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## **Factors influencing equine gut microbiota: current knowledge**

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## Abstract

Gastrointestinal microbiota play a crucial role in nutrient digestion, maintaining animal health and welfare. Various factors may affect microbial balance often leading to disturbances that may result in debilitating conditions such as colic and laminitis. The invention of next generation sequencing technologies and bioinformatics has provided valuable information on the effects of factors influencing equine gut microbiota. Among those factors are nutrition and management (e.g. diet, supplements, exercise), medical substances (e.g. antimicrobials, anthelmintics, anaesthetics), animal-related factors (breed and age), various pathological conditions (colitis, diarrhoea, colic, laminitis, equine gastric ulcer syndrome) as well as stress-related factors (transportation and weaning). The aim of this review is to assimilate current knowledge on equine microbiome studies, focusing on the effect of factors influencing equine gastrointestinal microbiota. Decrease in microbial diversity and richness leading to decrease in stability; decrease in *Lachnospiraceae* and *Ruminococcaceae* family members, which contribute to gut homeostasis; increase in *Lactobacillus* and *Streptococcus*; decrease in lactic acid utilising bacteria; decrease in butyrate-producing bacteria that have anti-inflammatory properties may all be considered as a negative change in equine gut microbiota. Shifts in *Firmicutes* and *Bacteroidetes* have often been observed in the literature in response to certain treatments or when describing healthy and unhealthy animals; however, these shifts are inconsistent. It is time to move forward and use the knowledge now acquired to start manipulating the microbiota of horses.

## Keywords

Horse, gut microbiota, equine, NGS, microbiome, 16S rRNA gene sequencing

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## 1. Introduction

Microbiota are usually referred to as the assemblage of microorganisms present in a gastrointestinal environment, while microbiome is the complex of biotic and abiotic factors, the entire habitat, including microorganisms and their genomes [1]. Intestinal microbiota appear to play an important role in the maintenance of health and the pathophysiology of several diseases in equids [2]. In addition to their role in digestive function (breakdown of feed particles)[3], intestinal microbiota are also known to play a role in immune function (protecting against pathobiont overgrowth, neutralizing toxins) [4-8], gut-brain connectivity and behavior [9], diabetes and obesity [10-12]. Pathobiont or opportunistic enteropathogen in microbiota-related research is defined as potentially a pathogenic microorganism, which usually lives as a non-harming symbiont; however, it may become an enteropathogen under certain circumstances [13].

Despite the clear importance of intestinal microbiota there is no comprehensive and systematic knowledge on equine gut microbial communities; moreover, our understanding of what constitutes 'normal' and 'abnormal' is currently very limited. Domestication and captivity have reshaped equine intestinal microbiota and greatly reduced fecal bacterial diversity compared to their wild-living counter-parts [14]. Determining the equine gut microbial composition of a healthy horse is important for the measurement of the impact of metabolic disorders and diseases. However, client-owned animals used or donated for research often come from different management conditions and after being fed various diets and being euthanized for specific reasons such as age, osteoarthritis, behavioral problems, neurological problems and other non-GIT (gastrointestinal tract) related conditions [15]. Taking into consideration that the GIT microbiome of the horse is likely to be affected by different states or management influences, not only intestinal and metabolic, some of the research data may not be applicable as an accurate reference for healthy animals. Conducting

crossover randomised controlled studies when possible should be the priority; however, the sample size in these cases is often smaller. Variability in equine gut microbiota composition among various breeds and age groups, within different gut compartments, as well as individual variability makes it difficult to generalise and make assumptions; hence, broader assessment of horse populations is required to gain a better understanding of what is a normal healthy gut microbiota [16]. Moreover, methodological differences during DNA extraction and library preparation protocols also make comparison between studies challenging.

Another aspect that is of relevance is that metagenomics studies are unable to identify exact functional pathways of microbial communities [17]. Hence, causation between alterations in the bacterial relative abundance at different taxonomic levels and actual physiological changes in the horse cannot be established without additional metabolomics analysis and relating any changes in microbial abundance to other factors. Metabolomics uses analytical approaches to determine metabolite profile in a given environment [1] and provides a direct functional readout of cellular activity, thus it has the potential to enable a better understanding of the physiological meaning to the host.

## 2. Factors influencing the equine gut microbiome

The aim of equine microbiome studies is to describe the function of microbial communities in health and disease states, and to identify changes in microbial abundance in relation to various factors. Large number of microbiome studies have been done to identify merely composition and structural differences occurring according to investigated factors with no reference to the function of microbial communities. To date, the role of the microbes within the hindgut of the horse remains largely unknown as well as their interactions.

The aim of the current review is to assimilate our current knowledge on equine microbiome studies, focusing on the effect of factors influencing equine gastrointestinal microbiota.

### 1.1 Nutrition and Management

#### 1.1.1 Diet

A systematic response of equine gut microbiota to feeding forage-based diets and high-concentrate diets was observed through the compositional and functional changes of the microbiota [18-21]. Stability of equine gut microbiota can be disrupted by dietary changes such as a sudden ingestion of high amounts of starch/fructan, leading to proliferation of lactic acid-producing bacteria in the hindgut, which may lead to lactic acidosis, colic, laminitis, and even death [2]. High nutrient availability (e.g. inclusion of grains rich in starch to fibre-based diet) reduces the microbial diversity [20-22] and according to ecological theories, decreased diversity leads to less stable microbial communities and potentially gastrointestinal dysbiosis [17]. Feeding hay alone, which contains less nutrients that are degraded at a slower rate compared to a hay and oat diet, which provides higher levels of nutrients that are degraded at a faster rate, has been reported to promote higher levels of microbial diversity and stability [22]. This can be explained by over-representation of propionic acid-producing bacterial family (*Porphyromonadaceae*) when horses were fed hay + oats diet [22]. Meanwhile, fiber substrate fermentation is known to increase acetic acid production [23], which is associated with a wider range of bacteria [22]. In agreement with the aforementioned study, Willing, VÖRÖS [20] found that a fiber-only diet resulted in more stable microbial communities with lower lactic acid bacteria (LAB), which is associated with GI disorders. Conversely, a traditional forage-concentrate diet resulted in the increase of *Clostridiaceae* family members and the presence of *Lactobacillus ruminis*, which was absent in the feces of horses fed a fiber-only diet. Daly, Proudman [19] demonstrated that the proportion of fibrolytic bacteria, genus *Fibrobacter* and

