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ORIGINAL ARTICLE

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Caecal microbiota in horses with trigeminal-mediated headshaking

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Abstract

Background: Trigeminal-mediated headshaking (TMHS) in horses is a form of neuropathic pain of undetermined cause that often results in euthanasia. The role of microbiota in TMHS has not been investigated in diseased horses.

Objective: To investigate if gastrointestinal microbiota in the cecum is different in horses with TMHS compared to a control population, during a summer season with clinical manifestations of disease.

Animals: Ten castrated horses: five with TMHS and five neurologically normal controls.

Methods: All horses were sourced from our institution and kept under the same husbandry and dietary conditions. All horses were fed orchard grass hay for 30 days and then were euthanized due to chronic untreatable conditions including TMHS and orthopedic disease (control group). Caecal samples for microbiota analysis were collected within 20 min after euthanasia. Sequencing was performed using an Illumina MiSeq platform and the microbiome was analyzed.

Results: The caecal microbiota of horses with TMHS was similar to control horses in terms of diversity but differed significantly with *Methanocorpusculum* spp. having higher abundance in horses with TMHS.

Conclusions and clinical importance: *Methanocorpusculum* spp. was more abundant in the cecum of horses with TMHS. However, its role in disease is unknown. Furthermore, it could also represent an incidental finding due to our small population size.

KEYWORDS
headshaking, microbiome, neuropathic, pain, trigeminal

1 | INTRODUCTION

Trigeminal-mediated headshaking (TMHS) in horses is a form of neuropathic pain that compromises performance and quality of life, often resulting in euthanasia (Pickles et al., 2014; Ross et al., 2018). This disorder commonly manifests as sudden violent vertical shakes, snorting, rubbing of the nose and anxious facial expression (grimace, nostril flare, wide-eye, ears pulled back) suggesting pain (Lane & Mair, 1987; Madigan & Bell, 1998). A variety of treatments with different mechanisms of action and variable results have been used in attempts to alleviate pain in suspected cases (Madigan & Bell, 2001; Pickles et al., 2014). Treatment has included antidepressants, anticonvulsants,

channel blockers, antihistamines, corticosteroids, dietary management, facial physical devices, neurectomy or compression of the infraorbital nerve and percutaneous infraorbital nerve electrostimulation (Madigan & Bell, 2001; Mills et al., 2002; Pickles et al., 2014; Roberts et al., 2020). The trigeminal complex is responsible for facial sensation and consists of central (brainstem with relay to the cerebral cortex and first spinal cord segments) and peripheral (trigeminal ganglia and nerve with its multiple branches) parts (Aleman et al., 2013). Through somatosensory and sensory nerve conduction studies of the trigeminal complex, it was reported for the first time that the infraorbital nerve has a low threshold for activation in horses with TMHS compared to normal horses (≤10 mA vs. >10 mA, respectively) (Aleman et al., 2013, 2014). Based on these studies and the lack of histological abnormalities of the trigeminal complex, TMHS was determined to be a functional disorder of likely multifactorial etiology (Aleman et al., 2013, 2014). Because the disorder usually affects young adult geldings (75% of cases) in a seasonal fashion with 59%–68% of cases occurring in the spring and summer months, with some horses triggered or worsened with natural sunlight; hormonal, dietary and environmental factors are suspected to play a role (Madigan & Bell, 2001; Madigan et al., 1995; Mills & Geering, 1997; Ross et al., 2018). Recent studies have shown that supplementation of magnesium and boron can alter the frequency and severity of headshaking (Sheldon et al., 2019). The microbiota within the gastrointestinal tract of the horse can vary depending on diet and season of the year (Salem et al., 2018). The role of microbiota in health and disease has been documented in human and veterinary medicine (Leclere & Costa, 2019; Wang et al., 2017). Since TMHS appears to be a multifactorial disorder, the role of microbiota needs investigation. Therefore, the purpose of the study was to investigate the microbiota from the cecum from horses with TMHS and a matched control population maintained under similar environmental and dietary conditions during a season with clinical manifestations of disease. We hypothesize that horses with TMHS have a different caecal microbiota profile than unaffected horses.

