EFFECTS OF ACUTE EXERCISE AND EXERCISE TRAINING ON THE GUT MICROBIOME OF THE STANDARDBRED RACEHORSES

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ABSTRACT OF THE DISSERTATION

Effects of Acute Exercise and Exercise Training on the Gut Microbiome of the Standardbred Racehorses

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The digestive systems of humans and animals contain an extraordinary diversity of microbes. These microbes constitute a functional organ contributing to physiological and health processes of the host. While many of these associations are hypothesized, correlations have been made between host health and specific bacterial groups, and the details of these associations are being revealed. Members of the phylum *Bacteroidetes* have been correlated with the development of early infant immunity, and members of the phylum *Firmicutes* have been shown to provide future protection against pathogenic bacteria. Exercise has been found to impact bacterial communities in the digestive tracts of human and laboratory animals leading to increasing levels of *Bacteroidetes* and decreasing levels of *Firmicutes*. Moreover, exercise related changes in the gut microbiome (GM) were found to either provide protection against certain

illness conditions or worsen the symptoms of other conditions. Moreover, some bacterial supplementations were found to increase performance in rodent models. Here, our studies tested the hypotheses that acute exercise and exercise training would alter the GM in Standardbred racehorses.

To identify the bacterial communities of the GM a good choice of DNA extraction protocol should be elected to ensure getting the right results. Therefore, we modified a phenol/chloroform extraction method plus insertion of an inhibitor removal solution. The results showed that our modifications generated clean with high concentrated DNA products as was revealed by illumine Miseq.

Next, we tested the hypothesis that acute intense exercise would alter the GM in horses. Eight horses were used to run on treadmill or serving as standing control. Rectal fecal samples were taken 24 hours 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 the pre-training standing control trial (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 the relative abundance of the genus *Clostridium*, *Fusicatenibacter saccharivorans*, and *Treponema zioleckii* might particularly be responsive to anticipatory effects associated with watching and hearing exercise.

Then, we examined the hypothesis that exercise training may alter the GM in Standardbred Racehorse. In this study, 8 horses (4 mares, 4 geldings) were exercise trained for 12 weeks, and 4 additional mares were used as a parallel seasonal control. To identify bacterial community changes over time for both groups, rectal fecal samples were collected, DNA was extracted, and the 16S rRNA gene (V3-V4) was sequenced using the IIllumina Miseq platform. The exercise training group showed significant changes in the levels of *Bacteroidetes*, *Proteobacteria*, and *Spirochaetes* phyla (P<0.05), while there were no changes in the gut microbiota of the seasonal control group through the three months of the study (P>0.05). Moreover, with training two genera significantly changed in their relative abundance over time, namely *Clostridium* and *Dysgonomonas* (P<0.05). *Dysgonomonas* spp was significantly changed in abundance during the exercise training period (P<0.05). *Treponema* spp. showed significant changes during the exercise training period (P<0.05). Shannon diversity index was decreased (P<0.05), in the exercise group at the beginning of the study, but then returned to pre-training levels. Principle coordinate analysis showed significant separation between time points of the exercise training group as far as the levels of genera and species (P<0.05) represented. Our results show that exercise training influences the gut microbiota especially at the beginning of training.

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INTRODUCTION

The gastrointestinal tracts (guts) of humans and other animals are characterized by diverse microbial communities made up of bacteria, protozoa, and fungi that promote complex functions and interactions (Mackie et. al., 1999). The number of bacteria inside the human colon has been estimated at 10^{11} cell/g (Mackie et. al., 1999; Hsiao et. al., 2008) and ~$12.3X10^{8}$ colony forming units/g in horse colon (Berg, 1996; Mackie and Wilkins, 1988). The equine hindgut is the primary site of bacterial breakdown and fermentation of plant material. Bacterially derived fermentation products such butyrate, acetate, and propionate provide as much as 65% of a horse's energy (Al Jassim and Andrews, 2009). There are multiple ways the GM benefits the host including metabolism of recalcitrant substrates, production of vitamins, and the biotransformation of toxins. For example, polychlorinated biphenyls (PCBs) related changes in gut microbiome of rodent were attenuated by voluntary exercise performed by these animals (Cerdá et al., 2016; Al Jassim and Andrews, 2009; Choi et al., 2013).

One of the factors that can influence the GM is exercise training which has been found to change the composition of microbial communities in the GIT of humans, mice, and rats (Santacruz et al., 2009, Potera, 2013, Queipo-Ortuño et al., 2013, Evans et al., 2014). Another factor that can influence the GM 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) or social disruption (SDR) (Bailey et al., 2011) has been shown to cause significant changes in specific members of the GM community. High fat diets can also alter the GM in ways that are similar to feeding stress (Evans et al., 2014). Furthermore, exercise training appears to induce beneficial GM 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 GM (Evans et al., 2014; Mika et al., 2015; Liu 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; Mika et al., 2015; Liu et al., 2015) respectively.

CHAPTER 1: Journal Review Article (Submitted for review)

REVIEW OF LITERATURE

Gut Microbiome: Importance and Effecters

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Abstract

The digestive systems of humans and animals contain an extraordinary diversity of microbes. These microbes constitute a functional organ contributing to physiological and health processes of the host. While many of these associations are hypothesized, correlations have been made between host health and specific bacterial groups, and the details of these associations are being revealed. Members of the phylum Bacteroidetes have been correlated with the development of early infant immunity, and members of the phylum Firmicutes have been shown to provide future protection against pathogenic bacteria. Exercise has been found to impact bacterial communities in the digestive tracts of human and laboratory animals leading to increasing levels of Bacteroidetes and decreasing levels of Firmicutes. Moreover, exercise related changes in the gut microbiome were found to either provide protection against certain illness conditions or worsen the symptoms of other conditions. In this review, we will focus on what is known about the gut microbiota (GM) and exercise in human and mouse model studies and how the gut microbiome may impact athletic performance in humans and horses.

Introduction

The gastrointestinal tracts (guts) of humans and other animals are characterized by diverse microbial communities made up of bacteria, protozoa, and fungi that promote complex functions and interactions (Mackie et. al., 1999).

The number of bacteria inside the human colon has been estimated at 10^{11} cell/g (Mackie et. al., 1999; Hsiao et. al., 2008) and ~$12.3X10^{8}$ colony forming units/g in horse colon (Berg, 1996; Mackie and Wilkins, 1988). The equine hindgut is the primary site of bacterial breakdown and fermentation of plant material. Bacterially derived fermentation products such butyrate, acetate, and propionate provide as much as 65% of a horse's energy (Al Jassim and Andrews, 2009). Next generation sequencing techniques utilizing platforms such as Illumina and Ion torrent have become standard methods for cataloging the composition of the GM (Koboldt et al., 2013).

There are multiple ways the GM benefits the host including metabolism of recalcitrant substrates, production of vitamins, and the biotransformation of toxins. For example, polychlorinated biphenyls (PCBs) related changes in gut microbiome of rodent were attenuated by voluntary exercise performed by these animals. These beneficial effects attributable to the GM have been reviewed elsewhere (Cerdá et al., 2016; Al Jassim and Andrews, 2009; Choi et al., 2013). In the present article we review current thinking about the interactions between the GM and immunity and GM and exercise and the downstream effects of these interactions in human and mouse model studies, and how these interactions could play a role in the performance of the equine athletes.

What happens when the microbiome is altered by the challenge of acute and chronic exercise? Are there protective effects associated with exercise? Do the long-term challenges of exercise training disrupt the host-microbiome relationship?

Importance of the GM in Immune System Development

Immunity is the first line of importance when discus the effects of exercise related changes in GM on hosts. Although and according to our knowledge there are no studies of the effects of acute exercise or exercise training on the GM in horses, it is important to review the literature in details to increase our understanding of GM relationship to immunity in different animal and human models. Based on this built up knowledge, links and interactions between exercise and immunity via the changes in the GM could be studied to enhance the health, nutrition, and performance of athletic horses.

Neonates obtain their first GM from their mothers during the delivery process via the microbiome of the birth canal (Orrhage and Nord, 1999). Years ago, researchers started to give their attention to the GM and its advantageous effects in the development of host immunity. Cebra (1999) compared conventionalized to germ-free mice to examine the positive impact of the microbiota has in boosting the immune system of the host. That study colonized Morganella morganii, a gut commensal bacterium, into the germ-free mice and the researchers found that after an initial high density dissemination of this bacterium to the spleen and lymph nodes, growth was restricted by the developing immune response, and subsequently cleared to low numbers only in the intestinal mucosa. That study also found that administration of segmented filamentous bacteria, commensal bacteria in the mouse gut, to germ-free mice,

resulted in a boost to murine resistance against a lethal oral dose of *Listeria monocytogenes*. Later studies confirmed that oral administration of small doses of dietary antigens activated the gut mucosal non-injurious immune response, stimulating the production of T-helper3 (Th3) cells in the Peyer's patches and thus modulating subsequent immune events in the surrounding areas of the gut mucosa between symbiotic microbiota and pathological antigens (Rautava and Isolauri, 2002). Some species of *Bifidobacteria*, commensal bacteria in the gut, have the ability to boost the immune response by activation of the anti-inflammatory processes via increased levels of IL-10 produced by dendritic cells (DNCs) (Hart et al., 2004; Lammers et al., 2003). The increase in IL-10 decreased the levels of co-stimulatory CD80, along with the levels of interferon- γ (Hart et al., 2004; Lammers et al., 2003). Moreover; Young et al., (2004) supplied a variety of strains of these bacteria to dendritic cells originating from cord blood to quantify immune cell marker expression and found that Bifidobacterium bifidum, B. longum, and B. pseudocatenulatum elevated the levels of CD83 and activated the levels of IL-10 generated by these cells. They also observed that the concurrent administration of B. infantis did not induce such effects, an observation that suggests that these species play a role in the development of the immune system in infants (Young et al., 2004). Rakoff-Nahoum et al. (2004) discovered that gut epithelial homeostasis in mice was promoted by the unique recognition of the GM by Toll-like receptors (TLRs). TLRs are mammalian receptors that recognize lipopolysaccharide (LPS) and lipoteichoic acid (LTA) of microbial species, which shows the role of the GM in the later development of

immune system in the gut and its ability to protect against future pathological infections as well as the ability to stimulate injury-based healing effects. The authors suggested that this protection is generated by bacterial commensal activation of TLR signaling via the MyD88 component and they concluded that the presence of these two elements is important to protect against injurious events to the intestinal epithelia.

Commensal bacteria of the intestine also have a close relationship with a variety of host pathways that generate local epithelial immune defenses against future injurious incidents. For example, gut wound proliferation healing events of colonic epithelial progenitors (ColEPs) have been found to be disturbed in commensal germ free (GF) and Myd88 knockout mice (Pull et al., 2005). The authors found that the GF and Myd88 deficient mice had limited numbers of ColEPs around ulcers induced by dextran sodium sulfate (DSS) treatment with a concurrent decrease in the presence of macrophages in the crypt. DNCs, IL-10, and TLR have been found to be activated due to Bifidobacteria abundance increasing upon oral administration of fructo-oligosaccharides (FOS) to patients with Crohn's disease (Lindsay et al., 2006). The mouse gut mucosal lymphoid tissue represented by the solitary intestinal lymphoid tissue (SILT) is activated when GF mice colonized with commensal bacteria resulting in more increase in immunity via the chemokine receptor CCR7 (Pabst et al., 2006). Nutten and co-workers (2007) tested the effects of amoxicillin administration on rat pups observing that antibiotic administration resulted in changes in the GM, gut mucosal mast cell number, and caused increases in mast cell protease

production. The authors thus suggested that early life administration of antibiotics has detrimental effects on immunity and some hormonal induction. Regenerating islet-derived protein 3 (Reg3), an ant- infection factor in Peyer's Patches, has been found to be linked and activated by the TLR-2 signaling pathway (Dessein et al., 2009). In addition, they found that Reg3 was important factor in the clearance of local bacteria found in Peyer's Patches. Moreover; they also demonstrated that giving TLR-2 agonists helped to restore the protective action of Reg3 against certain pathogens (Dessein et al., 2009). The microbiota may also alter T-cell function and Galdeano et al., (2009) detailed the observation that a nuclear factor of activated T-cells (NFAT) is turned on by the enzyme calcineurine. Calcineurine works upon the microbiota by contacting the gut defense system cells which leads to the activation of more T helper (Th) cells.

There are important links between the microbiome and inflammation, both in the gut and systemically (Maslowski et al., 2009). Alterations in the microbiota can alter the normal inflammatory response causing it to be prolonged via the activation of inflammatory signaling pathways (Maslowski et al., 2009). For example, Maslowski and co-workers (2009) found that upon contacting dietary-microbial produced short-chain fatty acids (SCFAs) to G-protein-coupled receptor 43 (GPR43) at the gut epithelial cells activates inflammatory responses that is disrupted in GF or GPR43 deficient mice . Mechanistically, the disruption of the above mentioned inflammatory response has been linked to deactivation of Th-17 cell differentiation (Cha et al., 2010). The deactivation of Th-17 cell differentiation occurred when the microbial community in the gut of mice was

altered by deprivation of retinoic acid, a metabolite of vitamin A (Cha et al., 2010).The polysaccharide A (PSA) of Bacteroides fragilis, a human gut commensal bacterium, has been found to activate CD4(+) T cells into Foxp3(+) regulatory T cells which leads to the production of IL-10 and maintain gut epithelial lineage homeostasis and treatment of colitis in animals (Round and Mazmanian, 2010). Heimesaat et al., (2010) have shown using TLR9-/- mice that TLR9 activation through alternation of GM increases the chances of undesirable inflammatory responses and cell death. In Nod1-/- mouse study by Clarke et al. (2010), mice were more susceptible to infection; moreover; wild mice diminished from microbiota had more resistance to infection when they were given Nod1 ligands. This suggests that microbiota and Nod1 are important for the development of early life gut immunity. B2 cell development and blood Igs have been found to be boosted upon exposure to microbiota in the gut of mice which gives evidence that microbiota enhances humoral immune system development (Hansson et al., 2011). TLR5-/- mice have revealed disruption in the gut mucosal immune homeostasis which suggests an important immune signaling pathway used by microbiota to develop the infant immunity (Carvalho et al., 2012). Commensal microbiota induces the activation of GPR15 which controls Tregs subsequent events; in addition; this process has been detected to boost the gut mucosal immunological homeostasis (Kim et al., 2013).

The microbes in the gastrointestinal tract also appear to have beneficial effects. For example, studies have documented that supplementation with probiotics and prebiotics have a beneficial effects on the immunity of the

intestinal niches including the activation of a variety of host pathways In another study, it was demonstrated that treatment with probiotics resulted in an enhancement of the immune response and an elevation of IgA- in the gut mucosa of unimmunized chickens (Haghighi et al., 2006). Studies of humans have shown that administration of Lactobacillus gasseri CECT5714 and Lactobacillus coryniformis CECT5711, both lactic acid-probiotic bacteria, results in a boost to macrophage phagocytic activity in healthy volunteers exposed to food nutrient deprivation (Olivares et al., 2006). Another beneficial effect of the microbiome is related to the modulation of autoimmune and allergic related disorders. For example, allergic conditions such as atopic-IgE associated allergy and eczema, have been found to be reduced in infants treated with probiotics (Kukkonen et al., 2007). The beneficial link between the GM and the immune system was demonstrated by Fink and co-workers (2007) who showed that the GM activates natural killer cells (NK), gamma interferon (IFN-γ), and Th-1 cells. Zeuthen and co-authors (2008) used DNCs from mice to test the immune-activation properties of lactic acid bacteria (LAB). As a result they recognized this process to be linked to TLR-2 receptors and mechanistically an association with nucleotide-binding oligomerization domain-2 (NOD2). This is a domain of the intracellular like receptor family that detects the muramyl dipeptide (MDP) of Gram-positive and peptidoglycan (PGN) of Gram-negative. Their results suggest that LAB play an important role in boosting host immunity against pathogens (Zeuthen et al., 2008). Guilmeau and coworkers, (2008) reported that Protein O-fucosyltransferase 1 (Pofut1), a ligand signaling pathway component, is

suppressed in gut mucosal epithelial cells of mice following alternations of the GM during enterocolitis. This was interpreted to indicate that Pofut1 is important for maintenance of gut homeostasis so as to insure and evidence that the presence of normal commensal bacteria is important for maintaining strong immunity (Guilmeau etal., 2008). The administration of probiotics were shown by Gori et al., (2011) to enhance gut commensal composition which in turn strengthened NK cell and T-cell activities in HIV patients. Finally, wound healing may be enhanced by the beneficial microbes found in the GM (Poutahidis et al., 2013). For example, lactic acid bacteria in the microbiota have been linked to activation of the hormone oxytocin-T cell regulatory pathway and this has been found to increase wound healing and decrease its time (Poutahidis et al., 2013). One can certainly summarize that the microbiota provides beneficial effects to the host immune system especially when it comes to gastrointestinal mucosal immunity and that this enhanced ability aids in the ability to prevent a variety of diseases, Figure 1. The above changes in the GM and the interaction between GM and the host immunity may show the same effects in horses and could be further studied to understand and enhance better health, immunity, and performance of racehorses. The question of importance to this review is what happens when the microbiome is altered by the challenge of acute and chronic exercise? Are there protective effects associated with exercise? Do the long term challenges of exercise training disrupt the host-microbiome relationship? The next sections of this review will address some of the current findings

regarding the horse gut microbiome followed by a review of the effects of exercise training on the microbiome in rodents and humans.

Horse Microbiome Surveys

The equine hindgut is the primary site of bacterial breakdown and fermentation of plant material. Bacterially derived fermentation products such butyrate, acetate, and propionate provide as much as 65% of a horse's energy (Al Jassim and Andrews, 2009). Total bacteria numbers in the equine cecum are estimated to be $1X10^{11}$ CFU/g using culture or quantitative real-time PCR methods respectively (Costa and Weese, 2012). Since many of these bacteria evade cultivation, it is hard to estimate the real numbers of these microorganisms (Staley and Konopka, 1985; Rappé and Giovannoni, 2003), however, the use of molecular techniques such as next generation sequencing (NGS) have enabled the detection of rare and novel bacterial taxa.

Shepherd et al., (2012) found that the relative abundances of Firmicutes, Proteobacteria, and Bacteroidetes were 43.7%, 3.8%, and 3.7% respectively, while Costa et al., (2012) showed that the relative abundance of Firmicutes, Proteobacteria, and Bacteroidetes were 68%, 10%, and 14% respectively. They found that phylum Spirochaetes and Clostridia class were more abundant in horses with colitis (Costa et al., 2012). The bacterial community of the horse stomach has been shown to be dominated by Proteobacteria, Bacteroidetes, and Firmicutes, but interestingly, no Helicobacter spp. was detected (Perkins et al.,

2012). Dougal et al., (2013) found that Lactobacillaceae, Lachnospiraceae, or Prevotellaceae groups are abundant in the ileum, proximal large intestine, or distal large intestine respectively. Dougal et al., (2014) also reported no differences in the microbial community profiles when the fecal microbiota of adult horses was compared to elderly horses. In another study, Liu et al., (2014) found that donkey feces to be dominated by Firmicutes, Bacteroidetes, Verrucomicrobia, Euryarchaeota, Spirochaetes, and Proteobacteria.

Costa et al., (2015a) studied horse digestive system bacterial profiles and found that Firmicutes are present in all the compartments of the gastrointestinal tract. They found that the species profile varies with location with the stomach dominated by Lactobacillus spp. and Sarcina spp., Streptococcus spp. dominant in the duodenum, Actinobacillus and Clostridium sensu stricto dominant in the ileum, and '5 genus incertae sedis'/Verrucomicrobia dominant in the colon and feces (Costa et al., 2015a). In another study, Costa et al., (2015b) studied the fecal bacterial profiles of foals from the first day of their lives until the age of 9 months and recognized that newly born foals had high levels of Firmicutes. After 2 days and until 30 days of age, their GM profiles were dominated by Akkermansia spp and they observed that the Fibrobacteres had high levels in the bacterial profiles after the age of weaning (Costa et al., 2015b).

Disruptions of the Horse Microbiome

Changing the diet of horses has been reported to alter the GM and its products (Daly et al., 2012; Moreau et al., 2014; Milinovich et al., 2006; Fernandes et al., 2014). Daly et al., (2012) studied the effects of dietary changes on bacterial communities in horses fed either grass, concentrate, or concentrate with simple colonic obstruction and distension (SCOD) initiated by diet. They identified GM changes that were correlated with diet and disease and reported specifically that there were increases in the levels of Lachnospiraceae, Bacteroidetes, and Lactobacillus-Streptococcus groups. Moreover, there was a decrease in the level of Ruminococcaceae (Daly et al., 2012).

Moreau et al., (2014) showed that the bacterial profile of the cecum suffered alternations after oral carbohydrates infusion. Their study revealed increases in the relative abundances of Lactobacillus sp., Streptococcus sp., Veillonella sp., and Serratia sp. In another paper, Milinovich et al., (2006) reported that bacterial members from the genus Streptococcus were abundant in fecal samples collected after oligofructose administration to horses. Moreover, those horses later developed laminitis (Milinovich et al., (2006). The authors suggest a link between laminitis incident and increasing abundance of this bacterium (Milinovich et al., 2006). A study reported by Fernandes et al., (2014) found that switching from forage-grain diet to pasture feeding in thoroughbred horses caused differences in the microbial communities included alternations in the genera: BF311/Bacteroidaceae, CF231/Paraprevotellaceae, and alterations in the relative abundance of bacteria from the order Clostridiales and family

Lachnospiraceae. Still another paper reported that after 6 weeks of amylase-rich malt extract dietary supplementation to thoroughbred horses (Proudman et al., 2015). In that experiment, Proudman and co-workers (2015) found that fecal abundances of Clostridia decreased and Bacteroidia increased. They also reported changes in the profiles of volatile organic compounds with significant decreases in acetic acid, propanoic acid, butanoic acid, 2-methyl propanoic acid, 3-methyl butanoic acid, and acetone.

Horse Microbiome and the Immune System

Some members of the gut microbiota may play an important role in increasing immune protection against gastrointestinal bacterial infections in horses. For example, Tanabe et al., (2014) administered Lactobacilli and Bifidobacteria, isolated previously from thoroughbred horses, to mouse splenocytes stimulated by IL-6 and growth factor-β. They found that the pro-inflammatory IL-17 production was decreased (Tanabe et al., 2014). Moreover, administration of these same bacterial strains to neonatal thoroughbred horses was later found to dominate fecal bacterial profiles (Tanabe et al., 2014). The authors also found clinically relevant benefits associated with the administration of the bacteria and they reported that the foals were protected from diarrhea with an incidence of 30.7% in the treated group compared to 75.9% in the control group (Tanabe et al., 2014). As in humans and other animal models, bacterial community alteration

may hold promise for enhancing immune protection in horses against specific illness, but the field of targeted probiotics is in its infancy.

Horse Microbiome in Normal and Ill Horses

Microbial communities appear to differ in horses with specific diseases or syndromes when compared to normal horses. Elzinga et al., (2016) found that horses with equine metabolic syndrome (EMS) had a less diverse GM, with greater abundance of subdivision 5 of Verrucomicrobia incertae sedis, while the control horses had higher levels of Fibrobacter. Other researchers have reported that administration of systemic antimicrobial agents to horses affects the GM by decreasing the abundance of bacterial members such as Verrucomicrobia (Costa et al., 2015c). Weese et al., (2015) studied the gut microbial profile of pregnancy related stages in mares. They compared the fecal microbial profiles in samples collected from pre- and post-pregnancy, pre to post-partum colic, and non-pregnant mares. There were no differences in the relative abundances of bacterial phyla when comparisons were made of samples collected before and after parturition and the non-pregnant mares. The samples were dominated by Firmicutes, Verrucomicrobia, Actinobacteria and Proteobacteria. However, pre-parturient mares showed higher levels of Roseburia, Treponema, and Papillibacter when compared to non-pregnant mare samples. Sample bacterial profiles of pre-postpartum colic were higher in Proteobacteria relative abundance when compared to non-colic mares. The authors found that a profile where the

relative abundance of Firmicutes was ≤ 50% and Proteobacteria was>4% preceded colic (Weese et al., 2015). They also revealed that the fecal samples of mares that suffered large colon volvulus were dominated by Lachnospiraceae and Ruminococcaceae (Weese et al., 2015). Destrez et al., (2015) found that alimentary stress caused by high starch feeding to horses increased the relative abundance of anaerobic, lactate-utilizing, and amylolytic bacteria with a concurrent occurrence of stress related behavioral symptoms in these horses (Destrez et al., 2015). The microbiome status in normal and ill horses may help finding important bacterial members that could play a very important role in improving these illnesses.

Horse Microbiome and Probiotics

A promising field in the equine industry is the development and utilization of probiotic supplements that can be used to treat a variety of diseases such as acute enterocolitis, diarrhea, and Salmonella infection or as a way to increase digestibility of various feedstuffs given to horses. Lactobacillus, Enterococcus, Bifidobacterium, and Streptococcus and Saccharomyces are the main microorganism strains used as probiotics in livestock (Coverdale, 2016). Interestingly, Jouany et al., (2008) found that if they supplied horses with Saccharomyces cerevisiae (SC) they could increase cellulose digestibility in the treated horses. Another report by Respondek et al., (2008) showed that supplying horses with short-chain fructooligosaccharides (scFOS) protected the

large intestine from the diet-based bacterial changes seen in control horses where there were increases in the relative abundances of total anaerobes, Lactobacilli, Streptococci, and lactate-utilizing bacteria. A review by Coverdale, (2016) highlighted the observation that feeding horses with supplements containing Lactobacillus acidophilus, Lactobacillus casei, Bifidobacterium bifidum, and Enterococcus faecium combined with a SC fermentation product increased dry matter digestibility in these horses. This may have a practical implication for improving the ability to provide nutrients to the performance horse and in the next sections of the paper will discuss how exercise training may affect various bacterial commensals and how that may increase performance in humans and animal models.

Physiological response to exercise

The physiological response to acute exercise involves the integration of several organ systems and a coordinated response to protect the internal environment of the host; for example Chen and co-workers (2011) found that the left ventricular myocardium of short-term exercised rats was protected against apoptosis caused by intermittent hypoxia. Changes in autonomic control, fluid and electrolyte shifts, decreases in blood flow to the splanchnic region are a few of the major adjustments that have the potential to affect the microbiome because they alter the environment of the gastrointestinal tract. Withdrawal of parasympathetic tone and increases in sympathetic drive results in alterations to

gut motility. Internal shifts of fluid can and fluid losses associated with thermoregulation can compromise plasma volume, cardiac filling pressure and mean arterial pressure, Figure 2. Researchers have long suggested that the internal defense of plasma volume involves enhanced uptake of water and electrolytes from the gut during endurance exercise (Hodgson et al., 2014). However, there are limits to these acute responses and repeated exercise (training) induces adaptations that allow the animal to better tolerate subsequent exercise challenges.