*Ruminococcaceae* family were decreased, while members of *Lachnospiraceae* family, the *Bacteroidetes* assemblage and *Bacillus–Lactobacillus–Streptococcus* group, were increased in horses receiving a concentrate-supplemented diet compared to a forage-based diet. *Streptococci* are major lactic acid producing bacteria associated with the development of hindgut acidosis [24, 25], while *Lachnospiraceae* family are the most abundant member of the microbial core in the proximal large intestine [26], and are a major VFA producer, which have been shown to ferment lactate to acetate and propionate. Lower abundance of *Lachnospiraceae* and *Streptococci* in the gut of the horses fed a forage diet may be explained by the lower fermentation rate of forage compared to cereal grains [18]. It appears that when horses are fed diets rich in starch (> 1 g/kg BW per meal), a substantial amount of starch escapes enzymatic amylolytic digestion in the foregut [27]. When reaching the hindgut, starch is fermented and increased amounts of lactic acid are produced [28]. As a result, pH in the large intestine may drop creating undesirable conditions for beneficial acid-intolerant fibrolytic bacteria to proliferate, which can represent a risk factor for digestive health [29]. However, the study of Daly, Proudman [19] had limitations, which are common to other equine gut microbiota studies. The precise dietary formulations for the experimental animals were unknown. The samples were taken after anaesthesia or euthanasia. Schoster, Mosing [30] later showed that ketamine and isoflurane, commonly used for anaesthesia, impact on the composition and structure of equine gut microbiota 24 h and 48 h after recovering from anaesthesia. Daly, Proudman [19] removed gut contents from the large intestine 10 minutes after death or surgery, hence it is unlikely that ketamine or isoflurane would affect the composition of microbiota that promptly. The effect of barbiturate used for euthanasia requires further investigation. Similarly, Warzecha, Coverdale [21] reported greater abundance of *Paraprevotellaceae*, *Veillonellaceae*, *Lactobacillaceae* family members and trend of *Streptococcaceae* family to increase, in the cecum of the horses fed a high-starch diet compared to those fed a diet low in starch, while a decrease in *Ruminococcaceae* and a trend for *Fibrobacter* to decrease was also observed. Hence, a decrease in caecal and colonic pH can be attributed to an increase in bacterial taxa that metabolize starch to produce lactate.

The equine microbiome project, where 184 horse owners participated by submitting fresh faecal samples from their horse using a provided kit and via filling out a survey questionnaire [31] revealed differences between pasture-based feeding and concentrate-forage diets. *Christensenellaceae*, *Oscillospira*, and *Prevotella* taxa were more abundant in the feces of horses maintained on pasture while *RFN20*, *Streptococcus*, and *Lactobacillus* taxa were represented in higher proportions in concentrate-forage diets. Though it is unclear how precisely horse owners followed the standard protocol when collecting equine feces, to the best of the authors' knowledge the sample size used in the aforementioned study remains one of the largest in equine microbiota related research. Structural differences in microbial communities highlighted in these studies may direct future research into how aforementioned structural differences translate into functional changes.

It appears that not only is it high-starch concentrate inclusion in the diet that alters equine gut microbiota, but that the botanical source of starch appears to have a differential effect on those microbial changes [32]. For instance, the study of Harlow, Lawrence [33] suggests that the addition of oats to the diet resulted in increased *Lactobacilli* and decreased gram-positive cocci, while these effects were reversed with the addition of maize to the diet. This could be due to higher pre-ileal digestibility of oats compared to maize in horses [34]. This can be also due to the differences in the ratio of amylose/amylopectin, morphology of starch granules, presence/absence of non-starch components in the grains (e.g. high levels of  $\beta$ -glucans in oats that have a prebiotic effect), susceptibility to gelatinization, and the retrogradation of starch molecules in maize and oats [35]. Moreover, Destrez, Grimm [36] demonstrated that high-starch diets not only induce changes in hindgut microbiota, but they also linked those changes with behavioural stress, often described as alimentary stress. In agreement with the study of Destrez, Grimm [36], Bulmer, Murray [37] established a correlation between faecal microbial alterations induced by the high-starch diet and behavioral reactivity of the ponies. In human-related research GIT microbes have been shown to

modulate eating behavior of the host [38, 39]. For example, microbes may have an affinity to certain substrates (e.g. *Prevotella* has substrate preference for carbohydrates) and have developed strategies to manipulate host behaviour through cravings and dysphoria until the desired substrate to maintain their fitness is consumed. The potential mechanisms to manipulate host behavior include microbial manipulation of reward pathways, production of hormone-like neurochemicals that alter emotional state, changes to taste receptors, and hijacking of neurotransmission *via* the vagus nerve [38, 40]. The alteration in behavior of the horses could be triggered by neuroendocrine changes caused by dietary induced changes to gut microbiota [37]. Moreover, behavioral cues can be potentially used to predict and prevent hindgut disorders caused by nutritional management of the horse [36].

Strong evidence exists that abrupt changes in the diet are also associated with microbial disturbances in the equine gut [41-45]. The impact of diet on equine hindgut microbiome was discussed in more details in the review of Julliand, Grimm [46].

### 1.1.2 Supplements

Digestive supplements, such as prebiotics and probiotics, have been shown to affect digestibility *in vivo* and *in vitro* and have been used to stabilize the microbiome in the large intestine of the horse; however, studies show conflicting results [43, 47-63]. Nevertheless, probiotics and prebiotics are extensively used in farm animal and equine feeding practices to modulate the balance and activities of GIT microbiota [64, 65]. The Food and Agricultural Organisation of the United Nations define probiotics as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” [66]. Microorganisms usually used as probiotics include yeasts and bacteria. Currently there are three *Saccharomyces cerevisiae* commercial products approved for use in horses in the European Union under the category of zootechnical additives under the claim of ‘digestive enhancers’ and one bacterial product containing *Escherichia coli* registered under the claim of ‘gut flora stabiliser’ [67]. A summary of some of the experiments studying the effects of yeast on equine gut microbial populations is outlined in Table 1. The findings of the studies that used probiotics to manipulate gut microbiota in domestic and farm animals (except horses) have been previously reviewed by O’Callaghan, Ross [40]. Overall, addition of probiotics to high-starch diets may help to prevent drop of caecal pH by decreasing lactic acid production, and create beneficial conditions for cellulolytic bacteria (higher pH and anaerobic environment) [68, 69]. There is a need for further research to target appropriate probiotic-substrate combinations taking into account dosage and animal-related factors, such as age (probiotics may have a different effect on immature microbiota), breed, body condition score and keeper-status, physiological state (e.g. pregnancy, lactation) [70].