2 | MATERIALS AND METHODS

2.1 | Animals

A total of 10 geldings donated to our institution for chronic non-treatable medical conditions requiring euthanasia were enrolled for the study (recurrent seasonal headshaking $n = 5$, chronic orthopedic lameness noticed at the trot in circles $n = 5$). All horses underwent a physical and neurological examination. Healthy control horses had no history of headshaking and were screened for signs of headshaking during the study. Age in the control group ranged from 4 to 21 years old (mean 12.2 years, SD 7.8, median 9) of Thoroughbred ($n = 3$) and Warmblood ($n = 2$) breeds. These horses received no medication and were euthanized during the spring and summer months.

A clinical diagnosis of TMHS was mandatory in the affected group. A diagnostic workup consisting of oral, ophthalmic and otoscopic examinations, complete blood count (CBC), serum biochemistry, skull radiographs and upper airway endoscopy including guttural pouches was performed to exclude other causes of headshaking. None of these horses had apparent cervical or nuchal pain based on palpation, extension and flexion of the neck. A full body necropsy further supported status as TMHS or control. The TMHS group consisted of geldings, ages 12 to 19 years old (mean 14.4 years, SD 1.9, median 15) of Quarter Horse breed. Headshaking was observed during the study while resting or eating in the stall, at a walk, and exacerbated by light exercise (trot in a circle for 2 min). These horses were euthanized during the summer.

2.2 | Experimental design

Horses were housed in covered box stalls bedded with wood shavings, had free access to water, and a diet consisting of orchard grass hay fed for a minimum of 30 days to allow environmental and dietary adaptation (Leclere & Costa, 2019). At the end of this period, horses were euthanized after sedation with xylazine hydrochloride at 0.25 mg/kg IV. Euthanasia consisted of a solution (Euthasol, Virbac AH, Inc., Fort Worth, TX) of pentobarbital sodium (390 mg/ml) and phenytoin sodium (50 mg/ml) IV at a dosage of 77–109 mg/kg (body weight ranged from 498 to 591 kg) for a total volume of 100 ml. The study was approved by an institutional animal care and use committee blinded for review.

2.3 | Sample collection and DNA extraction

All samples were collected within 30 min of euthanasia. A 20 cm skin incision at the mid aspect of the right side of the abdomen was made to access the cecum using new rectal clean gloves for the collection of caecal contents. A total of 60–100 g of caecal material was collected, placed in sterile plastic containers on ice within 2 min of collection. Samples were transported and placed in a −80°C freezer within 1 h of collection.

Total nucleic acid was extracted from the faecal samples using a QIAcube HT a semi-automated nucleic acid workstation (Qiagen, Valencia, CA) according to the manufacturer's instructions for the QIAamp 96 DNA QIAcube HT Kit (Qiagen, Valencia, CA).

2.4 | High throughput shotgun sequencing

One microgram of each of the DNA samples was incubated with 50 units RNase If (New England Biolabs, Ipswich, MA) for 15 min at 37°C to remove RNA contamination, before shearing the samples on an E220 Focused Ultrasonicator (Covaris, Woburn, MA). The sheared DNA samples were size-selected with a double-sided solid phase reversible immobilization (SPRI)-protocol with KapaPure beads (Kapa Biosystems-Roche, Basel, Switzerland) using bead-to-sample ratios of 0.6× and 0.76×. The size-selected DNA samples were converted to barcode-indexed shotgun sequencing libraries using the

Kapa Hyper Library Preparation Kit (Kapa Biosystems-Roche). The libraries were polymerase chain reaction (PCR) amplified, cleaned with a SPRI-protocol, and analyzed via micro-capillary gel electrophoresis on a LabChip GX system (PerkinElmer, Waltham, MA). The sequencing libraries were quantified by fluorometry on a Qubit instrument (LifeTechnologies, Carlsbad, CA), and pooled at equimolar ratios. The pool was quantified with a Kapa Library Quant kit (Kapa Biosystems-Roche) on a QuantStudio five real-time PCR system (Applied Biosystems, Foster City, CA) and shotgun sequenced on an Illumina Novaseq 6000 (Illumina, San Diego, CA) run with paired-end 150 bp reads.

