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Wageningen Academic
P u b l i s h e r s

P.O. Box 220
6700 AE Wageningen
The Netherlands
cep_cr@wageningenacademic.com
Tel: +31 317 476516
Fax: +31 317 453417

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The effects of acute strenuous exercise on the faecal microbiota in Standardbred racehorses

A.H.D. Janabi[1,2], A.S. Biddle[3], D.J. Klein[4] and K.H. McKeever[5*]

[1]Microbial Biology Graduate Program, Rutgers – State University of New Jersey, New Brunswick, NJ 08901, USA; [2]Department of Veterinary Internal Medicine, College of Veterinary Medicine, Al-Qadisiya University, Diwaniya, Iraq; [3]Department of Animal and Food Sciences, University of Delaware, Newark, DE 19716, USA; [4]Nutritional Sciences Graduate Program, Rutgers – State University of New Jersey, New Brunswick, NJ 08901, USA; [5]Equine Science Center, Department of Animal Science, Rutgers – State University of New Jersey, New Brunswick, NJ 08901, USA; mckeever@aesop.rutgers.edu

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RESEARCH ARTICLE

Abstract

While exercise has been found to change the faecal microbiome (FM) in laboratory animals exposed over weeks, no studies have identified immediate changes in the FM associated with short spans of intense exercise, ~5 min. The purpose of this study was to test the hypothesis that acute intense exercise would alter the FM in horses. Each horse performed two rounds of testing undergoing both a graded exercise test (GXT) and a parallel standing control (SC) trial before (GXT1 and SC1) and after (GXT2 and SC2) 12 weeks of exercise training. Rectal faecal samples were taken 24 h before and after testing. Bacterial community analysis was done by sequencing the 16s rRNA (V3-V4) region via Illumina Miseq. The relative abundance of the genus *Clostridium* significantly decreased in SC1 ($P<0.05$), with a concurrent decrease in the Shannon diversity index at the species level ($P<0.05$). At both the genus and species levels the principle coordinate analysis (PCoA) showed significant separation when the samples collected before SC1 were compared to those collected after SC1 ($P<0.05$). Interestingly, we found that *Fusicatenibacter saccharivorans*, a bacteria found to be decreased in ulcerative colitis patients, and *Treponema zioleckii*, a bacteria found to degrade fructan in sheep rumen, were significantly decreased when the samples collected before SC1 were compared to those collected after SC1 ($P<0.05$). None of the changes observed in SC1 happened in SC2 ($P>0.05$). Our results indicate that very intense acute exercise does not alter the faecal microbiome of the Standardbred race horse and that 12 weeks of exercise training does not alter that response.

Keywords: horse, intense exercise, microbiome

1. Introduction

The gut microbiome is considered to be the 'second brain' of animals and humans (Foster, 2013). Diverse microbial communities of bacteria, protozoa, fungi, and yeast (Mackie *et al.*, 1999) inhabit the gastrointestinal tract (GIT) of animals and humans. The extent to which the microbiome communicates with host cells and influences the regulation of host processes is an active area of research. Hindgut fermenters like horses have specialised compartments such as the caecum and large colon, where microbes supply the enzymes to ferment complex plant material into useable short chain fatty acids (Bjursell *et al.*, 2006; Costa and Weese, 2012; Desvaux, 2005; Hethener *et al.*, 1992; Zhang *et al.*, 2014).

One of the factors that can influence the faecal microbiome (FM) is exercise training which has been found to change the composition of microbial communities in the GIT of humans, mice, and rats (Evans *et al.*, 2014; Potera, 2013; Queipo-Ortuño *et al.*, 2013; Santacruz *et al.*, 2009). Another factor that can influence the FM is psychological stress mediated by the autonomic nervous system and its influence on the GIT (Carabotti *et al.*, 2015). For example, in mice, psychological stress caused by deprivation of food, water and bedding (Tannock and Savage, 1974)

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or social disruption (Bailey *et al.*, 2011) has been shown to cause significant changes in specific members of the FM community. High fat diets can also alter the FM in ways that are similar to feeding stress (Evans *et al.*, 2014). Furthermore, exercise training appears to induce beneficial FM alternations in mice fed a high fat diet (HFD) when compared to those fed the HFD alone (Evans *et al.*, 2014). This suggests that exercise training may have a protective effect. However; recent studies of rodents documented that it takes weeks of exercise training to initiate changes in the FM (Evans *et al.*, 2014; Liu *et al.*, 2015; Mika *et al.*, 2015). Those studies reported a training-induced alteration in *Bacteroidetes* and *Proteobacteria* in mice, *Bacteroidetes* and *Firmicutes* in the wheel running juvenile rats, and alternations in *Firmicutes* and *Proteobacteria* in rats (Evans *et al.*, 2014; Liu *et al.*, 2015; Mika *et al.*, 2015).

To the best of our knowledge, no studies have been published on the effects of a single bout of acute intense exercise on the faecal microbiome of the horse or other species. Furthermore, we are unaware of any published study that has examined the effects of exercise training on that response to acute exertion. The physiological rationale for this study centres on the well-recognised responses to intense exercise that alter the internal milieu of the gastrointestinal tract. These short term responses to intense exertion include, inter-compartmental shifts of fluid, changes in autonomic tone and increases in sympathetic drive that alter motility, as well as the well-documented in a decrease in blood flow to the splanchnic region that allows an increase in blood flow to the skin, muscles, and other vital organs in humans, dogs, and horses (McKeever and Lehnhard, 2014; Rowell, 1983). This temporary decrease in blood flow to the mesenteric region leads to a temporary decease in oxygenation to the intestinal mucosa and an increase in the abundance of the anaerobic bacterial genus *Clostridium* (Smith-Slatas *et al.*, 2006). The horse is an athletic animal and, like humans, it is well recognised that for the horse, exercise training has many beneficial effects related to the adaptive physiological response to the repeated acute challenge of exertion. The present study was preformed to test the hypothesis that acute intense exercise would alter the FM and secondarily, that the adaptive effects of exercise training would alter the response to acute exercise in the horse.