Exercise related changes in the composition of bacterial communities

Exercise has been shown to positively affect the structure and function of the gut microbiome in both human and animal models. In rats, voluntary exercise training resulted in increased levels of cecal n-butyrate as well as a 1.5-fold increase of the wall thickness of the cecum (Matsumoto et al., 2008). N-butyrate was found to be effective in protection against some diseases of the digestive tract such as colon cancer and inflammatory bowel disease by altering the activation of cellular NF-□B (Perrin et al., 2001; Boutron-Ruault et al., 2005). In humans, physical activity and a calorie restricted diet have been shown to alter the GM causing increases in the abundance of Bacteroides fragilis and Lactobacillus spp., and concurrent decreases in the relative abundances of Clostridium coccoides, Bifidobacterium longum, and Bifidobacterium adolescentis (Santacruz et al., 2009). They suggested that these changes in the body weight

condition are controlled by the physical activity and the microbial profile of the GM (Santacruz et al., 2009). In another study, rats that were exposed to exercise exhibited changes in the composition of the GM with increases in the abundances of Lactobacillus, Bifidobacterium and B. coccoides-Eubacterium rectale and concurrent decreases in the serum ghrelin concentration and increases in serum leptin concentration (Queipo-Ortuño et al., 2013). These changes in the appetite related hormones were found to be linked to the composition of the GM and the ways that it may affect the nutritional physiological condition of these hosts (Ducrotte, et al., 2008; Fetissov et al., 2008). The later would suggest interplay between the GM and the endocrine control of energy homeostasis. In another study, exercise training altered the GM in obese rats by increasing the abundance of the genera Pseudomonas and Lactobacillus (Petriz et al., 2014). Similar changes could be of importance to the health and well-being of horses. For example butyrate is composes 65% of the energy source fueled by the GM and levels of butyrate may have a protective effect against inflammatory bowel diseases and may decrease downstream conditions such as laminitis in horses (Al Jassim and Andrews, 2009).

Exercise related changes in microbial diversity

Exercise appears to affect the richness and/or evenness of bacterial communities, resulting in an alteration of microbial diversity. One study (Allen et al., (2015) compared the effects of voluntary (running wheel access) versus

forced moderate exercise training (40 min/day/5 days/6 weeks at 8-12 m/min on 5% grade treadmill) on the GM of mice. They showed that the two treatments affected the GM in different ways according to clustering patterns in a principle coordinate analysis (PCoA) plot, and a decrease in the bacterial richness in the voluntary exercised group due to a decrease in the relative abundance of Turicibacter spp., a bacterium important in immunity and bowel disease. Lambert et al., (2015) found that 6 weeks of exercise training increased the abundances of Firmicutes and Bacteroidetes/Prevotella spp. in normal and Type 2 diabetic mice and increased Bifidobacterium spp. in exercised normal mice. These bacterial changes in the obese mice may be important as if they either enhance or worsen the health status of these rodents. Clarke et al., (2014) tested the gut bacterial profile of rugby athletes and found that those highly trained athletes had high bacterial diversity of 22 recognized phyla. The profiles of the athletes were also correlated with positive patterns of protein consumption and with creatine kinase concentration (Clarke et al., 2014). Mika et al., (2015) found that voluntary exercise training changed the GM in juvenile and adult rats, and those alternations were more profound in juvenile rats compared to adult rats. Moreover, lean body mass showed persistent elevations in the juvenile rats (Mika et al., 2015). Some of the alternations in juvenile rats included high levels of Bacteroidetes, low levels of Firmicutes, high levels of Blautia spp., high levels of Anaerostipes spp., and high levels of Methanosphaera spp. These important exercise related changes in the above studies using other animal models may occur in horses and could enhance or worsen the physiological status of the

horses. Unfortunately, no studies have been published on the effects of acute exercise or exercise training on the GM of the horse.

Exercise related changes in GM and obesity

Researchers are only just beginning to understand the interplay between exercise, the GM, and health factors such as obesity. Reports suggest that exercise has a protective and/or preventative role in obesity that could be moderated by the GM. Denou et al., (2016) examined the effects of exercise training on the GM in mice where a high fat diet was utilized to induce obesity, showing that exercise training boosted the Bacteroidetes/Firmicutes ratio with a predictable increase in bacterial metabolism of the microbial community. The authors suggested that this increase in the metabolic rate of the GM bacteria may help prevent obesity by utilizing the energy in fat prior to host uptake (Denou et al., 2016). Another study by Welly and co-workers (2016) demonstrated that voluntary exercise training of obese rats resulted in increasing abundances of cecal Streptococcaceae and S24-7 with concurrent decreases in a Rikenellacea genus. These exercise related changes in GM composition coincided with both insulin resistance improvement, decrease in LDL cholesterol, and increased function of mitochondria of the brown adipose tissue. Campbell et al., (2016) found that obese mice had normal villi in the duodenum and ileum with concurrent presence of Faecalibacterium prausnitzi, Clostridium spp., and Allobaculum spp after 12 weeks of exercise using voluntary wheel running

indicating that GM is important in the health and integrity of the intestinal epithelia, especially in obese mice. Exercise related changes in GM are correlated with positive health indicators against the effects of obesity. We are unaware of any published study of the effects of chronic exercise training on the GM of the horse. These changes related to exercise might increase butyrate producing bacteria resulting in protection against damaging epithelia of the intestine by environmental factors such as bacterial toxins and eventually provide protection against metabolic syndromes in horses (Evans et al., 2014).

Exercise stimulates protective responses of the GM

Exercise related changes in the gut microbiota may provide protection against harmful environmental agents and the effects of poor diet. While oral administration of polychlorinated biphenyls (PCBs) alters the GM profile of mice causing a decrease in the phylum *Proteobacteria*, it has been reported that voluntary exercise training of rodents weakened the PCB related changes in the GM (Choi et al., 2013).

The effects of a poor diet on the GM may be ameliorated by training. Voluntary exercise trained mice fed a high fat diet showed alternations in the composition of the GM with increases in butyrate producing bacteria such as *Bacteroidales*, *Clostridiaceae*, *Lachnospiraceae* and *Ruminococcaceae* and decreases in body weight (Evans et al., 2014). The authors of that study suggested that butyrate acted to improve intestinal epithelial cell health in

response to the GM, environmental stressors, and ischemia via heat shock protein 70 (Hsp70) (Evans et al., 2014). These protection effects associated with training-induced changes in the GM may be important to horses can be exposed to harmful environmental substances while grazing outdoors.

Exercise stimulates protective responses of the GM against illness

Exercise training related changes in the GM have been suggested to improve health and protect the body against illnesses, but the research has been inconsistent. Cox-York and co-investigators (2015) used low and high aerobic capacity female rats prior to ovariectomization. The authors showed that the high aerobic capacity animals developed protection against adipose tissue and liver triglyceride accumulation after 9 weeks post-surgery (Cox-York et al., 2015) Their animals showed no alternation in the GM despite a decrease in SCFA production. The study suggested that exercise may prevent illnesses following changes in physiology such as menopause or post-surgery, but demonstrate no change in the GM (Cox-York et al., 2015). In a contrasting study, Liu et al., (2015) showed that when ovariectomized rats performed either low or high aerobic capacity voluntary exercised for 11 weeks, the result was a decrease in the body and fat mass in the low aerobic capacity animals and an increase in the high aerobic capacity animals respectively. Bacterial PCoA plots from this study showed differential clustering of the low aerobic capacity exercised ovariectomized rats when compared with the other groups (Liu et al., 2015).

Moreover, the low aerobic capacity exercised ovariectomized rats showed a decrease in the relative abundance of Firmicutes and increases in Proteobacteia and Cyanobacteria (Liu et al., (2015).

Exercise training-related changes in the GM have been associated with the maintenance of mental as well as physical health. Kang et al., (2014) found that feeding a high fat diet (HFD) caused increasing anxiety in mice when tested using the Light/Dark exploration and distance travelled assays. In their study, the increase in markers of anxiety was not weakened by exercise training, but the combination of HFD and exercise training resulted in changes to the GM with increases in the abundance of Streptococcus (OTU115) Kang et al., (2014). The abundance of Streptococcus (OTU115) was undetectable for mice exposed to exercise alone Kang et al., (2014). In addition, exercise alone decreased the relative abundances of Bacteroidetes and Tenericutes in mice fed a normal diet Kang et al., (2014). In another study, Shukla et al., (2015) studied the effects of maximal exercise at 15 minutes, 48 hours, and 72 hours on symptoms measured in patients with myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS), a disease not caused by physical activities. They found that exercise decreased the relative abundance of fecal Actinobacteria in those patients, and at the 72h time point there was a decrease in bacterial clearance from blood of those patients (Shukla et al., 2015). The authors suggested that high intensity exercise worsens the symptoms of this disease, and the speculated that this could have been due to the GM changes measured in these patients.

An excellent review of the literature by Van Dijk and Matson (2016) synthesized the current findings on the effects of exercise on the immune system with a focus on relationships to changes in the GM. They concluded depending on the level of exertion, exercise training could increase lymphocytes as well as decrease natural killer cells and saliva immunoglobulin A (SIgA) for hours and that that effect correlated with changes in the GM (Van Dijk and Matson, 2016). Based upon the literature, it appears that exercise training can induce changes to the gut microbiota that may protect against certain systematic physiological changes in the host. Depending on the intensity and volume, exercise training may also exacerbate certain health conditions associated with changes in the GM, Figure 3. The exercise related changes in the GM observed in studies using rodent and humans may also occur in horses. Unfortunately, we are unaware of any papers reporting the effects of acute or chronic exercise on the GM of the horse.

The Gut Microbiome and Performance

The GM may play a role to increase the athletic performance of humans and animals through the downstream effects of the bacterial metabolites on the physiological status of the host (Shing et al, 2014). Shing et al., (2014) studied the effects of 4 weeks of probiotic supplementation (45 billion CFU of Lactobacillus, Bifidobacterium and Streptococcus strains) on the performance of male runners. The probiotic treated group increased run time until fatigue at 80%

of their aerobic capacity (Shing et al, 2014). In another study, Hsu et al., (2015) found that specific pathogen-free (SPF) and Bacteroides fragilis (BF) gnotobiotic mice swam for longer times compared to Germ free (GF) mice, and they also observed that SPF mice showed higher concentrations of serum glutathione peroxidase and catalase with higher activity of superoxide dismutase than GF mice. The authors suggested that the GM of the SPF mice played a role in increasing the antioxidant enzymes which enabled the observed increases in swimming performance seen in these mice (Hsu et al., 2015). In another experiment, Chen et al., (2016) examined the effects of Lactobacillus plantarum TWK10 supplementation on mouse swimming performance. The bacterial supplement increased swimming time, relative muscle mass, and type I fibers in gastrocnemius muscle, while decreasing total body weight. In addition, following the bacterial supplementation, there were decreases in serum albumin, blood urea nitrogen, creatinine, and triacylglycerol (Chen et al., 2016).

In conclusion, exercise has potential to alter the bacterial communities in the gut and this interaction may play an important role in the health of athletic horses like their human and rodent counterparts. In horses, exercise performance requires more energy and understanding the effects of acute and chronic exercise on the GM is important because a significant contribution of their energy profile is made up of the SCFAs (such as butyrate) that are produced by the gut microbiota.

Summary

More work is needed to determine if acute exercise and exercise training generate healthy changes in the bacterial profiles of the athletic horse. Studies in human and other animal models suggest that the composition and activity of the GM is positively modulated by exercise to protect against the effects of obesity, disease, poor diet, and toxins. Studies of the equine gut microbiota suggest links to the host physiological, immunological, and nutritional statuses since a major part of the energy needed by for daily activities is provided by bacterial fermentation processes that produce short chain fatty acids such as butyrate. Horses have been found to have a very diverse gut bacterial profile that is vital to the health of the animal. The equine microbiome could be especially sensitive to exercise that these changes in the microbiome might bring more beneficial effects on the host such as increasing performance. It seems that gut microbiome is in many ways as important as other organs in the bodies of humans and other animals including horses. The activities of these microbes may influence the host's health in positive or negative manners. Furthermore, in horses, Studying these bacteria using high throughput techniques such as next generation sequencing methods has improved our knowledge about the behaviors and the participation of these microorganisms in the health of the horses.

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Figure Captions

Figure 1: Development of intestinal immunity via gut microbiota (Kamada et al., 2013).

Figure 2: Effect of Exercise on autonomic nervous system and related organs in horses.

Figure 3: Exercise - Gut microbiome – immunity - Brain Axis (Bermon et al., 2015)

Figure 1.

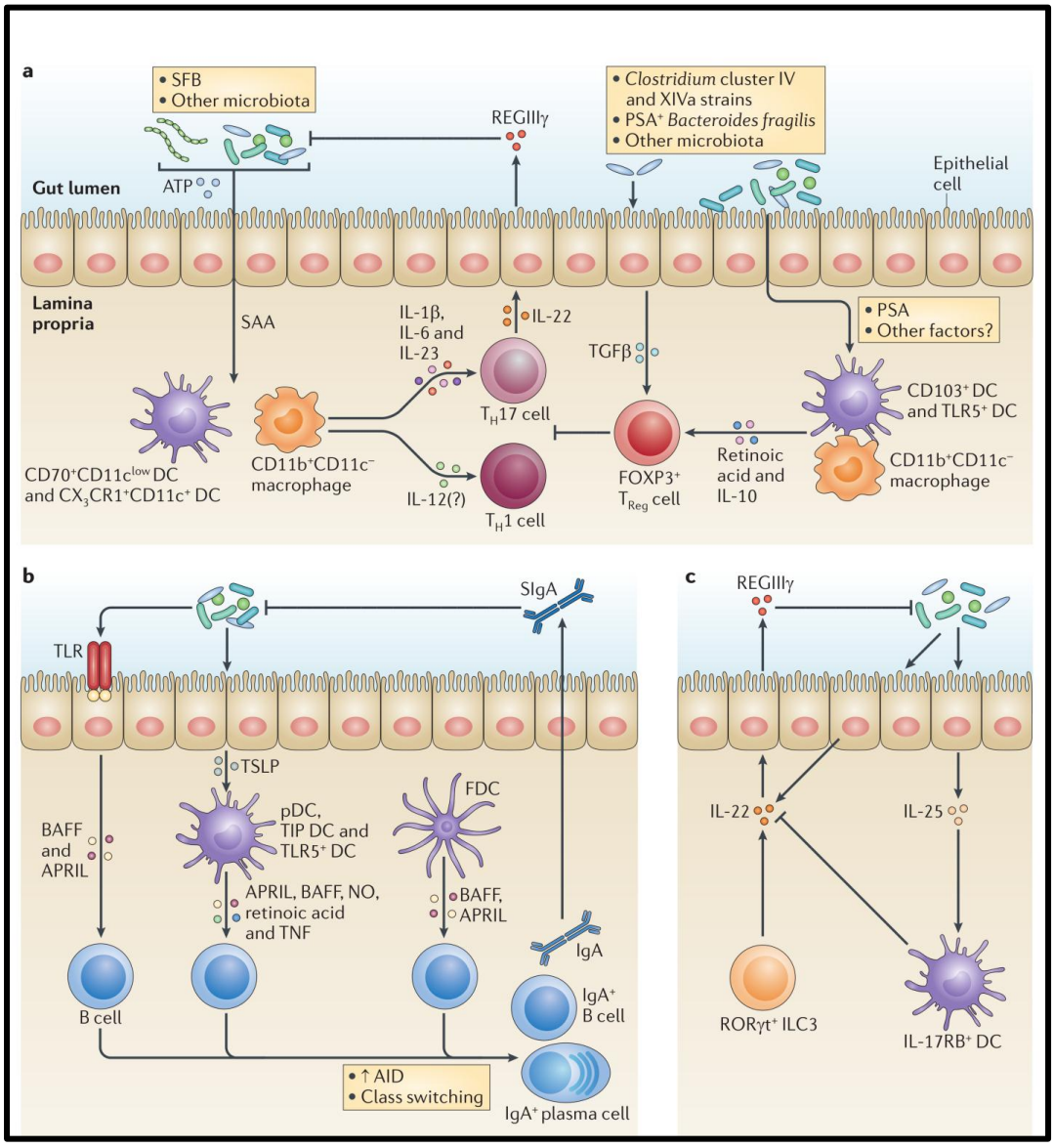

a
• SFB
• Other microbiota
REGIIIγ
• Clostridium cluster IV and XIVa strains
• PSA+ Bacteroides fragilis
• Other microbiota
Epithelial cell
Gut lumen
ATP
Lamina propria
SAA
IL-1β, IL-6 and IL-23
IL-22
TGFβ
• PSA
• Other factors?
CD103+ DC and TLR5+ DC
CD11b+CD11c− macrophage
TH17 cell
FOXP3+ TReg cell
Retinoic acid and IL-10
CD70+CD11clow DC and CX3CR1+CD11c+ DC
CD11b+CD11c− macrophage
IL-12(?)
TH1 cell
b
SIgA
TLR
TSLP
FDC
pDC, TIP DC and TLR5+ DC
BAFF and APRIL
APRIL, BAFF, NO, retinoic acid and TNF
BAFF, APRIL
IgA
B cell
IgA+ B cell
• ↑ AID
• Class switching
IgA+ plasma cell
c
REGIIIγ
IL-22
IL-25
RORγt+ ILC3
IL-17RB+ DC

Figure 2.

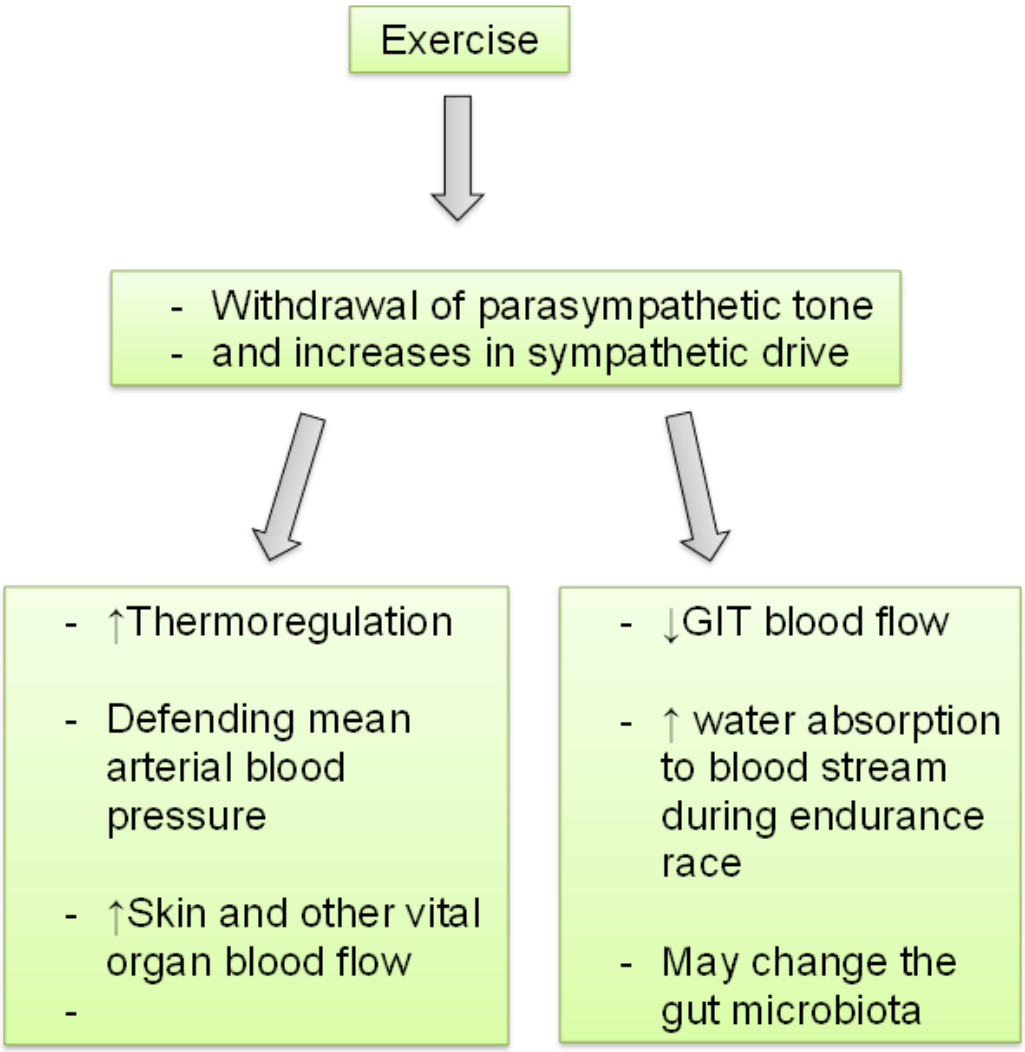
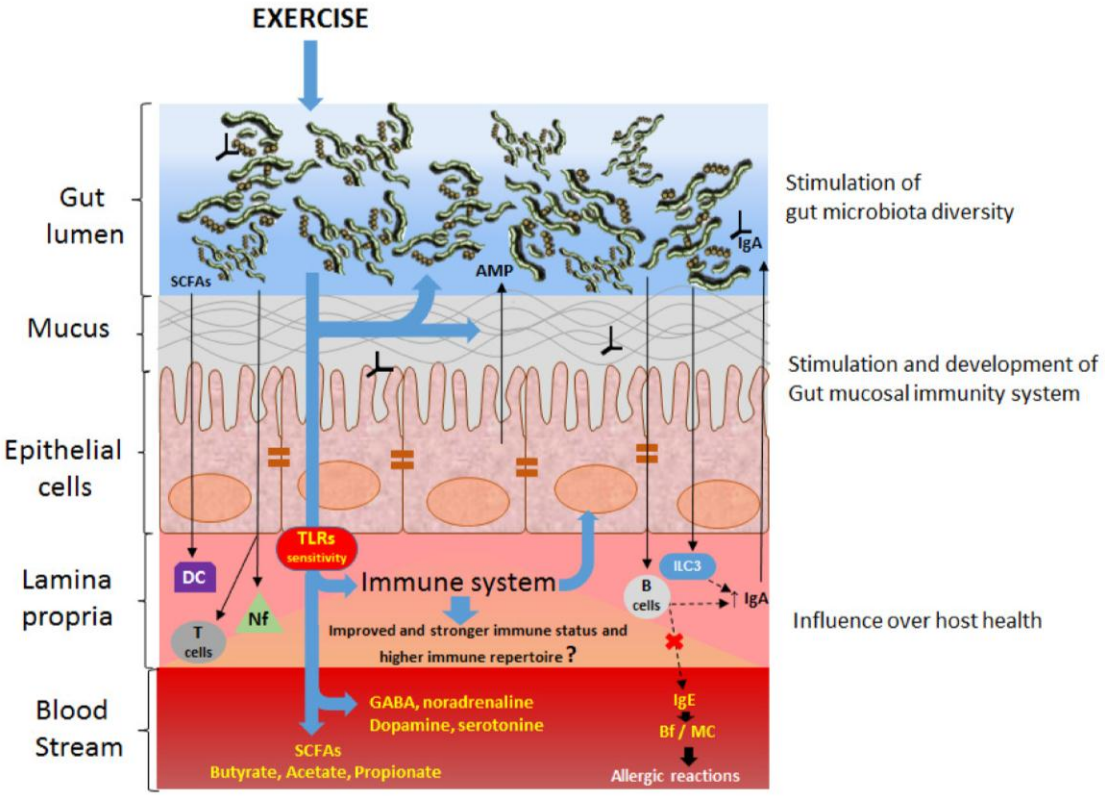

Exercise
- Withdrawal of parasympathetic tone
- and increases in sympathetic drive
- ↑Thermoregulation
- Defending mean arterial blood pressure
- ↑Skin and other vital organ blood flow
-
- ↓GIT blood flow
- ↑ water absorption to blood stream during endurance race
- May change the gut microbiota

Figure 3.


EXERCISE
Gut lumen
Mucus
Epithelial cells
Lamina propria
Blood Stream
SCFAs
AMP
IgA
Stimulation of gut microbiota diversity
Stimulation and development of Gut mucosal immunity system
TLRs sensitivity
DC
Immune system
ILC3
B cells
IgA
T cells
Nf
Improved and stronger immune status and higher immune repertoire ?
Influence over host health
GABA, noradrenaline
Dopamine, serotonine
IgE
Bf / MC
SCFAs
Butyrate, Acetate, Propionate
Allergic reactions

COMMON ABBREVIATIONS

GM-gut microbiota

Ph/Chl+C3-phenol/chloroform extraction method plus inhibitor removal solution (C3)

MoBio-K-MoBio kit

NGS-Next Generation Sequencing

PCoA-Principle Coordinate Analysis

GXT-graded exercise test

SC1 or SC2- standing control

SC-Seasonal control

ExTr-Exercise training

Research Hypotheses

1) Test the hypothesis that acute exercise may alter the gut microbiota in Standardbred racehorses

2) Test the hypothesis that exercise training may alter the gut microbiota in Standardbred racehorses

3) Test the hypothesis that 12 weeks of exercise training may affect the response by the gut microbiota of the Standardbred racehorses to the acute strenuous exercise.

CHAPTER 2: Journal Article (Published)

Comparison of a modified phenol/chloroform and commercial-kit methods for extracting DNA from horse fecal material.

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Abstract

There are many choices for methods of extracting bacterial DNA for Next Generation Sequencing (NGS) from fecal samples. Here, we compare our modifications of a phenol/chloroform extraction method plus an inhibitor removal solution (C3) (Ph/Chl+C3) to the PowerFecal® DNA Isolation Kit (MoBio-K). DNA quality and quantity coupled to NGS results were used to assess differences in relative abundance, Shannon diversity index, unique species, and principle coordinate analysis (PCoA) between biological replicates. Six replicate samples, taken from a single ball of horse feces manually collected from the rectum, were subjected to each extraction method. The Ph/Chl+C3 method produced 100x higher DNA yields with less shearing than the MoBio-K method. To assess the methods, the two method samples were sent for sequencing of the bacterial V3-V4 region of 16S rRNA gene using the Illumina MiSeq platform. The relative abundance of Bacteroidetes was greater and there were more unique species assigned to this group in MoBio-K than in Ph/Chl+C3 ($P<0.05$). In contrast, Firmicutes had greater relative abundance and more unique species in Ph/Chl+C3 extracts than in MoBio-K ($P<0.05$). The other major bacterial phyla were equally abundant in samples using both extraction methods. Alpha diversity and Shannon Weaver indices showed greater evenness of bacterial distribution in Ph/Chl+C3 compared with MoBio-K ($P<0.05$), but there was no difference in the OTU richness. Principle coordinate analysis (PCoA) indicated a distinct separation between the two methods ($P<0.05$) and tighter clustering (less variability) in Ph/Chl+C3 than in MoBio-K. These results suggest that the

Ph/Chl+C3 may be preferred for research to identify specific Firmicutes taxa such as Clostridium, and Bacillus. However; MoBio-K may be a better choice for projects focusing on Bacteroidetes abundance. The Ph/Chl+C3 method required less time, but has some safety concerns associated with exposure and disposal of phenol and chloroform. While the MoBio-K may be better choice for researchers with less access to safety equipment like a fume hood.