Live bacterial probiotics have mainly been assessed on their efficacy to treat and prevent gastrointestinal disease [64]. Use of bacterial probiotics (*Lactobacillus* and *Bifidobacteria*) to reduce the incidence of diarrhoea in foals has been previously studied with some of the research showing benefits from the use of these probiotics [71], while another study reported no differences with the probiotics administration on diarrhoea incidence [72]. Schoster, Guardabassi [73] determined that multi-strain *Lactobacilli* have a limited effect on gastrointestinal microbiota, which combined with the lack of clinical improvements may indicate that live bacteria as probiotics may be incapable of improving/preventing diarrhoea in foals. Nevertheless, in hospitalized horses it has been demonstrated that with *Salmonella* faecal shedding was reduced 2 days after hospitalization with the administration of a probiotic (*Lactobacillus spp* and *Enterococcus faecium*) [74], suggesting this therapeutic approach could be utilized to decreased the risk, or progression, of salmonellosis outbreaks. Evaluating the effects of probiotics on the microbiome as a whole, including fungal and viral populations, using shotgun metagenomics analysis instead of 16S rRNA gene sequencing would also be of interest and warrants further investigation.

Prebiotics are defined as “a substrate that is selectively utilized by host microorganisms conferring a health benefit” [75]. The potential of prebiotics to influence gastrointestinal microbiota in animal nutrition has been previously reviewed [76, 77]. Prebiotics most commonly used in animal nutrition include oligofructose, inulin, fructooligosaccharides and mannanoligosaccharides. Yeast fermentation products and yeast cell walls, nonviable preparations of *Lactobacillus acidophilus*, have also been used as a source of prebiotics [47]. Prebiotics such as short-chain fructooligosaccharides have been shown to mitigate decreases in digestibility encountered by senior horses [78], and have reduced the microbial disruption in the hindgut associated with sudden ingestion of barley (starch overload) [79]. Similar to probiotics, conflicting results in the literature exist on the efficacy of prebiotics. The appropriate pairing of probiotics and prebiotics should be investigated further to help achieve more consistent beneficial effects [47].

The effect of enzyme supplementation on the equine GIT has not been well studied to date. The study of Proudman, Darby [80] revealed no change in richness expressed in a number of operational taxonomic units (OTUs) observed 6 weeks post amylase enzyme dietary supplementation. However, substantial changes occurred in the relative abundance of two most populated phyla – *Bacteroidetes* (34% pre-supplementation, 44% post-supplementation) and *Firmicutes* (60% pre-supplementation, 53% post-supplementation). Moreover, increased relative abundance of lactate-utilizing *Veillonellaceae* family coupled with decrease in total pool of short chain fatty acids may suggest that enzyme supplementation may affect intestinal health in equids, likely by increasing the amount of starch digested in the small intestine and thereby reducing the amount of starch entering the hindgut [81].

The use of probiotics in equine gastrointestinal disease has been reviewed by Schoster [64]. A useful two-part review with the second part focusing on the potential influence of the microbiome to prevent disease and promote health in humans has recently been published [82, 83]. The review reveals recent findings on opportunities to modulate human gut microbiota through the use of probiotics, prebiotics and fibre, and it discusses the impact of environmental conditions and life-stage on gut microbiota and the dietary interventions to improve health.

**Table 1 *S. cerevisiae* doses used in *in vivo* experiments to determine the effect of probiotic supplementation on the equine gut microbiome**

Reference	Horses	Sample origin	Microbes targeted	Microbial evaluation method	<i>S. cerevisiae</i> dose	Results
Medina, Girard [84]	Fistulated mature geldings	caecum and colon	Total viable anaerobic bacteria Cellulolytic Lactic acid utilizing <i>Streptococci</i> <i>Lactobacilli</i>	Culture techniques	10 g/day 4.5 x 10 <sup>9</sup> CFU/g	Decreased total anaerobes and <i>Streptococci</i> when HF diet fed in colon Increased Lactobacilli in caecum when HF diet fed
Jouany, Medina [57]	Fistulated mature geldings	caecum and colon	1. Total anaerobic, cellulolytic, <i>Streptococci</i> 2. Lactic acid utilizing and lactobacilli	Culture techniques	10 g/day 4.5 x 10 <sup>9</sup> CFU/g	1. No effects 2. Increased in the caecum but not colon (P>0.1)
Taran, Gobesso [85]	Intact gelding miniature horses	feces	<i>Fibrobacter succinogenes</i> , <i>Lactobacillus</i> , <i>Ruminococcus flavefaciens</i>	Real-time PCR	10, 20 and 30 g/day with 5x10 <sup>8</sup> CFU/g	No effects
Murray, Brown [60]	Intact mature mares	feces	<i>Fibrobacter succinogenes</i> <i>Ruminococcus flavefaciens</i> <i>Streptococcus equinus</i> <i>Streptococcus bovis</i>	Real-time PCR	4 g/day 1 x 10 <sup>9</sup> CFU/g	Reduced <i>F. succinogenes</i> with high starch diet
Gobesso, Pombo [54]	Intact Arabian mature geldings	feces	<i>Fibrobacter succinogenes</i> <i>Lactobacillus</i> <i>Ruminococcus flavefaciens</i>	Real-time PCR	7.5 g/meal 1.5x 10 <sup>10</sup> CFU/g	Decreased <i>Lactobacillus</i> counts



### 1.1.3 Exercise

Gastrointestinal health is crucially important for equine athletes in training and competition. Exercise has been shown to affect digestibility and passage rate in horses [86]; however, a limited amount of studies have explored equine gut microbiota in relation to exercise. Almeida, Feringer [87] showed that intense training lasting 42 days can induce changes in microbiota composition, while alpha-diversity indices (inverse Simpson index) remained unchanged. Moreover, the samples collected at the beginning of the experimental period were closer to the samples collected at the end of the study according to principal coordinate analyses (PCoA). All of this suggests that gut microbiota adapt to the exercise programme, indicating that while exercise may alter gut microbiota it may be that it is the changes in exercise that may be a more important factor in influencing microbiota compared to the exercise *per se*. The study of Plancade, Clark [88] suggests that gut microbiota was not correlated to the biochemical and metabolomic blood parameters during the endurance race, and it could not be used as a biomarker to predict endurance performance. However, gut microbiota was functionally and compositionally divided into two distinct communities associated with type of feeding. As mentioned previously, diet is the major factor that influences microbial communities, hence greater sample size or controlled dietary conditions may be required to achieve statistical and functional significance that links the effect of other factors such as exercise on gut microbiota composition and function. Further research establishing the link between gut microbiota and exercise would enable future manipulation of equine GIT microbiota to enhance athletic performance.

