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Fecal microbiome and functional prediction profiles of horses with and without crib-biting behavior: A comparative study

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ABSTRACT

Crib-biting is a stereotyped oral behavior with poorly understood etiology and pathophysiology. The relationship between the gut microbiome and brain function has been described in behavioral disorders such as schizophrenia, depression and anxiety in humans. In horses, studies of behavioral problems and the microbiome are very limited. This study aimed to characterize the fecal microbiome and the predicted functional profile of horses with and without aerophagia. Fecal samples were collected from 12 Colombian Creole Horses of both sexes, divided into two groups: group 1, composed of six horses with crib-biting (3 females and 3 males), average body weight of 330 ± 10 kg, age of 7.0 ± 1.2 years and body condition score (BCS) of $5/9 \pm 1$ and group 2, consisting of six horses without crib-biting (3 females and 3 males), average body weight of 335 ± 5 kg, age 6.5 ± 1 years and BCS of $6/9 \pm 1$. From each horse in both groups fecal total DNA was obtained and 16S ribosomal RNA gene amplicons were sequenced to characterize the bacterial community structure. Community structure and differential abundance analyses revealed significant differences between the two conditions ($p < 0.05$). Specifically, the fecal microbiota at the family level in crib-biting horses, showing a decrease in *Bacteroidales* and an increase in *Bacillota* and *Clostridia*, differed from that of healthy horses without crib-biting, consistent with findings from previous studies. Furthermore, metagenome prediction suggests metabolic profile changes in bacterial communities between both conditions in horses. Further studies are required to validate the role of the microbiota-gut-brain axis in the etiology of crib-biting and other abnormal and stereotyped behaviors.

1. Introduction

Aerophagia is a stereotyped oral behavior characterized by muscular flexion and contraction of the neck and the emission of a noise originating from air suction with (crib-biting) or without (sucking) the support of the upper incisor teeth on surfaces [1]. This behavior has a prevalence exceeding 13 % both in confined and grazing animals [1,2], which may vary depending on breed, physical activity, sports discipline, and management conditions [2–7]. However, despite several predisposing factors having been identified, and different hypotheses having been postulated to understand this behavior, the etiology and pathophysiology behind crib-biting are not well understood. Several studies have linked changes in neurotransmitter concentration and stress-related hormones with this stereotypy [8–11]. Also, the

antioxidant levels and antioxidant defenses against lipid peroxidation are reduced in horses exhibiting crib-biting behavior, indicating that oxidative stress may be involved in its pathophysiology, as has been observed in various psychiatric disorders in humans, such as depression and anxiety [12,13].

Several human studies have described the relationship between the gut microbiome and brain function, demonstrating the association of changes between the gut microbiome and brain functionality [14,15]. Therefore, the gut microbiota affect mammalian behavior, as well as influencing inflammatory reactions within the brain through modulation of microglial activation and affecting myelination and neurogenesis in the adult brain [16–19]. On the other hand, gastrointestinal dysfunctions (irritable bowel syndrome, inflammatory bowel disease) are often accompanied by psychiatric comorbidities [20]; and the role of

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inflammation and the gut microbiome in depression and anxiety has also been described [21].

Recently, there has been an increased interest in studying the fecal microbiome and metabolome in equines [22–24], as it fulfills digestive, metabolic, protective, intestinal epithelium development, and immunological functions [23]. Similarly, several studies have linked the gastrointestinal microbiome to various pathological conditions in horses [25], such as inflammatory bowel disease, types of colic, asthma, gastric glandular disease, recurrent uveitis and behavioral problems, among others [26–34]. Additionally, modifying factors of the intestinal microbiota have been described (age, diet, environmental conditions, antibiotic therapy, stressing factors, among others) [23,32,35–37].

Given the importance of the intestinal microbiota to health and also to the gut-brain axis [38], this study aimed to characterize the fecal microbiome of horses with and without aerophagia. For this, a characterization of the microbiome composition of fecal samples from horses with and without crib-biting habit through 16S metagenomic sequencing was performed, followed by thorough bioinformatic and statistical analyses to find specific changes in the structure of the bacterial community which can be associated with aerophagia.

2. Materials and methods

2.1. Ethical approval

This study was approved by the Ethics Committee for Animal Experimentation of the University of Antioquia, protocol No. 147-09082022.