Introduction

The gut microbome (GM) of mammals is a diverse and complex community of microorganisms that are known to affect host health (Sekirov et al., 2010). Many of these microorganisms evade cultivation as demonstrated by plate count anomalies (Staley and Konopka, 1985) (Rappé and Giovannoni, 2003) but are detectable by Next Generations Sequencing (NGS) methods such as Illumina (Zhou et al., 2010). The ability of NGS to detect rare or recalcitrant bacteria depends on the extraction of a sufficient quantity (N20 ng/µl) of high quality, clean DNA from the entire microbial community. Inhibitors which co-purify during the extraction procedure, such as organic and phenolic compounds, divalent cations (e.g., Mg2+, Ca2+), and heavy metals can interfere with PCR based sequencing strategies (Wilson, 1997). This is important because animal feeds, especially for horses contain phenolic compounds (Dueñas et al., 2004; Naczk and Shahidi, 2006). Furthermore, fecal samples are generally rich with humic substances which can also affect the DNA quality and purity (Holben et al.,

1988). In this study, two DNA extraction methods were compared: a phenol/chloroform method (Kerkhof and Ward, 1993; McGuinness et al., 2006; Männistö et al., 2009), which was modified for the present experiment, and a commercial DNA extraction kit, (MoBio PowerFecal® DNA Isolation Kit, catalog# 12830-50; MoBio Laboratories Inc.). Our PCoA results demonstrate less variability upon extracting horse fecal DNA using the modified ph/Chl + C3 method, where a primary modification was the addition of an inhibitor removal solution (C3) that precipitates non-DNA organic and inorganic substances such as humic acid, cell debris, and proteins (MoBio Laboratories Inc., Catalog# 12830-50-3). This suggests clean and stable DNA products were generated after inserting our modifications and using the inhibitor removal solution (C3) in the phenol chloroform extraction method.

Materials and Methods

Samples

Biological replicates were collected from one horse and the same fecal ball at the same time. The collection was made from the inner end of the rectum of the horse, ~45 cm of distance, using the rectal palpation method with aseptic procedures (Mueller and Moore, 2000). The fecal ball was placed on a sterile surface and opened with sterile forceps. After that, 0.25 g samples (n=6 for each extraction method) were collected from the inner part of the ball and then placed in collection tube containing beads (MP Biomedical; (sku# 116914100) that had been preloaded with 300 µL of buffer solution containing cetyltrimethyl

ammonium bromide (CTAB) (0.25M phosphate buffer (pH 8), 5% CTAB in 1 M NaCl). Samples were then snap frozen in liquid nitrogen and then place in -80oC for extraction.

DNA Extraction Methods

The (Ph/Chl+C3) method was modified as follows: Samples were subjected to 5 quick freeze/thaw cycles between liquid nitrogen and a 55oC water bath. After these freeze/thaw cycles, 100 µl of Solution 1 (50 mM glucose, 10 mM EDTA, 25mM Tris-Cl; pH 8.0), 50 µl of lysozyme solution (4 mg in 1 ml of Solution 1) and 50 µl of 500mM EDTA were added to the frozen samples. The samples were then thawed and quickly combine with 50 µl 10% SDS and 800 µl phenol: chloroform: isoamyl alcohol; 25:24:1 (>pH 7.0). Samples were then disrupted using vortexing for 3 minutes and followed by centrifugation at (~ 16,000 x g) for 3 minutes. After the aqueous phase was transferred to a new centrifuge tube, a second 800 ul phenol: chloroform: isoamyl alcohol; 25:24:1 (>pH 7.0) was performed. These tubes were vortexed for 1 minute at maximum speed and centrifuged at about 16,000 x g for 3 minutes. The aqueous layer was transferred to a new micro-centrifuge tube to which 200 ul of C3, inhibitor removal solution (MoBio Laboratories Inc., catlg# 12830-50-3) was added. The tubes containing the inhibitor removal solution and the DNA extract were vortexed briefly and then incubated in ice at 4oC for 5 minutes. Following incubation, the tubes were centrifuged at 13,000 x g for 1 minute (as indicated by the manufacturer protocol), and the supernatant containing DNA was transferred to a sterile, DNAse, and RNAse free tube. The DNA was then assessed for

purity and concentration using NanoDrop™ 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE). DNA was visualized for shearing on agarose gel. DNA was checked for amplification of the 16S rRNA gene using the universal bacterial primers: 27F: AGAGTTTGATCCTGGCTCAG and 1522r: AAGGAGGTGATCCAICCGCA. We will refer to this method by (Ph/Chl+C3) in the rest of this article.

For the MoBio-K extractions, we collected as described above (0.25 g of feces in 300 µL of buffer solution containing CTAB (0.25M phosphate buffer (pH 8), 5% CTAB in 1 M NaCl). We followed the exact and complete manufacturer's protocol for the Powerfecal kit.

Sequencing (Miseq)

To sequence the 16S rRNA genes within the fecal samples, the V3-V4 variable region of this gene was amplified with universal bacterial primers 341F, CCTACGGGAGGCAGCAG /785R, CTACCAGGGTATCTAATCC (Mühling et al., 2008), and barcodes on the forward primer in a 30 cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, USA). Amplifications were performed under the following conditions: 94°C for 3 minutes, followed by 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final elongation step at 72°C for 5 minutes was performed. PCR products were analyzed on a 2% agarose gel to identify the size and the relative intensity of bands. Samples were purified using calibrated Ampure XP beads. These purified PCR products were used to produce a DNA library using the Illumina TruSeq

DNA library preparation protocol. Sequencing was performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) using the Illumina MiSeq platform.

Bioinformatics

Sequence data were processed using MR DNA analysis pipeline (MR DNA, Shallowater, TX, USA). In short, paired reads were joined and trimmed of barcodes. Sequences <150bp 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).

Statistical Analysis

Alpha diversity was estimated by calculating relative abundance of bacteria at the phylum and species levels (n=6 replicates/extraction method). T tests were used to compare the two extraction methods ($\alpha = 0.05$). Statistical analyses were performed using Prism 6 and Prism 7 (GraphPad, Inc.). Mean±SEM was used to represent data. Shannon diversity index at the species level for each extraction method was calculated using the R software version 3.2.3 (Wooden Christmas Tree) with the packages: Vegan (Oksanen et al., 2016) and Mass (Venables and Ripley, 2002). The Shannon values were compared using t-test. Principle Coordinate Analysis (PCoA) was performed using the R software version 3.2.3 (Wooden Christmas Tree) with the package Vegan (Oksanen et al., 2016), and scores were compared using t-test. Images

generated in R software were converted to Tiff format using GIMP software version 2.8.16 (The GIMP team, 1997-2014). Unique species from each extraction method were identified by lists generated in Microsoft office excel.

Results

DNA Quantity and Quality

The DNA concentration, Figure 1A, generated by Ph/Chl+C3 was significantly higher, ($P< 0.0001$), than the DNA concentration generated by MoBio-K. The 260/280 and 260/230 ratios for Ph/Chl+C3 were 1.84 and 1.81 respectively, while for MoBio-K, they were 2.11 and 1.94 respectively. Evident DNA shearing was greater for the MoBio-K method compared with the Ph/Chl+C3 method, Figure 1B.

Bacterial Taxa Abundance

Phylum Bacteroidetes relative abundance, Figure 2A, was significantly greater, ($p = 0.0328$), in MoBio-K than in Ph/Chl+C3 (39.83 ± 0.91 vs 37.06 ± 0.49). Genus Barnsiella, which belongs to phylum Bacteroidetes, relative abundance was significantly higher ($p = 0.0003$) in Ph/Chl+C3 than in MoBio-K (1.13 ± 0.08 vs 0.73 ± 0.08), Figure 2B. In case of phylum Firmicutes, Figure 2E, showed significantly higher relative abundance ($p = 0.0178$) in Ph/Chl+C3 than in MoBio-K (34.35 ± 0.31 vs 26.57 ± 2.15). Moreover; the relative abundance in 2 of the Firmicutes genera, Clostridium and Bacillus, Figure 2F and 2G, were significantly higher ($p = 0.0022$ and 0.0321 respectively) in Ph/Chl+C3 than in

MoBio-K. Finally, there were no significant differences between the extraction methods in the relative abundances of Phylum Proteobacteria, Spirochaetes, or Fibrobacteres, Figure, 2C, 2D, and 2H ($p > 0.05$).

Shannon Diversity Index and Principle Coordinate Analysis (PCoA)

At the species level, Figure 3A, Ph/Chl+C3 showed significantly greater index ($p = 0.0161$) than MoBio-K (4.20 ± 0.02 vs 4.04 ± 0.04). The PCoA, Figure 3B, showed significant separation ($p = 0.0464$) between Ph/Chl+C3 and MoBio-K (0.017 ± 0.003 vs 0.228 ± 0.079). The 6 replicates from Ph/Chl+C3 clustered together. There were thirteen unique species observed in each extraction method. Unique species from Firmicutes (7), Actinobacteria (4), and β-and γ-Proteobacteria (2) were identified in Ph/Chl+C3; while; species from Bacteroidetes (5), α-Proteobacteria (3), Firmicutes (3), δ-Proteobacteria (1), and Planctomycetes (1) appeared only in MoBio-K. All were low abundance species (less than 1%), Table 1.

Discussion

The bacterial DNA extraction method will probably affect the observed bacterial community when analyzing fecal samples (Larsen et al., 2015). One criterion that should help decide whether a certain DNA extraction method is suitable is DNA yield. Some studies have shown that different modified phenol/chloroform methods yielded high amounts of DNA from different types of samples (Zhang et al., 2006; Kok et al., 2000). To our knowledge, no study has

attempted to use a phenol/chloroform method to extract DNA from horse fecal samples for DNA extraction method comparison purposes. In the present study, our Ph/Chl+C3 method yielded a higher DNA concentration and higher molecular weight product (Figure 1A) with less shearing in a shorter amount of time than the MoBio-K method (Figure 1B). Hart et al., (2015) observed that the MoBio Power Fecal kit produced low amounts of DNA extracted from horse fecal samples. In our study, the MoBio-K also yielded a low concentration of DNA from horse fecal samples. These higher DNA concentrations and molecular weights of the Ph/Chl+C3 method might be a result of no prolonged and complicated processes such as multiple centrifugations involved in this method (Steffan et al., 1988). The second criterion is the downstream application that is needed to be used, such as NGS. Our NGS results showed that the relative abundance of the phyla Bacteroidetes and Firmicutes were impacted by the DNA extraction methods used in this study. In our study, more Bacteroidetes and less Firmicutes were recognized from MoBio-K by the NGS. In contrast, Peng et al., (2013) observed lower levels of Bacteroidetes and higher levels of Firmicutes in feces of rats using the UltraClean™ Fecal DNA kit (MoBio). This difference in sequence results could be from fecal material from rats and horses being very different in their nature. In our study, the Shannon diversity index was significantly lower in MoBio-K than Ph/Chl+C3 (Figure 3A), indicating a major difference in overall DNA yield. However, both methods captured the same overall number of bacterial species. Peng et al., (2013) demonstrated using PCoA on fecal samples from rats that the UltraClean™ Fecal DNA kit (MoBio) behaved differently from

other methods they used in their comparisons. They showed that MoBio kit showed different clustering and different microbial pattern on the PCoA. This confirms our results when our PCoA plot (Figure 3B) showed significant separation between the two extraction methods and high scattering in the replicates of MoBio-K. For Ph/Chl+C3, replicates clustered closer together indicating higher reproducibility than MoBio-K. The Ph/Chl+C3 method requires a fume hood and hazardous material handling and disposal and fresh lysozyme solution needs to be prepared prior to each extraction. MoBio-K can be easily done on the benchtop with ordinary laboratory precautions, directly out of the box.

In conclusion, The Shannon and the PCoA analyses indicate that Ph/Chl+C3 is a reliable horse fecal DNA extraction method that could be used with high confidence of generating the same stable DNA extract products every round of extraction. This suggests that may be fewer factors could affect the DNA extracting during the use of this method. This research highlights the challenge in choosing a DNA extraction method for 16S rRNA sequencing that captures the rare and common bacterial taxa and is also economical and easy to implement.

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List of Tables

Table1. Unique species in Ph/Chl+C3 or MoBio-K (relative abundance <1%).

Ph/Chl+C3 unique species	MoBio-K unique species	Phylum
	Bacteroides barnesiae	*Bacteroidetes*
Tepidimonas spp.		b-*Proteobacteria*
	Marivirga tractuosa	*Bacteroidetes*
Williamsia serinedens		*Actinobacteria*
Catenibacterium spp.		*Firmicutes*
	Dongia mobilis	a-*Proteobacteria*
	Devosia spp.	a-*Proteobacteria*
Williamsia sp.		*Actinobacteria*
Proteinivorax tanatarense		*Firmicutes*
	Eubacterium saburreum	*Firmicutes*
Robinsoniella peoriensis		*Firmicutes*
	Desulfovibrio vulgaris	d-*Proteobacteria*
Pseudomonas putida		g-*Proteobacteria*
Metabacterium polyspora		*Firmicutes*
Cellulosilyticum lentocellum		*Firmicutes*
	Alkaliflexus imshenetskii	*Bacteroidetes*
	Petrimonas spp.	*Bacteroidetes*
	Dysgonomonas capnocytophagoides	*Bacteroidetes*
Propionibacterium acnes		*Actinobacteria*
	Eubacterium contortum	*Firmicutes*
	Planctomyces maris	*Planctomycetes*
	Moorella spp.	*Firmicutes*
	Afipia sp.	a-*Proteobacteria*
Oceanirhabdus sediminicola		*Firmicutes*
Tindallia texcoconensis		*Firmicutes*
Eggerthella spp.		*Actinobacteria*

Figure Captions

Figure 1. Comparisons of DNA quantity and quality between the DNA extraction methods. A. Comparison between the two DNA extraction methods in the DNA concentrations, n=6 replicates/method, (P value < 0.0001). Error bars are SEMs. Asterisk represents significant difference. B. Comparison between the two DNA extraction methods in DNA shearing.

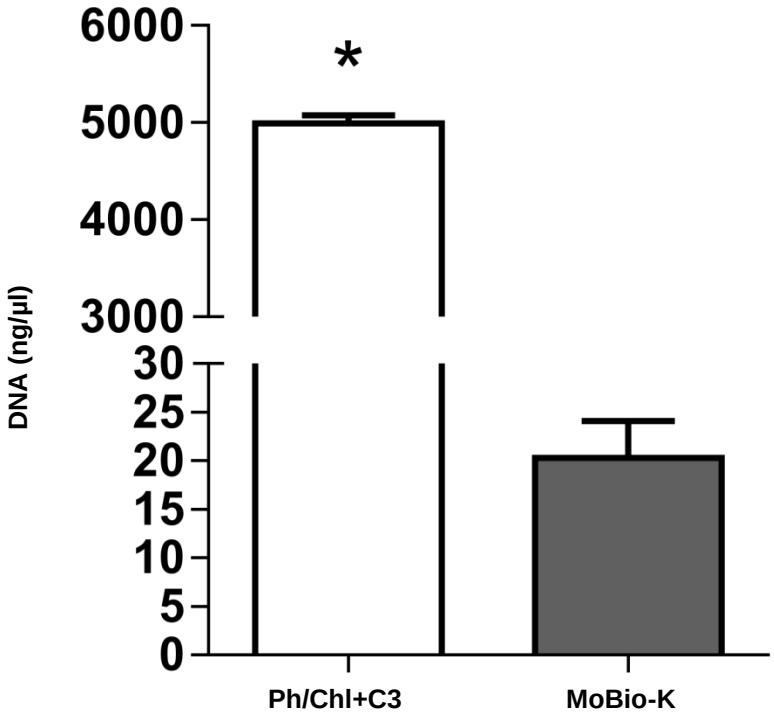
Figure 2. Comparison between the two DNA extraction methods in the relative abundance (%) of some bacterial phyla and genera. A. Bacteroidetes B. Genus Barnesiella. C. Proteobacteria D. Spirochaetes. E. Firmicutes. F. Genus Clostridium. G. Genus: Bacillus. H. Fibrobacteres. N=6 replicates/DNA extraction method. Error bars are SEMs. Asterisks represent significant differences.

Figure 3. Bacterial community ecological analyses. A. Shannon diversity index comparison between the two DNA extraction methods at the species level. N=6 replicates/DNA extraction method. Error bars are SEMs. Asterisk represents significant difference. B. Principle coordinate analysis (PCoA) at the species level of the two DNA extraction methods. N= 6 replicates/DNA extraction method.

Figures

Fig 1

A.



B.

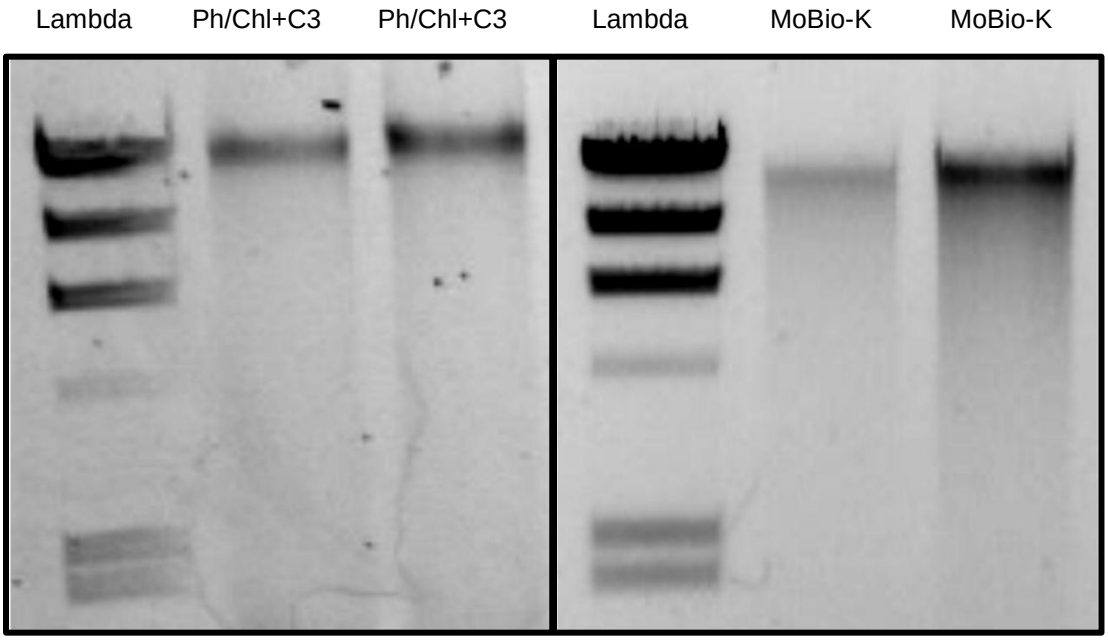


Fig 2

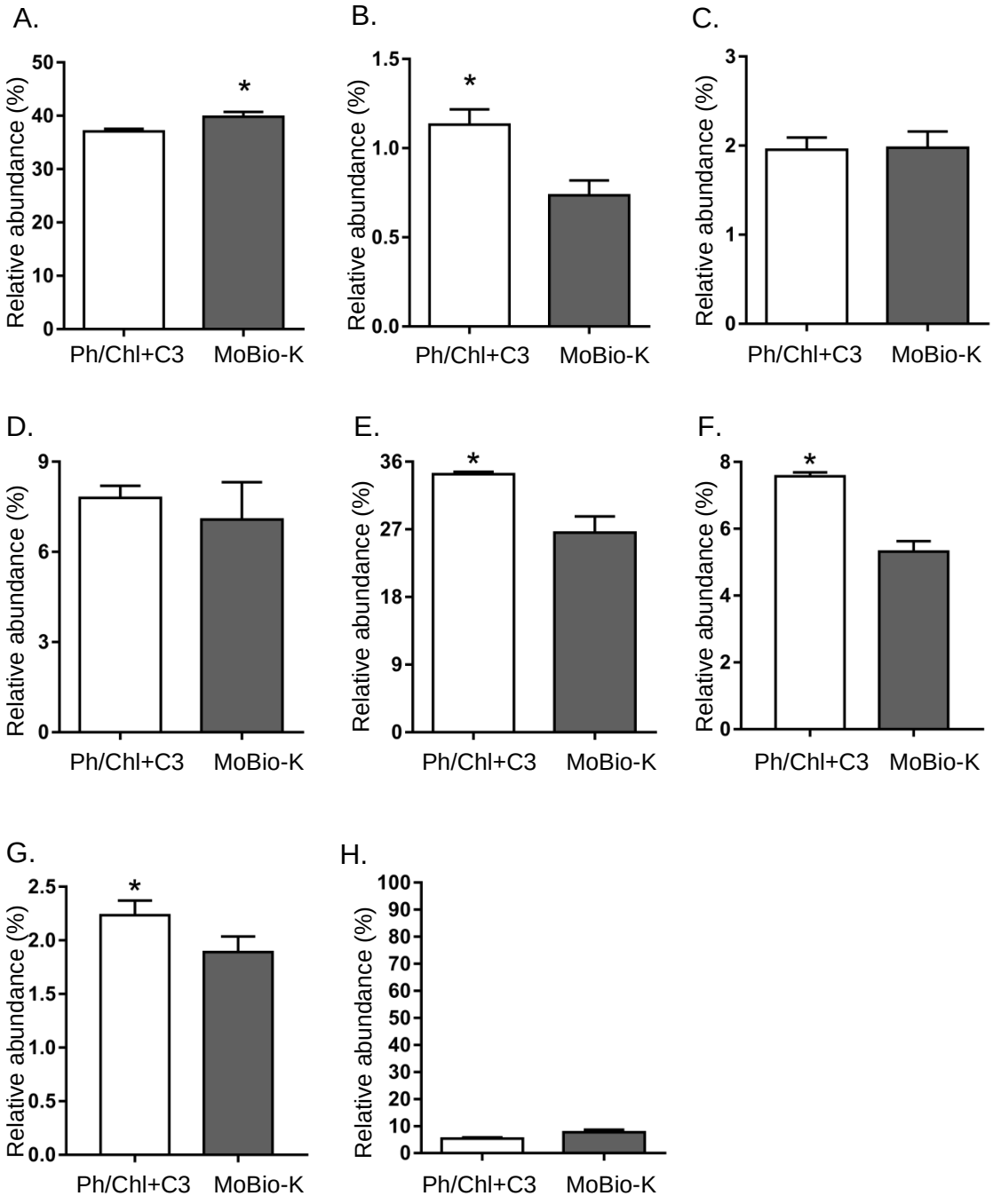
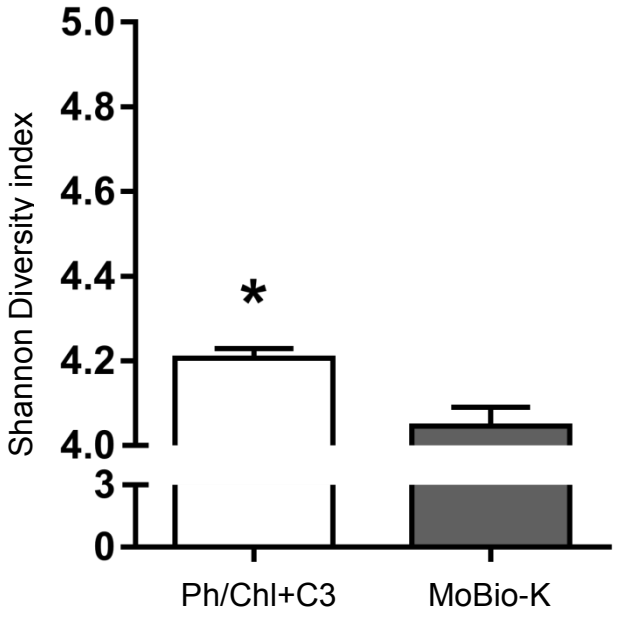
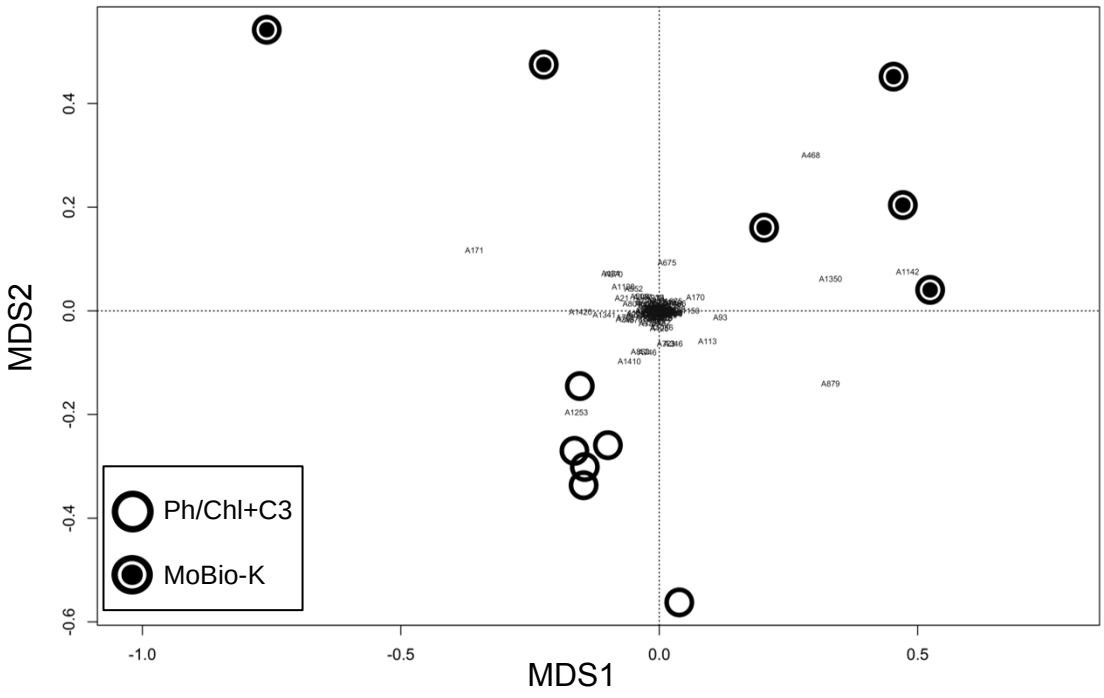


Fig 3

A.


5.0
4.8
4.6
4.4
4.2
4.0
3
0
Shannon Diversity index
*
Ph/Chl+C3
MoBio-K

B.


MDS2
MDS1
0.4
0.2
0.0
-0.2
-0.4
-0.6
-1.0
-0.5
0.0
0.5
Ph/Chl+C3
MoBio-K
A468
A171
A675
A1350
A1142
A170
A93
A113
A1410
A879
A1253

CHAPTER 3: Journal Article (Submitted for review)

The Effects of Acute Strenuous Exercise on the Gut Microbiota in Standardbred Racehorses

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Abstract

While exercise has been found to change the gut microbiome (GM) in laboratory animals exposed over weeks, no studies have identified immediate changes in the GM 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 GM 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 fecal samples were taken 24 hours 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 the relative abundance of the genus Clostridium, Fusicatenibacter saccharivorans, and Treponema zioleckii might particularly be responsive to anticipatory effects associated with watching and hearing exercise.

Introduction

The GM is considered to be the “second brain” of animals and human (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 specialized compartments such as the cecum and large colon, where microbes supply the enzymes to ferment complex plant material into useable short chain fatty acids (Costa and Weese, 2012; Zhang et al., 2014; Bjursell et al., 2006). During the cellulolytic process, some clostridial bacteria, e.g. Clostridium cellulolyticum, have been found to degrade cellulose and significantly produce less complex sugars (Desvaux, 2005; Hethener et al., 1992)

One of the factors that can influence the GM is exercise training which has been found to change the composition of microbial communities in the GIT of humans, mice, and rats (Santacruz et al., 2009, Potera, 2013, Queipo-Ortuño et al., 2013, Evans et al., 2014). Another factor that can influence the GM 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) or social disruption (SDR) (Bailey et al., 2011) has been shown to cause significant changes in specific members of the GM community. High fat diets can also alter the GM in ways that are similar to feeding stress

(Evans et al., 2014). Furthermore, exercise training appears to induce beneficial GM 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 GM (Evans et al., 2014; Mika et al., 2015; Liu 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; Mika et al., 2015; Liu et al., 2015) respectively.