2.5 | Data analysis

Raw sequencing reads were cleaned with BBDUK 37.68 with the following parameters; qtrim = rl trimq = 10 minlength = 80 (Bushnell, 2020). Taxonomy was assigned to the cleaned reads using Kaiju 1.7.3 with the following parameters (-a greedy -e 5 -E 0.05) using the National Research database from National Center for Biotechnology Information (Menzel et al., 2016). This information was processed using Kaiju reports at each taxonomic rank with a minimum report threshold of 0.5%. The reports were summarized using the combine_kaiju_reports.pl script (default parameters). The resulting matrix was imported into R 3.6.2, along with the sample metadata (Pinheiro et al., 2019). The raw counts were transformed into relative abundances to generate a Bray-Curtis distance matrix ordinated to generate a PCoA plot using phyloseq 1.30.0 (Mcmurdie & Holmes, 2013). The DESeq2 (1.26.0) package was used to identify significantly overrepresented taxa using the Wald test with a Benjamini–Hochberg correction for a parametric variable on a non-normalized count matrix to which pseudo-counts were added (Love et al., 2014).

For most analyses, the data were normalized as follows. The read counts for each taxon in each sample were divided by the sum of the read counts for that sample. This was repeated at each taxonomic level of interest.

3 | RESULTS

Both groups of horses were further confirmed as control or TMHS upon post-mortem evaluation. For TMHS horses, other causes of headshaking were ruled out. From the caecal samples, a total of 12.5 billion raw reads were sequenced, with an average of 401 million reads per sample. After quality trimming with BBDUK, a total of 12.3 billion cleaned reads were left for analysis (average 39 million reads per sample). The relative abundance of the 10 most common phyla and genera present in caecal samples from both groups of horses are presented in Figure 1. Beta diversity was examined via principal coordinate analysis (PCoA) of bacterial genus relative abundance (for controls vs. headshakers) and is shown in Figure 2. The genus *Methanocorpusculum* was significantly increased in the caecal content of horses with TMHS as compared to that of control horses (*P* value = 3.17E-20, Table 1).

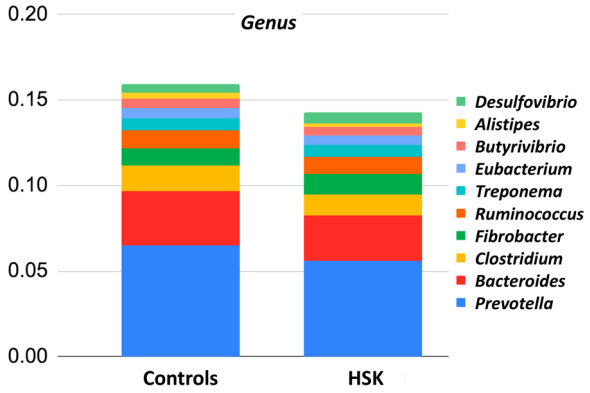

FIGURE 1 Overall normalized and pooled relative abundance of caecal contents in horses with TMHS and controls. HSK = Headshakers. Shown are only the top 10 taxa (ranked by abundance of each genus across all horses) that were identified to the genus level