2.2. Methodology

A total of 12 Colombian Creole horses of both sexes were divided into two groups: group 1, composed of six horses with crib-biting (3 females and 3 males), average body weight of 330 ± 10 kg, age of 7.0 ± 1.2 years and body condition score (BCS) of $5/9 \pm 1$ [39], and group 2, consisting of six horses without crib-biting (3 females and 3 males), average body weight of 335 ± 5 kg, age of 6.5 ± 1 years and BCS of $6/9 \pm 1$ [39]. All horses were selected from the same herd and were subjected to similar management and feeding conditions in a complete stabling system. The dietary regimen for these animals consisted of *ad libitum* access to grass hay (*Dichantium aristatum*), commercial concentrated feed (2kg/d) (Campeón Dorado®, Solla, Itagüí, Colombia) provided in two feedings, mineral supplement (approximately 60g/d) (Brio sales®, Itacol, Girardota, Colombia) and *ad libitum* access to drinking water. All horses had a complete and up-to-date health plan, with periodic dental care. The exclusion criteria for horse selection for this study were any antibiotic or anti-inflammatory drug treatments administered during the previous four (4) months and any incidence of gastrointestinal disturbance in the six (6) month period prior to fecal specimen collection.

The crib-biting horses in Group 1 were assigned to this group by their caregivers and the study authors, on the basis of demonstrating frequent daily episodes of this stereotypic behavior. The control group (Group 2) were medically diagnosed as clinically healthy, not exhibiting any abnormal locomotor oral behaviors, supported by observations by the owners and caregivers. Previously, the interviewees were trained on the stereotypies, for greater precision in their identification.

2.3. Sample collection and DNA extraction

Samples of fecal material of approximately 5g were taken directly from the rectal ampulla of each horse and kept under refrigeration (<5 °C) until further analysis (2 h). Fecal total DNA extraction was performed using the DNeasy PowerSoil kit (Qiagen® Toronto, ON, CA) on all specimens. The DNA samples were prepared with the GenXPro 16S rRNA-Seq Metagenomic Library Preparation Kit for Illumina Sequencing

tool, at the facilities of the company GenXPro GmbH (Frankfurt, Germany). The libraries were prepared according to the protocol described in the user manual provided in the kit. The DNA extracted was used for amplification and sequencing of the 16S rRNA, targeting the variable V3-V4 regions (Forward Primer=5'-CCTACGGGNGGCWGCAG-3', Reverse Primer=5'-GACTACHVGGGTATCTAATCC-3') resulting in amplicons of approximately ~460 bp. Dual indices and Illumina sequencing adapters using the Nextera XT Index Kit were attached to the V3-V4 amplicons. Subsequently, library quantification, normalization, and pooling were performed and MiSeq v3 reagent kits were used to load the samples for MiSeq sequencing. For more info, please refer to the guidelines of Illumina MiSeq System [40].

2.4. Bioinformatic analysis

Bioinformatic analysis was carried out utilizing QIIME 2 version 2023.9 [41]. Briefly, the quality of the sequences was inspected, followed by clustering into Amplicon Sequence Variants (ASVs) using DADA2 [42]. Taxonomic assignment was performed using the Naive Bayes classifier from the scikit-learn plugin in QIIME 2, with 16S rRNA V3-V4 primers and the SILVA 132 database compatible with QIIME 2 [43]. To normalize sampling efforts, ASV table rarefaction was performed using the sample with the lowest sequencing depth as the threshold for rarefaction and alpha diversity calculations. The strategy to handle low-abundance ASVs was to discard ASVs with less than 2 reads in the whole ASV table. Subsequently, the microeco R package version 1.1.0 was employed to perform diversity-based analyses as follows [44,45]. Community composition was assessed at the phylum, family, and genus taxonomic levels. Alpha diversity indices were calculated for each sample, and variations between conditions were assessed with a Kruskal-Wallis test ($\alpha = 0.05$).

To characterize the beta diversity of the samples, a Principal Coordinates Analysis (PCoA) was conducted using Bray-Curtis and Weighted Unifrac distances. Subsequently, a permutational test (PERMANOVA) was employed to assess significant differences between conditions ($\alpha = 0.05$). A Kruskal-Wallis test was performed to identify taxa with differential abundance between conditions, using a significance threshold of 0.05. The functional prediction was done using PICRUSt2, leveraging taxonomic information obtained from the ASVs table [46]. The identified Metacyc pathways [47], were subsequently compared between conditions through LEfSe analysis [48]. Following this, the pathways were filtered based on significance ($p < 0.05$, LDA threshold=2).

3. Results

3.1. Sequencing and taxonomic features

The bacterial communities within fecal samples from horses under both conditions were thoroughly characterized. A total of 1,029,773 reads were obtained from the 12 samples and 5,038 ASVs were identified, comprising a cumulative frequency of 228,308, with a median frequency of 19,026 per sample. Following rarefaction to 2,215 OTUs per sample, 11.64 % of the initially identified ASVs were retained. Notably, rarefaction curves illustrate that the subsampling strategy effectively captured the microbial diversity present in the samples (Fig. 1).

The fecal bacterial communities of horses with and without crib-biting conditions exhibited a predominance of specific phyla, namely *Bacillota* (*Firmicutes*), *Bacteroidota*, *Spirochaetaota*, and *Kiritimatiellaota*. At the family level, *Lachnospiraceae*, *Ruminococcaceae*, and *Prevotellaceae* were always present (Fig. 2). At lower taxonomic levels, such as genus, a complete classification was not possible; however, genera such as *Treponema*, *Marvinbryantia*, *Lactobacillus*, *Streptococcus*, *Ruminococcus*, and *Prevotella* were present (Fig. 3).