To the best of our knowledge, no studies have been published on the effects of a single bout of acute intense exercise on the gut 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 horse is an athletic animal and, like humans, it is well recognized that for the horse, exercise training has many beneficial effects related to the adaptive physiological response to the repeated acute challenge of exertion. Therefore, the present study was performed to test the hypothesis that acute intense exercise would alter the GM and secondarily, that the adaptive effects of exercise training would alter the response to acute exercise in the horse.

Materials and methods

Animals, ration, and housing

All methods used in this study were approved by the Rutgers Institutional Animal Care and Use Committee. Eight healthy, unfit Standardbred horses (3-8 yrs; ~500 kg; 4 mares and 4 geldings) were used in the experiment. All horses were acclimated to the Rutgers Equine Science Center training personal, 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 (6kg/day) and pelleted grain supplement (3kg/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 (GXT)

During the GXT maximal oxygen uptake (VO2max) 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, Lexington, KY) 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 60s (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 (VO2) and carbon dioxide production (VCO2) were measured every 10 seconds using the open flow indirect calorimeter (Oxymax-XL, Columbus Instruments, Columbus, OH).

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.

Logistically only four horses could be tested on a single day so 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 standing serving as standing controls. The same assignment was maintained for the post-training testing and sampling.

For the standing control, each of the horses stood in a stall inside the treadmill laboratory where they could hear and observe the exercise tests performed by the other horses. For the twelve week exercise training protocol, the eight horses were exercised in a free stall motorized exercise machine 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 VO2max with duration and speed adjusted upward each week, Table 1.

Sample collection

Fecal samples were obtained from all horses 24 hrs before and 24 hrs after GXT1 (or SC1) and GXT2 (or SC2). Fecal samples were collected via rectal palpation by the same investigator (Ali Hassan Daghir Janabi). Samples were obtained following the clearance of feces 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 (MP Biomedical; SKU# 116914100) and 300 uL of buffer solution containing CTAB. Each tube was snap frozen in liquid nitrogen and then stored at -80o C for later analysis.

DNA Extraction

DNA was extracted using a protocol from (Janabi et al., 2016). Briefly, samples were subjected to five quick freeze/thaw cycles with liquid nitrogen (-80o C) and 55o C hot bath. To the frozen samples, 100 µl of Solution 1 (50 mM glucose, 10 mM EDTA, 25mM Tris-Cl; pH 8.0), 50 µl of lysozyme solution (4 mg in 1 ml of Solution 1) and 50 µl of 500mM 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

minutes. Samples were then spun down in micro-centrifuge at (~ 16,000 x g) for 3 minutes. The top phase was transferred to a new centrifuge tube preloaded with 800 ul phenol: chloroform: isoamyl alcohol; 25:24:1 (>pH 7.0). Tubes were vortexed for 1 minute at maximum speed and then centrifuged for 3 minutes at (~ 16,000 x g). The top layer was then transferred to a new micro-centrifuge tube, and 200 ul of C3, inhibitor removal solution (MoBio Laboratories Inc., catlg# 12830-50-3), was added to each tube. The tubes containing the C3 solution and the DNA extract were vortexed briefly and then incubated in 4 o C for 5 minutes in ice. Following incubation the tubes were spun at 13000 xg for 1 minute (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), 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, USA) under the following conditions: 94°C for 3 minutes, followed by 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final

elongation step at 72°C for 5 minutes 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. Then the purified PCR product was used to prepare DNA library by following Illumina TruSeq DNA library preparation protocol. 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 MR DNA analysis pipeline (MR DNA, Shallowater, TX, USA). Briefly, paired reads were joined and trimmed of barcodes. Sequences <150bp 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 analyze and graph data. Data are presented in a series of graphs as mean ± SEM. The effects of acute exercise vs. control and the effect of training data were analyzed using a 2-

Way-ANOVA for repeated measures via Prism 6 (GraphPad, Inc., La Jolla, CA). 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 analyzed and plotted using repeated measured 2-way-ANOVA in Prism 6 (GraphPad, Inc., La Jolla, CA). 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 analyzed using 2-way-ANOVA for repeated measures that was performed on the group scores using Prism 6 (GraphPad, Inc, La Jolla, CA). To explain the decrease in the Shannon diversity index of the species level in the after-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 before-and after-SC sampling of the GXT1 using Excel, Prism 6 (Graphpad Inc., La Jolla, CA), and online Venny version 2.1, website (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 before-and after-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 (graphpad Inc., La Jolla, CA).

Results

Data collected before and after the incremental exercise tests and standing controls are presented below to document the effects of acute exercise and the effects of 12 weeks of moderate exercise training. To that end, twelve weeks of exercise training resulted in an increase in maximal aerobic capacity (VO2max) 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 ($P=0.027$) 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/SC2) sets of tests (Figure 2B). At the genus level, exercise training had

no effect (P > 0.05) on the response to the acute exercise as far as the relative abundance of Clostridium and Dysgonomonas (Figure 2A and 2B).

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 Figure 4). However, the relative abundance of Proteobacteria, in samples collected following the standing control trials (after-SC1 and after-SC2) were significantly different (P = 0.0049) suggesting an effect of 12 week-training on the acute response to observing the GXT in the standing control horses (Figure 3A). There was no effect of training (P>0.05) on other phyla, including Bacteroidetes, Spirochaetes, Firmicutes, and Fibrobacteres (Figure 3B and 3C) and (Figure 4A and 4B) respectively.

At the level of species, Fusicatenibacter saccharivorans, (P = 0.031) and Treponema zioleckii (P = 0.048), were among 56 bacterial species that significantly decreased in the pre-training standing control (SC1) (Figure 9). There were no alterations (P>0.05) in these species following GXT1, GXT2, or SC2 (Data not shown). Interestingly, Fusicatenibacter saccharivorans and Treponema zioleckii showed higher relative abundances (P = 0.039) and (P = 0.0156) 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

Interestingly there was a significant decrease in the species diversity in the following the pre-training standard control trial (SC1) with the index dropping (P = 0.0219) 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 = 0.0038) 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 either 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=0.0179) 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=0.0156) 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 analyzed at bacteria genus or species level (Figure 6A and 6B) and (Figure 7A and 7B) respectively.

Shared and unique species in the SC 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 versus 53 unique species after), Figure 8. The names of these unique species are not shown (Data not shown). Additionally, there were significant decreases in the relative abundances of 56 species after the SC horses watched the GXT horses running on the treadmill (Figure 9). P values of these changes are shown in Table 2.

Discussion

The central hypotheses of the present study were that intense acute exercise would induce a change in the GM 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-recognized responses to intense exercise that alter the internal milieu of the gastrointestinal tract. 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 (Hodgson et al., 2014). 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 GM after weeks of submaximal endurance exercise training including changes in Bacteroidetes and Proteobacteria in mice, changes in Bacteroidetes and Firmicutes in the wheel running juvenile rats, and changes in Firmicutes and Proteobacteria in rats (Evans et al., 2014; Mika et al., 2015; Liu et al., 2015) respectively. 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 GM 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.

A notable finding of the present study was that after ~5 min of standing while watching or listening to the horses running 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 watching the horses run on the treadmill. While purely speculative, the anticipatory response in the unfit but acclimatized horses could have been related to an increase in gastric acid secretion which has been shown to increase when horses are brought in from paddocks and placed in stalls (Hunter et al., 1999). In other species, decreases in gastric pH have been associated with decreases in the abundance of Clostridium spp. (Dial et al., 2005). However, we should emphasize 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 and any suggestion that watching the horses run on the treadmill altered the physiology of the horses standing in the stalls is pure speculation. Studies of rodents subjected to non-exercise stressors have documented that major changes in the hypothalamic pituitary adrenal (HPA) axis and the Autonomic Nervous System lead to increases in the circulating cortisol and catecholamines (Carabotti et al., 2015), and the authors speculate that these molecules react with the GM leading to changes in the bacterial profile with substantial increase in detrimental Clostridial species (Freestone, 2013; Evans et al., 1948). In the present study we found a decrease in the abundance of the genus Clostridium suggesting that the horses of the present study were not undergoing stress as defined in the rodent studies cited above. This is supported by previously published studies where we have demonstrated that there are no changes in cortisol, heart rate, and blood pressure in the standing control horses (Gordon et al., 2007). Interestingly, there was no decrease in the abundance of genus Clostridium during the post-training standing control (SC2).

Another interesting 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 imparts 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 GM for horses (Evans et al., 2014; Mika et al., 2015; Liu et al., 2015).

An unexpected finding of the present study was the observation that the pre-training standing control trial (SC1) showed broader species based alternations. There were reductions in the number of unique species when the horses underwent the standing control where they either stood quietly or ate while watching or listening to the horses running 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 disturbance occurred in the GM system. In other species, chronic changes in the GM that were not related to acute exercise or exercise training have been shown to have the potential to affect the health of the host and have been linked to alterations in immune function, inflammatory bowel disease and ulcerative colitis (Piknova et al., 2008; Takada et al., 2013; Kanai et al. 2015) . However, the changes observed in the present study were transient 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 all this is all pure speculation as we only examined the response to acute intense exercise before and after the horses underwent the 12 weeks of exercise conditioning.

Summary

The observed changes in the pre-training standing control are interesting but we have no data to support any physiological mechanism or biological implications associated with this response. One can speculate that if such changes were to last long term there would be potential for detrimental effects on the host. As an effect of exercise training, ultimately the responses seen in the first battery of standing control trials were not a factor after 12 weeks of exercise conditioning. On a practical level, the present study provides information benefiting those seeking ways to improve the health and well-being of the athletic horse. Furthermore, acute intense exertion does not appear to alter the gut microbiome of the horses running on the treadmill.

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List of Tables

Table 1. Exercise Training Protocol Groups and Standard Exercise Procedure (SEP)

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)

Table 2. *P* values corresponding to Figure 12 of the species changes following the pre-training standing control trial (SC1). The empty cells mean that the corresponding species relative abundance is zero in all horses.

Before-SC	After-SC	P-Value
Syntrophococcus sp.		**0.034917**
Clostridium propionicum		**0.045813**
Bacteroides coprocola	*Bacteroides coprocola*	**0.024703**
Tepidimonas spp.		**0.019374**
Bacteroides nordii		**0.038033**
Cellulosilyticum spp.	*Cellulosilyticum spp.*	**0.02904**
Natranaerovirga pectinivora	*Natranaerovirga pectinivora*	**0.038723**
Magnetococcus spp.		**0.043531**
Bacteroides stercorirosoris	*Bacteroides stercorirosoris*	**0.036849**
Eggerthella lenta	*Eggerthella lenta*	**0.018087**
Rhodobacter sp.		**0.018743**
Parasutterella secunda	*Parasutterella secunda*	**0.046473**
Anaerovorax odorimutans	*Anaerovorax odorimutans*	**0.031656**
	Rubellimicrobium spp.	**0.029097**
	Bacteroides oleiciplenus	**0.036549**
Eubacterium rangiferina		**0.033162**
Sporocytophaga sp.		**0.008085**
Treponema parvum	*Treponema parvum*	**0.038601**
Collinsella intestinalis		**0.027308**
Desulfuromonas spp.	*Desulfuromonas spp.*	**0.034666**
Dehalobacterium spp.	*Dehalobacterium spp.*	**0.015799**
Ornatilinea apprima	*Ornatilinea apprima*	**0.02322**
Paludibacter sp.	*Paludibacter sp.*	**0.006865**
Treponema saccharophilum		**0.040727**
Psychrobacillus psychrodurans		**0.01641**
Clostridium hylemonae	*Clostridium hylemonae*	**0.005602**
Hespellia porcina		**0.040039**
Bacteroides plebeius	*Bacteroides plebeius*	**0.039659**
Mogibacterium diversum	*Mogibacterium diversum*	**0.001104**
Spiroplasma culicicola	*Spiroplasma culicicola*	**0.018601**
Opitutus spp.	*Opitutus spp.*	**0.047812**
Geofilum rikenellaceae bacterium	*Geofilum rikenellaceae bacterium*	**0.038019**
Butyrivibrio spp.		**0.035821**
Lachnospira pectinoschiza	*Lachnospira pectinoschiza*	**0.006011**
Fusicatenibacter saccharivorans	*Fusicatenibacter saccharivorans*	**0.031455**
Nodosilinea bijugata	*Nodosilinea bijugata*	**0.001205**
Cloacibacterium normanense	*Cloacibacterium normanense*	**0.022815**
Erysipelothrix sp.	*Erysipelothrix sp.*	**0.035712**
Bacteroides luti		**0.04311**
Desulfosporosinus meridiei	*Desulfosporosinus meridiei*	**0.014811**
Garciella nitratireducens		**0.045875**
Hallella spp.		**0.009493**
Pelospora spp.	*Pelospora spp.*	**0.007583**
Sphingobacterium multivorum	*Sphingobacterium multivorum*	**0.030345**
Clostridium fusiformis	*Clostridium fusiformis*	**0.038359**
Spiroplasma velocicrescens	*Spiroplasma velocicrescens*	**0.020527**
	Paracoccus aminovorans	**0.034546**
Mucinivorans rikenellaceae bacterium		**0.030052**
Treponema succinifaciens	*Treponema succinifaciens*	**0.036667**
Cloacibacterium sp.		**0.043173**
Treponema zioleckii	*Treponema zioleckii*	**0.048365**
Gordonibacter pamelaeae		**0.04663**
Prevotella melaninogenica		**0.042427**
Adlercreutzia spp.		**0.050395**
Clostridium citroniae		**0.05405**
	Brevundimonas vesicularis	**0.049326**

Figure Captions

Figure 1. The Experimental design. A. Pre-training, GXT1 and SC1. B. Post-training, GXT2 and SC2.

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. Levels of genus *Dysgonomonas.* No changes in all ($P > 0.05$).

Figure 3. Acute exercise and exercise training effects at the phylum level. A. *Proteobacteria* there was only significant effect ($P = 0.0049$) 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 ($P > 0.05$).

Figure 5. Acute exercise and exercise training effects on Shannon Diversity Index at the species level. Values indicate mean ± standard error. *Species diversity index has significantly changed in the SC group due to GXT1 watching ($P = 0.0219$). ** Significant effect ($P = 0.0038$) of the exercise training on the response to the post-training acute exercise.

Figure 6. Principle Coordinate Analysis (PcoA) of the GXT and SC groups at the genus level. A. Pre-training. B. Post-training. PcoA of the pre-training showed significant separation between Before and After-SC ($P = 0.0179$).

Figure 7. Principle Coordinate Analysis (PcoA) of the GXT and SC groups at the species level. A. Pre-training. B. Post-training. PcoA of the pre-training showed significant separation between Before and After-SC ($P = 0.0156$).

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.

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

Figure 1

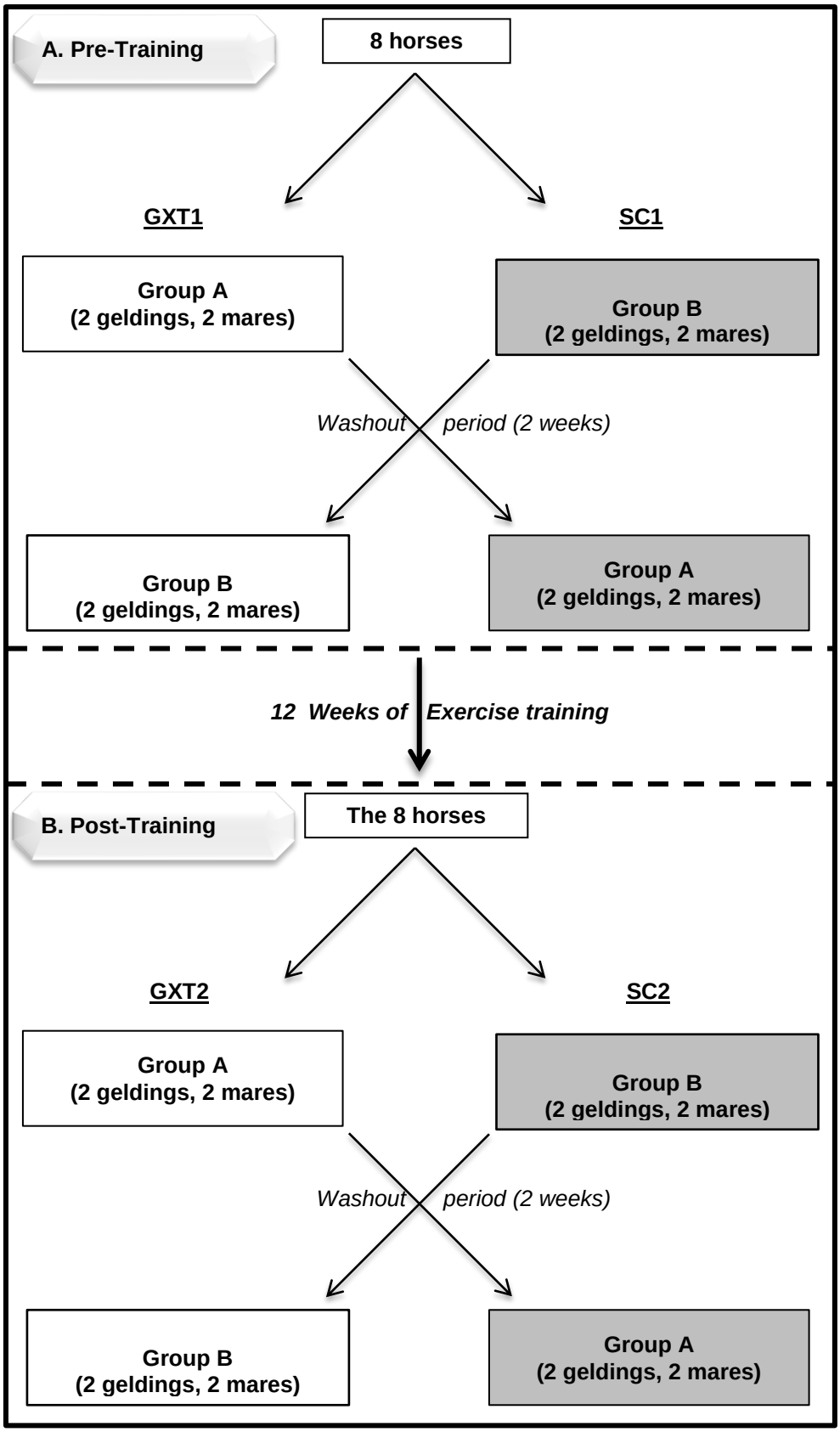
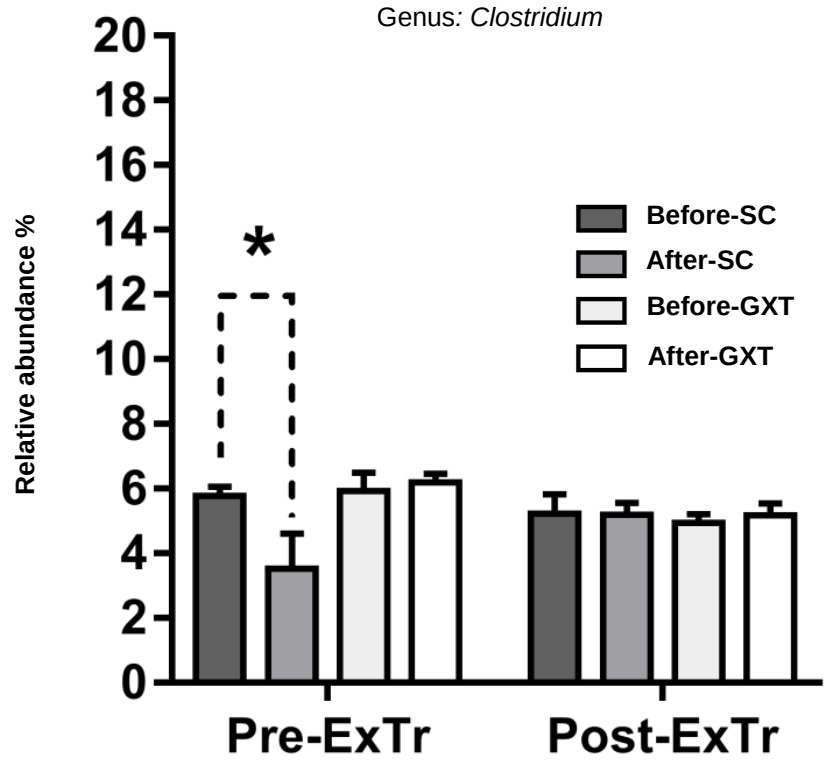

A. Pre-Training
8 horses
GXT1
SC1
Group A
(2 geldings, 2 mares)
Group B
(2 geldings, 2 mares)
Washout period (2 weeks)
Group B
(2 geldings, 2 mares)
Group A
(2 geldings, 2 mares)
12 Weeks of Exercise training
B. Post-Training
The 8 horses
GXT2
SC2
Group A
(2 geldings, 2 mares)
Group B
(2 geldings, 2 mares)
Washout period (2 weeks)
Group B
(2 geldings, 2 mares)
Group A
(2 geldings, 2 mares)

Figure 2.

A.


Genus: *Clostridium*
Relative abundance %
20
18
16
14
12
10
8
6
4
2
0
*
Before-SC
After-SC
Before-GXT
After-GXT
Pre-ExTr
Post-ExTr

B.

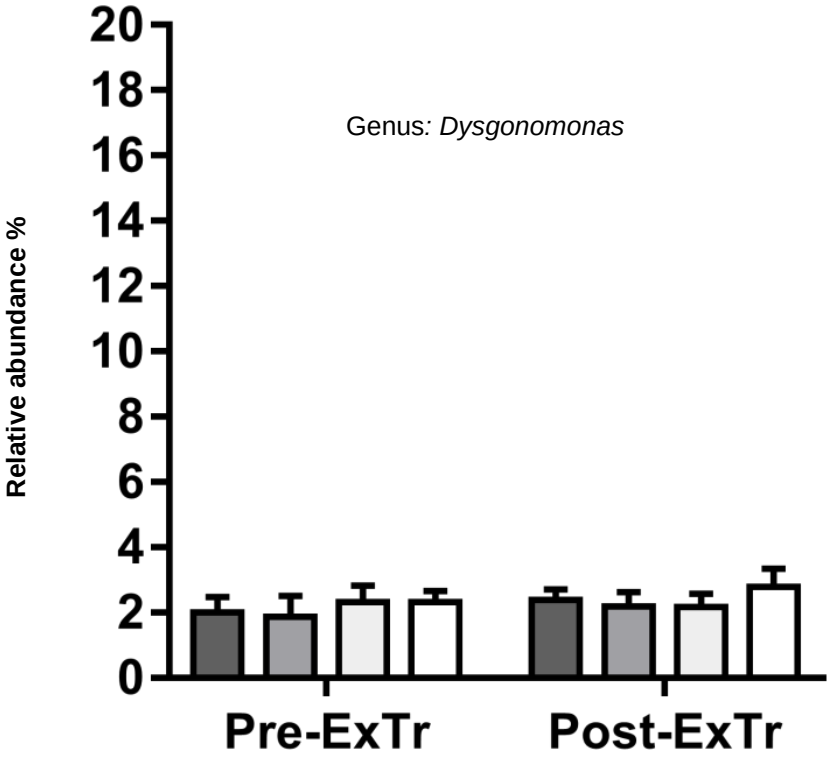
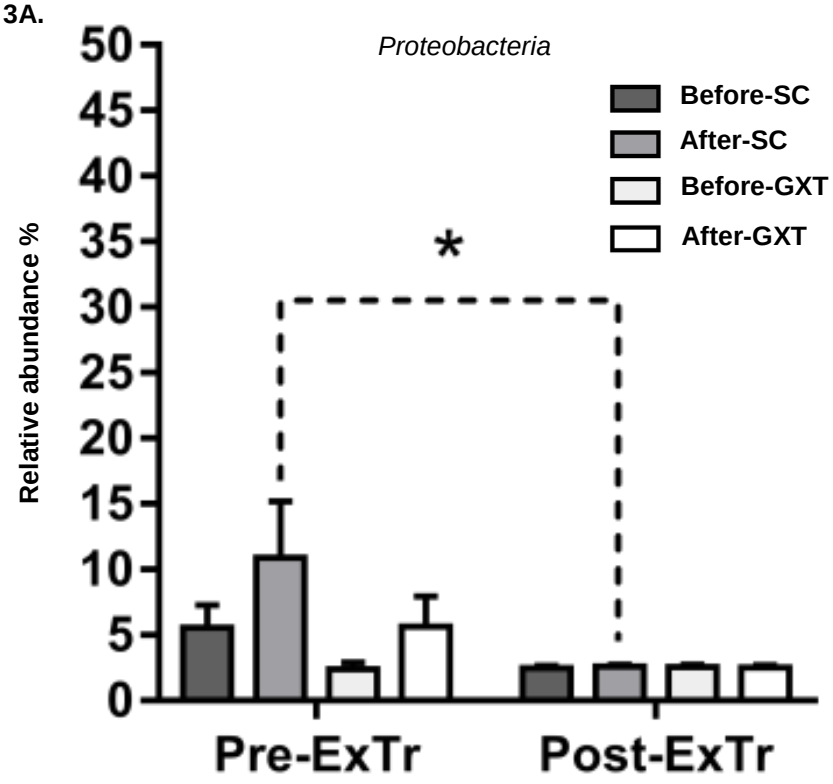

Genus: *Dysgonomonas*
Relative abundance %
20
18
16
14
12
10
8
6
4
2
0
Pre-ExTr
Post-ExTr

Figure 3.

3A.



3B.

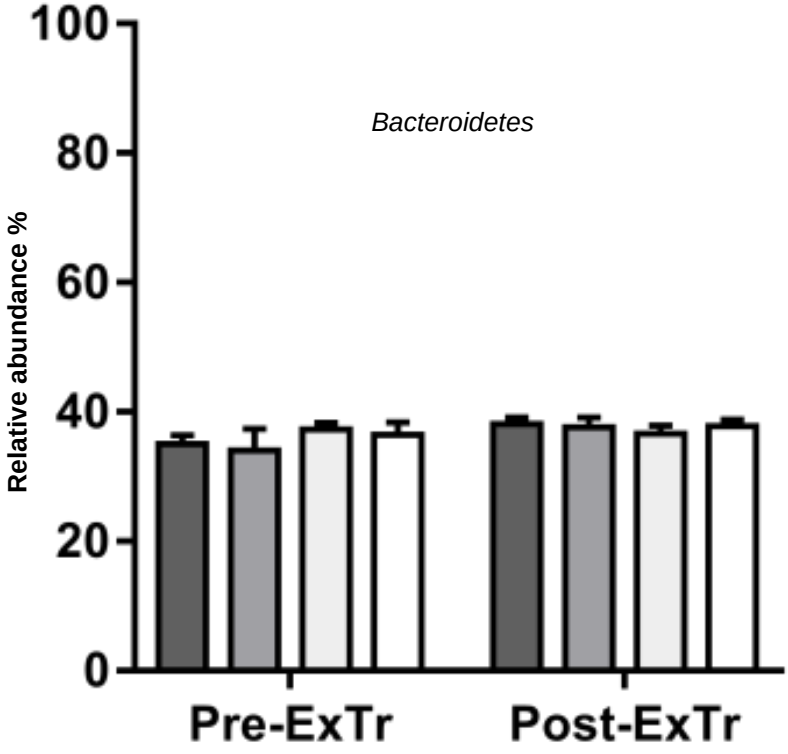


Figure 3C.