### 1.1.4 Obesity and Equine Metabolic Syndrome (EMS)

Recently, with high obesity levels in domesticated horses, EMS has become an increasingly important problem [89] as it may lead to metabolic states, including insulin dysregulation and resistance, an increased risk of laminitis [90], heat intolerance, reduced performance and joint problems [10]. Obesity and EMS are discussed under the nutrition and management section of this review due to the close relation of these problems to the nutrient dense diet and feeding management that domesticated horses are currently experiencing. Diet is the major driving factor in the development of obesity and EMS [12] and as it was discussed previously in this review it influences GIT microbial profile of the horse. On the other hand, gut microbiota composition and structure influences the efficiency of energy utilization from the diet, hence the availability of excess energy for storage, and finally the development of obesity [12, 91]. There is clearly an association between gut microbiota composition and EMS [89], obesity [10] and keeper status [92]; however, “chicken and egg” causality dilemma exists, that needs to be further addressed.

Biddle, Tomb [10] identified differences in the microbiome of lean, normal and obese horses that were related to blood analytes associated with body condition score (BCS). *Firmicutes/Bacteroidetes* ratio was higher for obese horses compared to lean horses and those with normal body condition score. Diversity and richness were greater for obese horses compared to their lean and normal counterparts. The samples were collected from university herds and private horse owners; meta-data collected from each horse included diet, and diet was categorised during analysis. However, the study was not controlled for the diet, which is known to be primary driver of microbial composition.

In the study of Elzinga, Weese [89] there were differences in overall community structure between EMS horses and healthy horses. EMS was associated with increased *Clostridium* cluster XI, unclassified members of the phylum *Verrucomicrobia*, *Lactobacillus*, *Cellulosilyticum* and *Elusimicrobium* and decreased unclassified members of families *Lachnospiraceae*, *Flavobacteriaceae* and *Rhodospirillaceae*, and *Anaerovorax*, *Fibrobacter*, and *Saccharofermentans*. Moreover, EMS

horses exhibited a decrease in faecal microbial diversity. Although this study identified differences in fecal microbiome composition between horses with EMS and healthy controls, it is unknown whether these differences persist when lean and obese horses are maintained on the same diet.

Weems [92] studied the microbiome of easy and hard keeper horses and identified that *Firmicutes*, *Bacteroidetes*, *Fibrobacteres*, *Tenericutes* phyla were affected by keeper status according to the LEfSe analysis with 32 differentially abundant taxa revealed within aforementioned phyla. Relative abundance of *Clostridiales* order (were members of *Lachnospiraceae* family reside) was higher for easy keepers. *Lachnospiraceae* family is a polysaccharide-degrading and short-chain fatty acids-producing family [92]; hence, Weems [92] suggests that easy-keepers may have a greater ability to transform dietary nutrients from plant material to energy. This study identified differences in the composition of the gut microbiome between lean and obese subjects, but again it was not been controlled with respect to diet.

Morrison, Newbold [12] conducted a diet controlled study with a single pony breed and suggested that the fecal microbiome of obese horses was significantly more diverse in terms of bacterial species, which is in agreement with the study of Biddle, Tomb [10]. Moreover, lower evenness in the distribution of bacterial species when compared to control animals was observed. On the contrary, the study results of Morrison, Newbold [12] revealed an increased abundance of *Bacteroidetes* and *Actinobacteria* in the obese group, while the study of Biddle, Tomb [10] report a decrease of the aforementioned taxa. These differences could be due to having a more heterogeneous population and diverse diet in the study of Biddle, Tomb [10], while Morrison, Newbold [12] controlled the study design more strictly. In mice it has been shown that performing fecal transplants from healthy individuals with normal body weight to obese animals improved metabolic syndrome symptoms [93]. This suggests that the microbiome may play an important role in the physiology of weight regulation. Manipulation of the gut microbiome with faecal transplants in equids warrants further investigation to determine if similar effects are evident.

### 1.1.5 Seasonal, spatial and social interactions

Geographical area, seasonal variations and social interactions seem to play an important role in the establishment of the gut microbiome and these related factors should be considered when designing and interpreting studies on equine gut microbiota [94]. In human research, there are differences in the phylogenetic composition of fecal microbiota between individuals living in different countries [95]. Recently, the influence of spatial structuring and social interactions on gut microbial composition of semi-feral ponies was characterised [96]. The results suggest that microbiota composition is multi-level structured (individual, group, spatial). Maternal relationship had a significant effect on microbiota similarity; moreover, mares had significantly more similar microbial composition to the dominant stallion in the herd than the other mares in their herd. Shared diet and environment as well as mother-offspring, stallion-mare relationships accounts for microbiome similarities; however, individual identity remains the main predictor of microbiome composition.

Salem, Maddox [97] were the first to study seasonal variations in the faecal microbiome of horses maintained on pasture over a 12 months period with minimum changes to their management. Season of the year, weather (temperature and rainfall) and additional forage (haylage) were associated with microbial changes. The results of this study suggest that microbial communities of the equine gut are highly dynamic and responsive to environmental changes. A future study over at least a 24-36 month period warrants investigation to determine whether these effects are consistent over the seasons.