2. Materials and methods

Animals, ration, and housing

This paper is a companion paper to our previous article that sampled the same eight horses every two weeks over the course of a 12 week training period (Janabi *et al.*, 2016a). All methods used in this study were approved by the Rutgers Institutional Animal Care and Use Committee. Eight healthy, unfit Standardbred horses (3-8 years; ~500 kg; 4 mares and 4 geldings) were used in the experiment. All horses were acclimated to the Rutgers Equine Science Center training personnel, housing, and training equipment for at least two months before the experiments. All of the horses had been dewormed and vaccinated per standard veterinary practice and all had been examined by a veterinarian for soundness and health status. Animals were fed a maintenance ration of alfalfa/grass hay *ad libitum* (6 kg/day) and pelleted grain supplement (3 kg/day). Water and mineral blocks were provided *ad libitum*. They were housed in groups of 4 in their respective 2-acre dry lot paddocks.

Incremental exercise test

During the incremental exercise test (GXT) maximal oxygen uptake (VO_{2max}) and physiological markers of exercise performance were measured as previously described (Kearns and McKeever, 2002). Each horse ran on a high-speed horse treadmill (Sato I; Sato, Lexington, KY, USA) at a fixed 6% grade. The GXT started at initial speed of 4 m/s for 1 min and increased by increments of 1 m/s every 60 s (omitting 5 m/s) until the horses reached fatigue. Fatigue was defined as the point where the horse could not keep up with the treadmill despite humane encouragement. At this point, the treadmill was stopped and the horse walked to the safety stocks. Oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were measured every 10 seconds using the open flow indirect calorimeter (Oxymax-XL, Columbus Instruments, Columbus, OH, USA).

Experimental design

The general experimental design is depicted in Figure 1. In short, each horse performed two rounds of testing where they underwent both a graded exercise test (GXT) and a parallel standing control (SC) test prior to (GXT1 and SC1) and following (GXT2 and SC2) 12 weeks of exercise training. The pre-training and post-training testing used a crossover design where the horses performed both the GXT and the SC. There was a 2 week washout period before conducting the opposite treatment. All horses were handled the same for each trial and all received the same feeding and watering protocol during the day of the GXT and SC trials. All other variables such as laboratory temperature, time of day for the trials, etc. were controlled in a similar identical fashion.

The equine exercise physiology laboratory contains four 3×3 m stalls and logistically that meant that only four horses could be tested on a single day. Therefore; for the first round, the 8 horses were randomly assigned into 2 groups (Group A or Group B) with four horses running a GXT and four horses placed in the stalls within the treadmill lab to serve as standing controls on their given day. The same stall and group assignment was maintained for the post-training testing and sampling.

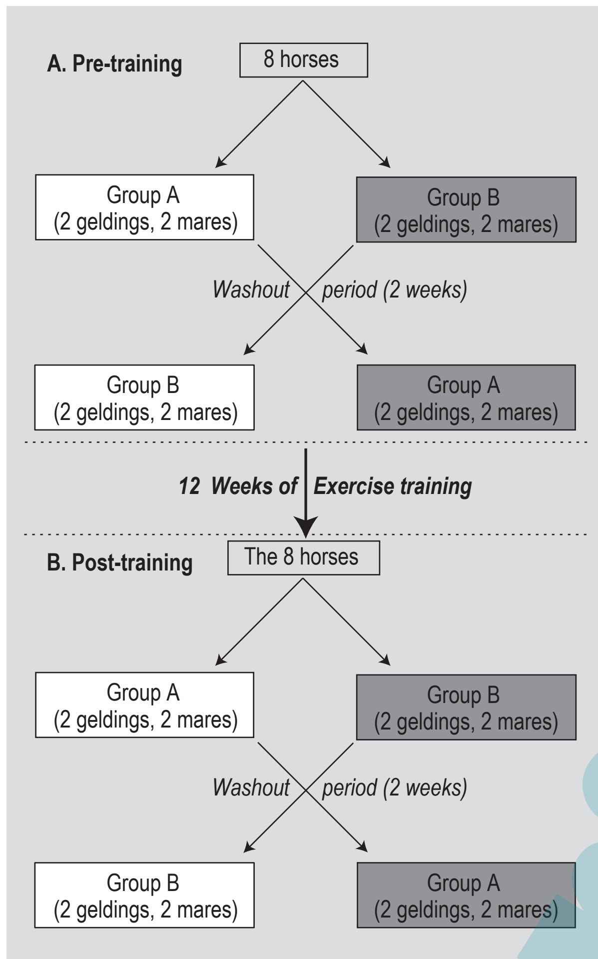

Figure 1. The experimental design. GXT = graded exercise test; SC = standing control.

For the standing control, each of the horses stood quietly in a stall inside the treadmill laboratory where they could hear and observe the exercise tests performed by the other horses. For the 12 week exercise training protocol, the eight horses were exercised in a free stall motorised exercise machine (Equi-ciser, Calgary, Canada)4 days a week and ran on the treadmill trough a simulated GXT one day per week. Submaximal exercise intensity was set at ~60% of VO_{2max} with duration and speed adjusted upward each week (Table 1).

