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Short Communication

Bacillus subtilis Strain PB6 Demonstrates Growth Inhibition Toward Equine-Specific Bacterial Pathogens

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ABSTRACT

This study determined the antagonistic activity of a probiotic *Bacillus subtilis* strain PB6 (PB6) toward six bacterial pathogens of equine origin. Antimicrobial activity of PB6 was evaluated using two different in vitro methodologies. A streak line assay resulted in measurable zones of clearing between growth of PB6 and *Clostridium difficile*, *Clostridium perfringens*, *Rhodococcus equi*, and *Streptococcus equi*. A broth micro dilution assay using cell-free supernatant from PB6 culture demonstrated inhibition of *Salmonella* Typhimurium and *R. equi* growth. The results indicate the potential for PB6 to be a beneficial probiotic for use in the equine industry.

1. Introduction

With the widespread overuse of antibiotics for the treatment of animal ailments and the emergence of antibiotic-resistant pathogens [1], direct-fed microbials, or probiotics, are becoming more popular as natural means to treat and prevent animal disease. It is becoming more recognized that changing the microbial balance of the gastrointestinal tract environment through diet or supplementation can have significant health benefits in the animal [2]. Probiotics are defined as microorganisms which when administered provide health benefits to the host through modulation of the microbial balance [3]. Characteristics of beneficial probiotics include the ability to adhere to mucosal tissue/cells and intestinal mucus, competitively exclude and displace enteropathogens, elicit an immune response, and secrete antimicrobial compounds. Previous in vitro and in vivo research conducted using *Bacillus subtilis* PB6 demonstrated antagonistic activity against various human and livestock pathogens, such as *Clostridium* species [4–6].

Probiotics have gained interest as a means to prevent diarrhea in horses and foals; however, to date there have been few published clinical trials showing positive effects of probiotics in equine [7]. Bacteria that are typically associated with enterocolitis and acute diarrhea in horses and neonatal foals are *Salmonella* spp., *Clostridium perfringens*, and *Clostridium difficile* [8]. Salmonellosis is one of the most common cause of diarrhea in horses and the severity of the infection ranges from mild to severe diarrhea and death. *Clostridium* spp. are spore-forming toxin producing gram-positive bacteria that are common inhabitants of the intestines of equine. Antibiotic use, however, can cause a shift in microbial populations leading to the overgrowth and subsequent toxin production of these bacteria [9,10]. Although less common than *Salmonella* and *Clostridium*, both *Escherichia coli* and *Rhodococcus equi* can may also cause diarrhea in foals [11–13]. There is little research on the use of probiotics to prevent infections not associated with the intestinal tract. *Streptococcus equi* is a highly

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contagious bacterium and the causative agent of strangles and bastard strangles in young horses and foals [14]. It was included in the study to evaluate the potential activity, which could lead to investigating alternative administration of probiotics.

The aim of this study was to evaluate *B. subtilis* strain PB6 as a potential probiotic for equine by screening for antagonistic activity against six bacterial pathogens isolated from equine patients.

2. Materials and Methods

2.1. Pathogenic Bacteria

All equine bacterial pathogens were purchased from the Veterinary Diagnostic Laboratory at Iowa State University, Ames, IA. *Clostridium perfringens* #1984 was isolated from the feces of an adult horse presenting with colic and diarrhea. *Clostridium difficile* #42081 was isolated from the feces of a 2-year-old horse with diarrhea and colic. *Salmonella* Typhimurium #06-767 was isolated from the organs of a foal presenting with diarrhea. The foal had failure of passive transfer of colostral immunoglobulin and died from septicemia. *Escherichia coli* # 07-1690 was isolated from the feces of a neonatal (<7 days) foal presenting with diarrhea. No other pathogens were present in the fecal material. *Rhodococcus equi* #45 was isolated from a 1-year-old horse with a history of *R. equi* positive tracheal wash samples, which led to the suspicion that this animal had respiratory abscesses. However, at necropsy, *R. equi* was isolated from an abscess located in the abdomen. *Streptococcus equi* #43006 was isolated from a nasal swab and the mandibular lymph nodes of a 1-year-old horse from a farm with a history of *S. equi* problems.