## 1.2 Medication

### 1.2.1 Antimicrobial and Non-steroidal anti-inflammatory drugs

The balanced interaction between the host and gut microbiome is important to maintain digestive and overall health. When the balance is compromised, the dysbiosis leads to proliferation of pathobionts [16]. In clinical practice, oral administration of antibiotics may induce dysbiosis, which often results in antimicrobial-associated diarrhoea and antimicrobial-associated colitis in horses [98, 99]. Even though the incidence of antimicrobial-associated diarrhoea is low, antimicrobial treatment may still represent a significant risk to equine health due to the presence of enteropathogens, e.g. *Clostridium difficile* [99]. In human-related research common sequelae of antibiotic-induced dysbiosis include reduced microbial diversity and increased risk of development diseases associated with pathobionts [100]. Altered microbial community structure may lead to a deterioration of the protective mucosal layer structure that serves as a protective barrier for the host; however, functional connection between changes in microbial structure and what effect this has on the host is not well understood [101]. Costa, Stämpfli [102] studied intramuscular administration of procaine penicillin and ceftiofur sodium, and oral administration of trimethoprim sulfadiazine, on equine faecal microbial populations. All antimicrobial drugs tested had some effect on the microbiota, with trimethoprim sulfadiazine resulting in more pronounced changes; such as, reduced bacterial species richness (total number of taxa present in the environment), diversity (richness and evenness/distribution of species), and differences in population structure, predominantly in *Verrucomicrobia* phylum. Similarly, Collinet, Grimm [98] found that oral administration of trimethoprim sulfadiazine resulted in decreased short-term cellulolytic bacteria concentrations, while amylolytic bacteria increased progressively. The authors report that it took fecal microbial communities approximately 28 days to return to their basal state following antibiotics administration.

Harlow, Lawrence [103] found that cellulolytic bacteria in feces of the horses administered with trimethoprim–sulfadiazine or ceftiofur decreased during antibiotic administration (1 week) and were still decreased compared to the controls after withdrawal period (2 weeks after antibiotic administration). Moreover, the results of the study suggested that antibiotics disrupted the GIT microbiota and allowed proliferation of *Salmonella* and *Clostridium difficile* commonly associated with equine diarrhoea even after antibiotic treatment was discontinued. Arnold, Isaiah [104] found that metranidazole administered intracecally decreased  $\alpha$ -diversity metrics obtained from both fecal and cecal samples, decreased relative abundance of *Christensenellaceae*, *Campylobacteriaceae*, and *Spirochaetaceae* family members and altered two cecal metabolites. Thus, a reduction in cellulolytic bacteria with antimicrobial drugs therapy suggests that antibiotic administration could decrease the total dietary energy derived from forages that is available to the horse [98, 103]. Moreover, a decrease of bacterial diversity and cellulolytic bacterial communities that normally inhabit the GIT of the horse may lead to an increased proliferation of pathogenic bacteria. It would appear from the literature to date that a prolonged period of recovery (up to 30 days) is needed before equine gut microbiota returns to a baseline state after antimicrobial drug administration.

Non-steroidal anti-inflammatory drugs (NSAID) such as phenylbutazone are routinely used in horses to alleviate pain associated with colic syndrome; however, NSAID have been also associated with gastropathy, enteropathy or NSAID associated colitis [105-107], and inhibition of the recovery of mucosal barrier function [108]. Whitfield-Cargile, Chamoun-Emanuelli [105] showed that therapeutical dosages of NSAID resulted in transient dysbiosis of the fecal microbiota of healthy horses. These microbial changes were characterized by a decrease of the *Firmicutes* phylum, and the families *Lachnospiraceae*, *Clostridiaceae*, and *Ruminococcaceae*, no matter the type of NSAID used (phenylbutazone or firocoxib), although the implications of this dysbiosis remain unclear.

### 1.2.2 Helminthes and Anthelmintics

Gastro-intestinal parasites inhabit the same environment as bacteria, archaea, and fungi. They interact with each other and their interaction plays an important, yet not completely understood role in the regulation of immunity and inflammation [109, 110]. In mice, abundance of *Lactobacilli* has been shown to positively correlate with intestinal nematode parasite infestation and immune response of the host, showing complex host-bacteria-parasite interaction [111].

In horses, anthelmintic treatment is a recognised colic risk factor [112], which may be due, at least in-part, to the anthelmintic treatment altering the GIT microbiome. Specific changes in gut microbiota post-treatment include a decrease in relative abundance of *Bacteroidetes* [109, 113]. Sirois [113] found that *Bacteroidetes* (43%) phylum was the most abundant phylum in the feces of horses prior to treatment with an anthelmintic (fenbendazole, over the course of five days followed by ivermectin on the fifth and final day) followed by *Firmicutes* (27%). After the treatment *Firmicutes* was the most prevalent phylum (35%) followed by *Bacteroidetes* (32%). The shift in bacteria was notable and the drop of *Bacteroidetes* was 11%; however, the results are based on only 12 samples (n=6, samples collected pre- and post-treatment). The study of Walshe, Duggan [109] (n=31) reports similar results – a decrease in *Bacteroidetes* phylum, no matter of the age group (older or younger animals) and treatment (fenbendazole or moxidectin). Apart from the direct effect of anthelmintics on bacterial populations, helminths may alter mucin production, which may lead to changes in the nutritional environment for gut bacteria [114]. Clark, Salle [115] studied the response to *Strongyle* infestation of resistant and susceptible ponies and found that susceptible ponies showed a reduction of *Ruminococcus*, *Clostridium XIVa* genera and members of the *Lachnospiraceae* family. Overall, parasite infection increased relative abundance of *Paludibacter*, *Campylobacter*, *Bacillus*, *Pseudomonas*, *Clostridium III*, *Acetivibrio* in the faecal profiles. Authors suggested that members of *Ruminococceae* and *Lachnospiraceae* families are mostly butyrate-producing bacteria and butyrate is known for its anti-inflammatory properties, concluding that *Strongyle* infestation may contribute to inflammation in the gut.

Increased richness and diversity has been associated with helminth infestation, while a decrease in alpha-diversity has been shown to be associated with the administration of anthelmintics [109, 116, 117]. Walshe, Duggan [109] found a decreased alpha- and beta-diversity post-fenbendazole and moxidectin treatment on day 7 post-treatment, and this time point coincides with degradation of helminths. The changes were associated with an increase of inflammatory biomarkers and were reversed on day 14 post-treatment. There was no difference between anthelmintic treatments; however, the difference between age groups was observed. The most recent study of Kunz, Reed [116] suggests that moxidectin and praziquantel decreased microbial diversity (Shannon index), while beta-diversity using weighted UniFrac remained unchanged. Regardless of the significant differences discovered in the abundance of twenty-one taxonomic groups, the authors suggest no major impacts on the GIT microbiota after anthelmintic treatment. Similarly, Daniels, Leng [117] reported moxidectin did not significantly alter bacterial diversity, while 13 different OTUs differed between treatment and control groups.