Sample collection

Faecal samples were obtained from all horses 24 h before and 24 h after GXT1 (or SC1) and GXT2 (or SC2). The sample was collected at 24 h as good turnover of bacterial populations would be significant by that time post exercise (Yan *et al.*, 2012). Faecal samples were collected via rectal palpation by the same investigator (A.H.D. Janabi). Samples were obtained following the clearance of faeces from the lower GI tract with the grab sample obtained ~45 cm from the inner end of the rectum of the horse. Each sample was placed on sterile paper and opened using sterile techniques to collect 0.25 g samples. The samples were placed into collection tubes containing beads (SKU# 116914100; MP Biomedical, Illkirch, France) and 300 µl of buffer solution containing cetyl trimethylammonium bromide. Each tube was snap frozen in liquid nitrogen and then stored at -80 °C for later analysis.

DNA Eextraction

DNA was extracted using a protocol from (Janabi *et al.*, 2016b). Briefly, samples were subjected to five quick freeze/thaw cycles with liquid nitrogen (-80 °C) and 55 °C hot bath. To the frozen samples, 100 µl Solution 1 (50 mM glucose, 10 mM EDTA, 25 mM Tris-Cl; pH 8.0), 50 µl of lysozyme solution (4 mg in 1 ml of Solution 1) and 50 µl of 500 mM EDTA were quickly added. To the thawed samples, 50 µl 10% SDS and 800 µl phenol: chloroform: isoamyl alcohol; 25:24:1 (>pH 7.0) were quickly added. Samples were disrupted using vortexing for 3 min. Samples were then spun down in micro-centrifuge at (~16,000×g) for 3 min. The top phase was transferred to a new centrifuge tube preloaded with 800 µl phenol:chloroform:isoamyl alcohol; 25:24:1, v/v/v (>pH 7.0). Tubes were vortexed for 1 min at maximum speed and then centrifuged for 3 min at (~16,000×*g*). The top layer was then transferred to a new micro-centrifuge tube, and 200 µl of C3, inhibitor removal solution (catlg# 12830-50-3; MoBio Laboratories Inc., Carlsbad, CA,

Table 1. Exercise training protocol groups and standard exercise procedure.

Day of the week	Group A (2 geldings, 2 mares)	Group B (2 geldings, 2 mares)
Sunday	paddock (free-exercise)	paddock (free-exercise)
Monday	light (walk, trot, canter)	light (walk, trot, canter)
Tuesday	moderate (walk, trot, canter, slow gallop)	light (walk, trot, canter)
Wednesday	light (walk, trot, canter)	moderate (walk, trot, canter, slow gallop)
Thursday	heavy (walk, trot, canter, gallop to fatigue)	light (walk, trot, canter)
Friday	light (walk, trot, canter)	heavy (walk, trot, canter, gallop to fatigue)
Saturday	paddock (free-exercise)	paddock (free-exercise)

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USA), was added to each tube. The tubes containing the C3 solution and the DNA extract were vortexed briefly and then incubated in 4 °C for 5 min in ice. Following incubation the tubes were spun at 13,000×*g* for 1 min (as indicated by the manufacturer protocol), and the supernatant containing DNA was transferred to a sterile and DNAse and RNAse free tube. The DNA was measured for purity and concentration using NanoDrop™ 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA), and checked for amplification of the 16s rRNA gene using universal bacterial primers: 27F: AGAGTTTGATCCTGGCTCAG and 1522r: AAGGAGGTGATCCAICCGCA.

Sequencing (Miseq)

The 16S rRNA gene V3-V4 variable region was amplified using PCR primers 341F, CCTACGGGAGGCAGCAG /785R, CTACCAGGGTATCTAATCC, which are mentioned by (Mühling *et al.*, 2008), with barcodes on the forward primer in a 30 cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA, USA) under the following conditions: 94 °C for 3 min, followed by 28 cycles of 94 °C for 30 s, 53 °C for 40 s and 72 °C for 1 min, after which a final elongation step at 72 °C for 5 min was performed. PCR products were further investigated in 2% agarose gel to detect the success of amplification and the relative intensity of bands. Purification of samples was done by the use of calibrated Ampure XP beads (Beckman Coulter, Indianapolis, IN, USA). Then the purified PCR product was used to prepare DNA library by following Illumina TruSeq DNA library preparation protocol (Illumina, San Diego, CA, USA). Sequencing was carried out at MR DNA (www.mrdnalab.com; Shallowater, TX, USA) on a MiSeq following the manufacturer's guidelines.

Bioinformatics

Sequence data were processed using the MR DNA analysis pipeline. Briefly, paired reads were joined and trimmed of barcodes. Sequences <150 bp and those with ambiguous base calls were removed. Sequences were denoised, operational taxonomic units (OTUs) generated and chimeras removed. OTUs were defined by clustering at 3% divergence (97% similarity). Final OTUs were taxonomically classified using BLASTn against a curated database derived from GreenGenes, RDPII and NCBI (www.ncbi.nlm.nih.gov, DeSantis *et al.*, 2006, http://rdp.cme.msu.edu). Data were processed using MR DNA software.

Statistical analysis

Bacterial phyla, genera, and species were used to statistically analyse and graph data. Data are presented in a series of graphs as mean ± standard error of the mean. The effects of acute exercise vs. control and the effect of training data were analysed using a 2-way ANOVA for repeated measures via Prism 6 (GraphPad, Inc., La Jolla, CA, USA). The null hypothesis was rejected when $P<0.05$. Shannon diversity index was calculated in each horse in each group using R software version 3.2.3 (wooden Christmas tree) with Vegan package (Oksanen *et al.*, 2016) and Mass package (Venables and Ripley, 2002). Then, the Shannon index values were analysed and plotted using repeated measured 2-way ANOVA in Prism 6. Principle Coordinate Analysis (PCoA) was used to show distance and richness between groups before and after GXT1 (or SC1) and GXT2 (or SC2). Average of the individuals in each group was used to plot PCoA using R software version 3.2.3 (wooden Christmas Tree) with Vegan package. PDF based images generated from R software were converted to Tiff type images using GIMP software version 2.8.16 (The GIMP team, 1997-2014). PCoA data were analysed using 2-way ANOVA for repeated measures that was performed on the group scores using Prism 6. To explain the decrease in the Shannon diversity index of the species level in the post-SC sampling of the GXT1 and in order to check which species were affected in the SC group, we identified shared and unique species in the Pre-and Post-SC sampling of the GXT1 using Excel (Microsoft, Redmond, WA, USA), Prism 6, and online Venny version 2.1 (http://bioinfogp.cnb.csic.es/tools/venny) to plot the species in Venn diagram. Moreover; we checked the differences in the relative abundance of each species alone between pre-and post-SC sampling in the pre-training standing control trial (SC1). We have calculated these differences using T *test* in Excel and plotted using Prism 6.