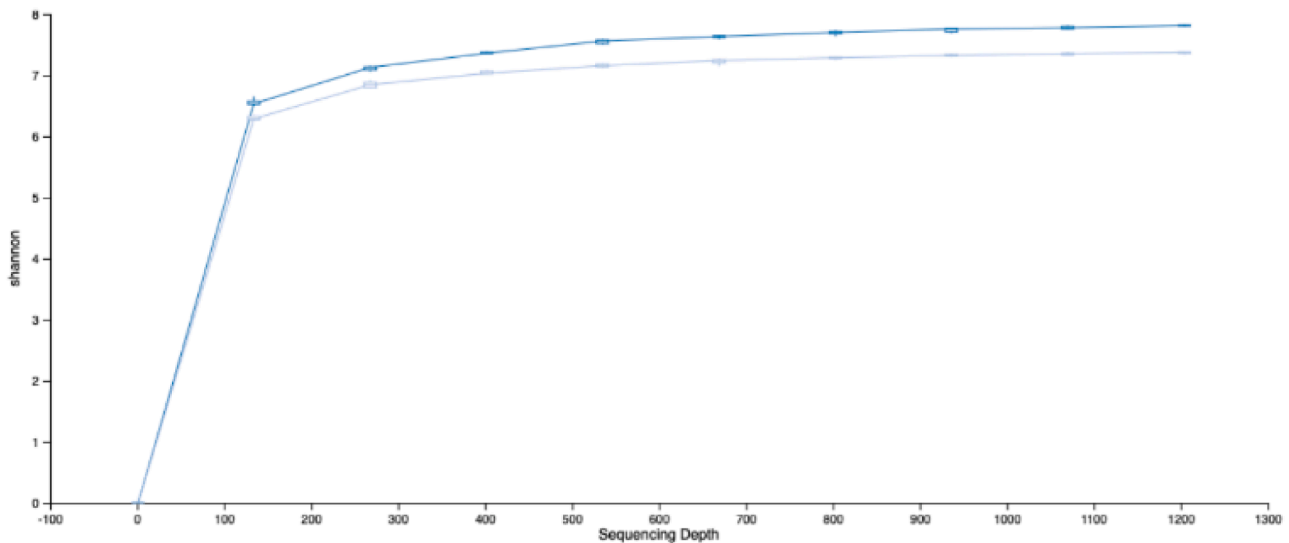


Fig. 1. Alpha rarefaction curves. Accumulation curves of Alpha diversity (Shannon Index) based on sampling depth after rarefaction at 2215. Control (dark blue) exhibits a trend towards higher diversity compared to Crib-biting (light blue). Both conditions reach a diversity plateau at a sampling depth of approximately 100.

3.2. Diversity of bacterial communities in horses' fecal samples

Main alpha diversity metrics were calculated; however, no significant differences between conditions were found ($p > 0.05$). Nonetheless, it is worth noting that the control group tended to have a higher alpha diversity (Table 1). The evaluation of beta diversity using Bray-Curtis dissimilarity distances and PCoA accounted for 31.2 % of the total variation and revealed differences between crib-biting and non-crib-biting conditions on the bacterial communities' structure ($p < 0.05$). However, when the analysis was performed using UniFrac distances, 60 % of the total variation was accounted for, and no significant differences were found ($p > 0.05$) (Fig. 4).

3.3. Differential abundance test

Furthermore, differential abundance analysis between the two conditions showed significant differences ($p < 0.05$). Specifically, the crib-biting horses exhibited greater relative abundance in phylum *Bacillota*, class *Clostridia*, order *Clostridiales*, and family *Ruminococcaceae*. In contrast, the control group displayed significantly greater relative abundances of phylum *Bacteroidota*, order *Bacteroidales*, class *Bacteroidia*, and family *Lachnospiraceae* (Fig. 5).

3.4. Functional prediction

The functional profile of fecal samples of horses was predicted using PICRUSt2. LEfSe analysis revealed a total of 32 pathways exhibiting significant differences between conditions, encompassing 16 over-represented pathways in each condition. Notably, pathways associated with biotin biosynthesis, formaldehyde assimilation, vitamin B6 biosynthesis, phosphopantothenate biosynthesis, formaldehyde oxidation, gluconeogenesis, carbohydrate biosynthesis, quinol, and quinone biosynthesis were overrepresented in non-crib-biting condition. Conversely, pathways linked to butanediol biosynthesis, amidine, amine, and polyamine degradation, polysaccharide degradation, isopentenyl diphosphate biosynthesis, diterpenoid biosynthesis, peptidoglycan biosynthesis, L-methionine biosynthesis, L-lysine biosynthesis, proteogenic amino acid biosynthesis, mixed acid fermentation (lactic acid, acetate, succinate), S-adenosyl-L-methionine biosynthesis, inosine-5'-phosphate biosynthesis, and L-methionine biosynthesis were prominent in the crib-biting condition (Fig. 6).