### 1.2.3 Anaesthetics and fasting

Anaesthesia and fasting are another group of stress factors which may have an impact on equine gut microbiota, and which may be supported by increased prevalence of diarrhoea, colitis and colic onset post-surgery [118]. Schoster, Mosing [30] studied the effects of fasting, premedication/anaesthesia on equine faecal microbiota and results showed that alpha-diversity metrics were similar; however, bacterial community and structure were altered after anaesthesia. Isoflurane [119], a commonly used halogenated inhaled anaesthetic for many species, including horses, was used with neonatal male rats. At 42 day isoflurane-exposed rats showed increased

relative abundance of *Firmicutes* and *Proteobacteria* phyla coupled with a decreased abundance of *Bacteroidetes* and *Actinobacteria* compared to controls. Neurotoxic effects of anaesthetics can be potentially explained by induced gut dysbiosis [120]. After fasting, there appears to be a trend for decreased relative abundance of *Clostridiales*. Indeed, *Clostridiales* order has been previously reported to decrease in horses with digestive upsets (e.g. acute colitis) [94]. However, because transport, fasting and anaesthesia were included in the study design of Schoster, Mosing [30], it is difficult to identify microbial alterations specific to each of the treatments. Despite some methodological flaws or limitations, the aforementioned studies add to the body of evidence in the field.

### 1.3 Animal-related factors

A significant inter-horse variability in equine gut microbiota has been previously reported [81, 97, 121]. There are various physiological factors that may affect the equine gut microbiome: breed, sex, age, pregnancy, parturition, lactation are just some of them. The difference in microbiomes of mares and stallions has been reported [122]; moreover, the fecal microbiota in mares during late pregnancy has also been reported to be different from non-pregnant control mares [123]. It can be speculated that these differences may be attributable to the alteration in intestinal capacity of pregnant mares, hormonal changes occurring during the pregnancy, and stress related to changes happening in the management of the mare prior to parturition. Intrinsic factors such as breed are reported to be predisposing factors for certain clinical conditions. For instance the Arabian breed is known to be one of the breed risk factors associated with prevalence of colic in horses [124], while pony breeds tend to have a propensity to be at more risk of glucose-intolerance compared to horses [90], and finally Thoroughbreds are the breed that appear to be most affected by crib-biting behaviour [125]. The authors suggest that differences in management practices of different breeds may increase risk of colic or incidence of stereotypic behaviour, while some pony breeds are more genetically predisposed to obesity and metabolic syndrome. However, to the best of the authors' knowledge, there are only a few studies describing inter-breed variations in equine gut microbiota [126].

#### 1.3.1 Age

Another area of particular interest is investigating the initial colonization of the equine GIT from birth to weaning to adulthood until senior age [127]. Foals are developing in a uterine environment with no symbiotic microbes in their GIT and the colonization process starts at birth and lasts until weaning [127, 128]. A recent study provides evidence of the prenatal exposure of the fetus to microbial components from the mare microbiomes [129]. Quercia, Freccero [129] analysed shared OTUs in microbial ecosystems of meconium, amniotic fluid and mare feces and they highlighted the importance of mare microbiomes in the establishment of the gut microbiome of the foal. The authors suggest possible existence of internal vertical transmission of microbiome components from the mare to the fetus. After birth, the external route of transmission initiates colonization of the foal's GIT with ingestion of bacteria from its mother's vagina, udder, skin, hair, and environment overall. All of these bacteria compete to colonize the foal's GIT as it represents a highly beneficial environment for microbial existence. Hence, the microbiota that inhabit the newborn foal's GIT is richer, more diverse, and more dynamic compared to those of the adult animals [16]. As the newborn foal grows, its digestive microbiota is constantly adapting to the changes in dietary needs, changes in type of food consumed such as changes in mare's milk composition or transition from milk to a forage-based diet [130]. Earing, Durig [127] suggested that a mature microbial community in foals is present by around six weeks post-parturition, while Faubladiere, Sadet-Bourgeteau [131] reports the microbiome of the hindgut changes rapidly in early

life from birth up until about 30 days of age. Furthermore, Costa, Stampfli [128] suggests this process takes twice longer (60 days). The recent study of Lindenberg, Krych [132] indicated that relative stability of the gut microbiota was reached within 50 days post-partum. The authors suggest that higher diversity in gut microbiota between Day 7 and Day 20 compared to Day 50 and after weaning indicates that instabilities in the gut microbiota composition in the early period; however, there was no sampling performed between Day 20 and Day 50. Different findings obtained by four research groups might be due to study design and type of analyses undertaken. Earing, Durig [127] analysed samples from mare and foal pairs from parturition until 12 weeks of age using polymerase chain reaction (PCR) of 16S rRNA gene followed by denaturing gradient gel electrophoresis. The researchers further compared gel bands using pairwise similarities between mares and foals. Faubladiet, Sadet-Bourgeteau [131] analyzed fecal samples of 5 foals from birth until 1 year of age using Automated Ribosomal Intergenic Spacer Analysis (ARISA). ARISA profiles were then compared using analysis of similarity (ANOSIM). Costa, Stampfli [128] used samples from 11 mare-foal pairs from parturition up to 9 months of foal's age. Illumina MiSeq next generation sequencing of 16S rRNA gene followed by bioinformatics data analyses in mothur software. Lindenberg, Krych [132] used 16S rRNA gene MiSeq Illumina next generation sequencing and the separation between the time groups was tested with Permutational Multivariate Analysis of Variance (PERMANOVA). In addition, all the aforementioned studies differ in sampling times and feeding practices, making direct comparisons challenging. Moreover, equine gut microbiota might not be stable even in adult animals. Indeed, within each individual pony fed a constant high-fiber diet, a significantly different microbial population was found after 11 weeks on the same diet [121]. At early stages of the development, a foal's microbiota is affected by the intestinal microbiota of the mother (taking into account coprophagy foals are prone to), colostrum and milk, introduction of fiber and other nutrient sources in the foal's diet [16].

