*, and unclassified Firmicutes (Figure 6B).

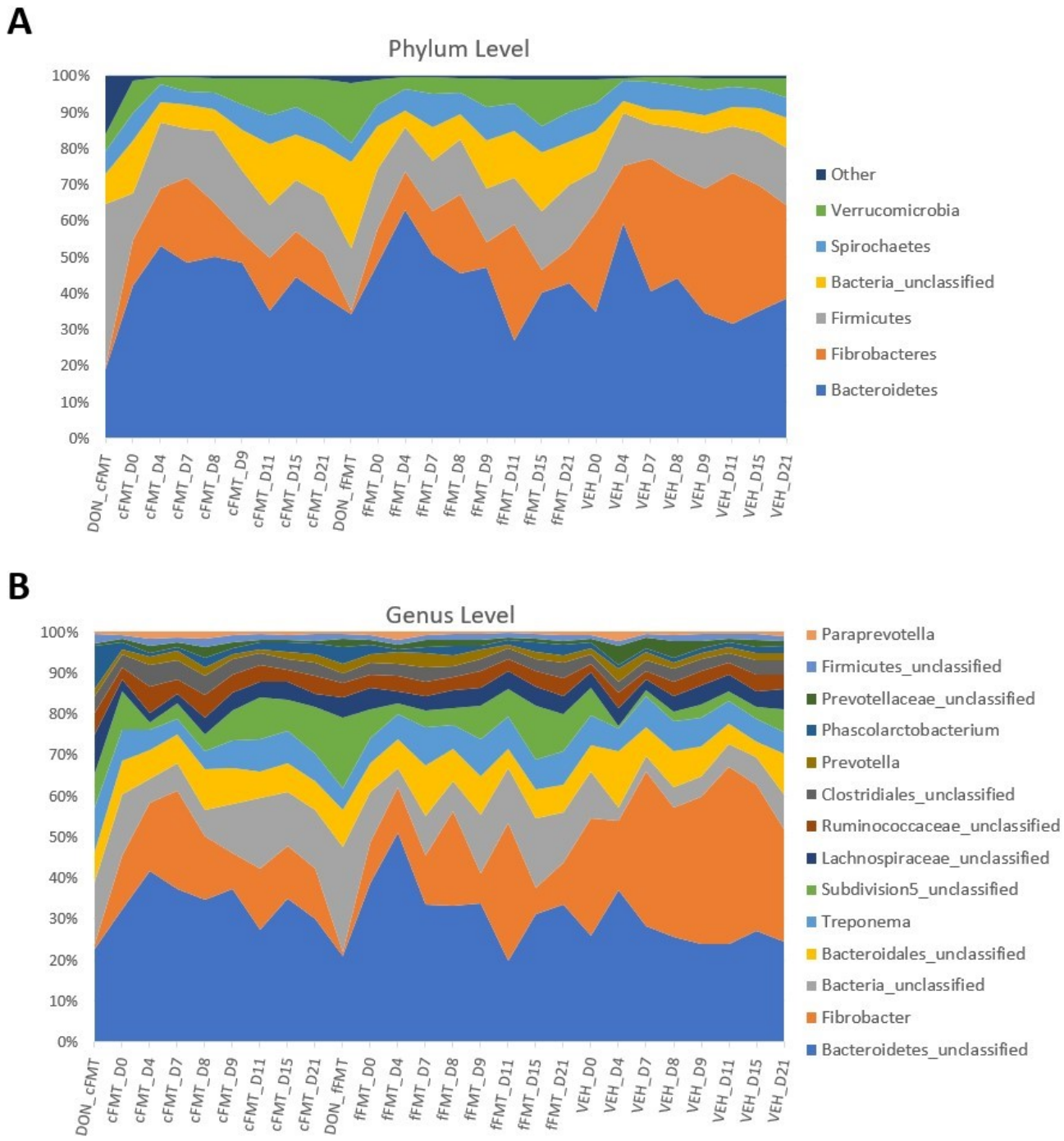

Figure 6. – Relative abundance of predominant bacteria at the phylum (A) and genus (B) levels. Recipients before and after antibiotic administration, after transplantation, and vehicle recipients are represented. Only the 6 most common phyla and 14 most common genera are represented.

When comparing specific relative abundances between cFMT and fFMT donor fecal suspensions, and between treatment groups after transplantation, the genus *Fibrobacter* was significantly decreased in the cFMT and fFMT at D9 compared to VEH ($P = 0.63$, unpaired Student's t-test of DON_cFMT vs. DON_fFMT, Figure 7A; $P < 0.05$, one-way ANOVA of fFMT vs. cFMT vs. VEH, Figure 7B). The relative abundance of the *Escherichia* genus was significantly increased in the cFMT donor fecal suspension, but this was not represented in the cFMT group on D9 ($P = 0.009$, unpaired Student's t-test of DON_cFMT vs. DON_fFMT, Figure 7C; $P = 0.69$, one-way ANOVA of fFMT vs. cFMT vs. VEH, Figure 7D). A significant increase in the relative abundance of the unclassified subdivision 5 genus was observed in the fFMT donor fecal suspension, however no significant difference was observed in the treatment groups ($P = 0.01$, unpaired Student's t-test of DON_cFMT vs. DON_fFMT, Figure 7E; $P = 0.07$, one-way ANOVA of fFMT vs. cFMT vs. VEH, Figure 7F).