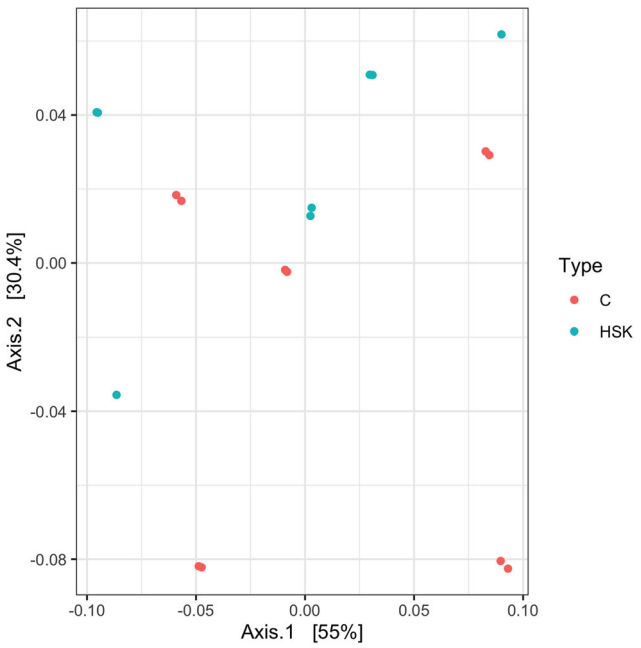

FIGURE 2 Principal coordinate analysis (PCoA plot) of bacterial levels at the genus by horses. C, Control; HSK, Headshaker

4 | DISCUSSION

This study evaluated the caecal microbiota in horses with TMHS and control horses. The caecal microbiota was similar between the two groups except for *Methanocorpusculum* spp. which was significantly higher in horses with TMHS. *Methanocorpusculum* has been shown to be the main inhabitant of the equine cecum and found to be part of the highest two archaeal clades in the faecal microbiota in horses (Fernandes et al., 2014; Murru et al., 2018). *Methanocorpusculum* reduces carbon dioxide to methane using hydrogen (Morvan et al., 1996). It is thought that the methane production by monogastrics is less than that of ruminants (Murru et al., 2018). Furthermore, there are lower counts of methanogenic archaea in the equine cecum

TABLE 1 Normalized pooled genus abundance data in horses from caecal samples. Controls and headshakers (HSK)

Genus	Controls	HSK
Unclassified*	60.83%	63.36%
Cannot be assigned to a genus	12.58%	11.72%
Genus < 0.5% of all reads	7.97%	7.63%
Prevotella	6.48%	5.62%
Bacteroides	3.19%	2.64%
Clostridium	1.51%	1.19%
Ruminococcus	1.07%	1.00%
Fibrobacter	0.96%	1.21%
Treponema	0.71%	0.68%
Eubacterium	0.59%	0.58%
Butyrivibrio	0.55%	0.50%
Desulfovibrio	0.50%	0.63%
Roseburia	0.42%	0.38%
Faecalibacterium	0.40%	0.39%
Alistipes	0.37%	0.22%
Parabacteroides	0.31%	0.25%
Lachnoclostridium	0.28%	0.23%
Blautia	0.25%	0.21%
Lactobacillus	0.22%	0.27%
Paraprevotella	0.15%	0.15%
Alloprevotella	0.12%	0.16%
Phascolarctobacterium	0.10%	0.03%
Oscillibacter	0.09%	0.09%
Streptococcus	0.06%	0.12%
Butyricicoccus	0.05%	0.02%
Porphyromonas	0.05%	0.00%
Coprococcus	0.05%	0.05%
Akkermansia	0.04%	0.00%
Pseudobutyrivibrio	0.04%	0.08%
Campylobacter	0.04%	0.20%
Viruses	0.03%	0.02%
Bacillus	0.00%	0.03%
Coprobacillus	0.00%	0.02%
Dermatophilus	0.00%	0.07%
Enterococcus	0.00%	0.10%
Lysinibacillus	0.00%	0.00%
Methanobrevibacter	0.00%	0.00%
Methanocorpusculum	**0.00%**	**0.09%**
Mycoplasma	0.00%	0.00%
Paenibacillus	0.00%	0.00%
Paludibacter	0.00%	0.00%
Staphylococcus	0.00%	0.05%