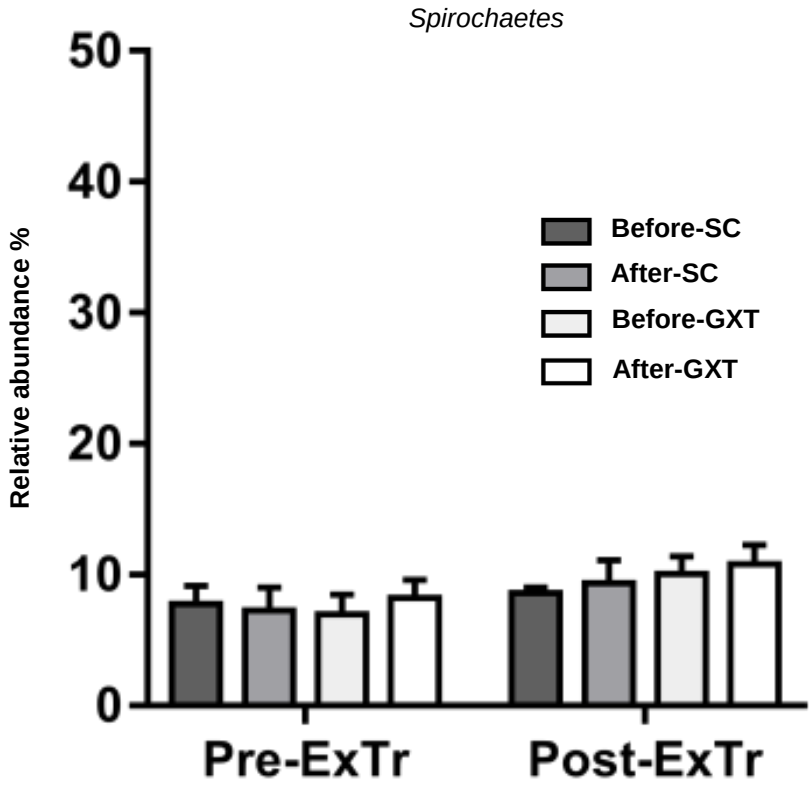
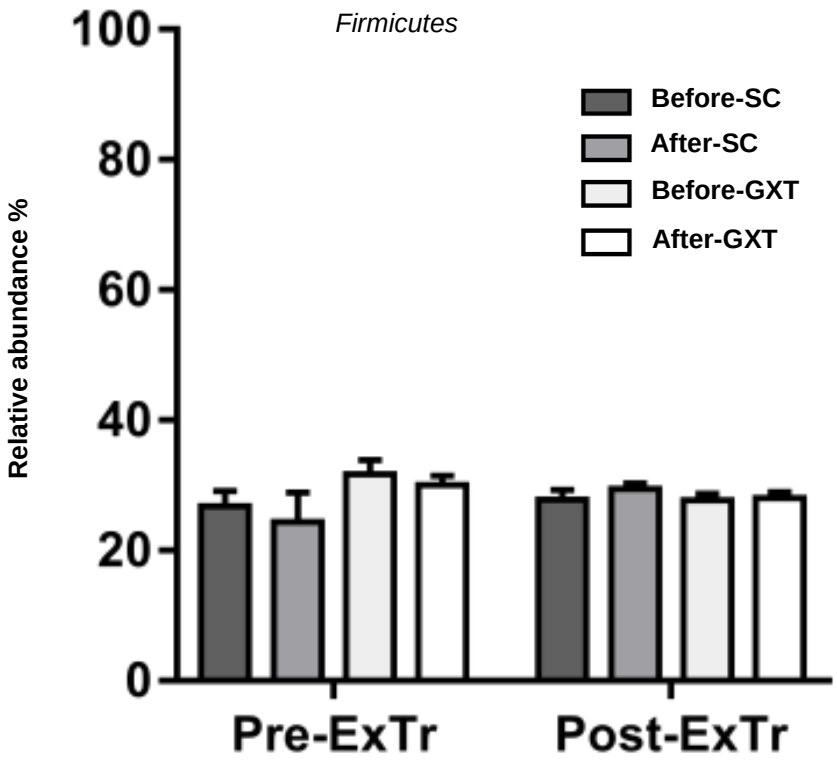

Spirochaetes
Relative abundance %
50
40
30
20
10
0
Before-SC
After-SC
Before-GXT
After-GXT
Pre-ExTr
Post-ExTr

Figure 4.

A.


Firmicutes
Before-SC
After-SC
Before-GXT
After-GXT
Relative abundance %
100
80
60
40
20
0
Pre-ExTr
Post-ExTr

B.

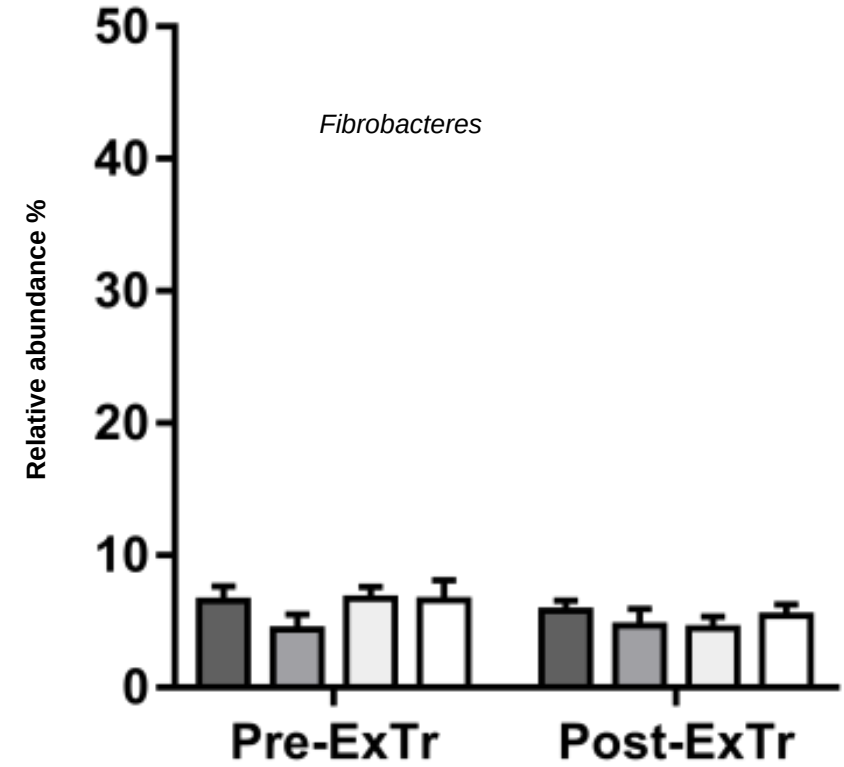

Fibrobacteres
Relative abundance %
50
40
30
20
10
0
Pre-ExTr
Post-ExTr

Figure 5.

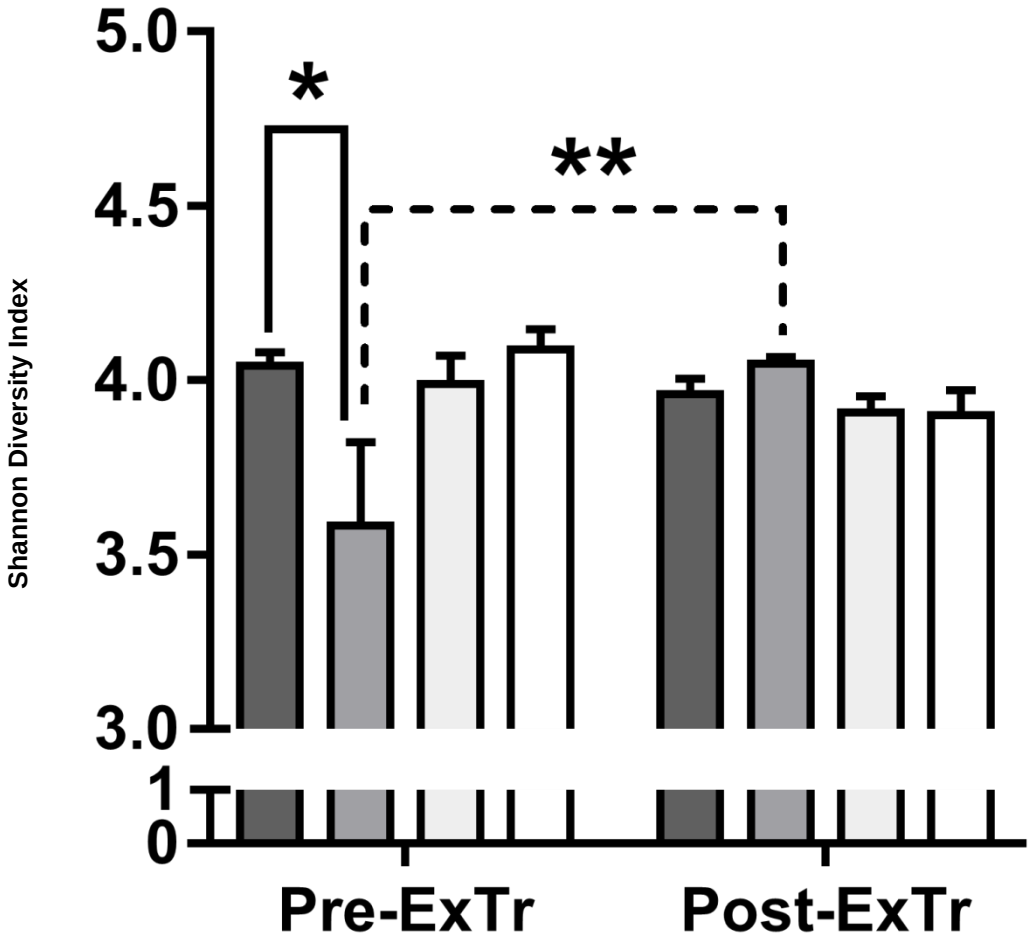
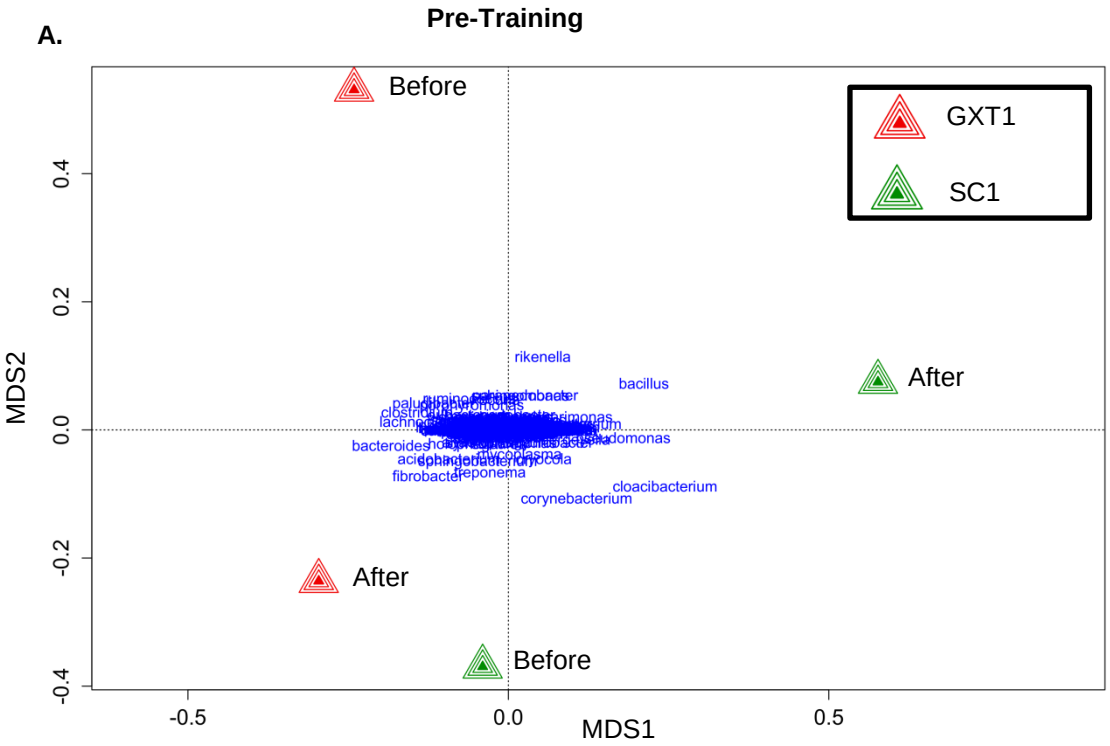


Figure 6.

A.


Pre-Training
Before
GXT1
SC1
After
rikenella
bacillus
bacteroides
fibrobacter
treponema
cloacibacterium
corynebacterium
After
Before
MDS1
MDS2
-0.5
0.0
0.5
0.4
0.2
0.0
-0.2
-0.4

B.

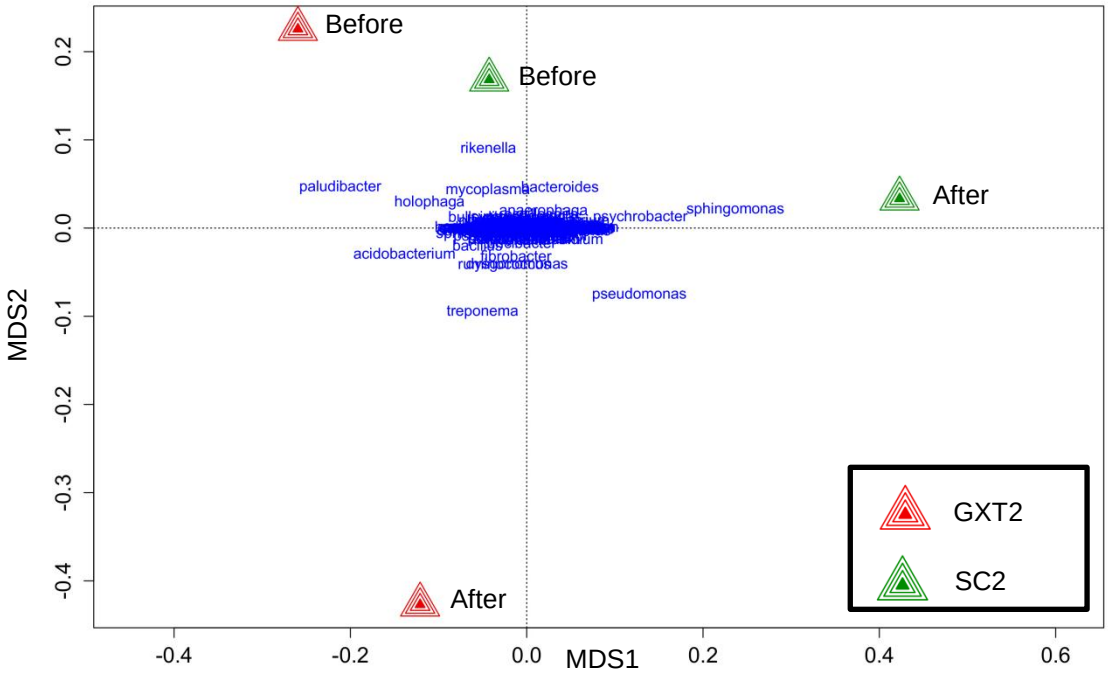

Post-Training
Before
Before
rikenella
paludibacter
mycoplasma
bacteroides
holophaga
sphingomonas
psychrobacter
After
acidobacterium
fibrobacter
pseudomonas
treponema
GXT2
SC2
After
MDS1
MDS2
-0.4
-0.2
0.0
0.2
0.4
0.6
0.2
0.1
0.0
-0.1
-0.2
-0.3
-0.4

Figure 7.

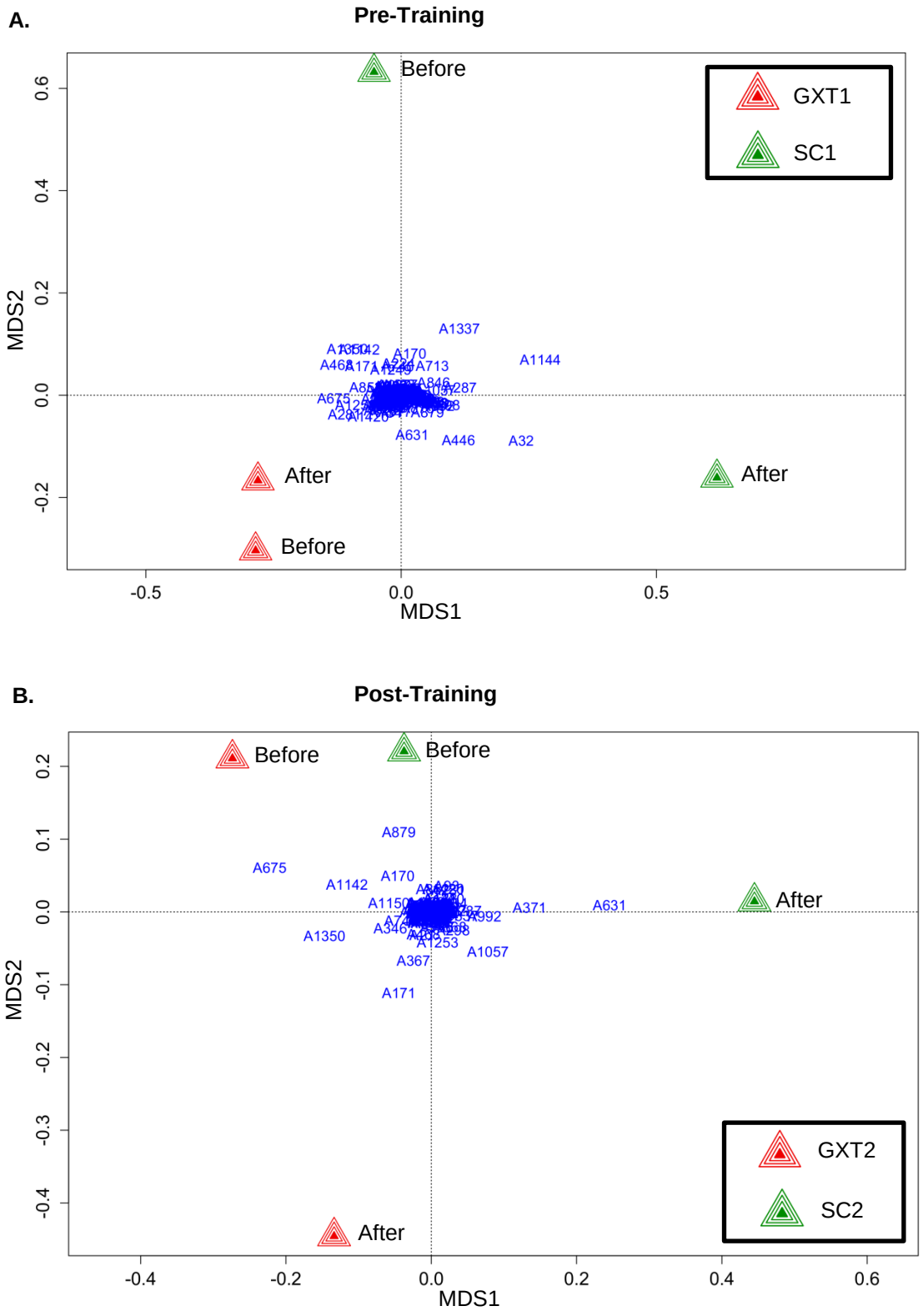

A.
Pre-Training
Before
GXT1
SC1
A1337
A1144
A631
A446
A32
After
After
Before
MDS2
MDS1
0.6
0.4
0.2
0.0
-0.2
-0.5
0.0
0.5
B.
Post-Training
Before
Before
A879
A675
A1142
A170
A371
A631
A1350
A1253
A1057
A367
A171
After
After
GXT2
SC2
MDS2
MDS1
0.2
0.1
0.0
-0.1
-0.2
-0.3
-0.4
-0.4
-0.2
0.0
0.2
0.4
0.6

Figure 8.

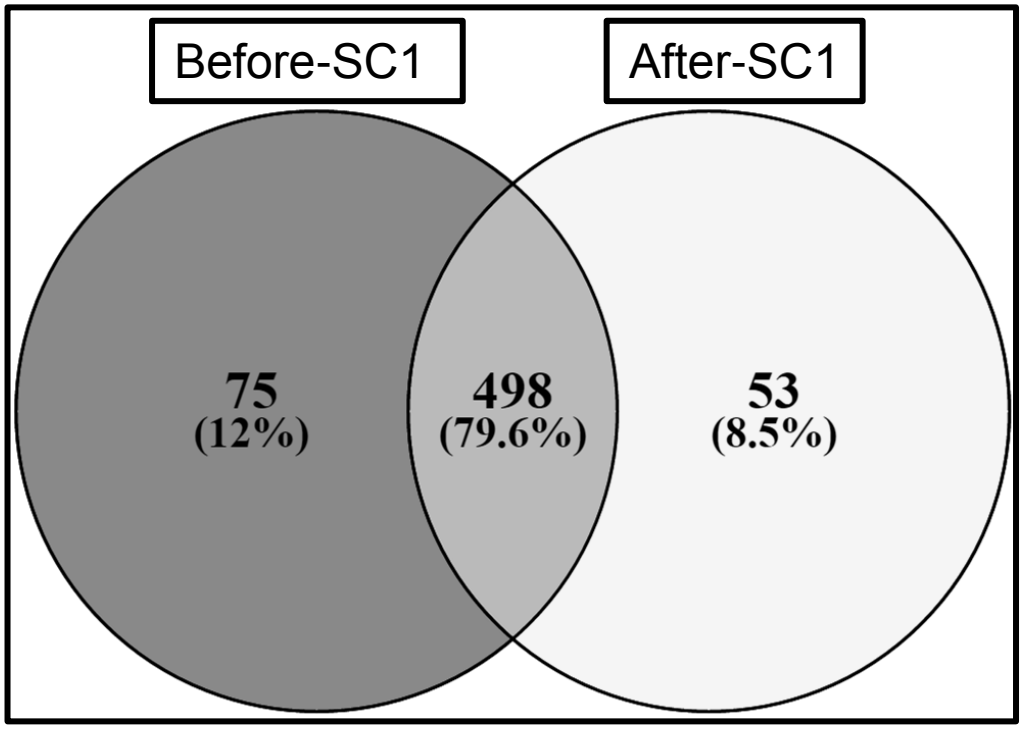
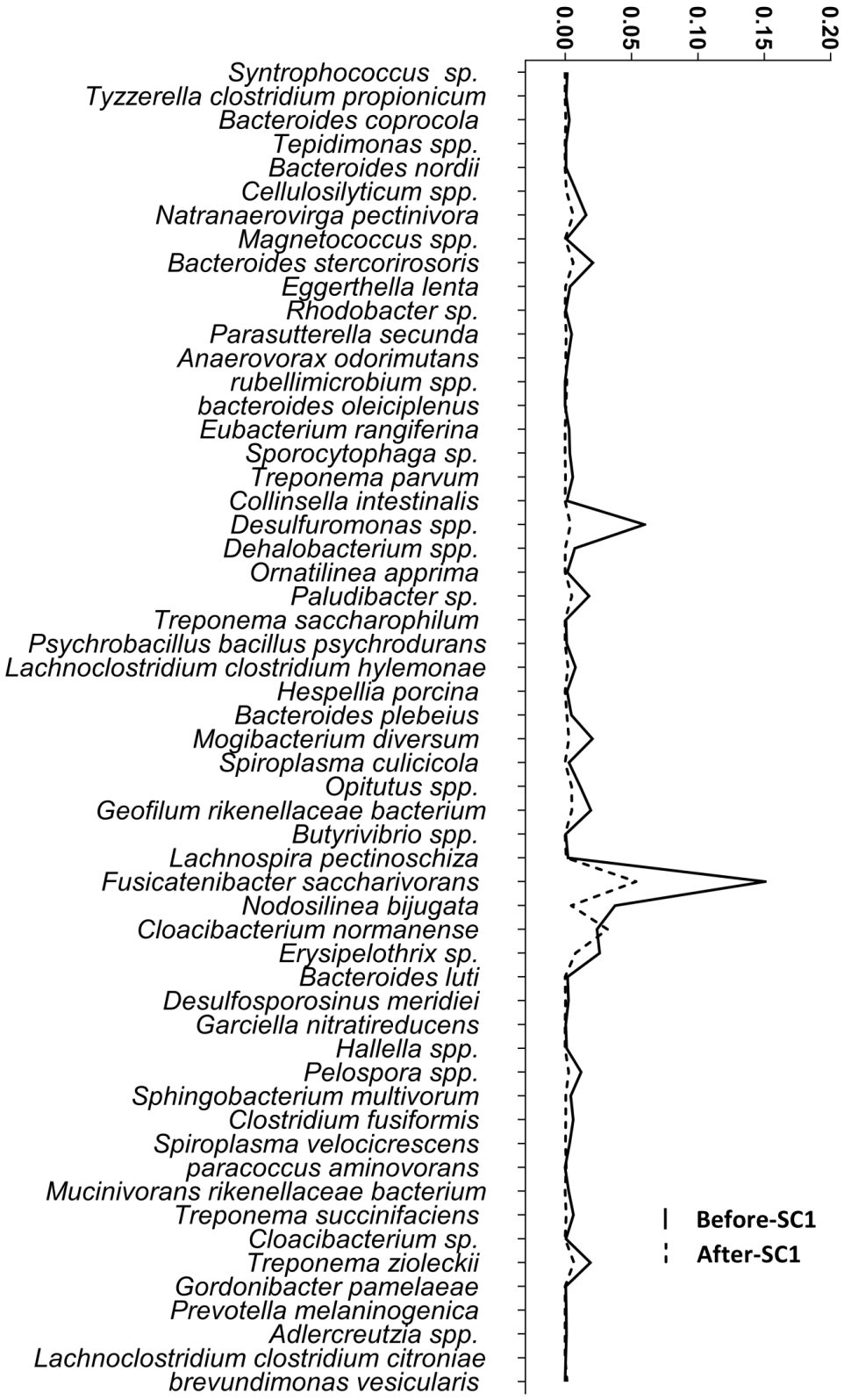


Figure 9.