3. Results

Data collected before and after the GXT and SC are presented below to document the effects of acute exercise and the effects of 12 weeks of moderate exercise training. To that end, 12 weeks of exercise training resulted in an increase in maximal aerobic capacity (VO_{2max}) from 148±6 ml/kg/min before training to 162±3 ml/kg/min after training.

Relative abundance

Interestingly, the relative abundance of the genus *Clostridium* decreased ($P=0.03$) in the horses during the standing control (SC1) trial conducted before training (Figure 2A). There were no observed changes ($P>0.05$) in the genus *Clostridium* following either GXT1 or GXT2; moreover; there were no changes in the genus *Clostridium* when the horses stood for the parallel control following the 12 weeks of training (Figure 2A). It was also observed that there were no changes ($P>0.05$) in the abundance of the genus *Dysgonomonas* when comparisons were made using samples collected before and after acute exercise or parallel control for both the pre-training (GXT1/SC1) and post-training (GXT2 and SC2) sets of tests (Figure 2B). At the genus level, exercise training had no effect ($P>0.05$)

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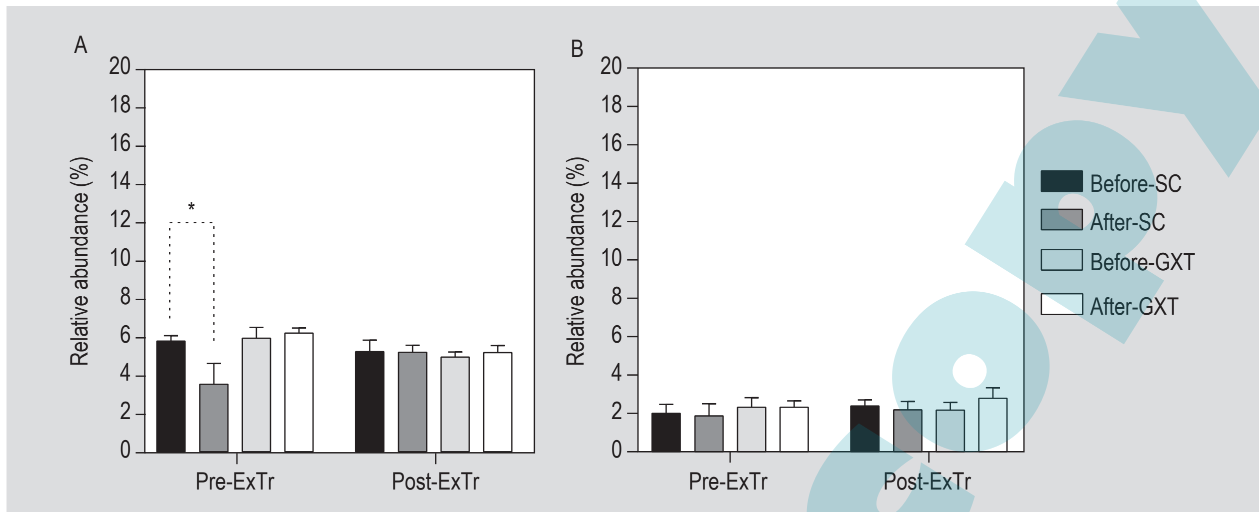

Figure 2. Acute exercise and exercise training effects at the genus level. Values indicate mean ± standard error. (A) Genus *Clostridium* level changes. *Clostridium* level was significantly decreased following SC1 (*P*=0.027). (B) Genus *Dysgonomonas* level changes. No changes in all (*P*>0.05).

on the response to the acute exercise as far as the relative abundance of *Clostridium* and *Dysgonomonas* (Figure 2A and B).

There were no differences (*P*>0.05) in the relative abundance of *Proteobacteria, Bacteroidetes, Spirochaetes, Firmicutes*, and *Fibrobacteres*, when comparisons were made using samples collected before and after acute exercise or parallel control for both the pre-training (GXT1 and SC1) and post-training (GXT2 and SC2) sets of tests (Figure 3 and 4). However, the relative abundance of *Proteobacteria*, in samples collected following the standing control trials (after-SC1 and after-SC2) were significantly differenced (*P*=0.01), suggesting an effect of 12 weeks of training on the acute response in the horses when they underwent the standing control (Figure 3A). There was no effect of training (*P*>0.05) on other phyla, including *Bacteroidetes*, *Spirochaetes*, *Firmicutes*, and *Fibrobacteres* (Figures 3B, 3C, 4A and 4B, respectively).

At the level of species, *Fusicatenibacter saccharivorans* (*P*=0.031) and *Treponema zioleckii* (*P*<0.05) were among 56 bacterial species that were significantly decreased in the pre-training standing control (SC1) (See Figure 9). There were no alterations (*P*>0.05) in these species following GXT1, GXT2, or SC2 (data not shown). Interestingly, *F. saccharivorans* and *T. zioleckii* showed higher relative abundances (*P*=0.04) and (*P*=0.02) respectively in the following the post training standing control (SC2) when compared to the samples collected following the pre-training standing control trial (SC1).