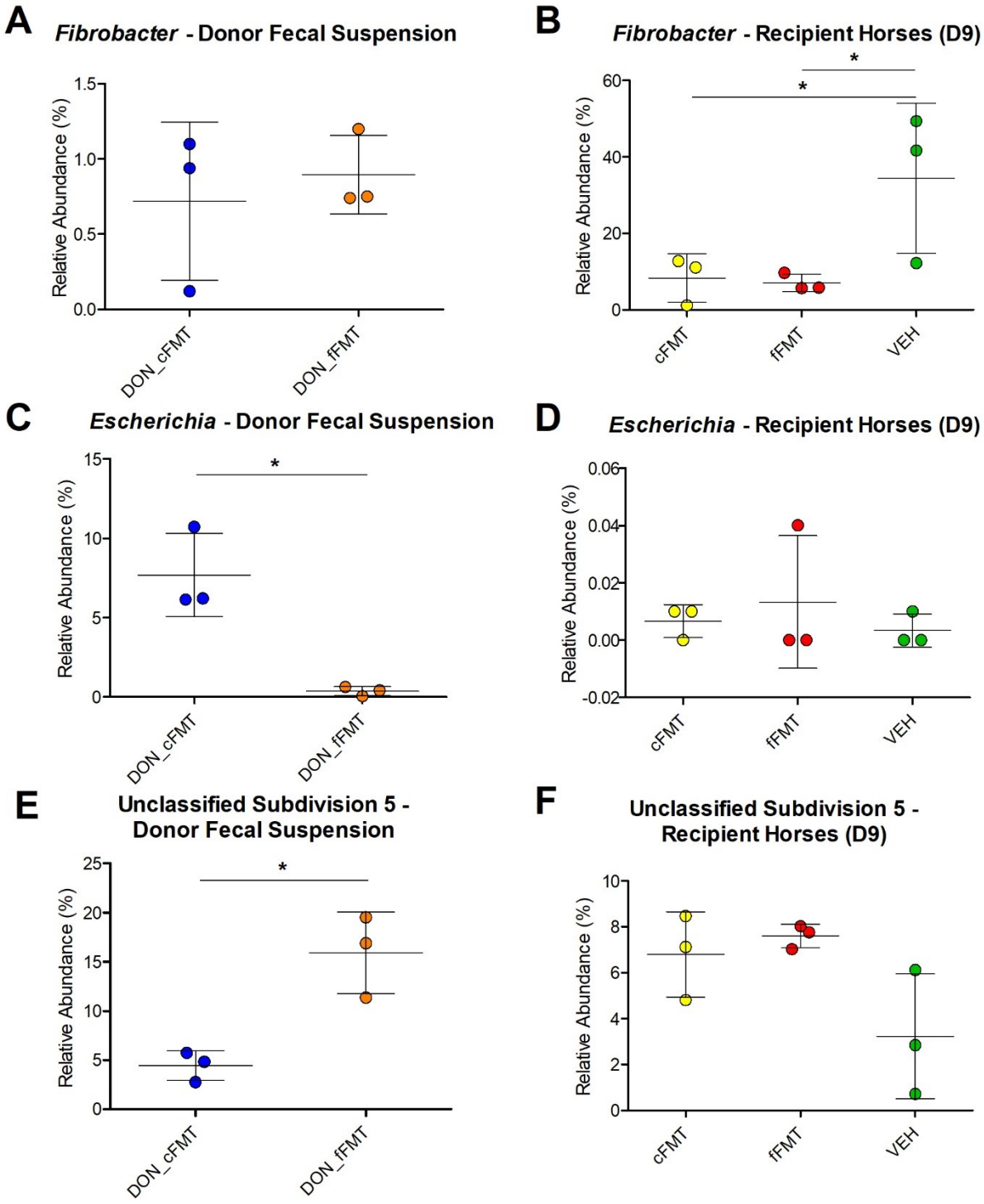

Figure 7. – Relative abundances of genera in donor fecal suspensions and treatment groups. Relative abundances in the cFMT donor fecal suspension (DON_cFMT) and fFMT donor fecal suspension (DON_fFMT) of *Fibrobacter* **(A)**, *Escherichia* **(C)** and unclassified subdivision 5 **(E)**

on D7, D8, and D9. Relative abundances in the cFMT recipients (cFMT), fFMT recipients (fFMT), and vehicle recipients (VEH) of *Fibrobacter* **(B)**, *Escherichia* **(D)**, and unclassified subdivision 5 **(F)** on D9. Note that the scale of relative abundances (y axis) is different between each picture.