0.00
0.05
0.10
0.15
0.20
Syntrophococcus sp.
Tyzzerella clostridium propionicum
Bacteroides coprocola
Tepidimonas spp.
Bacteroides nordii
Cellulosilyticum spp.
Natranaerovirga pectinivora
Magnetococcus spp.
Bacteroides stercorirosoris
Eggerthella lenta
Rhodobacter sp.
Parasutterella secunda
Anaerovorax odorimutans
rubellimicrobium spp.
bacteroides oleiciplenus
Eubacterium rangiferina
Sporocytophaga sp.
Treponema parvum
Collinsella intestinalis
Desulfuromonas spp.
Dehalobacterium spp.
Ornatilinea apprima
Paludibacter sp.
Treponema saccharophilum
Psychrobacillus bacillus psychrodurans
Lachnoclostridium clostridium hylemonae
Hespellia porcina
Bacteroides plebeius
Mogibacterium diversum
Spiroplasma culicicola
Opitutus spp.
Geofilum rikenellaceae bacterium
Butyrivibrio spp.
Lachnospira pectinoschiza
Fusicatenibacter saccharivorans
Nodosilinea bijugata
Cloacibacterium normanense
Erysipelothrix sp.
Bacteroides luti
Desulfosporosinus meridiei
Garciella nitratireducens
Hallella spp.
Pelospora spp.
Sphingobacterium multivorum
Clostridium fusiformis
Spiroplasma velocicrescens
paracoccus aminovorans
Mucinivorans rikenellaceae bacterium
Treponema succinifaciens
Cloacibacterium sp.
Treponema zioleckii
Gordonibacter pamelaeae
Prevotella melaninogenica
Adlercreutzia spp.
Lachnoclostridium clostridium citroniae
brevundimonas vesicularis
Before-SC1
After-SC1

CHAPTER 4: Journal Article (Accepted)

Exercise Training-Induced Changes in the Gut Microbiota of Standardbred Racehorses

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Abstract

Exercise has a significant effect on different physiological systems in the body of human and animals. Only limited numbers of published studies in laboratory animals or humans have shown the effect of exercise on the gut microbiota, and no studies have shown this effect in horses. In this study, 8 horses (4 mares, 4 geldings) were exercise trained for 12 weeks, and 4 additional mares were used as a parallel seasonal control. To identify bacterial community changes over time for both groups, rectal fecal samples were collected, DNA was extracted, and the 16S rRNA gene (V3-V4) was sequenced using the IIllumina Miseq platform. One-way ANOVA, Shannon diversity index, and Principle Coordinate Analyses were used to identify differences between and among samples. The exercise training group showed significant changes in the levels of Bacteroidetes, Proteobacteria, and Spirochaetes phyla ($P<0.05$), while there were no changes in the gut microbiota of the seasonal control group through the three months of the study ($P>0.05$). Moreover, with training two genera significantly changed in their relative abundance over time, namely Clostridium and Dysgonomonas ($P<0.05$). Dysgonomonas spp was significantly changed in abundance during the exercise training period ($P<0.05$). Treponema spp. showed significant changes during the exercise training period ($P<0.05$). Shannon diversity index was decreased ($P<0.05$), in the exercise group at the beginning of the study, but then returned to pre-training levels. Principle coordinate analysis showed significant separation between time points of the exercise training group as far as the levels of genera

and species (P<0.05) represented. Our results show that exercise training influences the gut microbiota especially at the beginning of training.

Introduction

The gut microbiota (GM) is an important “micro-organ” in the gastrointestinal tract (GIT) of animals and humans, hosting a unique and complex population of microbial communities including bacteria, protozoa, and fungi (Clarke, 1977; Dehority, 1997; Hespell et al., 1997). Bacterial numbers have been estimated to be 1011 colony forming units/g inside the human colon and ~12.3X108 colony forming units/g in horse colon (Berg, 1996; Mackie and Wilkins, 1988). Horses are hindgut fermenters, and as herbivores they depend more heavily on the fermentation products of billions of naturally present bacteria and protozoa to generate volatile fatty acids; and therefore, energy. (Al Jassim and Andrews, 2009). Some gut microbial communities benefit the host by breaking down chemical toxins into simple molecules that do little or no harm (Swann et al., 2009). For instance, hydrazine, a potent toxic substance found in the environment, has been shown to be less toxic for conventional rats, than germ-free (GF) rats as indicated by increasing urine concentrations of 2-aminoadipate (2-AA), argininosuccinate, creatine, Na-acetylcitrulline, and taurine, and by decreasing the concentrations of citrate, 2-oxoglutarate, succinate, creatinine, TMAO and hippurate (Swann et al., 2009). Gut microbial communities accelerate food energy availability through bacterial metabolism, converting complex nutrients into forms of energy that can be easily absorbed by the host

via the fermentation of complex carbohydrates into short chain fatty acids (SCFAs) (Krajmalnik-Brown et al., 2012). In some instances, the GM can have detrimental effects on a host. For example, studies of mice have shown that alterations in the GM affect monosaccharide absorption, fat storage and insulin resistance which, in turn, may play a role in the onset of obesity and Type II diabetes (Backhed et al, 2004). Obesity and insulin resistance are important health concerns for owners of horses, especially during summer months when access to high quality pasture may be coupled with less physical activity (Giles et al., 2014). In an in vitro horse model, starch enrichment of fecal samples resulted in increased propionate and acetate production along with increases in Megasphaera elsdenii (Biddle et al., 2013). Those observations are similar to the changes seen, in vivo, when horses are induced with laminitis (Garner et al., 1975; Milinovich et al., 2008).

While the GM can be affected, disturbed, or altered by different factors, such as various drugs and hydration status, diet and exercise have been shown to have major impacts on gut microbial communities (Imhann et al., 2016; Mai, 2004; Redondo et al., 2015; Evans et al., 2014, Destrez et al., 2015). Human subjects exposed to physical activity and a calorie restricted diet showed increases in the abundance of Bacteroides fragilis and Lactobacillus spp., and lowering of Clostridium coccoides, Bifidobacterium longum, and Bifidobacterium adolescentis (Santacruz et al., 2009). Exercise exposed rats showed increases in the abundance of Lactobacillus, Bifidobacterium and B. coccoides-Eubacterium rectale groups of different correlations with ghrelin and leptin plasma levels

(Queipo-Ortuño et al., 2013). Another twelve week study of exercised trained-high fat diet fed-mice showed alternation in the profile of the GM and decreased the body weight gain (Evans et al., 2014). In obese rats, exercise was associated with increased abundance of Pseudomonas and Lactobacillus (Petriz et al., 2014). We are unaware of any studies that have looked at the effects of exercise training on the gut microbiome of healthy horses. The present study tests the hypothesis that exercise training would alter the gut microbiome of the unfit horse.

Materials and Methods

Animals

All methods used in this study were approved by the Rutgers Institutional Animal Care and Use Committee. Twelve unfit, healthy, Standardbred horses (8 mares and 4 geldings) were used for this project. The exercise training group (ExTr) consisted of eight mature (3-8 yrs; 446-517 kg) horses (4 mares and 4 geldings). The horses in the ExTr group were acclimated to the Rutgers Equine Science Center training personal, housing, and training equipment for at least 2 months before the experiment. After acclimation, the horses underwent initial testing followed by exercise training for 12 weeks. An additional 4 mares (24-25 yrs, ~500 kg) served as a parallel seasonal control (SC) group. All of the horses were dewormed and vaccinated per standard veterinary practice. Animals were fed a maintenance ration of alfalfa/grass hay ad libitum (6kg/day) and pelleted grain supplement (3kg/day). Water and mineral blocks were provided ad libitum. The

ExTr group performed their daily morning exercise followed by turnout in groups of 4 in their respective 2-acre “dry lot” paddocks overnight. The SC group was housed in an adjacent “dry lot” paddock for the duration of the experiment.

Exercise Training and Incremental Exercise Test

The general experimental design for the ExTr horses included three phases. The first phase was a pre-training incremental exercise test (GXT) where the horses ran on the treadmill (Sato I, Lexington, KY) to measure markers of fitness and exercise performance. This was followed by 12 weeks of exercise training, where each week the horses were trained 4 days/week in a motorized free-stall exercise machine (Equi-ciser, Calgary, Canada) and 1 day on the treadmill (Table 1). After the 12 weeks of training the horses performed a second GXT to document the effects of training.

Incremental Exercise Test (GXT)

Maximal oxygen uptake (VO2max) and markers of exercise performance were measured as described in Kearns and McKeever (2002). Briefly, each horse ran on a high-speed horse treadmill (Sato I, Lexington, KY) at a fixed 6% grade, starting at an initial speed of 4 m/s for 1 min. Speed was then increased to 6 m/s, followed by incremental increases of 1 m/s every 60s (omitting 5 m/s) until the horses reached fatigue, defined as the point in which a horse could no longer 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 (VO2) and carbon dioxide production (VCO2) were measured every 10 seconds using the open flow indirect calorimeter (Oxymax-XL, Columbus Instruments, Columbus, OH) during each GXT.

Fecal Sample Collection

Fecal samples were obtained from all horses at the same time of day at the following time points over the course of the experiment: 24 hrs before and after the pre-training GXT; at two week intervals during the training period; and 24 hrs before and after the post-training GXT. Sampling during the training period occurred on Mondays as none of the horses exercised on Saturdays or Sundays. Fecal samples were collected via rectal palpation by the same investigator (Ali Hassan Daghir Janabi). Feces in the lower GI tract were first cleared, and samples were taken from the inner end of the rectum, ~45 cm of distance. Fecal samples were placed on sterile paper and opened using sterile techniques to collect 0.25 g samples. The samples were placed into collection tubes containing beads (MP Biomedical; (SKU# 116914100) and 300 uL of buffer solution containing CTAB. Each tube was snap frozen in liquid nitrogen and then stored at -80o C.

DNA Extraction

DNA was extracted using a protocol (Kerkhof and Ward, 1993; McGuinness et al., 2006; Männistö et al, 2009) modified as follows. Samples were subjected to five quick freeze/thaw cycles with liquid nitrogen (-80 o C) and 55o C hot bath. To

the frozen samples, 100 µl of Solution 1 (50 mM glucose, 10 mM EDTA, 25mM Tris-Cl; pH 8.0), 50 µl of lysozyme solution (4 mg in 1 ml of Solution 1) and 50 µl of 500mM 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 minutes. Samples were then spun down in micro-centrifuge at (~ 16,000 x g) for 3 minutes. The top phase was transferred to a new centrifuge tube preloaded with 800 ul phenol: chloroform: isoamyl alcohol; 25:24:1 (>pH 7.0). Tubes were vortexed for 1 minute at maximum speed and then centrifuged for 3 minutes at (~ 16,000 x g). The top layer was then transferred to a new micro-centrifuge tube, and 200 ul of C3, inhibitor removal solution (MoBio Laboratories Inc., catlg# 12830-50-3), was added to each tube. The tubes containing the C3 solution and the DNA extract were vortexed briefly and then incubated at 4 o C for 5 minutes in ice. Following incubation the tubes were spun at 13000 xg for 1 minute (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), and checked for amplification of the 16s rRNA gene using universal bacterial primers: 27F: AGAGTTTGATCCTGGCTCAG and 1522r: AAGGAGGTGATCCAICCGCA.

Sequencing (Miseq)

The V3-V4 variable region of the 16S rRNA gene was amplified using universal bacterial 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, USA) under the following conditions: 94°C for 3 minutes, followed by 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final elongation step at 72°C for 5 minutes 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. Then the purified PCR product was used to prepare DNA library by following Illumina TruSeq DNA library preparation protocol. 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 MR DNA analysis pipeline (MR DNA, Shallowater, TX, USA). Briefly, paired reads were joined and trimmed of barcodes. Sequences <150bp 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

Relative abundance of bacteria at phyla, genera, and species levels for ExTr (n=8 horses / time point) and SC (n=4 horses / time point) groups were analyzed and graphed separately. Mean ± SEM were calculated. One way ANOVA for Repeated measures was used to analyze the data within each group (α=0.05). For multiple comparisons between time points, post-hoc analysis included an F test to identify significant differences between time points with significance set at a P value <0.05. Statistical analysis and graphs were performed using Prism 6 and Prism 7 respectively (GraphPad, Inc., La Jolla, CA). In order to test for bacterial diversity changes the Shannon diversity index was calculated for each horse at each time point using R software version 3.2.3 (Wooden Christmas Tree) with Vegan (Oksanen et al., 2016) and Mass packages (Venables and Ripley, 2002). The Shannon values were analyzed and plotted using one-way repeated measured ANOVA in Prism 6 and Prism 7 respectively (GraphPad, Inc.). Principle Coordinate Analysis (PCoA) was used to show distance between time points. The relative abundance of the community profiles of the horses in each time point were averaged and used to perform just the PCoA plot using R software version 3.2.3 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). To show if the separation between time points in PCoA were significant or not, one-way repeated measured ANOVA was performed on the site (time point) scores using Prism 6 (GraphPad, Inc.), ExTr group n=8 horses/time point, SC group n=4 horses/time point. To make over look of which bacteria have gone up or down during the study period, the mean and SEM of each time point from phyla, genera, and species members that suffered changes by ExTr were plotted using one graph for each of those taxa levels. Means and SEM were calculated using Excel.

Results

Twelve weeks of exercise training resulted in an increase ($P<0.05$) in maximal aerobic capacity (VO2max) from 148 ± 6 mL/kg/min before training to 162 ± 3 mL/kg/min after training. The results for the ExTr horses and the SC horses are presented in a series of graphs that depict changes if any, the phyla, genera, and species levels. Data are presented for the Shannon Diversity Index, Principal Coordinate Analysis, and Same Time Changes in the bacterial taxa.

Phyla Changes in Relative Abundance

Training resulted in changes in the relative abundance of major bacterial phyla. In the ExTr group, the relative abundance of the Bacteroidetes changed significantly over time (Figure 1A and Table S1A). Levels from week 0 to week 2 were increased significantly ($P<0.0001$), but then were not changed between week 2 and week 4 ($P>0.05$). From week 4 to week 6, levels dropped

significantly below week 4 values (P = 0.0261). The relative abundance of the Proteobacteria changed significantly over time (Figure 1B and Table S1B). Levels were stable for the first 2 weeks, but then significantly increased from week 4 to week 6 (P = 0.0115), and from week 6 to week 8 (P = 0.0254). It was also observed that training had a significant effect on the relative abundance of Spirochaetes changed significantly over time (Figure 1C and Table S1C). Levels of this phylum significantly increased from week 2 to week 4 (P = 0.0148), then dropped from week 8 to week 10 and week 12 below week 8 levels (P = 0.0381). There was no effect (P>0.05) of training on the two common phyla Firmicutes and Fibrobacteres (Figure 2A and B respectively).

At the phyla level, we observed no changes (P>0.05) in the relative abundance of the Bacteroidetes, Proteobacteria, Spirochaetes (Figure 3A, 3B, and 3C) or the relative abundances of the Firmicutes and Fibrobacteres (Figure 4A and 4B) in the seasonal control horses at any point during the experiment.

Genera Changes in Relative Abundance

The relative abundances of the Clostridium genus significantly changed in the ExTr group with an increase at week 2 (P = 0.0352). However, relative abundances of Clostridium then decreased significantly by week 4 (P = 0.0004) (Figure 5A and Table S1D). Bidirectional changes in the relative abundances of the Dysgonomonas genus over time during training. From week 2 to week 4 the level of these bacteria increased (P = 0.0004), but by week 6 it had decreased compared to week 4 levels (P = 0.0035) (Figure 5B and Table S1E).

The relative abundances of the Clostridium and Dysgonomonas genera were not changed (P>0.05) over the course of the experiment in the seasonal control horses (Figure 6A and B).

Species Changes in Relative Abundance

The relative abundances of the Treponema spp. were increased at week 4 (P = 0.0063) compared to week 2 and decreased between week 8 to week 10 (P = 0.023) (Figure 7A and Table S1F). The relative abundances of Dysgonomonas spp. increased from week 2 to week 4 (P = 0.0014) and then decreased in week 6 (P = 0.0014) and stayed at this decreased level until the end of the experiment which is almost the same as the pre-training levels (Figure 7B).

For the control horses we observed the levels of Treponema spp and Dysgonomonas spp were unchanged during the period of the study (P>0.05) (Figure 8A and 8B).

Shannon Diversity Index

There was a biphasic change in the Shannon Diversity Index in the ExTr group at the phylum level. Shannon diversity index decreased in week 2 when was compared to week 0 (P = 0.0384), but then increased from week 4 to week 6 (P = 0.0355) (Figure 9A and Table S2). No change was seen for the Shannon Diversity Index at the genera and the species levels (P>0.05) (Figure 9B and 9C).

The Shannon diversity index of phyla, genera, and species was unchanged over the course of the study in the SC group (P>0.05) (Figure 10A, 10B, and 10C).

Principle Coordinate Analysis

At the bacterial phyla level the PCoA of the ExTr group showed no separation between time points during the experimental period, (P > 0.05) (data not shown). However at the genus level the ExTr group showed separation between time points with significant separations of week 0 and week 2 (P = 0.0490), week 0 and week 6 (P = 0.0276), week 0 and week 10 (P = 0.0133), and week 0 and week 12 (P = 0.0127) which are presented in Figure 11. Finally, at the species level of the ExTr horses the PCoA showed significant separation between the time points in the ExTr group for week 0 and week 6 (P = 0.0103), week 0 and week10 (P = 0.0173), week 0 and week12 (P = 0.0094), and week 6 and week 10 (P = 0.0095), Figure 12.

There was lack of separation (P>0.05) in the SC group for all levels for the course of the study (data not shown).

Same Time Changes of Bacterial Taxa

To see which phylum, genus, and species members were going up or down at the same time point, we plotted the means and SEM in one graph for each of the taxa members (Figure 13). In week 4, Bacteroidetes, Proteobacteria, and Spirochaetes were significantly changed together, regardless whether they increased or decreased, Figure 13A. In case of genus members, in week 4,

Clostridium decreased significantly while Dysgonomonas significantly increased, Figure 13B. At the level of species, Treponema spp. and Dysgonomonas spp. significantly increased together in week 4 (Figure 13C). All the P values are in Table S1. No changes were found in the SC group (data not shown).

Discussion

Exercise Training

Exercise training has been reported to affect the GM in mouse models (Evans et al., 2014) specifically altering Bacteroidetes and Proteobacteria. Exercised rats also showed changes in bacterial phyla such as Firmicutes and Proteobacteria (Liu et al., 2015). To our knowledge this is the first study to show the effect of chronic exercise on GM of horses. Our study results show significant effects of ExTr on GM at the phyla, genus, and species levels, observations which agree with the above mentioned studies that utilized rodents.

Relative Abundance

In the present study training resulted in changes in the relative abundance of bacteria in the phylum Bacteroidetes, a major member of the microbial community in the gut of human and animals (Thomas et al., 2011). These bacteria play a very important role in development of intestinal mucosal immunity by initiation of T-Cell responses and prevent colonization by pathogenic bacteria. Moreover; some of these bacteria produce butyrate which has antineoplastic activity (Thomas et al¬., 2011). In the present study training altered

Bacteroidetes levels over time, with both significant increases and decreases in the relative abundance. The increases in the levels of these bacteria observed in the samples taken at week 2 and week 4 were the same, which may be related to an increased demand for bacterial products such as butyrate as an energy source for the horse hosts. It could also be related to an increased need for enzymes to break down indigestible polysaccharides (Bjursell et al., 2006). By week 6 the level of Bacteroidetes had gone down and stayed at the same level for the remaining weeks of training, suggesting that the demand for related beneficial bacterial products had plateaued at levels that, while greater than before training was less than that observed at weeks 2-4. While purely speculative, this continuation of the increase associated with training may be an adaptation related to a continued need for the bacterial products related to energy production. Increases in energy sources are required to cover the increased cost of exercise training and thus, it is logical that more bacterial fermentation is required to generate more SCFAs (Samuel and Gordon, 2006).

Another major observation in the present study was the change in the relative abundance of the Proteobacteria phylum. The Proteobacteria are Gram negative bacteria that include many species that can be pathogenic causing several illnesses (Gupta, 2000). The Proteobacteria also include member species that are symbiotic bacteria with eukaryotic cells of the host (Gupta, 2000). It has been reported that when Proteobacteria abundance decreases, one will observe a concurrent increase in Bacterodetes abundance (Pérez-Cobas et al., 2013). Thus, the observed decrease in Proteobacteria in the beginning of the

present study may have been a reason for the increase in the abundance of the Bacteroidetes observed during the same points during training. This may be an important adaptive response to training and protection against the pathogenic Proteobacteria (Devkota, et al., 2012). Similar protective alterations in “good” and “bad” bacteria have been observed in mice when fed a diet high in milk fat (Devkota, et al., 2012). It has been reviewed by Shin and co-workers, (2015) that increasing the abundance of Proteobacteria might happen as a result of non-illness states such as the neonatal period and after elective gastric bypass surgery. In the early newborn life, Proteobacteria abundance dominates the GM profile. After this period of life, Bacteroidetes dominates the bacterial profile. In the case of gastric bypass surgery, changing the pH and bile flow may expanse the relative abundance of Proteobacteria. In this study, the final three time points, staying higher than first three time points could indicate transient elevation in the abundance of this phylum that might decrease later or could be stable at the higher level.

Another finding of the present study was that exercise training altered the abundance of the Spirochaetes phylum and one of its species members, namely Treponema spp. Some members of these bacteria are symbiotic and inhabit the GIT of animals (Stanton and Canale-Parola, 1980). For example, Stanton and Canale-Parola (1980) found that in bovine, ruminal Spirochaetes, Treponema bryantii, grew in co-culture with cellulolytic bacteria and enhanced cellulose degradation in the presence of Bacteroides succinogenes. We speculate that the changes in the levels of Spirochaetes phylum observed over time with training

could be an indication of increasing demands for some species that belong to the Spirochaetes phylum. This change may help in the degradation of some complex food compounds such as cellulose which in turn generates energy to meet the energetic needs of the bacteria, for example species from the Bacteroidetes phylum (Samuel and Gordon, 2006). At the same time this process results in the generation of more SCFAs by the bacteria which can be used by the horse to cover the increased cost of exercise (Samuel and Gordon, 2006). Until the end of the study, the abundance of this phylum remained elevated over the pre-training levels suggesting a continuous need for cellulose degrading species to help provide the horse host with greater sources of energy. The observed alterations of Treponema spp. levels seen with training appear to occur at the same time points as the changes that occurred in levels of the Spirochaetes phylum. This concurrent change in both the phylum and its species may be important because of the above mentioned association with energy substrate production. Moreover, there were no changes in other taxa belonging to the phylum Spirochaetes, because the increase in the phylum Spirochaetes being due to the increase in Treponema spp.

Interestingly, another finding was that the abundance of the genus Clostridium was altered by exercise training. These bacteria occupy an important part of the GM of humans and other animals (Wells and Wilkins, 1996) which characterized for their degrading enzymes that produce SCFAs. We the cause of this increase is that many of these bacteria are also butyrate producers (Barcenilla et al., 2000). Horses, especially during training, need more energy.

Alternative sources of energy, such as SCFAs like butyrate are absorbed from the cecum and colon and they represent around 70% of the fuel needed for the horse in training (Bergman, 1990). As training progressed there was a decrease in the abundance of this genus, which may reflect the adaptation of the gastrointestinal tract and the GM to training (Brouns and Beckers, 1993).

Another observation was the finding that there were alternations in the abundance of Dysgonomonas spp. over the course of exercise training. These organisms are members of Bacteroidetes (Minard et al., 2015). Interestingly, Dysgonomonas spp., found in the gut of formosan subterranean termites, have been shown to produce and provide vitamin B12 to the termite host (Husseneder et al., 2009). There are no studies on the effects of exercise training in horses that recommend vitamin B12 supplementation (Hodgson et al., 2014). However; blood flow to the working muscles, skin, and other vital organs is highly important therefore there is a high demand for more functional RBCs supported by B12. Because the alternations in the abundances of the genus Dysgonomonas and its species, Dysgonomonas spp. occurred at the same time point (week 4) and because both of them had almost the same relative abundance, we speculate that the increases could indicate that these bacteria produce more bacterial B12, which in turn may aid erythropoiesis in the horse.

Shannon Diversity Index

This is the first study to report training associated changes in the Shannon Diversity Index in the horse. The index is used to measure evenness and

richness of microbial communities (Hill, 1973). Exercise training had a clear impact on the diversity of the GM of the horses used in the present study. We observed a decrease in the Shannon Diversity index due to changes in the relative abundances of Bacteroidetes, Proteobacteria, and Spirochaetes affecting the evenness of the communities. The return of the index and bacterial group abundances to pre-training levels (after 4 weeks of training) indicates the resilience of equine gut communities to perturbation, and may reflect adaptation to training.

Taxa Abundance Over Time

The gut microbiome is a complex living system and it is important to understand the complex interplay of the organisms that interact in the intestinal tract of the host. Plotting taxa abundances over a time course enables the tracking of community changes and identification of succession patterns, as well as competitive or cooperative interactions between bacterial taxa. Two specific taxa, Treponema spp. and Dysgonomonas spp, increased in abundance at week 2 and week 4 of exercise, suggesting cooperative interactions between these taxa in the beginning stages of training. Yang et al., 2014; Zhang et al., 2014 showed that bacteria isolated from termites gut such as species belong to Dysgonomonas have glucosidase genes that synthesizes cellulose degrading enzyme. Pramono et al., 2015 found that Dysgonomonas termitidis sp, isolated from the gut of subterranean termite Reticulitermes speratus is a cellulose degrading bacterium. It was found that rumen Treponema bryantii and

Bacteroides succinogenes act synergistically in degrading cellulose (Stanton and Canale-Parola, 1980). In this study, Treponema spp. and Dysgonomonas spp. increased significantly in week 2 and week 4, indicating that they also may act synergistically in degradation of complex polysaccharides.

Summary

In conclusion exercise training has a measurable effect on the gut microbiome of the horse and these changes, regardless of the bacterial taxa, started to appear in the early part of training. Interestingly, these changes were transient and reversed by the last weeks of the experiment. Further investigation is needed to determine whether the co-occurrence of bacteria such as Treponema spp. and Dysgonomonas spp. reflects syntrophic relationships. Elucidation of competitive or cooperative microbial interactions could guide probiotic supplementation strategies for greater energy availability and feed efficiency, and point to microbial interventions against inflammation and illnesses affecting the athletic horse.

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List of Tables

Table 1. Standard Exercise Procedure (SEP)

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)

Figure Captions

Figure 1. Phylum level changes by exercise training in the ExTr group. A. *Bacteroidetes*. B. *Proteobacteria*. C. *Spirochaetes*. Values indicate mean ± standard error. Only different letters over bars mean significant differences between time points, n=8 horses/time point. Significant differences ($P < 0.05$) are shown in Table S1.

Figure 2. Phylum levels after exposure to exercise training in the ExTr group. A. *Firmicutes*. B. *Fibrobacteres*. Values indicate mean ± standard error. N = 8 horses/time point. No changes ($P > 0.05$).

Figure 3. Seasonal effect on phylum levels during spring, and half of the summer in the SC group. A. *Bacteroidetes*. B. *Proteobacteria*. C. *Spirochaetes*. Values indicate mean ± standard error. N = 4 horses/time point. No changes ($P > 0.05$).

Figure 4. Seasonal effect on phylum levels during spring and half of the summer in the SC group. A. *Firmicutes*. B. *Fibrobacteres*. Values indicate mean ± standard error. N = 4 horses /time point. No changes ($P > 0.05$).

Figure 5. Genus level changes by exercise training in the ExTr group. A. *Clostridium*. B. *Dysgonomonas*. Values indicate mean ± standard error. Only different letters over bars mean significant differences between time points, n=8 horses/time point. Significant differences ($P < 0.05$) are shown in Table S1.

Figure 6. Seasonal effect on genus level during spring and half of the summer in the SC group. A. *Clostridium*. B. *Dysgonomonas.* Values indicate mean ± standard error. N = 4 horses /time point. No changes (P > 0.05).

Figure 7. Species level changes by exercise training in the ExTr group. A. *Treponema* spp. (P < 0.05) shown in Table S1. B. *Dysgonomonas* spp, week 2 vs week 4 (P = 0.0014), week 4 vs week 6 (P = 0.0014). Values indicate mean ± standard error. Only different letters over bars mean significant differences between time points, n=8 horses/time point.

Figure 8. Seasonal effect on species Levels during spring and half of the summer in the SC group. A. *Treponema* spp. B. *Dysgonomonas* spp. Values indicate mean ± standard error. N = 4 horses/time point. No changes (P > 0.05).