when compared to rumen samples from various ruminant species (Morvan et al., 1996). A cause and effect or contribution of higher concentrations of *Methanocorpusculum* to TMHS in horses was not investigated here. Although possible differences were noted in caecal samples between groups in *Phascolarctobacterium*, *Streptococcus* and *Campylobacter*; and *Roseburia*, *Oscillibacter*, *Bacillus*, *Coprobacillus* and *Lysinibacillus*, respectively; these were not statistically significant.

The microbiota varies greatly among the different anatomic compartments of the gastrointestinal tract with less variation between adjacent compartments and largest diversity in the distal gut (Costa et al., 2015). In our study, the 10 most common genera from the cecum in both groups of horses were *Prevotella*, *Bacteroides*, *Clostridium*, *Fibrobacter*, *Ruminococcus*, *Treponema*, *Eubacterium*, *Butyrivibrio*, *Alistipes* and *Desulfovibrio*. Similar to other reports, *Bacteroides* and *Firmicutes* were the most dominant caecal phyla (Costa et al., 2015; Arnold et al., 2020). The control caecal group had relative abundance of pooled phyla greater than 1% with *Bacteroides* at 11.8%, *Firmicutes* at 11.8% and *Proteobacteria* at 1.5%. The TMHS caecal group had relative abundance of pooled phyla greater than 1% with *Bacteroides* at 9.8%, *Firmicutes* at 9.9%, *Proteobacteria* at 1.6% and *Fibrobacter* at 1%. The top three phyla being *Bacteroides*, *Firmicutes* and *Proteobacteria* were similar to another study, however in that study, the relative abundance of these three phyla were in higher percentages than was found in our group of horses (Warzecha et al., 2017).

The intestinal microbiota plays an important role in health and disease (Wang et al., 2017). Some of the essential roles of the microbiota include extraction of energy from food, alteration of appetite signalling, provision of a physical barrier for pathogens and development of intestinal mucosa and immune system of the host (Macpherson & Harris, 2004; Rakoff-Nahoum & Medzhitov, 2008). The role of microbiota in health and disease, and its alteration following drug administration has been investigated in horses (Arnold et al., 2020; Leclere & Costa, 2019; Stewart et al., 2018, 2019; Tyma et al., 2019; Schoster et al., 2019). Bacterial composition is influenced by diet, environment, season, oxygen tension, physiological role and inflammation (Leclere & Costa, 2019; Salem et al., 2018). Inter-breed diversity and temporal dynamics of the faecal microbiota were investigated in a cohort of 189 healthy young horses of six breeds under similar conditions at two time points 8 months apart (Massacci et al., 2020). The authors concluded that despite an apparent microbial diversity and composition, breed exerted limited effects in microbiota (Massacci et al., 2020). Although there is no reported breed predisposition in horses with TMHS; Thoroughbreds, Quarter Horses and Warmbloods are the most represented breeds (Madigan & Bell, 1998, 2001). In our study, all horses with TMHS were of Quarter Horse breed; and controls were of thoroughbred and warmblood breeds. It is unknown if breed played a role in the results of this study.

Microbiota adaptation to dietary and environmental modification occurs in healthy horses (Costa et al., 2015; Fernandes et al., 2014; Leclere & Costa, 2019; Salem et al., 2018). Similar adaptations were not observed in horses with asthma which raises a possible role of the