Significant differences between donor fecal suspensions, and between recipients before and after antibiotic administration, and before and after transplantation were investigated using LEfSe analysis (Figure 8).

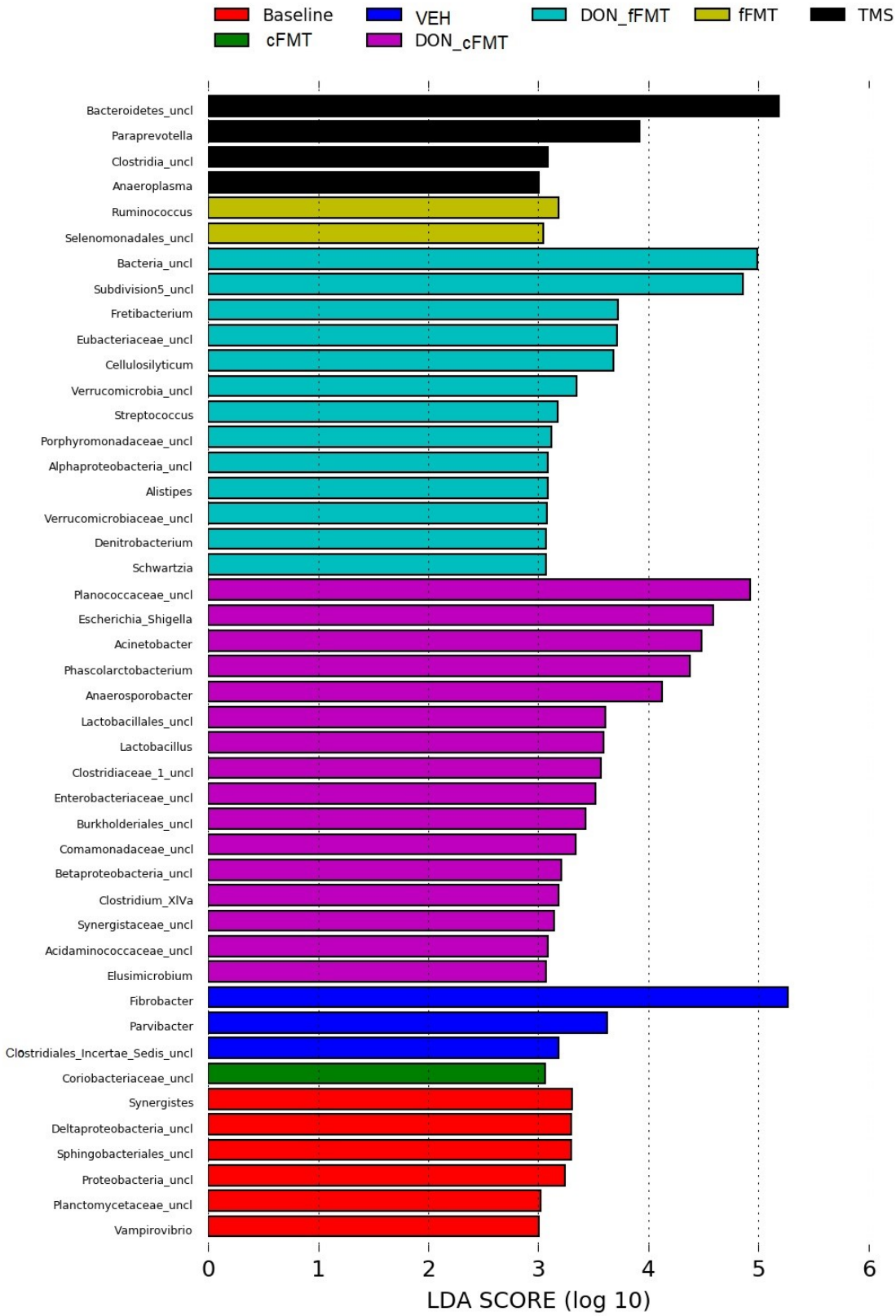
Baseline
VEH
DON_fFMT
fFMT
TMS
cFMT
DON_cFMT
Bacteroidetes_uncl
Paraprevotella
Clostridia_uncl
Anaeroplasma
Ruminococcus
Selenomonadales_uncl
Bacteria_uncl
Subdivision5_uncl
Fretibacterium
Eubacteriaceae_uncl
Cellulosilyticum
Verrucomicrobia_uncl
Streptococcus
Porphyromonadaceae_uncl
Alphaproteobacteria_uncl
Alistipes
Verrucomicrobiaceae_uncl
Denitrobacterium
Schwartzia
Planococcaceae_uncl
Escherichia_Shigella
Acinetobacter
Phascolarctobacterium
Anaerosporobacter
Lactobacillales_uncl
Lactobacillus
Clostridiaceae_1_uncl
Enterobacteriaceae_uncl
Burkholderiales_uncl
Comamonadaceae_uncl
Betaproteobacteria_uncl
Clostridium_XlVa
Synergistaceae_uncl
Acidaminococcaceae_uncl
Elusimicrobium
Fibrobacter
Parvibacter
Clostridiales_Incertae_Sedis_uncl
Coriobacteriaceae_uncl
Synergistes
Deltaproteobacteria_uncl
Sphingobacteriales_uncl
Proteobacteria_uncl
Planctomycetaceae_uncl
Vampirovibrio
0
1
2
3
4
5
6
LDA SCORE (log 10)