Figure 9. Shannon Diversity index of bacterial taxa of the ExTr group. A. phyla B. Genera. C. Species. Values indicate mean ± standard error. Only different letters over bars mean significant differences between time points, n=8 horses/time point. Significant differences (P values) are shown in Table S2.

Figure 10. Shannon Diversity index of the SC group during spring and half of the summer. A. phyla B. Genera. C. Species. Values indicate mean ± standard error. N = 4 horses/time point. No changes (P > 0.05).

Figure 11. Principle Coordinate Analysis (PCoA) of the exercise training (ExTr) group and its bacterial genera. ExTr group shows significant separation between time points, week 0 and week 2 (P = 0.0490), week 0 and week 6 (P = 0.0276), week 0 and week 10 (P = 0.0133), and week 0 and week 12 (P = 0.0127), n=8 horses/time point.

Figure 12. Principle Coordinate Analysis (PCoA) of the exercise training (ExTr) group and its bacterial species. ExTr group shows significant separation between time points, week 0 and week 6 (P = 0.0103), week 0 and week 10 (P = 0.0173), week 0 and week12 (P = 0.0094), and week 6 and week 10 (P = 0.0095), n=8 horses/time point.

Figure 13. Same time changes by exercise training represented by the mean and SEM. A. Phyla in the ExTr group. B. Genera in the ExTr group. C. Species in the ExTr group. ExTr n=8 horses/time point. Only different letters above or under the error bars represent significant changes between time points. ExTr group significant differences P values are shown in Table S1.

Figure 1.

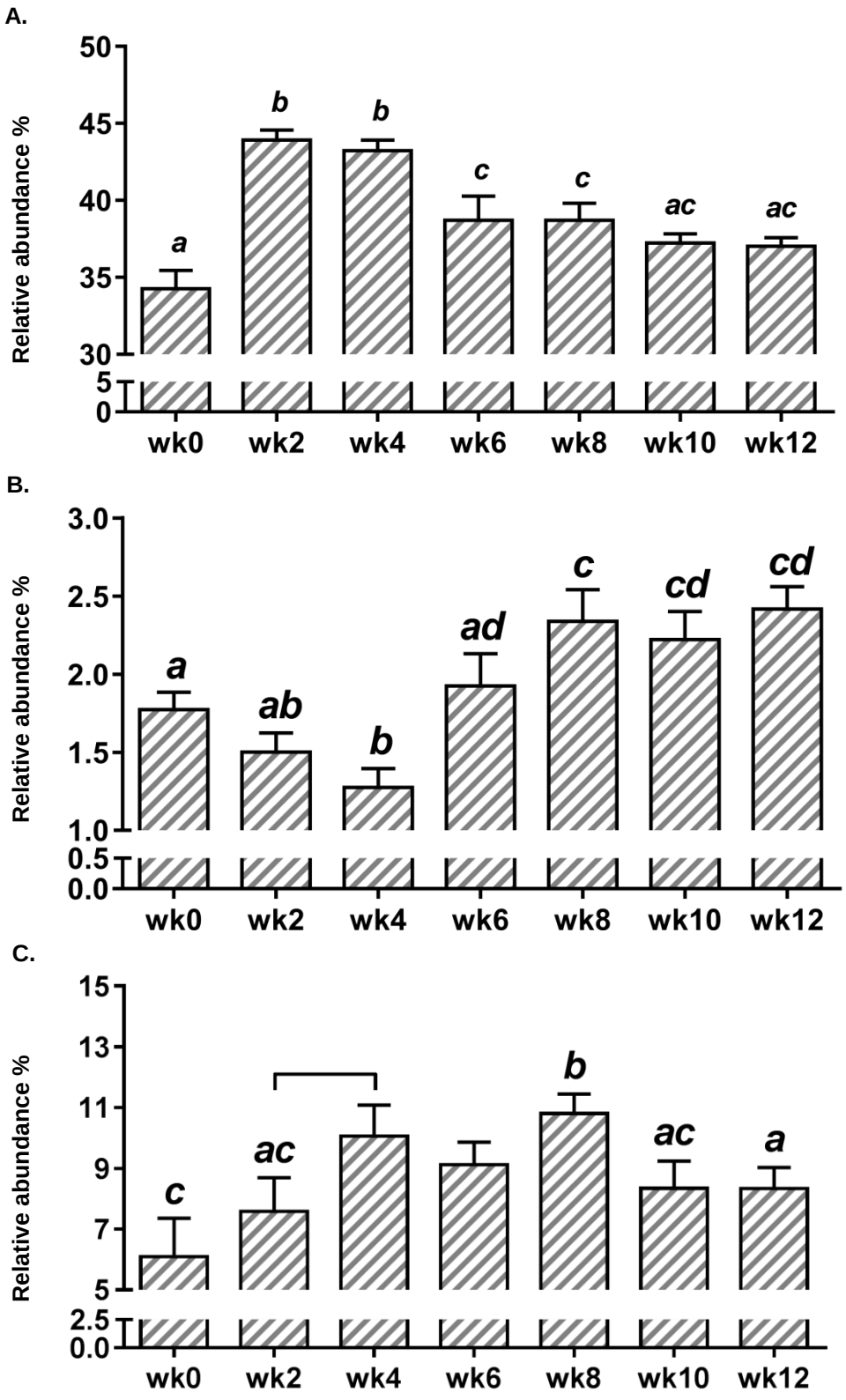
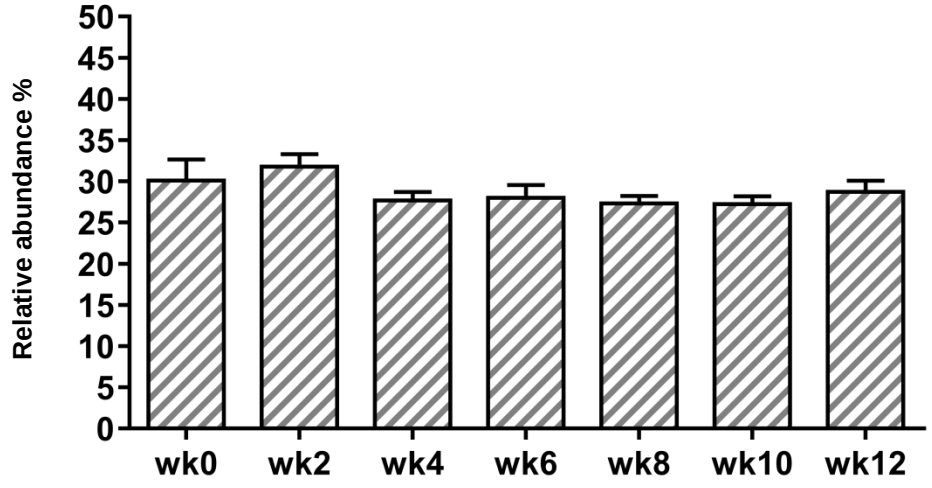

A.
Relative abundance %
50
45
40
35
30
5
0
a
b
b
c
c
ac
ac
wk0
wk2
wk4
wk6
wk8
wk10
wk12
B.
Relative abundance %
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
ab
b
ad
c
cd
cd
wk0
wk2
wk4
wk6
wk8
wk10
wk12
C.
Relative abundance %
15
13
11
9
7
5
2.5
0.0
c
ac
b
ac
a
wk0
wk2
wk4
wk6
wk8
wk10
wk12

Figure 2.

A.


Relative abundance %
50
45
40
35
30
25
20
15
10
5
0
wk0
wk2
wk4
wk6
wk8
wk10
wk12

B.

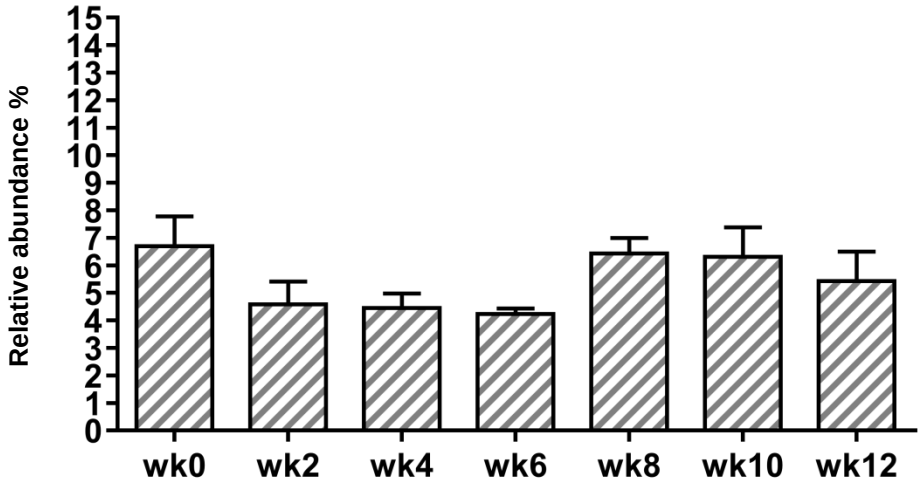
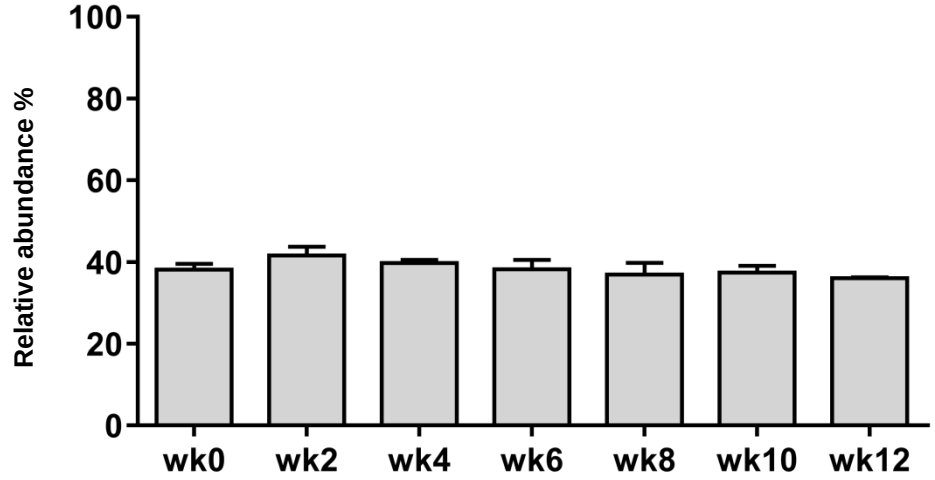
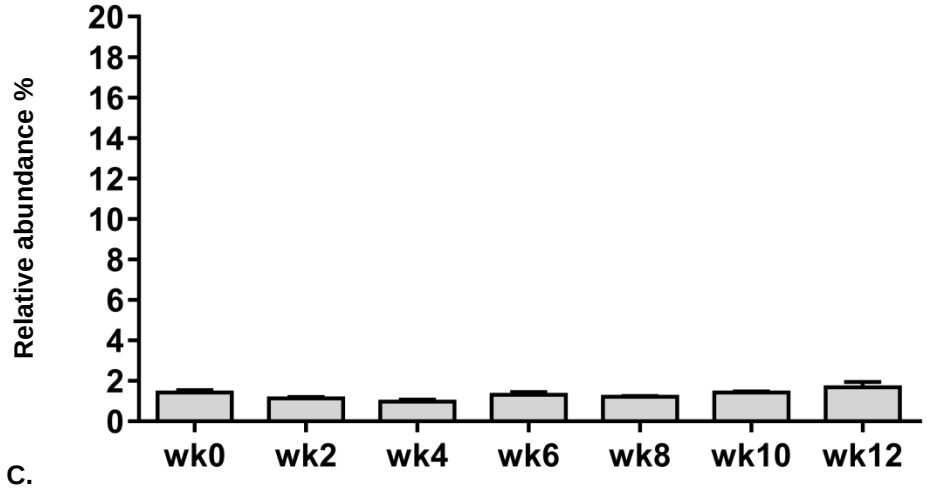

Relative abundance %
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
wk0
wk2
wk4
wk6
wk8
wk10
wk12

Figure 3.

A.


Relative abundance %
100
80
60
40
20
0
wk0
wk2
wk4
wk6
wk8
wk10
wk12

B.


Relative abundance %
20
18
16
14
12
10
8
6
4
2
0
wk0
wk2
wk4
wk6
wk8
wk10
wk12

C.

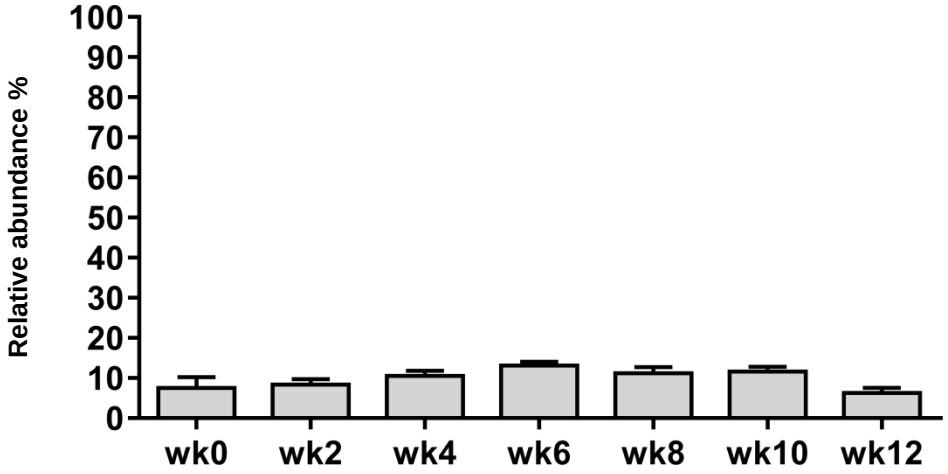
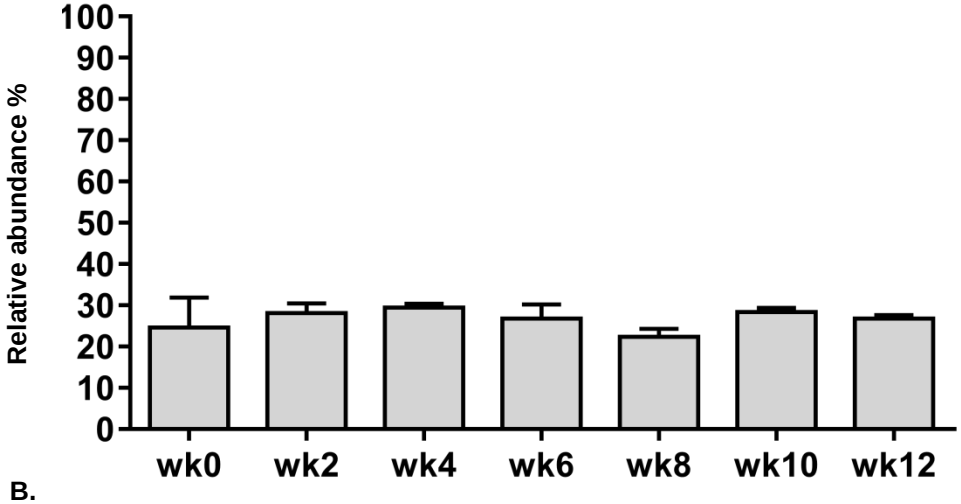

Relative abundance %
100
90
80
70
60
50
40
30
20
10
0
wk0
wk2
wk4
wk6
wk8
wk10
wk12

Figure 4.

A.


Relative abundance %
100
90
80
70
60
50
40
30
20
10
0
wk0
wk2
wk4
wk6
wk8
wk10
wk12

B.

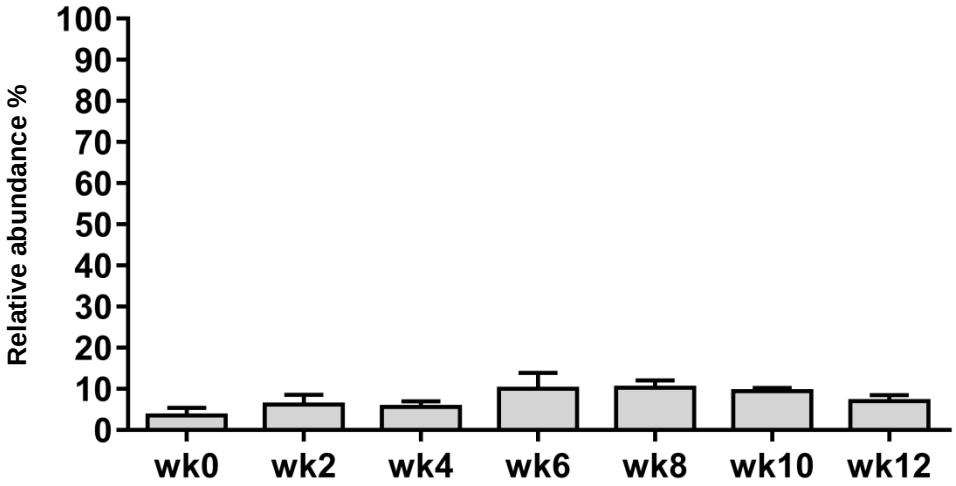
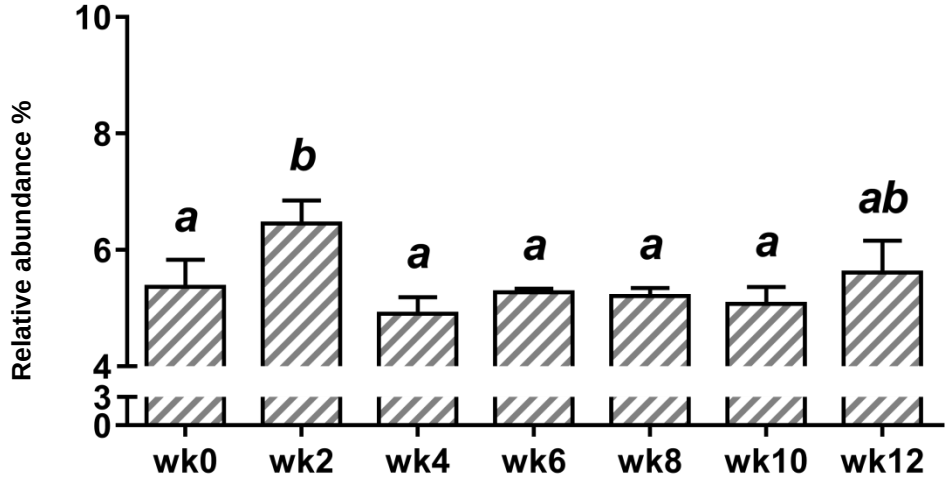

Relative abundance %
100
90
80
70
60
50
40
30
20
10
0
wk0
wk2
wk4
wk6
wk8
wk10
wk12

Figure 5.

A.


Relative abundance %
10
8
6
4
3
0
a
b
a
a
a
a
ab
wk0
wk2
wk4
wk6
wk8
wk10
wk12

B.

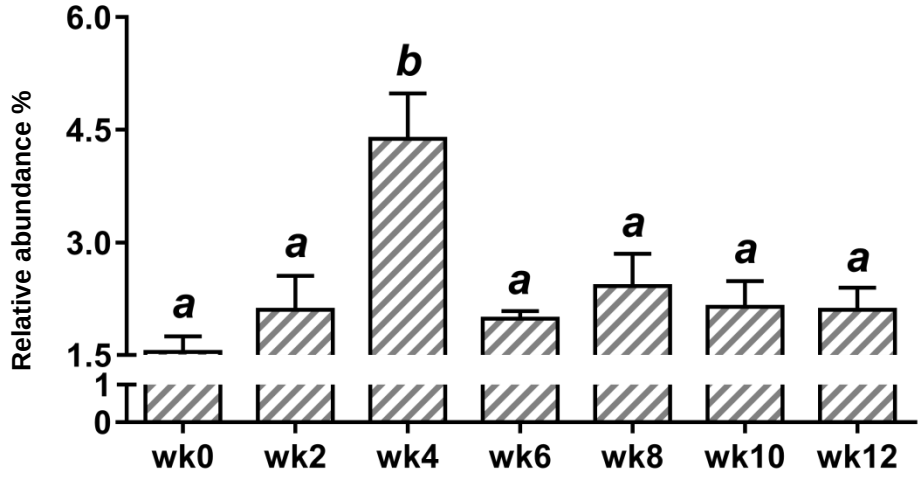
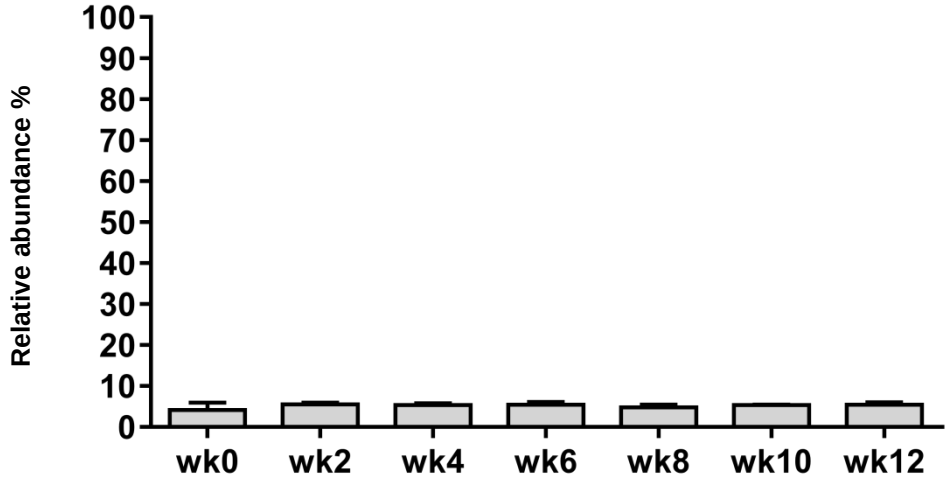

Relative abundance %
6.0
4.5
3.0
1.5
1
0
a
a
b
a
a
a
a
wk0
wk2
wk4
wk6
wk8
wk10
wk12

Figure 6.

A.


Relative abundance %
100
90
80
70
60
50
40
30
20
10
0
wk0
wk2
wk4
wk6
wk8
wk10
wk12

B.

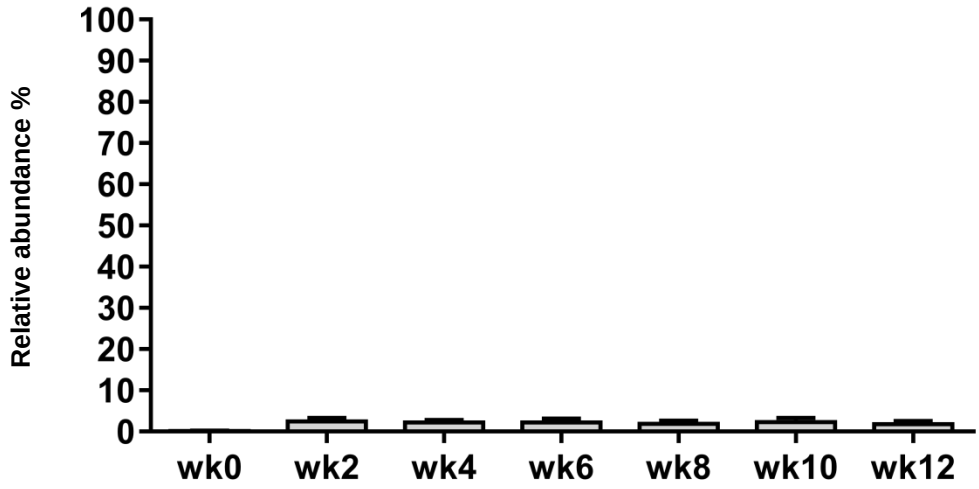
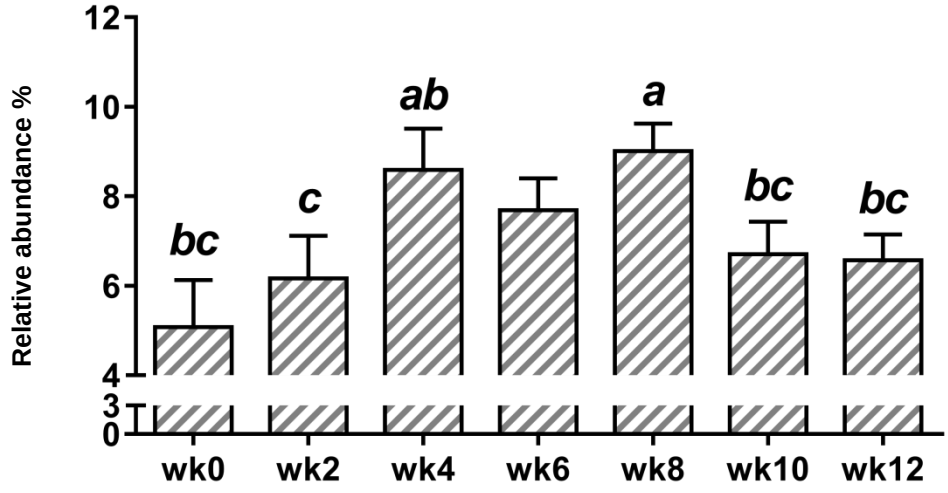

Relative abundance %
100
90
80
70
60
50
40
30
20
10
0
wk0
wk2
wk4
wk6
wk8
wk10
wk12

Figure 7.

A.


Relative abundance %
12
10
8
6
4
3
0
bc
c
ab
a
bc
bc
wk0
wk2
wk4
wk6
wk8
wk10
wk12

B.

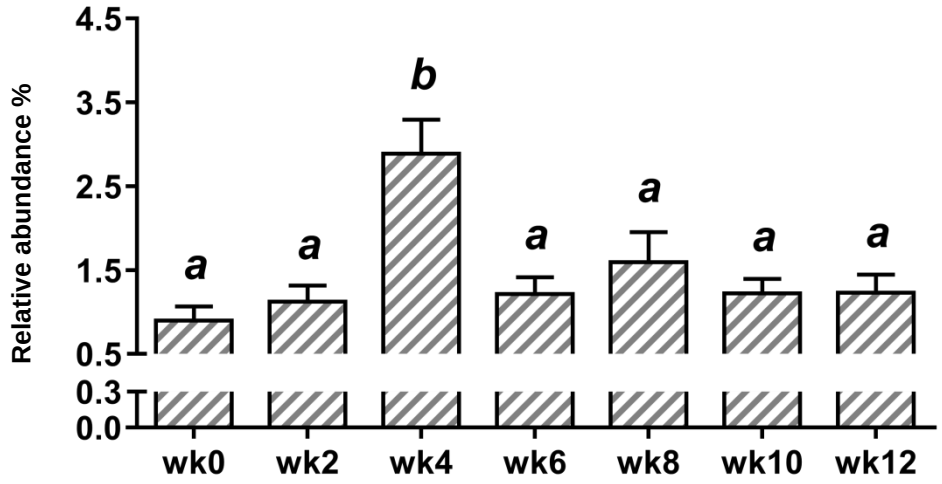
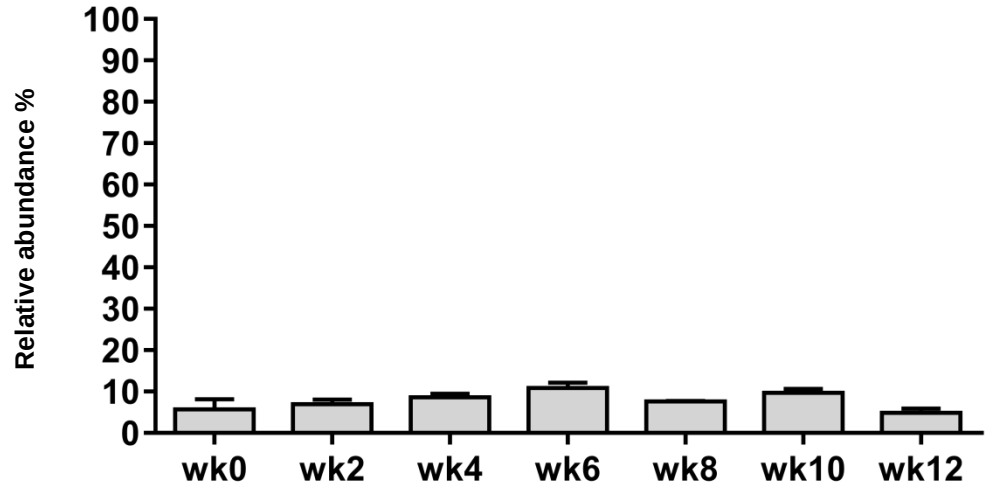

Relative abundance %
4.5
3.5
2.5
1.5
0.5
0.3
0.0
a
a
b
a
a
a
a
wk0
wk2
wk4
wk6
wk8
wk10
wk12

Figure 8.