Shannon diversity index

There was a significant decrease in the species diversity in the following the pre-training standard control trial (SC1) with the index dropping (*P*=0.02) from 4.042 in the samples collected before the standing control (SC1) to 3.584 in the samples after the pre-training standing control (Figure 5). There was no change (*P*>0.05) in the Shannon diversity index during the post-training standing control (SC2) (Figure 5). When the samples collected at the end of SC1 and SC2 were compared, the post-SC2 samples exhibited more bacterial diversity at the species level (*P*=0.004) when compared to the same time point in SC1 (Figure 5). For the GXT group, we observed no changes (*P*>0.05) in the diversity (Shannon diversity index) at the phylum, genus, or species level for either the pre-training or post-training GXT's (data not shown).

Principle coordinate analysis

At the bacterial genus level, there was a significant (*P*=0.02) separation observed when one compared samples collected before and after the pre-training standing control trial (SC1) (Figure 6A). The data regarding changes at bacterial species level, revealed a significant separation (*P*<0.02) when one compared samples collected before and after the pre-training standing control trial (SC1) (Figure 7A). No other changes (*P*>0.05) occurred following GXT1, GXT2, or SC2 when data analysed at bacteria genus or species level (Figure 6 and 7, respectively).

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Figure 3. Acute exercise and exercise training effects at the phylum level. (A) *Proteobacteria* – there was only significant effect (*P*=0.005) of exercise training on the response to the post-training acute exercise. (B) *Bacteroidetes*. (C) *Spirochaetes*. Values indicate mean ± standard error – No changes in all (*P*>0.05).

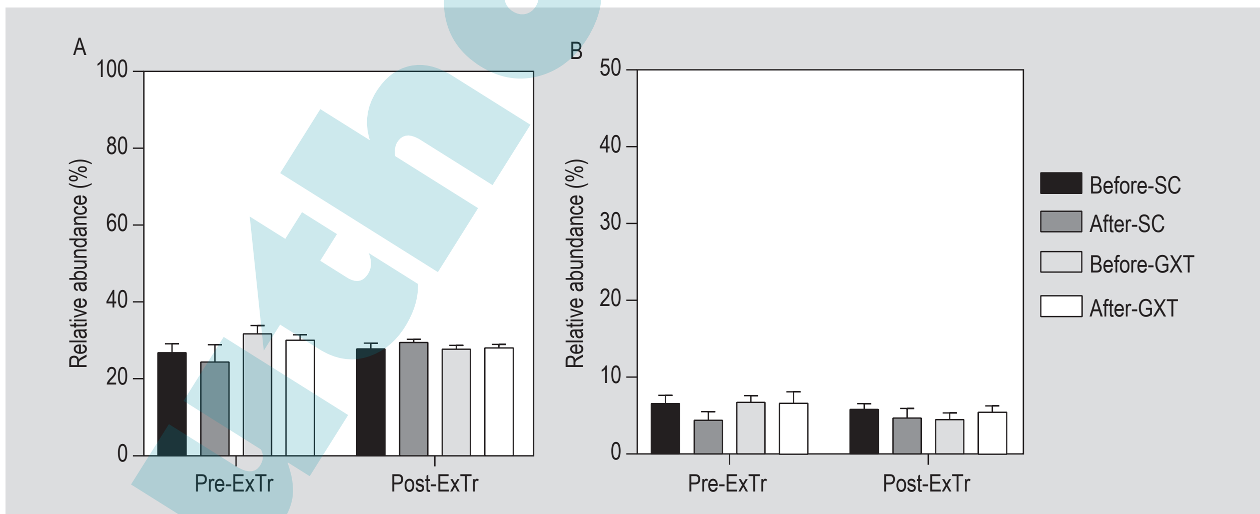

Figure 4. Acute exercise and exercise training effects at the phylum level. (A) *Firmicutes*. (B) *Fibrobacteres*. Values indicate mean ± standard error. No changes in all (*P*>0.05).

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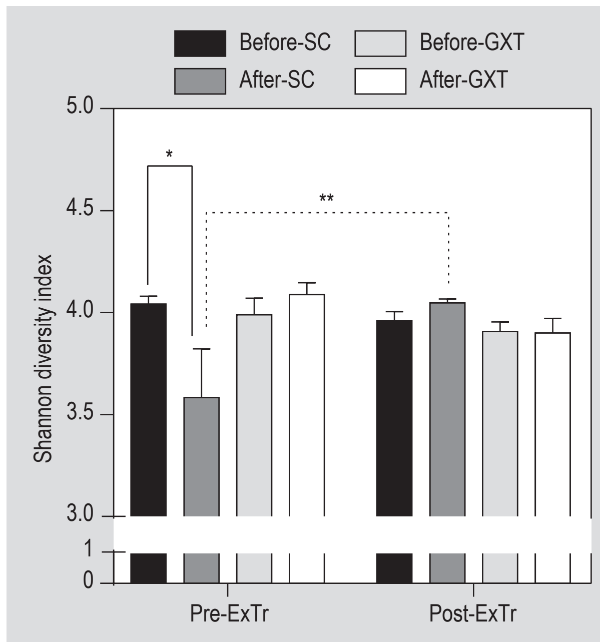

Figure 5. Acute exercise and exercise training effects on Shannon diversity index at the species level. Values indicate mean ± standard error. * Species diversity index significantly changed following SC1 (*P*=0.02). ** Significant effect (*P*=0.004) of the exercise training on the response to the post-training acute exercise.