In humans, the establishment of adult phylogenetic gut microbiota composition happens within the 3 years after birth no matter of the population studied; moreover, individual variation is greater between children compared to adults [83, 95, 133]. Dougal, de la Fuente [134] report that there were no differences in bacterial community structure between healthy adult (5-12 years) and elderly (19-28 years) horses; however, there was a reduction in fecal bacterial diversity associated with ageing. Reduced bacterial diversity in older horses, as in humans, may be attributed to physiological changes accompanying the ageing process [135], for example increased digesta transit time, deteriorating dentition, changes in diet consumed, and dietary energy requirements.

## 1.4 Disease

Disturbances of the equine gut microbiota are associated with conditions and diseases such as acidosis, diarrhoea, colitis [94], colic [123], laminitis [136], equine metabolic syndrome [89], and equine grass sickness [137]. Nevertheless, it is difficult to estimate what comes first and shapes the other; disease or altered microbiome. However, composition and diversity of the fecal microbiome does not always contribute to susceptibility of developing pathological conditions (e.g. clinical signs of *Rhodococcus equi* pneumonia) [138].

### 1.4.1 Colitis and diarrhoea

The main bacteria-associated causes of colitis known in horses are *Clostridium difficile*, *Clostridium perfringens*, *Salmonella spp.*, and *Neorickettsia risticii* [16]. The contribution of facultative pathogenic bacteria to diarrhoea is currently under investigation. Moreover, it is unclear whether certain bacteria are over-represented in the GIT of horses with colitis/diarrhoea because they contribute to disease outbreak or because they may be faster to proliferate in the affected gut.

In the study of Costa, Arroyo [94] *Firmicutes* were the dominant phylum (68%) in healthy horses followed by *Bacteroidetes* (14%), while in horses with colitis *Bacteroidetes* (40%) were increased compared to healthy horses and it was the most abundant phylum, followed by *Firmicutes* (30%). Moreover, healthy horses had a higher abundance of *Actinobacteria* and *Spirochaetes* while horses with colitis had more *Fusobacteria*. Finally, members of the *Clostridia* class, *Lachnospiraceae* family were more abundant in healthy horses, suggesting these may contribute to a healthy core microbiome. The alterations in GIT microbiota during colitis were not surprising as enteropathogenic bacteria have been previously associated with this disease; however, the cause often remains unclear [94, 139, 140]. Moreover, decreased intestinal permeability or “leaky gut” may also contribute to altered microbial population [141]. Pathogen-induced barrier dysfunction may lead to decreased/increased proliferation of other bacteria in the gut. The recent findings indicate that colitis is the disease related to major microbial gut disturbances and is not simply caused by a single pathogen [94].

Diarrhoea affects more than half of all foals during the first 6 months of their life with retrospective study of Frederick, Giguere [142] reporting *Rotavirus* being the most frequently detected agent, followed by *Clostridium perfringens*, *Salmonella* and *Clostridium difficile*. Schoster, Staempfli [143] observed a decreased bacterial richness in foals with diarrhoea compared to healthy foals; moreover, members of the *Lachnospiraceae* and *Ruminococcaceae* families were decreased in the affected foals. De la Torre, Henderson [130] found that on day 7 post-weaning in the non-diarrheic faecal samples of foals *Actinomycetales* and *Micrococcaceae* were more abundant according to LEfSe analysis compared to faecal samples originating from diarrheic foals. The role of these two microbial taxa have not been studied to date in horses. Rodriguez, Taminiau [144] reported that colonization of *Clostridium difficile*, which has been previously identified as an important cause of diarrhoea in horses, may be a transient bacterial member with no overgrowth to trigger infection having been noted. Species richness and bacterial evenness are lower in diarrhoeic horses. Moreover, similarly to the study of Costa, Arroyo [94] who found increased levels of *Fusobacteria* in horses with colitis, *Fusobacteria* has been found in horses with diarrhoea but not in any of the feces from non-diarrheic horses. *Fusobacteria* in faecal microbiota of diarrhoeic horses and horses with colitis requires further investigations, as it may act as an equine enteropathogen. Hence, future research should be focused on investigating functional characteristics of less abundant bacterial taxa and what their importance is to the host.

#### 1.4.2 Colic

A variety of equine diseases may manifest as colic/abdominal pain syndrome. Currently only a few studies evaluating the effect of colic on equine gut microbiota exist. Venable, Kerley [145] reported increased *Clostridium phytofermentans* and *Bacteroidetes* in all samples from horses during the colic state compared to those post-colic (30-90 days following discharge). Weese, Holcombe [123] suggested an association exists between *Firmicutes* (particularly *Lachnospiraceae* and *Ruminococcaceae* family members)/*Proteobacteria* ratio and the onset of colic (the higher the ratio, the less likely colic is to develop), which can be potentially used to predict and prevent colic. In the study of Stewart, Southwood [146] horses presenting for colic had reduced microbial diversity and richness compared with horses presenting for elective procedures, while relative abundance of commensal gut bacteria including *Prevotella* and *Lachnospiraceae* were decreased, whereas pathobionts including *Streptococcus* and *Sphaerochaeta* were increased in horses with colic compared with elective surgery patients. The decreased relative abundance of *Lachnospiraceae*, butyrate-producing family in horses with colic is consistent with other studies [94, 123]. Identifying faecal microbial shifts that occur early before manifestation of clinical symptoms and might be predisposing factors for colic may help with early identification of horses with increased risk of colic. Salem [147] proposed that manifestation of colic syndrome may be related to an inability of gut microbiota to adapt to changes in forage type, season and ambient weather, which may be specific

to individual horses. This might explain why these changes may increase the risk of colic in some of the horses, while others do not develop any symptoms. Indeed, Biddle, Black [148] identified that lactate-utilizing bacteria in an *in vitro* system react differently to the introduction of starch, which may elucidate why some horses are more susceptible to starch-induced colic and laminitis than others.

### 1.4.3 Laminitis

Various factors may contribute to the development of clinical laminitis in horses [2, 24, 25, 136, 149-156]. Those associated with starch overload from cereal-rich diets, and fructan overload associated with overgrazing lush pastures, are better researched. However, non-dietary conditions such as repeated hoof trauma, colitis-associated laminitis, retained placenta, use of glucocorticoids [153], and colic may also result in onset of laminitis [16].

It is suggested that the most prominent alteration to equine gut microbiome associated with carbohydrate-induced laminitis are credited to *Streptococci* and *Lactobacilli* changes [24, 136]. Moreover, when Milinovich, Trott [25] induced laminitis with oral administration of oligofructose mimicking pasture-induced laminitis they found that *Streptococcus bovis-Streptococcus equinus* complex may be involved in the development of laminitis. These results confirm the association of gram-positive lactic acid producing bacteria and sequential events leading to the development of fermentative acidosis and laminitis in horses [2].