4. Discussion

The horses involved in the study were sourced from the same property, with similar management and feeding systems, and strictly adhered to the established inclusion criteria, aiming to reduce variability in the results. This was done because there is a noticeable interindividual variation in the microbiome, which is influenced by several factors as described elsewhere [23,35,49,50]. Additionally, age was considered a factor, and the study groups were formed with horses aged between 7-8 years, as there are greater microbial population dynamics in the intestines of young and geriatric animals [51,52,53]. With regard to BCS and body weight, they were slightly lower in the crib-biting horses, although without significant differences. This finding may be related to this condition [54], since all the horses had a similar management plan.

In this study, several limitations were clearly identified, such as the use of a small number of individuals in each study group, which impacted the robustness of the results. Additionally, the genera and species of the bacterial community in each group were not completely determined, which may have influenced the interpretation of the results. It is important to consider exclusion criteria (antibiotics and anti-inflammatory), as these factors can affect the microbiome [28,35]. However, this study addressed aspects of metabolic profiles aimed at recognizing differences in the metabolome between horses with and without crib-biting, distinguishing itself from previous reports.

The study recently conducted by Arias-Esquivel et al. [34] analyzed horses with and without crib-biting, using two different treatments (placebo and supplement) and two different sample origins (gastric and fecal). Compared to their study, ours had the following differences: it precisely established the number of samples per treatment and focused specifically on horses with and without aerophagia, without supplementation. It also specified the sequencing technology used, along with all the details of sequence preprocessing. An ASV-based approach was employed, and the beta diversity analysis was performed using two dissimilarity matrices for higher taxonomic resolution. However, both studies contribute to the limited literature on the microbiome and aerophagy in equines.

The incidence of aerophagia in the utilized breed has been estimated at 5.27 % [6], which is considered high compared to other breeds in geographic regions other than the tropics. Additionally, Colombian creole horses are characterized by elevated serum cortisol levels, related to environmental and individual factors associated with temperament and management conditions [55]. Therefore, stress has been considered