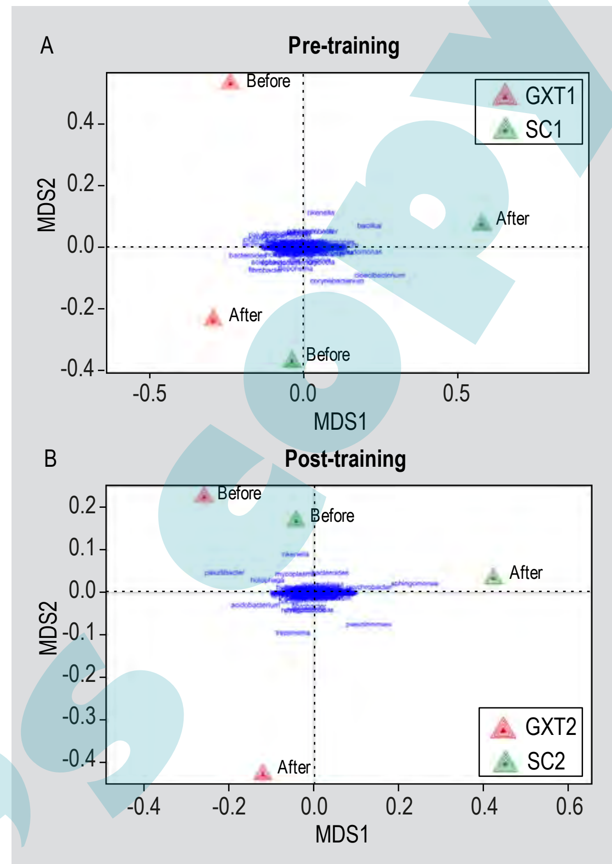

Figure 6. Principle Coordinate Analysis (PCoA) of the incremental exercise test (GXT) and standing control (SC) groups at the genus level. (A) Pre-training. (B) Post-training. PCoA of the pre-training showed a significant separation between before and after-SC1 (*P*=0.02).

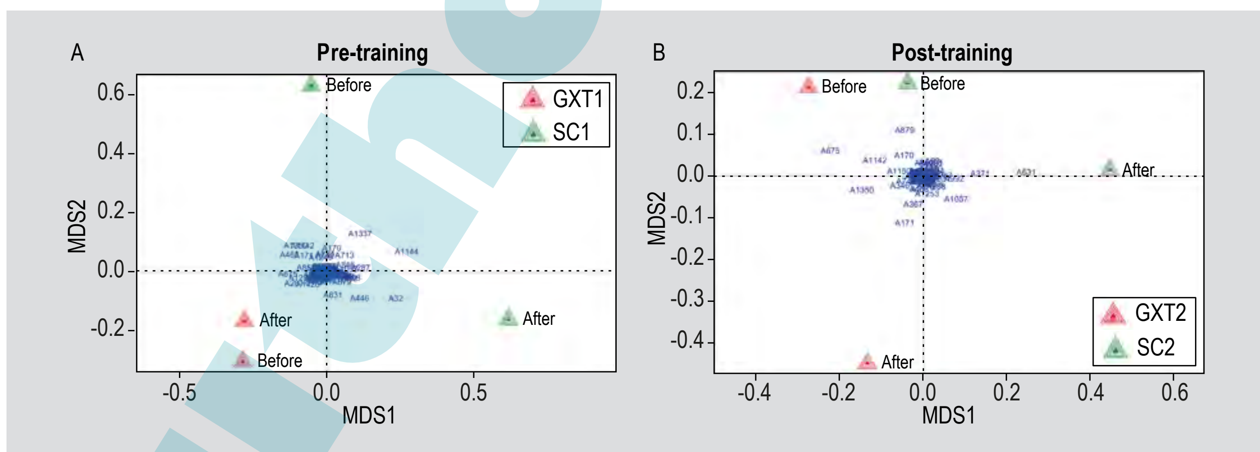

Figure 7. Principle Coordinate Analysis (PCoA) of the incremental exercise test (GXT) and standing control (SC) groups at the species level. (A) Pre-training. (B) Post-training. PCoA of the pre-training showed a significant separation between before and after-SC1 (*P*=0.02).

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Shared and unique species in the standard control group

There was a reduction in the total number of species from samples collected prior to the first standing control (SC1) compared with samples collected after (573 species vs 551 species; Figure 8). Following SC1 there was also a reduction in unique species (75 unique species in before vs 53 unique species after; Figure 8). The names of these unique species are not shown. Additionally, there were significant decreases in the relative abundances of 56 species after the SC horses stood quietly in the stalls within the treadmill laboratory (Figure 9). *P*-values of these changes are shown in Table 2.

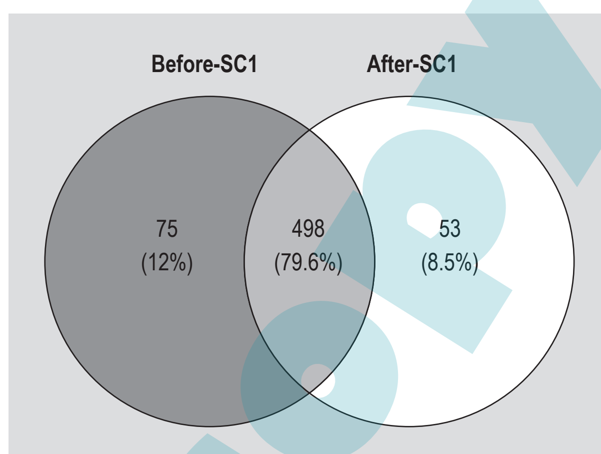

Figure 8. Species sharing in the standing control group of the pre-training. The figure shows the shared and not shared species in the samples collected before and after SC1.

Table 2. *P*-values corresponding to Figure 9 of the species changes following the pre-training standing control trial (SC1).