Figure 8. – Linear Discriminant Analysis Effect Size (LEfSe) of treatment groups and time points. LEfSe analysis showing taxa that were significantly overrepresented in cFMT donor fecal suspensions (DON_cFMT), fFMT donor fecal suspensions (DON_fFMT), in the recipients before antibiotic administration (Baseline), after antibiotic administration (TMS), in the cFMT recipient group (cFMT), fFMT recipient group (fFMT), and the vehicle recipients (VEH).

Discussion

The antibiotic TMS succeeded in causing intestinal dysbiosis in all 9 horses as seen by the decrease in richness and compositional changes. However, a decrease in diversity was lacking, which may be explained by TMS affecting mainly low abundant bacteria. This trend appears again in the analysis of beta-diversity where membership was significantly different after antibiotic administration, but structure was not. In contrast, a study by Costa et al. observed changes in richness and diversity, as well as membership and structure, after administering TMS to seven healthy horses twice a day for five consecutive days (8), but different primers targeting the V4 region of the 16rRNA gene were used, which might explain those differences. In addition, the low abundant bacteria seem to be affected greater in horses with asthma compared to healthy horses (39). Nevertheless, low abundant bacteria were shown to be important contributors to maintaining gut health (40).

In addition, the membership of one vehicle recipient remained different from all other recipients, suggesting that the horse remained in a dysbiotic state up to 17 days post-antibiotics. This result is similar to studies shown in horses and humans where the gut microbiota of individuals after antibiotic administration can take days or months to recover and return back to their original composition (8, 41, 42).

This fecal microbiota transplantation protocol that aimed to increase the chances of colonizing the gut microbiota by concentrating the bacteria from a healthy donor had a limited and transient impact, as did the protocol currently used. In addition, the gut microbiota of the horses treated with FMT did not resemble the bacteria found in the transplanted solution. Results of this study highlight the limitations of FMT in horses, likely because of the decreased bacterial viability caused by administration via the oral route and exposure to oxygen and freeze-thawing.

Noteworthy, this study was performed in healthy horses with antibiotic-induced dysbiosis, which might substantially differ from inflammation-driven dysbiosis, such as in cases of colitis. It is also possible that more proximal changes are not detected in feces.

Nevertheless, scientific evidences that FMT has clinical benefits in horses remain to be shown. The current FMT protocol proposed to correct dysbiosis in horses (32) failed to induce microbiota changes in 6 horses with diarrhea (Costa et al., under review). Another study in which FMT was administered to geriatric horses with diarrhea found a significant increase in alpha-diversity in 3 out of 5 horses, however, the study lacked a control group (29). In addition, another study reported clinical recovery in 4 horses with post-operatory diarrhea after FMT, however, no microbiota analysis was performed, and a control group was absent as well (30). Therefore, controlled studies with larger sample sizes demonstrating clinical and microbiological benefits of the procedure in horses remain to be performed.

The significant difference in membership after the fecal transplants (D4 vs. D9) in both treatment groups, and the lack thereof in vehicle recipients, suggests that perhaps some bacteria transiently colonized the large intestine of the treatment groups altering their microbiota composition. Furthermore, the difference in membership between the cFMT and fFMT treatment groups was also absent, indicating no advantages of concentrating bacteria in the correction of dysbiosis. Interestingly, the membership and structure of the donor's fecal microbiota clustered with the fecal microbiota of all recipients, indicating that their microbiota were similar before treatment even though the donor and recipients were housed at different facilities. The structure and membership of both cFMT and fFMT donor fecal suspensions did not cluster with the donor feces, suggesting a strong impact of handling feces in ambient air.

This study observed the most abundant phyla to be Bacteroidetes, followed by Fibrobacteres and Firmicutes. In comparison to other studies, horses with colitis had a high relative abundance of Bacteroidetes (40%), Firmicutes (30.3%), and Proteobacteria (18.7%), while healthy horses had a high relative abundance of Firmicutes (68.1%), Bacteroidetes (14.2%), and Proteobacteria (10.2%) (43). Differences can be due to the variation in methodologies used. One study using the same methods on the same horses found similar results to this present study (39).