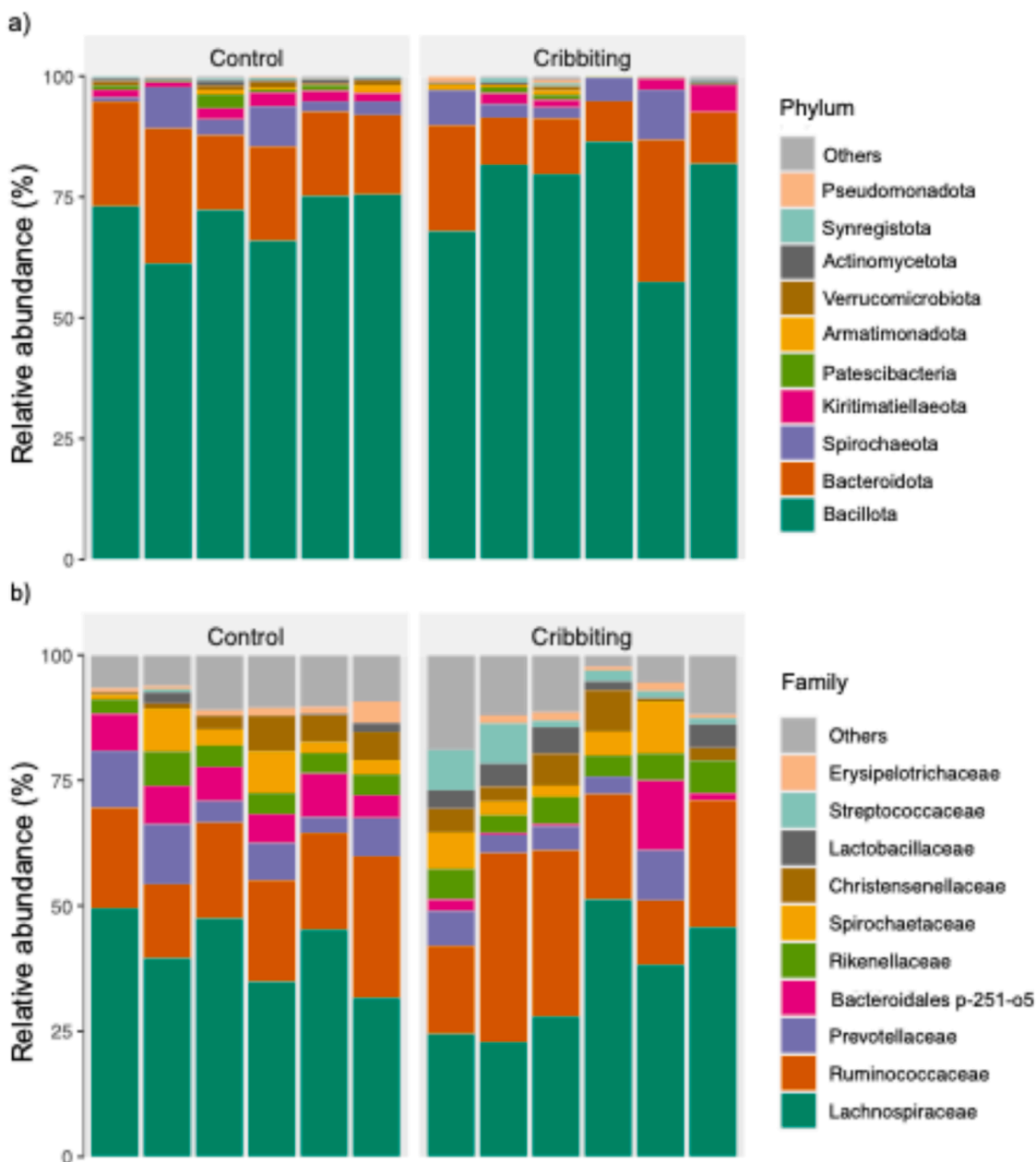


Fig. 2. Taxonomic composition of horses' fecal bacterial communities: A comparison between Control and Cribbiting condition. Relative abundance of ASVs at the phylum level (a) and family level (b). The top 10 most abundant taxa are displayed, while the remaining taxa are categorized as "Others".

a predisposing factor within the multifactorial nature of stereotypies, and recently, evidence has been obtained regarding the effect of stress on the dynamics of the intestinal microbiota associated with the gut-brain axis in behavioral patterns [38,56–58].

Stress caused by biotic and abiotic factors affects the intestinal microbiota through the gut-adrenal axis, describing different adaptation microbiota patterns [23–25]. These changes in the bacterial community are more evident in foals after weaning [53] and are most studied in horses subjected to strenuous exercise [22,32,59–61]. This is relevant as the intestinal microbiome is implicated in various medical conditions, behavioral alterations, and mood states [62]. However, in this study stress-related hormones (cortisol, ghrelin, dopamine, and catecholamines) were not measured. Nevertheless, it is known that this breed would present elevated serum cortisol levels [55].

The fecal microbiome structure and composition at different taxonomic levels in cribbiting horses significantly differed from those of healthy horses without stereotypies, consistent with previous studies

[34,38]. This suggests the possibility that the microbial composition of the intestine plays a role in the etiology of this oral stereotypy [38], as proposed in diseases such as anxiety and depression, which occur through the microbiota-gut-brain axis [21]. Additionally, studies suggest that dysbiosis in the intestinal microbiota leads to cribbiting, possibly through this axis [63]. However, the results of this study do not determine whether the change in microbiota in cribbiting horses is a cause or consequence of the stereotypy. Therefore, longitudinal studies considering the variables described as predisposing factors for cribbiting are needed.

The observational nature of the present study precludes answering this question. Here, we observed a structuring of bacterial communities based on Bray-Curtis distances but not with UniFrac distances. UniFrac incorporates phylogenetic information, and although we are seeing differences compared to Bray-Curtis, incorporating phylogenetic data in an environment with such specific conditions as the intestine might not reveal much phylogenetic variation, regardless of the condition (crib-

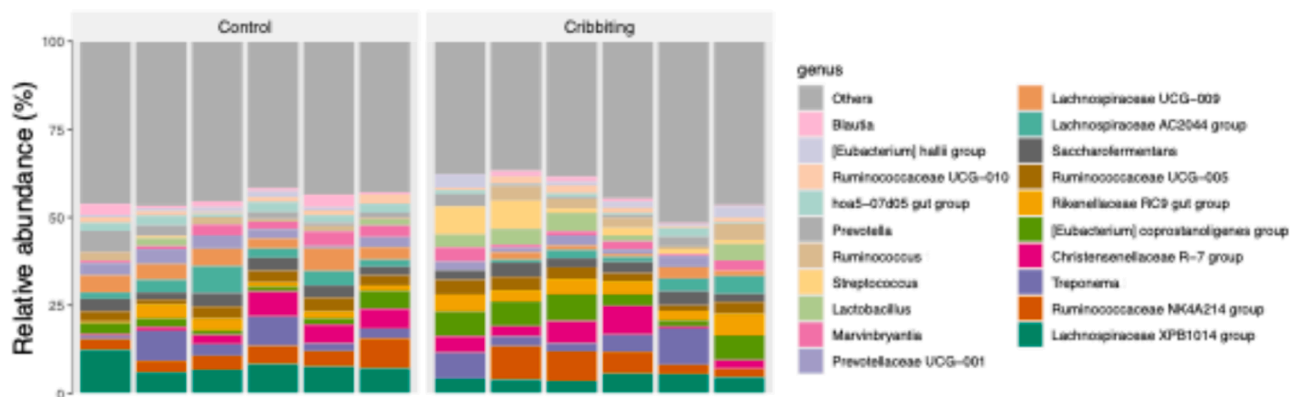


Fig. 3. Taxonomic composition of horses’ fecal bacterial communities: A comparison between Control and Crib-biting condition. Relative abundance of ASVs at the genus level. The top 20 most abundant taxa are displayed, while the remaining taxa are categorized as “Others”.