microbiota as a potential modulator of the allergic response (Leclere & Costa, 2019). Similar findings in germ-free mice and infants with dysbiosis that developed asthma support the observations made by LeClere and Costa (Frati et al., 2018; Leclere & Costa, 2019; Lynch & Boushey, 2016; Martinez & Guerra, 2018; Pisi et al., 2017). Asthma can be influenced by environmental factors such as presence of allergens due to husbandry practices or seasonal factors (Bond et al., 2018). Horses with seasonal TMHS manifest signs during spring, summer and early fall months (Madigan & Bell, 2001). This seasonal similarity to asthma in horses warranted investigation of microbiota in horses with TMHS (Bond et al., 2018). The higher number of *Methanocorpusculum* in caecal samples from horses with TMHS does not prove cause and effect, and might be incidental due to the small sample size. Seasonal dietary changes can occur as the result of soil conditions, forage composition and harvest management on which forage from winter crop to spring crop are different and coincide with the onset or exacerbation of headshaking (Sheldon et al., 2018). Dietary components influence changes in blood pH and ionized electrolytes such as calcium and magnesium which are essential for the modulation of nerve transmission (Sheldon et al., 2018). Whether microbiota adaptation to dietary changes and season of the year occurs in horses with TMHS is uncertain.

The contribution of microbiota in neurologic disease and pain in humans is well documented (Guo et al., 2019; Quigley, 2017). Gut microbiota influence chronic, visceral, inflammatory and neuropathic pain, headache and tolerance to opidoids (Guo et al., 2019; Holzer & Farzi, 2014). Various microbiota regulatory mechanisms such as modulation of dorsal root ganglia and neuroinflammation of the peripheral and central nervous systems have been described (Guo et al., 2019). Neuropathic pain is associated with dysesthesia (abnormal sensation) or allodynia (nonpainful stimuli-evoked pain) (Von Hehn et al., 2012). Presumably, horses with TMHS have both based on somatosensory studies (Aleman et al., 2013, 2014). Horses with acutely induced-laminitis resulted in microbiota alteration compared to healthy horses (Moreau et al., 2014; Tuniyazi et al., 2021). This effect was not investigated in our horses with orthopedic disease.

The main limitations of our study were the small number of animals and lack of investigation of microbiota composition of the various GI anatomical compartments. Although faecal samples would have been easier to collect; these were not collected due to being reported as remarkably similar despite variables such as individual history, breed or age (Kauter et al., 2019). The effect of breed was not investigated here and remained unknown if Quarter Horses might have more *Methanocorpusculum* in the cecum than other breeds and not as the result of TMHS. The source of control horses depended on the availability of horses being euthanized during the same season of TMHS; resulting in variability of breed, age and presenting lameness. Also, the effect of lameness in the caecal microbiota expression of *Methanocorpusculum* was not investigated. Another limitation is that control horses were not all fed in the same month as horses with TMHS (spring and summer vs. summer, respectively) due availability. However, major environmental differences were not noted due to our institution geographical location. Housing, management and dietary conditions were the same. Lastly, differences in microbiota adaptation throughout the seasons of the year was not investigated. Caution must also be considered when comparing studies on microbiota since different methodologies could have been used generating different results.

In conclusion, horses with TMHS had a significantly higher concentration of *Methanocorpusculum* spp. in caecal samples during the season of clinical manifestations compared to control horses. However, caution must be practiced in the interpretation of this finding due to the small sample size, and variability of breed and age in the control group. Therefore, the role of *Methanocorpusculum* in disease is unknown. Understanding the role of microbiota in TMHS throughout the seasons of the year might result in dietary and environmental modification in attempts to prevent clinical manifestations of disease, improve quality of life and avoid possible euthanasia.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

AUTHORS CONTRIBUTION

Study conception, hypothesis generation and experimental design: Aleman; organizing and conducting experiments: Aleman, Sheldon; interpreting and analyzing results: Jospin, Coil, Eisen; writing and revising the manuscript: Aleman, Sheldon, Jospin, Coil, Phelps, Eisen.

ETHICAL STATEMENT

The authors declare human ethics approval was not needed for this study.

DATA AVAILABILITY STATEMENT

Data available upon request.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1002/vms3.735

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