Furthermore, the Fibrobacteres phylum, and correspondingly, the *Fibrobacter* genus, were observed in significantly relatively high levels in vehicle recipients, compared to horses receiving cFMT or fFMT. *Fibrobacter* was also increased in healthy horses compared to horses with metabolic syndrome (44) and asthma (39) and was found to be part of the core gut microbiota of a horse (45), with abundances increasing with age in foals (46). Therefore, perhaps cFMT and fFMT recipients in this study with lower abundances of *Fibrobacter* compared to vehicle recipients began with a gut microbiota that was partially different than the vehicle recipients before beginning the experimental trial.

Interestingly, while the cFMT donor fecal suspension in this study had a high relative abundance of Proteobacteria (15.7%) mainly caused by increased *Escherichia* genus, the cFMT recipient horses had relatively low abundances of this phylum (< 1%). Proteobacteria are part of the gut microbiota of healthy horses (47, 48), but it has also been associated with dysbiosis (43, 49). Studies have reported that handling feces at room temperature in ambient air greatly decreases the abundance of anaerobic bacteria and increases the abundance of opportunistic facultative aerobic bacteria such as *E. coli* (50). In this study, the cFMT donor fecal suspension was exposed to ambient air for 3-5 hours longer than the fFMT donor fecal suspension with the addition of the centrifugation step. Furthermore, cFMT donor fecal suspensions underwent freeze-thawing, which was also shown to affect bacterial viability and composition (50). Although no side effects were observed in horses receiving the cFMT donor fecal suspension twice a day for 3 days, those were otherwise healthy animals with no intestinal disease, but the use of this protocol might be harmful in sick debilitated horses.

In addition, abundance of subdivision 5, which are also part of a healthy equine gut (39, 51), was higher in the fFMT donor fecal suspension compared to the cFMT donor fecal suspension. Subdivision 5 are bacteria belonging to the Verrucomicrobia phylum. Several sequences of this bacterium have first been described in 1998, but still remain to be fully classified, thus subdivision 5 is used as a provisory name (52). These results suggest that the relative abundance of subdivision 5 might be negatively affected by longer exposure to oxygen or freezing. Therefore, it might be important to minimize exposure time to oxygen to prevent the overgrowth of

potential pathogenic bacteria, but further studies investigating the best conditions for FMT preparation in horses are required.

Further, sodium bicarbonate is usually given to horses to prevent metabolic acidosis (53, 54). A study in which horses received sodium bicarbonate in their feed showed significantly lower fecal lactic acid compared to their controls (55). In addition, a study in which horses received sodium bicarbonate by a cæcal cannula showed increased cæcal pH (56). However, there is debate on the concentration of sodium bicarbonate that should be given to horses. This study administered 0.1 M (approximately 0.01 g/kg of body weight (bwt) for a horse of 500 kg) to all recipient horses before each transplant to increase the pH of the stomach (34), with the intent of increasing survival of the bacteria passing through to reach the large colon. However, studies have reported administering 0.5 g/kg bwt (54), or 1 g/kg bwt (56). Therefore, perhaps the concentration used in this study was not high enough to increase the pH significantly, preventing some bacteria from surviving the harsh conditions of the stomach.

As frequently observed in other studies investigating the microbiota of horses (29, 51, 57), the main limitation of this study was its small sample size. While studies of FMT in horses are limited, this study brings new information to guide future research on microbiota manipulation in horses. Furthermore, a great degree of interindividual variability is present in response to treatments aimed at manipulating the gut microbiota, such as FMT (58), probiotics (59), prebiotics (60), and dietary interventions (61), highlighting the importance of larger studies and the inclusion of control animals. In addition, the exposure to oxygen and freeze-thawing of the cFMT donor fecal suspensions may have led to decreased bacterial viability. Moreover, using a nasogastric tube for FMT only allows the bacteria to be administered to the stomach, where they must survive the gastric acid and enzymes present in the small intestine, and the fermentation present in the cæcum before reaching the large colon. Nevertheless, this is the current method of choice for FMT delivery in horses (29, 30).

Conclusions

Current recommendations for FMT in horses, as well as the concentrated solution, have limited impact to correct antibiotic-induced dysbiosis. The composition of the transplanted

solution greatly differed from the microbiota found in healthy horses, probably caused by oxygen exposure and freeze-thawing. More basic and clinical studies investigating the use of FMT in horses are necessary before the procedure can be recommended to treat and prevent dysbiosis in horses.

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Conflicts of Interest

The authors declare no conflicts of interest.

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Chapter 3 – General Discussion

The main objective of this research study was to develop a protocol to improve FMT in horses. One factor to consider for why studies fail to show clear evidence of FMT success in horses is the anatomy of their GI tract.

1.0 Challenges of FMT in horses

1.1 Delivery modality

The horse GI tract is composed of the stomach; the small intestine which includes the duodenum, jejunum, and ileum; the large intestine, composed of the cæcum and large colon; the small colon; and the rectum. The stomach helps break down proteins through enzymes such as pepsin, but the small intestine is the primary site for digestion and absorption. Horses are hindgut fermenters, meaning the majority of the fermentation happens in the large intestine, which contains a vast amount of bacteria to further digest the plants ingested and produce volatile fatty acids that are then absorbed by the horse for energy (5). As the majority of the bacteria in the GI tract of a horse are present in the large intestine, this is the site aimed to manipulate through methods such as FMT.