Table 1
Alpha diversity metrics for each sample and Kruskal-Wallis test comparisons among conditions.

Measure	Condition	Mean	SD	p-value
Observed	Control	163.17	65.73	0.52
	Crib-biting	143.67	83.61	
Chao1	Control	183.54	83.87	0.63
	Crib-biting	164.06	107.03	
ACE	Control	185.53	85.99	0.52
	Crib-biting	164.69	109.89	
Shannon	Control	4.74	0.51	0.52
	Crib-biting	4.49	0.72	
Simpson	Control	0.99	0.01	0.52
	Crib-biting	0.98	0.02	
Fisher	Control	77.08	46.91	0.52
	Crib-biting	66.10	59.20	
Coverage	Control	0.94	0.05	0.63
	Crib-biting	0.95	0.07	

biting or non-crib-biting). This may cause sources of variation to intertwine with the main phylogenetic effect, leading to limitations in the interpretation of the results [64].

The phyla found in higher proportions (*Bacillota*, *Bacteroidota* and *Spirochaetota*) were like those reported in other studies of the same nature and with the same stereotypy, conducted in different contexts and breeds, indicating consistency at this level of the equine microbiome regardless of the condition [34,38]. However, horses without aerophagia showed an increased relative abundance of taxa such as *Ruminococcaceae*, *Bacteroidales* and *Lachnospiraceae* consistent with previous

findings [34]. On the other hand, aerophagic horses differed completely from those reported by Arias-Esquivel et al. [34] and Mach et al. [38]. The fact that similarities were found between the microbiomes of healthy horses, while dissimilarities were observed among crib-biting horses across studies, coupled with the tendency for less diversity in crib-biting horses (indicating less homogeneous communities), suggests that this condition may destabilize the microbiome in specific ways. This is consistent with another study on athletic horses, where groups of bacteria and their metabolites were linked to behaviors associated with poor welfare [38].

In previous studies, aerophagia has been associated with an increase in pathogenic bacteria, such as *Desulfurispora*, *Helicobacter*, *Acinetobacter*, *Ruminobacter*, *Pseudobacteroides*, *Roseburia*, and the family *Marinilabiliaceae* [38], linked to the production of stress-derived catecholamines. However, in this study, most of these bacteria were not reported. Therefore, there is a difference in the bacterial clusters found in horses exhibiting this stereotypy, which is expected due to the different modifiers of the microbiota. However, when comparing horses under similar husbandry conditions, the difference in the alpha diversity between these animals is confirmed.

The outcomes of the functional prediction test of the bacterial clusters identified in each group of horses were different, confirming the differences in the composition and/or function of the intestinal microbiota of horses with and without crib-biting in this study. The mixed acid fermentation pathway was common to both groups, possibly due to similar husbandry conditions, although overrepresented in the crib-biting; additionally, multiple metabolic pathways of several proteogenic amino acids (L-methionine, L-lysine), were observed in the crib-

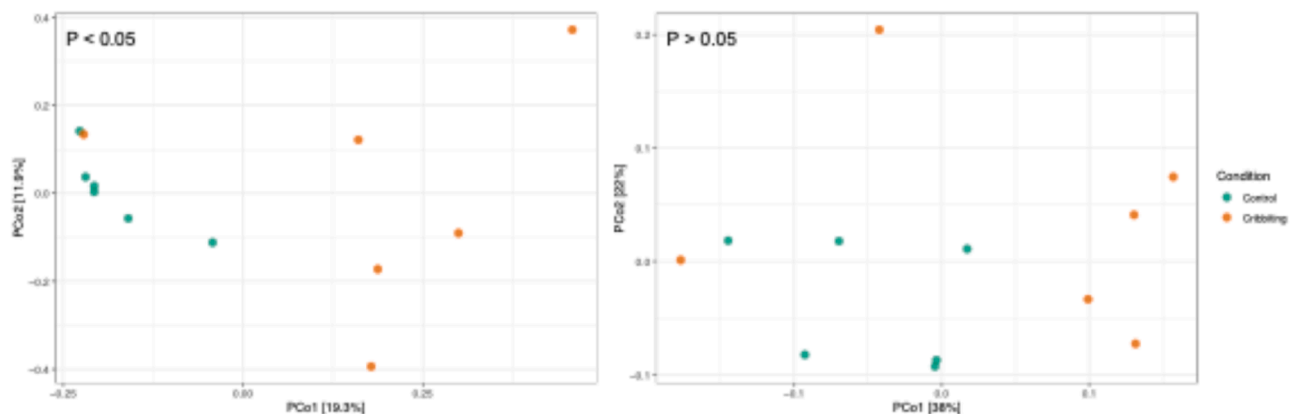


Fig. 4. Bacterial community structure of 12 samples from horse fecal samples. The dataset includes six (6) samples each from Control and Crib-biting conditions. Principal Coordinate Analysis (PCoA) was performed on 16S data using Bray-Curtis distances (left) and Weighted Unifrac distances (right). The significance of the differences was assessed using PERMANOVAs with 999 permutations and a significance level of $\alpha = 0.05$.