Species	Before SC[1]	After SC[1]	*P*-value	Species	Before SC[1]	After SC[1]	*P*-value
Adlercreutzia spp.	+		0.050	*Hallella* spp.	+		0.010
Anaerovorax odorimutans	+	+	0.032	*Hespellia porcina*	+		0.040
Bacteroides coprocola	+	+	0.025	*Lachnospira pectinoschiza*	+	+	0.006
Bacteroides luti	+		0.043	*Magnetococcus* spp.	+		0.044
Bacteroides nordii	+		0.038	*Mogibacterium diversum*	+	+	0.001
Bacteroides oleiciplenus		+	0.037	*Mucinivorans rikenellaceae* bacterium	+		0.030
Bacteroides plebeius	+	+	0.040	*Natranaerovirga pectinivora*	+	+	0.039
Bacteroides stercorirosoris	+	+	0.037	*Nodosilinea bijugata*	+	+	0.001
Brevundimonas vesicularis		+	0.049	*Opitutus* spp.	+	+	0.048
Butyrivibrio spp.	+		0.036	*Ornatilinea apprima*	+	+	0.023
Cellulosilyticum spp.	+	+	0.029	*Paludibacter* sp.	+	+	0.007
Cloacibacterium normanense	+	+	0.023	*Paracoccus aminovorans*		+	0.035
Cloacibacterium sp.	+		0.043	*Parasutterella secunda*	+	+	0.047
Clostridium citroniae	+		0.054	*Pelospora* spp.	+	+	0.008
Clostridium fusiformis	+	+	0.038	*Prevotella melaninogenica*	+		0.042
Clostridium hylemonae	+	+	0.006	*Psychrobacillus psychrodurans*	+		0.016
Clostridium propionicum	+		0.046	*Rhodobacter* sp.	+		0.019
Collinsella intestinalis	+		0.027	*Rubellimicrobium* spp.		+	0.029
Dehalobacterium spp.	+	+	0.016	*Sphingobacterium multivorum*	+	+	0.030
Desulfosporosinus meridiei	+	+	0.015	*Spiroplasma culicicola*	+	+	0.019
Desulfuromonas spp.	+	+	0.036	*Spiroplasma velocicrescens*	+	+	0.021
Eggerthella lenta	+	+	0.018	*Sporocytophaga* sp.	+		0.008
Erysipelothrix sp.	+	+	0.036	*Syntrophococcus* sp.	+		0.035
Eubacterium rangiferina	+		0.033	*Tepidimonas* spp.	+		0.019
Fusicatenibacter saccharivorans	+	+	0.031	*Treponema parvum*	+	+	0.037
Garciella nitratireducens	+		0.046	*Treponema saccharophilum*	+		0.041
Geofilum rikenellaceae bacterium	+	+	0.038	*Treponema succinifaciens*	+	+	0.037
Gordonibacter pamelaeae	+		0.047	*Treponema zioleckii*	+	+	0.048

[1] The empty cells mean that the corresponding species relative abundance is zero in all horses. A + mean the bacterial species was present.

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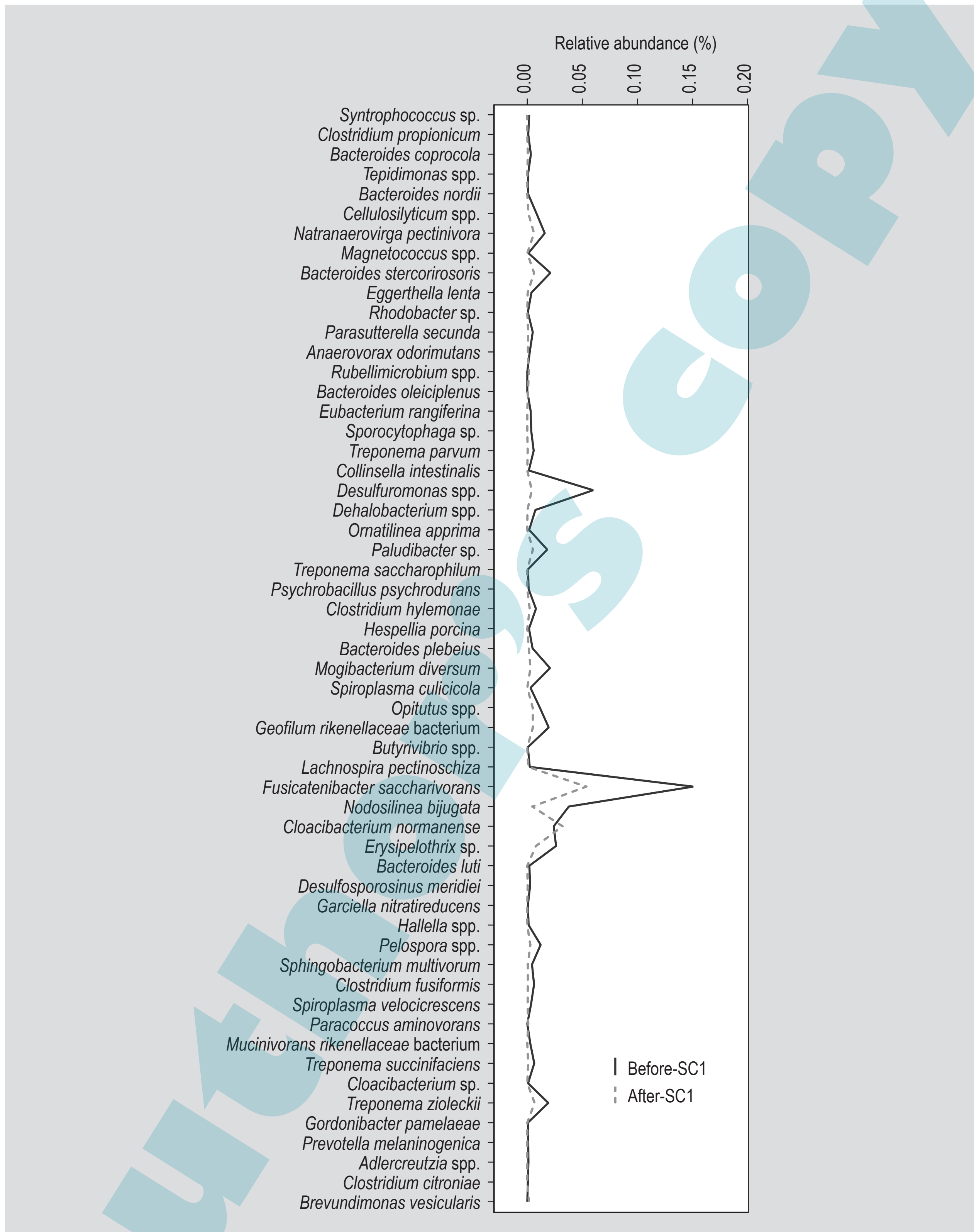