In humans, in order to manipulate the gut microbiota present in the large intestine, enemas and colonoscopies are largely used to carry out FMT procedures. Studies evaluating the lower GI route of administration for FMT delivery in patients with recurrent *Clostridioides difficile* infection (rCDI) have shown over a 90% resolution rate (15, 194, 195). Similarly, most studies performing FMT in dogs also use the lower GI route and have shown the resolution of symptoms in dogs with diarrhea (196), parvovirus (121), and IBD (197). Studies in humans have also conducted trials delivering FMT by the oral route using a nasogastric tube and gastroscopy, but these methods have seen a lower success rate (15, 194, 195). However, one study treating a dog with *C. difficile*-associated diarrhea saw improved stool consistency and absence of fecal blood, mucous and infection (181).

In horses, the length of their small colon, measuring approximately 3.5 meters, precludes the use of the lower GI route to deliver FMT. A minimally invasive option is delivery by the upper GI route using a nasogastric tube. However, this only allows the bacteria to be administered to the stomach, where they must survive the gastric acid and enzymes present in the small intestine, and the fermentation present in the cæcum before reaching their target destination: the large colon. Nevertheless, this is the current method of choice for FMT delivery in horses (23, 24).

1.2 Interindividual variability

Many studies attempting to manipulate the gut microbiota of healthy or ill individuals find controversial results, partly due to the interindividual variability present between hosts. For instance, a study in which the probiotics *Lactobacillus paracasei* DG was administered to healthy human volunteers found that those with low initial butyrate levels showed significantly increased fecal butyrate concentrations in their gut after probiotic intake, and in contrast, those with high initial butyrate levels revealed significantly decreased fecal butyrate concentrations after probiotic intake (198). The authors suggest that it may be the probiotic's ability to stabilize the levels of butyrate in the gut microbiota, as too much butyrate is associated with metabolic syndrome and child obesity (198).

Interindividual variability was also observed in studies administering prebiotics to participants. One study in which inulin was given to ten healthy volunteers found that participants with initial low levels of bifidobacteria showed a significant increase in these bacteria after prebiotic intake, compared to individuals with high baseline levels of bifidobacteria (199). Similar findings in bifidobacteria levels were detected in several other studies administering prebiotics to healthy volunteers (200-203).

Studies performing FMT have also seen a degree of interindividual variability in patient response. One study in which FMT was administered to 16 patients with rCDI through a nasoduodenal tube saw resolution of diarrhea in 13/16 of patients after one FMT, while the remaining patients received a second FMT from a different donor, and only 2/3 achieved resolution of diarrhea (204). Another study saw resolution of diarrhea in 85% of patients with rCDI after receiving FMT via enema (205). While these cure rates are promising, the lack of a 100%

diarrhea resolution rate may be due to interindividual variability in patient response, as each individual's baseline composition may respond differently to diverse donors. Similarly, resolution of diarrhea in horses after FMT treatment is variable. A study in which FMT was administered to 5 geriatric horses with diarrhea saw resolution in only 3 of the horses receiving feces from the same donor (23). In the present study interindividual variability was also present as the gut microbiota of recipient horses within the same group reacted differently to the transplants as some recovered quicker than others. In addition, the presence of interindividual variability in a small sample size like the one in this study could have hindered at obtaining significant results. Larger sample sizes and correlations between the baseline gut microbiota composition to the gut microbiota composition of the donor should be prioritized in future studies administering FMT to patients with intestinal diseases.

1.3 Donor selection

Donor selection is gaining increasing attention as several studies have observed a higher success rate in patients receiving FMT from certain donors over others. Studies performing FMT in patients with overgrowth of a certain pathogen do not seem to reveal any donor-specific effects (195, 206). However, patient response in individuals with more complex diseases such as IBD and metabolic syndrome has been more variable (169-171, 207, 208). For instance, one study in which FMT was administered to patients with active ulcerative colitis by enema found that one donor in particular (Donor B) led to more remissions compared to other donors (170). In addition, the microbiota profile of Donor B had a significantly higher relative abundance of Lachnospiraceae family and *Ruminococcus* genus compared to Donor A, who led to no remissions (170). Lachnospiraceae and *Ruminococcus* are part of the *Clostridium* clusters *IV* and *XIVa* and have been suggested to be part of the core microbiota in horses (32, 42, 43) and are important contributors to gut health (43, 80). Another study in which stool from up to seven donors was pooled together and administered by colonoscopy and enema to patients with ulcerative colitis found that patients given FMT from a batch that contained one specific donor had higher remissions rates than those patients who received stool from batches that didn't contain that donor (169). In the present study, only one donor was used for cFMT and fFMT groups. A single donor was chosen

over multiple donors to avoid adding another variable to the analysis due to the low number of animals available to us.