A.


Relative abundance %
100
90
80
70
60
50
40
30
20
10
0
wk0
wk2
wk4
wk6
wk8
wk10
wk12

B.

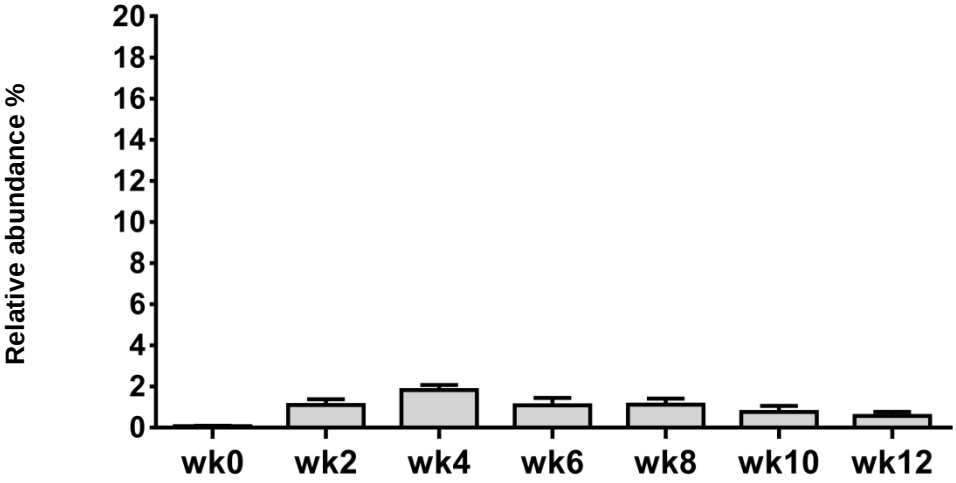

Relative abundance %
20
18
16
14
12
10
8
6
4
2
0
wk0
wk2
wk4
wk6
wk8
wk10
wk12

Figure 9.

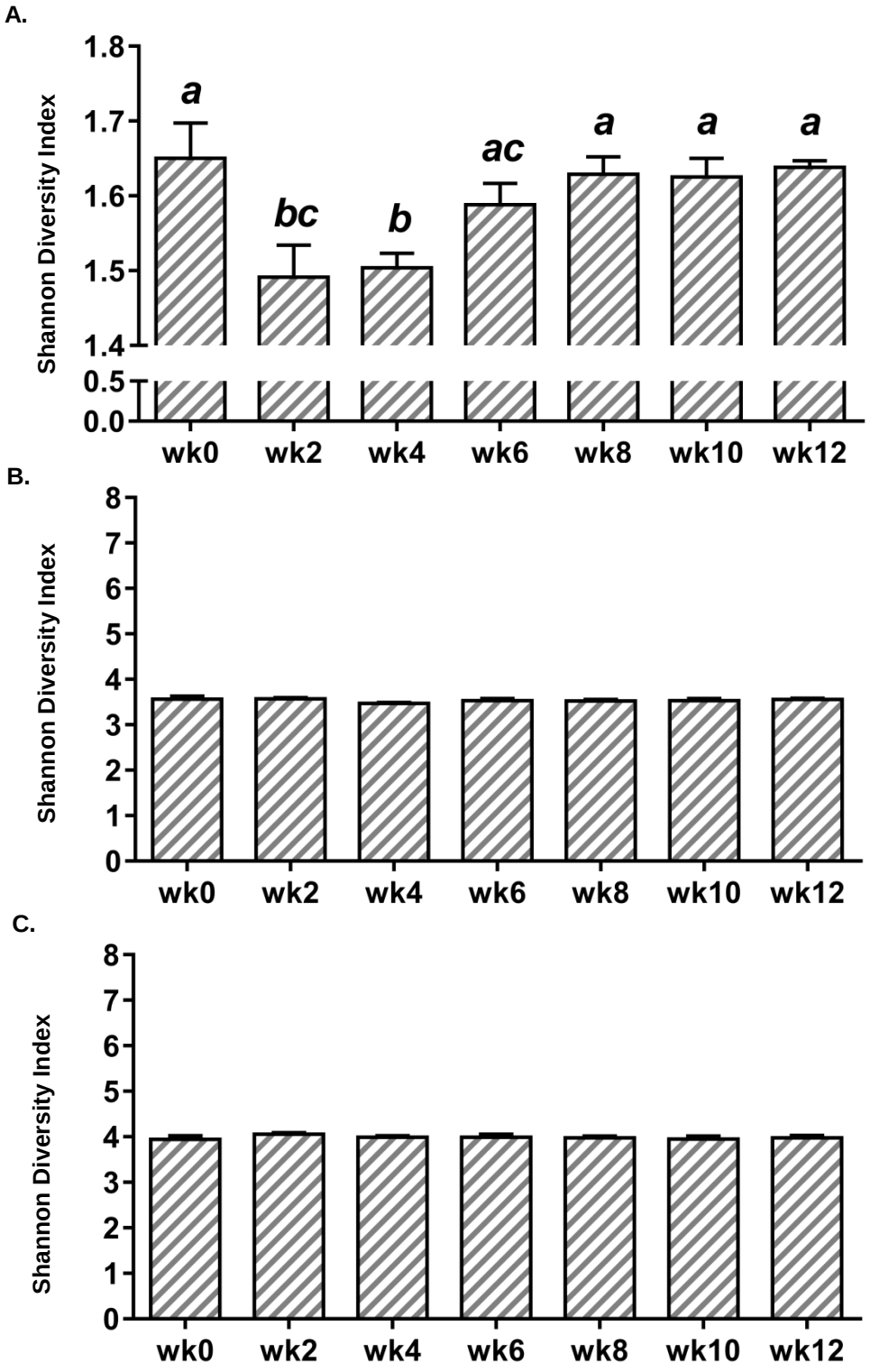
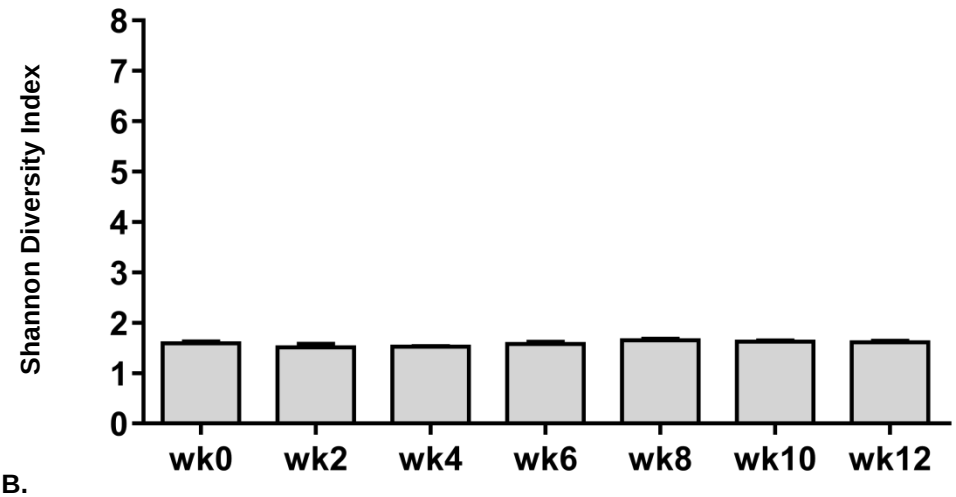
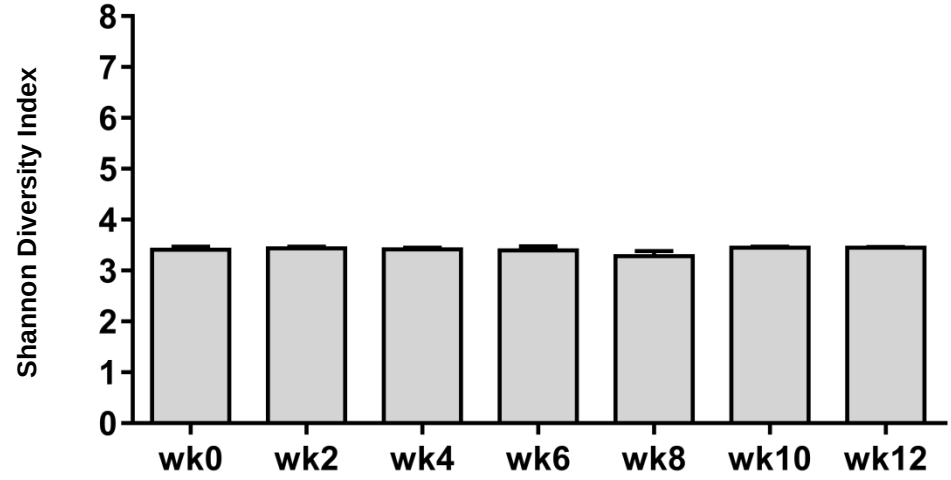

A.
Shannon Diversity Index
1.8
1.7
1.6
1.5
1.4
0.5
0.0
a
bc
b
ac
a
a
a
wk0
wk2
wk4
wk6
wk8
wk10
wk12
B.
Shannon Diversity Index
8
7
6
5
4
3
2
1
0
wk0
wk2
wk4
wk6
wk8
wk10
wk12
C.
Shannon Diversity Index
8
7
6
5
4
3
2
1
0
wk0
wk2
wk4
wk6
wk8
wk10
wk12

Figure 10.

A.



B.



C.

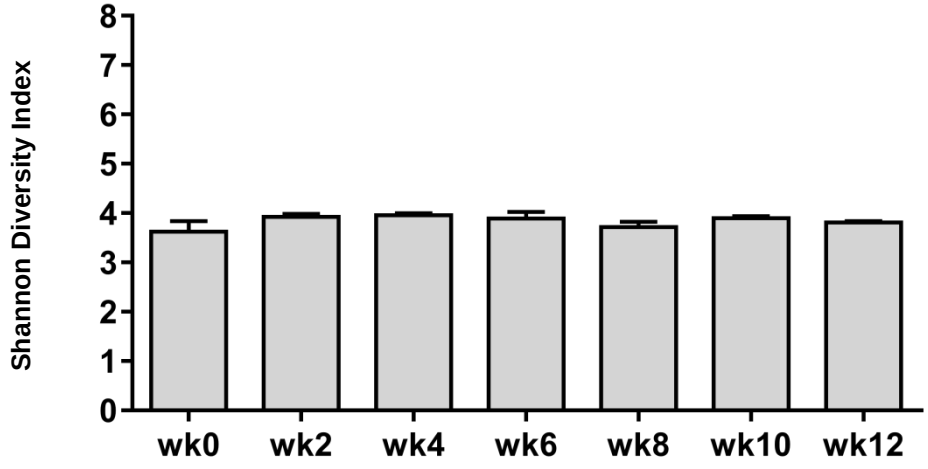


Figure 11.

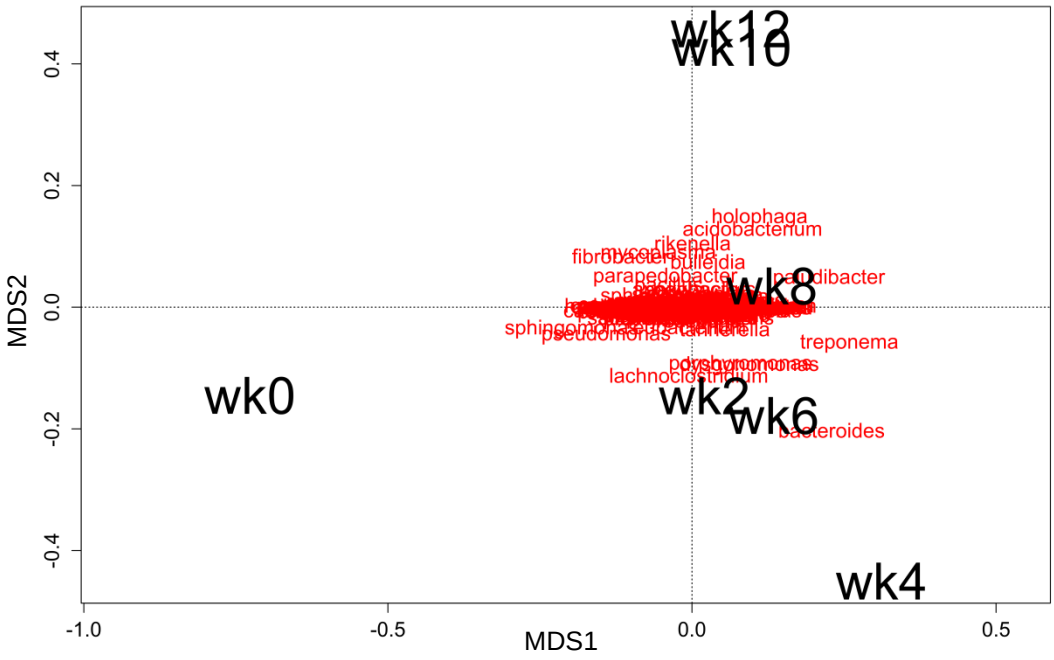

wk12
wk10
wk8
wk0
wk2
wk6
wk4
holophaga
acidobacterium
rikenella
bulleidia
parapedobacter
treponema
bacteroides
lachnoclostridium
MDS1
MDS2
-1.0
-0.5
0.0
0.5
0.4
0.2
0.0
-0.2
-0.4

Figure 12.

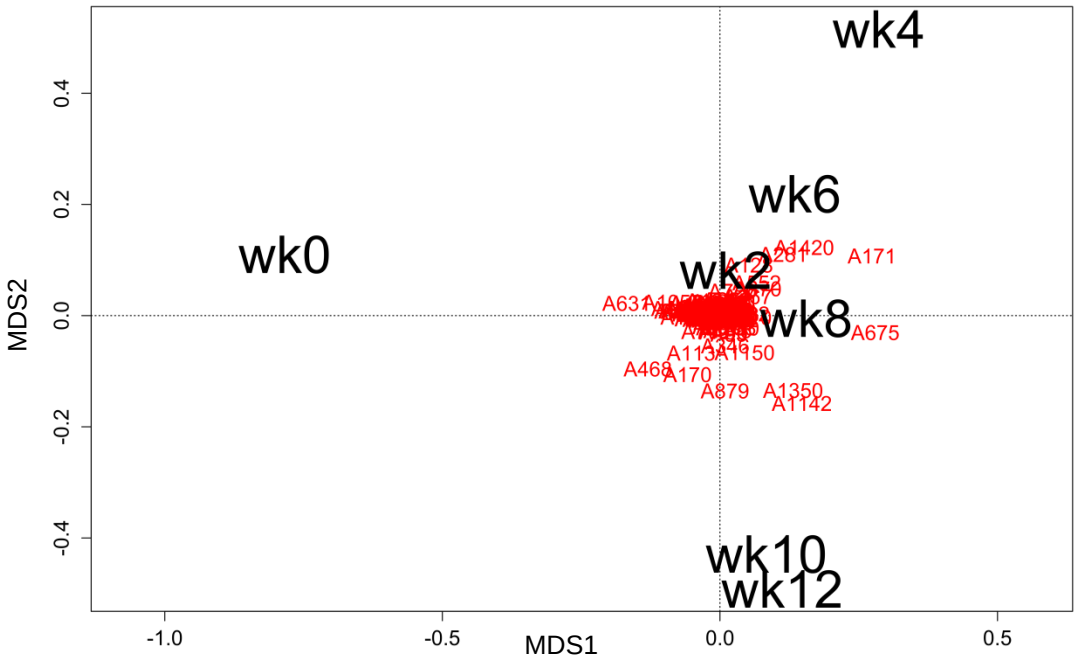
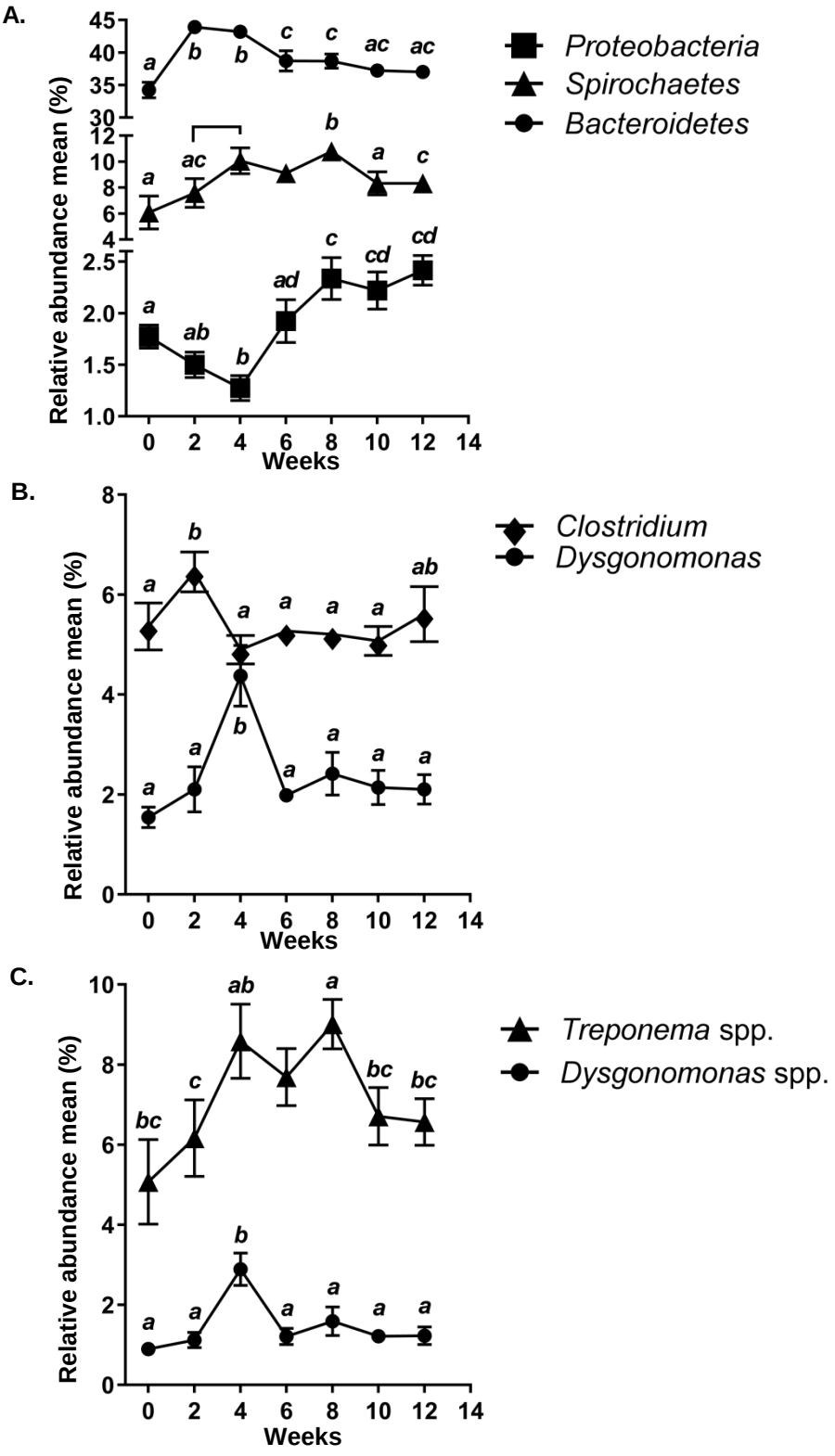

wk4
wk6
wk0
wk2
wk8
wk10
wk12
A1420
A171
A675
A1150
A170
A879
A1350
A1142
MDS1
MDS2
-1.0
-0.5
0.0
0.5
0.4
0.2
0.0
-0.2
-0.4

Figure 13.


A.
Relative abundance mean (%)
Weeks
Proteobacteria
Spirochaetes
Bacteroidetes
B.
Relative abundance mean (%)
Weeks
Clostridium
Dysgonomonas
C.
Relative abundance mean (%)
Weeks
Treponema spp.
Dysgonomonas spp.

Appendix Tables

Appendix Table S1. Probability (P) values of the time point comparisons of the exercise training shown in Figure 1, Figure 5, Figure 7.

	Time Point comparisons	Significance summary	P values
A. *Bacteroidetes*	wk0 vs. wk2	****	< 0.0001
	wk0 vs. wk4	***	0.0002
	wk0 vs. wk6	*	0.0229
	wk0 vs. wk8	*	0.0155
	wk2 vs. wk6	**	0.0051
	wk2 vs. wk8	**	0.0011
	wk2 vs. wk10	***	0.0002
	wk2 vs. wk12	****	< 0.0001
	wk4 vs. wk6	*	0.0261
	wk4 vs. wk8	*	0.0128
	wk4 vs. wk10	***	0.0007
	wk4 vs. wk12	***	0.0001
B. *Proteobacteria*	wk0 vs. wk4	**	0.0031
	wk0 vs. wk8	**	0.0089
	wk0 vs. wk10	*	0.015
	wk0 vs. wk12	*	0.0203
	wk2 vs. wk8	**	0.0083
	wk2 vs. wk10	**	0.0072
	wk2 vs. wk12	***	0.0003
	wk4 vs. wk6	*	0.0115
	wk4 vs. wk8	***	0.0001
	wk4 vs. wk10	**	0.0017
	wk4 vs. wk12	***	0.0005
	wk6 vs. wk8	*	0.0254
C. *Spirochaetes*	wk0 vs. wk8	**	0.0042
	wk0 vs. wk12	*	0.0483
	wk2 vs. wk4	*	0.0148
	wk2 vs. wk8	**	0.002
	wk8 vs. wk10	*	0.0381
	wk8 vs. wk12	*	0.0266

	Time Point comparisons	Significance summary	P values
D. *Clostridium*	wk0 vs. wk2	*	0.0352
	wk2 vs. wk4	***	0.0004
	wk2 vs. wk6	*	0.0173
	wk2 vs. wk8	*	0.0224
	wk2 vs. wk10	*	0.01
E. *Dysgonomonas*	wk0 vs. wk4	**	0.0022
	wk2 vs. wk4	***	0.0004
	wk4 vs. wk6	**	0.0035
	wk4 vs. wk8	**	0.0092
	wk4 vs. wk10	**	0.0021
	wk4 vs. wk12	**	0.0074
F. *Treponema spp.*	wk0 vs. wk8	**	0.0043
	wk2 vs. wk4	**	0.0063
	wk2 vs. wk8	***	0.0007
	wk8 vs. wk10	*	0.023
	wk8 vs. wk12	*	0.0172

Appendix Table S2. Probability (P) values of Phylum Shannon Diversity index Change during exercise training.

Time Point comparisons	Significance Summary	P values
wk0 vs. wk2	*	0.0384
wk0 vs. wk4	*	0.0123
wk2 vs. wk8	*	0.0438
wk2 vs. wk10	*	0.0345
wk2 vs. wk12	**	0.0097
wk4 vs. wk6	*	0.0355
wk4 vs. wk8	*	0.0127
wk4 vs. wk10	*	0.0156
wk4 vs. wk12	****	< 0.0001

SUMMART

More work is needed to determine if acute exercise and exercise training generate healthy changes in the bacterial profiles of the athletic horse. Studies in human and other animal models suggest that the composition and activity of the GM is positively modulated by exercise to protect against the effects of obesity, disease, poor diet, and toxins. Studies of the equine gut microbiota suggest links to the host physiological, immunological, and nutritional statuses since a major part of the energy needed by for daily activities is provided by bacterial fermentation processes that produce short chain fatty acids such as butyrate. Horses have been found to have a very diverse gut bacterial profile that is vital to the health of the animal. The equine microbiome could be especially sensitive to exercise that these changes in the microbiome might bring more beneficial effects on the host such as increasing performance. It seems that gut microbiome is in many ways as important as other organs in the bodies of humans and other animals including horses. The activities of these microbes may influence the host's health in positive or negative manners. Furthermore, in horses, Studying these bacteria using high throughput techniques such as next generation sequencing methods has improved our knowledge about the behaviors and the participation of these microorganisms in the health of the horses.

The observed changes in the pre-training standing control are interesting but we have no data to support any physiological mechanism or biological

implications associated with this response. One can speculate that if such changes were to last long term there would be potential for detrimental effects on the host. As an effect of exercise training, ultimately the responses seen in the first battery of standing control trials were not a factor after 12 weeks of exercise conditioning. On a practical level, the present study provides information benefiting those seeking ways to improve the health and well-being of the athletic horse. Furthermore, acute intense exertion does not appear to alter the gut microbiome of the horses running on the treadmill.

In conclusion exercise training has a measurable effect on the gut microbiome of the horse and these changes, regardless of the bacterial taxa, started to appear in the early part of training. Interestingly, these changes were transient and reversed by the last weeks of the experiment. Further investigation is needed to determine whether the co-occurrence of bacteria such as Treponema spp. and Dysgonomonas spp. reflects syntrophic relationships. Elucidation of competitive or cooperative microbial interactions could guide probiotic supplementation strategies for greater energy availability and feed efficiency, and point to microbial interventions against inflammation and illnesses affecting the athletic horse.

Curriculum Vita

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Year: 2005

Working: Faculty member in the College of Veterinary Medicine / Al-Qadisiya University

Year: 2006-

Publication:

1- Janabi AHD, Kerkhof LJ, McGuinness LR, Biddle AS, McKeever KH. Comparison of a modified phenol/chloroform and commercial-kit methods for extracting DNA from horse fecal material. Journal of Microbiological Methods. 2016; 129:14-19

2- Janabi A.H.D., Biddle A.S., Klein D., McKeever K.H., 2016. Exercise Training-Induced Changes in the Gut Microbiota of Standardbred Racehorses. "ACCEPTED in Comparative Exercise Physiology Journal"

3- Daghir, A.H., 2008. Isolation of Branhamella ovis from Intact Eyes of Calves. Al- Anbar J. Vet. Sci. 1:10-12.

4- Dawood, K.A., Chelab, K.G., Daghir, A.H., 2008. Pathological study of lamb coccidiosis in Diwaniya. AL-Qadisiya Journal of Veterinary Medicine Science. 7: 72-75.