Figure 9. Species level changes in the standing control group of the pre-training. The abundance levels of the species in the figure showed a significant decrease in the standing control (SC) horses (*P*<0.05).

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4. Discussion

The central hypotheses of the present study were that intense acute exercise would induce a change in the FM of the horse and secondarily that weeks of exercise training would alter said acute response. The rationale for this hypothesis was based on the well-recognised responses to intense exercise that alter the internal milieu of the GIT. These short term responses to intense exertion include dramatic changes in autonomic tone and increases in sympathetic drive that alter motility as well as the well-documented in a decrease in blood flow to the splanchnic region that allows an increase in blood flow to the skin, muscles, and other vital organs in horses (McKeever and Lehnhard, 2014; Rowell, 1983). This temporary decrease in blood flow to the mesenteric region leads to a temporary decease in oxygenation to the intestinal mucosa and an increase in the abundance of the anaerobic bacterial genus *Clostridium* (Smith-Slatas *et al.*, 2006). In the present study we saw no decrease in the genus *Clostridum* after the pre-training and post-training GXT's. Other studies have reported changes in the FM after weeks of submaximal endurance exercise training, including changes in *Bacteroidetes* and *Proteobacteria* in mice (Evans *et al.*, 2014), changes in *Bacteroidetes* and *Firmicutes* in the wheel running juvenile rats (Mika *et al.*, 2015) and changes in *Firmicutes* and *Proteobacteria* in rats (Liu *et al.*, 2015). None of the studies reported in the literature examined acute intense exercise as performed in the present experiment. The lack of an effect of very intense exercise on the FM in the present study has practical significance as it eliminates a concern for those caring for athletic horses where intense exercise is routinely performed during a racing career.

Another finding of the present study was that after ~5 min of standing control during the pre-training GXT1, there was a decrease in the abundance of the genus *Clostridium.* We do not have data to explain this change but it could be related to anticipatory effects of the horses wanting to run on the treadmill. However, we should emphasise that in the present study the horses had undergone extensive habituation to the laboratory and the holding stalls for months prior to the experiment. Furthermore, we observed that the horses were relatively calm when undergoing the parallel control trials. Previously published studies from our lab have featured parallel standing controls similar to the present study and have demonstrated that there were no changes in cortisol, heart rate, and blood pressure in the standing control horses (Gordon *et al.*, 2007). Studies of rodents subjected to non-exercise stressors have documented that major changes in the hypothalamic pituitary adrenal axis and the autonomic nervous system lead to increases in the circulating cortisol and catecholamines (Carabotti *et al.*, 2015), and the authors speculated that those molecules reacted with the FM leading to changes in the bacterial profile including substantial increases in detrimental *Clostridial* species (Evans *et al.*, 1948; Freestone, 2013). Thus, the observed decrease in the abundance of the genus *Clostridium* would, if anything, suggests that the horses of the present study were not undergoing stress as defined in the rodent studies cited above. Interestingly, there was no decrease in the abundance of genus *Clostridium* during the post-training standing control (SC2).

An unexpected finding of the present study was that there were decreases in the species diversity following the pre-training standing control (SC1), but not the post-training trial (SC2). This decrease may have been due to the changes that occurred in levels of 56 bacterial species explained in the next section. The observation that no change in Shannon diversity index occurred in the standing control horses after training suggests that training may impart a protective effect on the system. There were no changes occurring in the diversity (Shannon diversity index) at phylum, genus, or species level following short-term acute intense exertion. We cannot comment on what would occur during more prolonged submaximal exercise exposure where significant fluid and electrolyte losses could cause a change in the FM for horses (Evans *et al.*, 2014; Liu *et al.*, 2015; Mika *et al.*, 2015).

An surprising finding of the present study was the observation that there were minor reductions in the number of unique species when the horses underwent the pre-training standing control where they stood quietly while the other two horses ran on the treadmill. We have no explanation for this change as the horses had been taught for months to stand quietly in the treadmill lab while other horses were running on the treadmill. The decrease in the total number of species may be a serendipitous finding or it could indicate that a structural change in the FM system. However, the changes observed in the present study were not chronic as they were not observed following training. This may suggest that the changes observed in SC1 were only an unexplained transient phenomenon that had no lasting physiological effect on the host. We should caution that this is speculation as we only examined the response to acute intense exercise before and after the horses underwent the 12 weeks of exercise conditioning.

5. Conclusions

In summary, acute intense exertion does not appear to alter the faecal microbiome of the horse. Moreover; chronic exercise training does not appear to produce a change in this acute response to exercise. There are many supplements being sold to 'treat' the microbiome of the horse; therefore, on a practical level, the present study provides information benefiting those seeking ways to improve the health and well-being of the athletic horse.

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