These studies raise the question of the existence of a "super donor". Studies have shown that successful donors were associated with high bacterial diversity (207, 208), and high relative abundances of *Clostridium* clusters *IV* and *XIVa* (169, 209, 210) and *Bifidobacterium* (211). Studies performing FMT in horses have thus far only used a single donor (23, 24). Future studies investigating the different microbiota profiles of various donors in correlation with FMT success in horses are needed. Future studies should also evaluate the impact of pooling feces from multiple donor horses, as well as compare the colonizing potential between different donors.

1.4 Adverse events

Another limitation of FMT is the development of possible adverse events if a pathogen from the donor feces is transferred to the recipient. While adverse events are rare, a systematic review by Wang et al. (2016) revealed that 28.5% of patients receiving FMT for rCDI, irritable bowel syndrome, IBD, colitis, or antibiotic associated diarrhea experienced adverse events such as abdominal discomfort, diarrhea, fever, nausea, vomiting and constipation, of which 15% were likely related to FMT (212). However, data regarding adverse events in animals is lacking, therefore the safety of the procedure cannot be determined (213). Our horses were closely monitored by physical examination including changes in behavior, appetite, temperature, respiratory and cardiac frequency, GI motility, and stool consistency, and no side effects were recorded.

1.5 Long-term effects of FMT

In order for the microbiota transplanted from FMT to be successful in manipulating the gut microbiota of the recipient, the bacteria must remain attached to the mucosa to grow and become permanently established. However, studies have shown that microbiota alterations can vary from one host to another. For instance, a study in which FMT was given to patients with *C. difficile*-associated disease saw similarities between donor and recipient gut microbiota for 3 weeks, with similarities diminishing after a year (214). However, since rCDI is due to a pathogen, it is unlikely that disease will reoccur due to shifts away from the donor gut microbiota in the

host. In contrast, long-term establishment of FMT in the host may be more important in multifactorial diseases such as IBD. Supporting the establishment of the beneficial bacteria after FMT in these patients may be performed through diet modifications such as increased fiber intake to support the production of SCFAs (215). While the long-term effects of FMT have not been evaluated in horses, diet at time of treatment should be taken into consideration. Future studies should also focus on following horses with diarrhea and characterizing their fecal microbiota over several months to a year while performing weekly or biweekly treatments of FMT to determine whether long-term benefits are present or whether the benefits are transient and disappear once FMT is halted.

2.0 Protocol limitations

2.1 Bacterial viability

A limitation of the protocol of this study is the manipulation of feces in ambient air. One study has shown that the exposure of feces to oxygen reduced the amount of viable bacteria to 19% (190). In addition, a significant reduction in anaerobic bacteria was revealed, such as the important commensal butyrogenic species *Faecalibacterium prausnitzii*, *Subdoligranulum variable*, and *Eubacterium hallii* (156). Concordantly, the concentration of SCFAs like butyrate and acetate were also shown to be significantly reduced after oxygen exposure (120). Interestingly, bacterial viability decreased by 50% in feces processed under strict anaerobic conditions (190). Furthermore, a study in which FMT prepared under anaerobic conditions was administered to patients with ulcerative colitis had significantly higher remission rates compared to patients receiving FMT prepared in aerobic conditions (216). Moreover, freeze-thawing of feces was shown to decrease bacterial viability and composition (190). Studies comparing the effects of oxygen exposure and freezing on equine donor feces would be of interest to determine the levels of important beneficial bacteria lost during the manipulation and storage process, and the effects of the latter on FMT success in horses.

Further, sodium bicarbonate is usually given to horses to prevent metabolic acidosis (217, 218). A study in which horses received sodium bicarbonate in their feed showed significantly

lower fecal lactic acid compared to their controls (219). In addition, a study in which horses received sodium bicarbonate by a cæcal cannula showed increased cæcal pH (220). However, there is debate on the concentration of sodium bicarbonate that should be given to horses. This study administered 0.1 M (approximately 0.01 g/kg of body weight (bwt) for a horse of 500 kg) to all recipient horses before each transplant to increase the pH of the stomach (193), with the intent of increasing survival of the bacteria passing through to reach the large colon. However, studies have reported administering 0.5 g/kg (218), or 1 g/kg (220). Therefore, perhaps the concentration used in this study was not high enough to increase the pH significantly, preventing some bacteria from surviving the harsh conditions of the stomach.

An initial aim of this project was to perform CFU counts of the cFMT and fFMT fecal suspensions to determine the concentrations of bacteria. We also aimed to determine the absolute abundance of viable bacteria before and after freezing of the cFMT fecal suspension using propidium monoazide coupled with quantitative PCR (PMA-qPCR). However, due to the delays brought upon by the covid-19 pandemic, and the minimal impact of cFMT treatment on the gut microbiota of horses with antibiotic-induced dysbiosis, we decided not to move forward with those objectives.