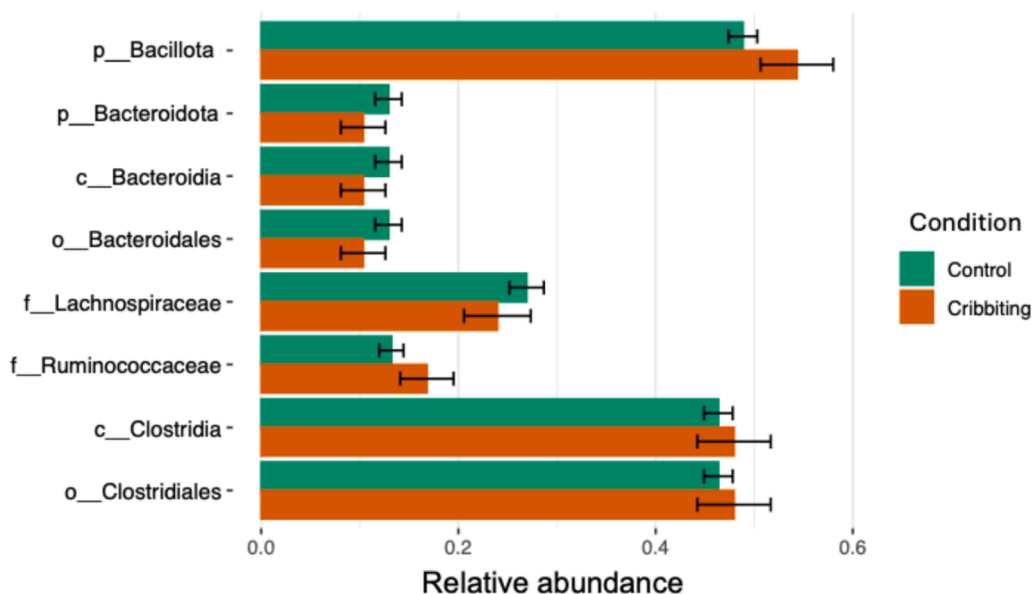


Fig. 5. Relative abundance of taxa with significant differential abundance. Kruskal-Wallis test was performed to evaluate bacterial differential abundance between control and crib-biting conditions in horse fecal samples. Differential abundance of bacterial taxa in both conditions (Control/crib-biting) ($p < 0.05$) are displayed.