Chronic laminitis in relation to the fecal microbiome was studied by Steelman, Chowdhary [155]. Horses with chronic laminitis had higher bacterial diversity compared to horses with no history of laminitis; moreover, the abundance of the two unassigned genera belonging to *Clostridiales* order were higher in the laminitis group compared to the control group. Although these data provide an initial starting point for future research of hindgut bacterial community alterations that may influence the development and progression of chronic laminitis, the authors fail to explain physiological meaning of the observed changes.

### 1.4.4 Equine grass sickness (EGS)

EGS is often a fatal disease in horses, which causes degenerative changes in the autonomic nervous system [157]. It can manifest in acute, subacute and chronic forms and is characterised by intestinal stasis. The association with *Clostridium botulinum* type C neurotoxin has been established [137]. Garrett, Brown [137] suggests there were fourteen species of *Clostridia* isolated from digestive contents of EGS horses, while there was a single species isolated from controls. However, this finding was not been confirmed by the metagenomics study of Leng, Proudman [158]; on the contrary, relative abundance of the bacterial family *Clostridia* where species *Clostridia botulinum* resides, was lower in EGS horses compared to their matched controls. The study also demonstrated an increase in *Bacteroidetes* and *Proteobacteria* phyla and a decrease of *Firmicutes* and *Verrucomicrobia* phyla in EGS horses. An earlier study by Leng, Proudman [159] reported an increase in *Bacteroidetes* and a decrease in *Firmicutes* phylum in horses with EGS; moreover, relative abundance of *Desulphovibrio* and *Veillonella* genera and *Veillonella parvula* were increased. Interestingly, *Clostridium botulinum* often associated with EGS was not increased in the feces of horses with EGS. Decrease in *Firmicutes* phylum and an increase in *Bacteroidetes*, as well as increase in relative abundance of lactic acid utilising *Veillonella*, were observed in both of the aforementioned studies; hence, this finding may be considered consistent in relation to faecal microbial changes in horses with EGS.



## 1.5 Stress

The research is limited on the effects of stress and change of management conditions on equine GIT microbiota; however, it is commonly proposed that stressful events can cause digestive upsets such as diarrhoea.

### 1.5.1 Transportation

Horses are frequently being transported; for example after purchasing, for competitions and training, pleasure rides outside of their residential area, for reproduction purposes and to medical facilities [160]. Transportation imposes stress-related risks on equine welfare and performance [161], especially for those horses that are not frequently transported. Faubladiere, Chaucheyras-Durand [160] studied the effects of yeast supplementation on the equine faecal microbiome when horses were subjected to 2 hours transportation. *Streptococci* counts tended to be affected by transportation, while faecal concentrations of cellulolytic bacteria, lactate-utilizing bacteria, and *Lactobacillus* remained unchanged. Yeast supplementation led to higher concentrations of lactate-utilizing bacteria and a trend for cellulolytic bacteria to be greater compared to control horses. Schoster, Mosing [30] reported that after 12 hours post-transportation, which lasted 1 hour, horses had a decreased abundance of class *Clostridia* and order *Rickettsiales* compared with baseline, while diversity indices remained unaffected. The authors acknowledged that a prolonged investigation period of 24-36 hours may be required to identify any changes post-stress. In the study of Perry, Cross [161] alpha-diversity (species richness and evenness) were decreased in horses after transportation compared to baseline; however, it was also decreased in the control group of horses, compared to baseline. This could be due to control horses being affected by the return of the travel group to the barn; this finding can also be due to the effect of time, not treatment. There was no effect of transportation observed on beta-diversity measured using weighted and unweighted UniFrac methods. At individual taxonomic level, *Bacteroidetes* phylum decreased significantly after transportation compared to baseline in travel group only, but not the control. Other taxa were also affected by transportation compared to baseline values; however, this was not significant when compared to the control group. These changes include an increase of acidophilic *Lactobacillus* and *Streptococcus spp.* during the travel; however, no changes in cecal pH were detected, and drop of undefined genera within the family *Ruminococcaceae* and *Lachnospiraceae*. The authors suggest that the lack of differences between the travel and the control group may be due to stress caused by separation (herd mates leaving). It appears that careful consideration of the study design (sample collection duration and intervals, assignment of the control and treatment groups) is required to estimate changes truly related to the treatment. However, confounding factors cannot always be eliminated from the analysis; moreover, in the normal management of the horse, changes that may affect the microbiome do not happen in isolation (e.g. transportation is related to change of temperature, humidity, air quality, feeding regime). Thus, combined treatments should be considered in study designs as long as they mimic real situations that horses may encounter in their daily routine.

### 1.5.2 Weaning

Mach, Foury [162] reports that, irrespective of the method (progressive or abrupt), weaning affected the composition of the foal's gut microbiota with the most considerable alterations noticed on the third day post-weaning. The relative abundances of the genera *Prevotella*, *Oscillibacter*, *Streptococcus*, *Anaerovibrio*, *Lactobacillus* and of members of the family *Lachnospiraceae incertae sedis* increased, while the relative abundances of the members of the genera *Fibrobacter*, *Clostridium XIVa*, *Ruminococcus*, *Treponema* and of the unclassified members of the family

Lachnospiraceae decreased. Overall, it took about 7 days for the foal's microbiome to return to relative stability and the changes observed post-weaning were thought to be related to stress. On the contrary, Lindenberg, Krych [132] report that weaning did not have a major impact on the microbial composition. Based on the inter-study differences a conclusion regarding the affect of weaning cannot be reached; however, the most significant changes in microbial composition occur within the first 1-2 months post-partum, before weaning, which corresponds to the introduction of solid fibrous feeds in foals.

### 3. Conclusion

Nowadays, drivers of equine gut microbiota structure are increasingly well described. However, the vast majority of studies on equine gut microbiota are merely descriptive with methodological limitations or differences present. Most of the researchers admit that the functional implications of their results requires further research, which comprises a major limitation in this field. Recently, more and more data are being generated focused on evaluation of existent practices to identify less stressful and more sustainable management techniques in every day practice for equids. It is time to move forward and use this knowledge to start manipulating the microbiota of horses as has already happened in human microbiota-related research.

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