2.2 Loss of metabolites

Another limitation of this study is the loss of metabolites through the centrifugation step aimed to concentrate the donor fecal suspension. Metabolites were shown to be important drivers of the neonatal mouse gut microbiota (221). Alterations in fecal metabolites were also shown to correlate with clinical improvement in patients with ulcerative colitis after receiving FMT (222). In addition, the fecal concentrations of SCFAs increased after FMT in a patient with dysbiosis due to anorexia nervosa (223). Furthermore, the metabolic profile in diarrheic dogs treated with FMT were significantly more similar to healthy dogs compared to dogs treated with metronidazole (196). To the author's knowledge, studies evaluating metabolic profiles in horses after FMT have not been performed. It is therefore important to consider the role of metabolites in altering the gut microbiota to a healthy state after FMT.

2.3 Fecal samples as a proxy of other intestinal compartments

Many of the studies evaluating changes in bacterial composition of the equine gut microbiota use fecal matter for analysis due to its easy acquisition and low level of invasiveness (34). Fecal samples have been shown to appropriately represent the microbiota of the large colon, but may not be suitable for evaluating the proximal intestinal tract (34, 182). Studies evaluating changes in the gut microbiota in humans also use endoscopic biopsy samples of the intestine, but this is invasive, requires bowel preparation, and may yield an insufficient amount of biomass for analysis (224). Studies may also use a catheter to aspirate samples from the luminal microbiota, which is also invasive and brings discomfort to the patient (224). However, this may not be feasible in horses as invasive procedures are time consuming and can cause stress which can further induce dysbiosis (43, 106). Studies evaluating different compartments in the equine GI tract have used horses euthanized for reasons unrelated to GI disease (34). Another alternative is to sample the cæcal microbiota using cecally cannulated horses (225). However, this surgical procedure may require horses to be anaesthetized and may cause complications such as colic and death (226). Therefore, the search for novel minimally invasive methods for sampling the equine proximal intestinal tract are warranted.

2.4 Straining of donor feces

The fecal suspensions in this study were obtained by straining the feces using a cheese cloth to remove large particles, such as the fibers present, as previously recommended (26). The bacteria adhered to the fiber were therefore lost during this step. However, the cheesecloth should not retain any bacteria present in the suspension. In addition, this method has been used for studies administering FMT in humans as well (227).

3.0 Future perspectives

3.1 Handling of Donor Feces and Storage Conditions

The next step to improve FMT in horses is to evaluate the impacts of manipulating donor feces under anaerobic conditions using an anaerobic chamber. The goal would be to increase viability of important anaerobic bacteria, which represent a large proportion of the equine large intestine

(228). Anaerobic bacteria are important producers of SCFAs such as acetate, propionate, and butyrate, which are absorbed by the epithelium to provide the horse with energy (5).

We would also like to evaluate the effects of various storage conditions on bacterial viability, such as different temperatures (-20°C vs -80°C), the addition or not of a cryoprotectant, and different storage times (days, weeks, or months). Storage temperatures have shown to impact bacterial viability of human feces intended for FMT (229). In addition, cryoprotectants have been shown to be an effective method at reducing bacterial lysis during storage of human feces (230, 231). Furthermore, the impact of storage periods of the donor stool on FMT efficacy have been evaluated for human feces (231). To the author's knowledge, these methods have not been evaluated in horses.

3.2 FMT Evaluation in Clinical Cases

Once the optimal manipulation and storage conditions have been determined, FMT should be evaluated in clinical cases of equine GI diseases such as colitis and metabolic syndrome to determine the efficacy of the treatment.

3.3 Probiotics as Personalized Therapeutics

Once FMT is shown to be effective in treating horses with GI disease, a next step would be to identify the bacterial species that are most beneficial in treating specific diseases and creating probiotics. Studies have been using high throughput sequencing technologies and multi-omics approaches (metagenomics, metatranscriptomics, metaproteomics, and metabolomics) to identify biomarkers of the gut microbiota involved in certain human (77, 78, 232) and equine (74, 233) GI diseases. Determining the presence of biomarkers in an individual with GI disease, and the corresponding specific bacterial species to target that potentially harmful biomarker, is a personalized therapeutic that may allow the gut microbiota to return to a healthy state efficiently.

3.4 Gut sampling using ingestible capsules

A novel approach to sample intestinal fluid is through the ingestion of capsules. One study developed a remote controlled capsule that can deliver drugs and sample the intestine at a target location (234). Another study tested a different ingestible capsule to sample the small and large

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intestine *in vivo* in pigs and primates (235). A limitation of this method is the need to recover the capsule in excreted feces, which could be easily missed, as what happened in the previous study in 5/8 rhesus macaques (235). Nevertheless, the use of this non-invasive method to precisely sample different locations of the gut could be of great interest in horses to characterize specific changes in different GI compartments after FMT.

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