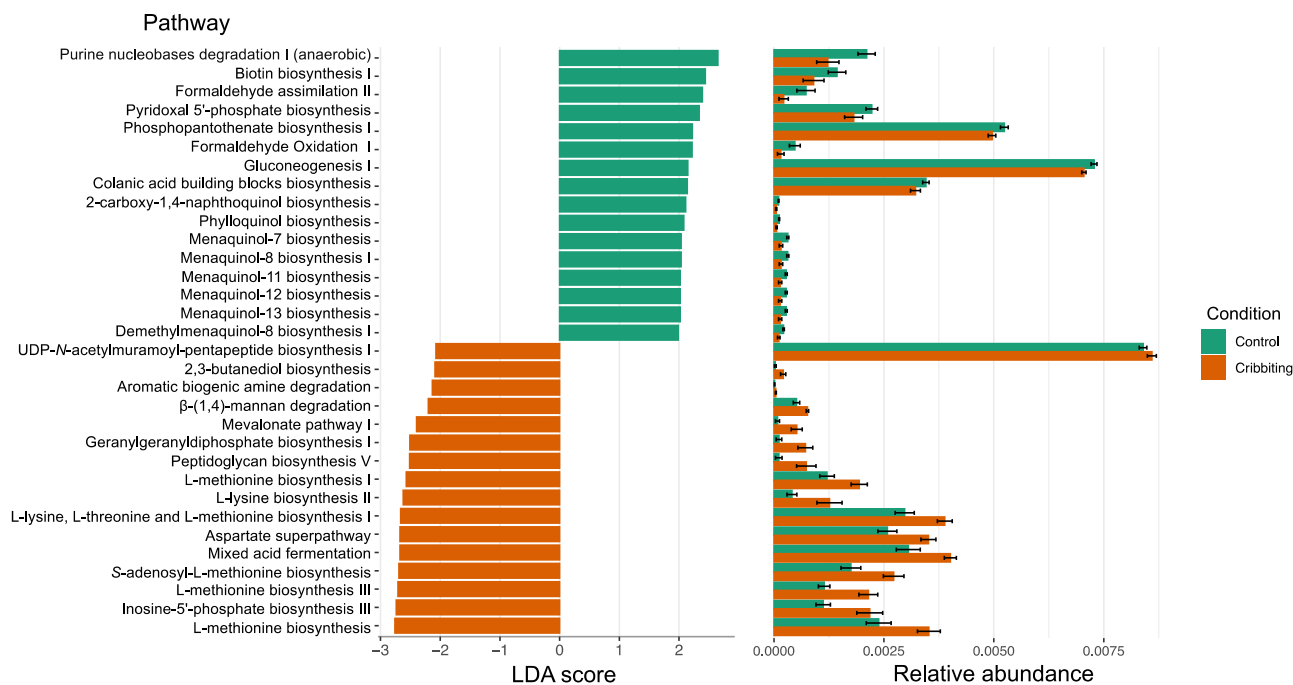


Fig. 6. Prediction of the functional profile in horses' fecal samples under crib-biting and control conditions. Left: LefSe analysis was performed to identify pathways significantly present in each condition, with pathways having an LDA score above the threshold of 2 considered significant for differential abundance between conditions. Right: relative abundance of significant differential pathways.

biting horses. Although this study did not have the scope to reveal the role of metabolites derived from the bacterial clusters of this group, specific amino acids generated by intestinal microbiota have been associated with health, brain function and welfare conditions [65,66]. Therefore, we can only speculate on the existence of various pathways of interaction that must be considered within the microbiota-gut-brain axis, to relate the cause or effect of this and other behavioral alterations. There is evidence of high absorption of amino acids and nitrogen from bacterial activity in the ileum and large intestine of equine [67,68], therefore, it is an interesting topic to explore.

The involvement of microbiota and their metabolites in behavioral

alterations has been extensively studied in mice. Bacteria such as *Roseburia*, a producer of butyrate and *Desulfurispora* producer of hydrogen sulfide (H₂S), both correlated beneficially with oral stereotypies, since butyrate has anti-inflammatory effects in the intestine and H₂S, a protector of the central nervous system [69,70], however, none of these bacteria were found in the present work. Nevertheless, studies aimed at understanding the microbiota-gut-brain axis are incipient in equines, therefore, the study of metabolic pathways and their interactions warrants further investigation. Despite progress in studying intestinal microbial populations in horses, the identification of various metabolites and interaction pathways remains limited, especially in comparison to

human research data [71].

In this regard, an imbalance in intestinal microbial composition has been associated with mental illnesses such as anxiety and depression [21,65], where experiments in rats have shown attenuation of these behaviors with fecal matter transplantation, indicating that bacterial metabolites may be involved [72,73]. However, the present analysis did not fully determine specific bacterial genera as did previous studies [34, 38], thus representing a limitation. Further studies on the microbiome at the genus level, specific bacterial functional profile, and its respective metabolome in aerophagic horses are warranted, as has already been explored in equines with other medical conditions, where the microbiome and metabolome are altered or are co-responsible for the onset of these disorders [74,75].

5. Conclusions

Differences in the fecal bacterial communities of crib-biting and non-crib-biting horses were evidenced, specifically in the abundance of certain families that differed between both groups of horses, concurring with previous studies. It is important to note that the number of individuals involved in each group influenced the statistical power of this study. Further studies are required to validate the role of the microbiota-gut-brain axis in the etiology of crib-biting and other abnormal and stereotyped behaviors, oriented to the search for therapeutic resources such as prebiotics and probiotics. Additionally, determining bacterial genera and their respective metabolome is necessary to identify the chemical mediators that interplay in each component of the microbiota-gut-brain axis and to recognize the degree of causality in aerophagia.

Ethics in publishing statement

This research presents an accurate account of the work performed, all data presented are accurate and methodologies detailed enough to permit others to replicate the work. This manuscript represents entirely original works and or if work and/or words of others have been used, that this has been appropriately cited or quoted and permission has been obtained where necessary. This material has not been published in whole or in part elsewhere. The manuscript is not currently being considered for publication in another journal. That generative AI and AI-assisted technologies have not been utilized in the writing process or if used, disclosed in the manuscript the use of AI and AI-assisted technologies and a statement will appear in the published work. That generative AI and AI-assisted technologies have not been used to create or alter images unless specifically used as part of the research design where such use must be described in a reproducible manner in the methods section. All authors have been personally and actively involved in substantive work leading to the manuscript and will hold themselves jointly and individually responsible for its content.

CRedit authorship contribution statement

José R. Martínez-Aranzales: Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Conceptualization. **Mateo Córdoba-Agudelo:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation. **Juan E. Pérez-Jaramillo:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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