2.2. Culture Preparation

Each of the pathogen isolates was subcultured into 10 mL brain heart infusion (BHI) broth (Difco, Becton Dickinson, Sparks, MD) and grown aerobically overnight at 37°C ± 2°C, except for *Clostridium* cultures which were grown under anaerobic conditions (GasPak EZ Anaerobic Gas Generating Pouch System, Difco, Becton Dickinson, Sparks, MD). Cultures were concentrated via centrifugation and the pellet was resuspended in a 50/50 mix of BHI and 20% glycerol. The concentrated cultures were aliquoted into 1.5 mL cryovials and frozen at −80°C.

2.3. Probiotic Organism

The direct-fed microbial product containing *B. subtilis* PB6 (CLOSTAT, Kemin Industries, Inc, Des Moines, IA) was used as the probiotic organism in the inhibition tests.

2.4. Cross-Streak Diffusion Assay

A cross-streak agar diffusion method was used to identify activity of PB6 against equine bacterial isolates. PB6 was inoculated in tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD) and incubated aerobically at 37°C ± 2°C for approximately 18 hours. The overnight culture was streaked perpendicularly onto prepared tryptic soy agar + 5% sheep blood plates (TSA + SB; Remel, Thermo Fisher Scientific, Lenexa, KS) in triplicate. Plates were incubated aerobically at 37°C ± 2°C for 18 hours. After overnight incubation, the PB6 cultures on the TSA + SB plates were inactivated by placing them in a closed jar with 50 mL chloroform for 2 hours. Each of the six equine isolates was inoculated into BHI broth. Cultures were grown aerobically overnight at 37°C ± 2°C, except for *Clostridium* cultures which were grown under anaerobic conditions. Overnight cultures were streaked perpendicular to the PB6 line at three locations (top, middle, and bottom). Plates were grown overnight as described previously for each particular pathogen. Inhibition was observed as zones of clearing occurring between PB6 and the pathogen, and were measured in millimeters.

2.5. Broth Microdilution Assay

PB6 was inoculated into TSB and incubated aerobically at 37°C ± 2°C for 20–24 hours while shaking at 130 rpm. The culture was centrifuged and the supernatant was filtered through a 0.22 μm in-line syringe filter (cell-free supernatant [CFS]). Equal aliquots of the challenge organism and CFS were dispensed into individual microtiter plate wells. A control consisted of each challenge organism in BHI without CFS addition. Optical density (OD) was read at 620 nm wavelength over 20 hours at 35°C ± 2°C. All results reflect the average OD measurements of four microtiter wells.

3. Results

The results of the cross-streak assay are depicted in Fig. 1 and zone of inhibition measurements are listed in Table 1. There was a measurable inhibitory activity of PB6 against *C. perfringens*, *C. difficile*, *R. equi*, and *S. equi*. No zones of inhibition were observed for *S.* Typhimurium or *E. coli*. The broth microdilution assay (Fig. 2) showed that molecules produced by PB6 were inhibitive to the growth of both *S.* Typhimurium (ST) and *R. equi* (RE). This was indicated by a lower OD in the wells containing the combined pathogen and CFS (•) compared with the pathogen alone (▲). After 20 hours of incubation, no inhibition was observed against *E. coli* (EC) or *S. equi* (SE) as noted by similar ODs in the wells containing the combined pathogen and CFS (•) compared with the pathogen alone (▲).

4. Discussion

B. subtilis strains are known to produce molecules that are inhibitory to many pathogens. These molecules, called bacteriocins, are proteinaceous in nature and stable to high heat, bile salts, and solvents [5,15]. In vivo poultry studies have demonstrated the efficacy of *B. subtilis* PB6 against *Clostridium* spp. and *E. coli* in addition to improving growth performance. In one study, broiler chickens were infected with a pathogenic strain of *E. coli*. After 42 days on trial, birds treated with PB6 had an improved feed:gain ratio, higher weight gain, and reduced mortality as compared with the infected/not treated group [6]. In another study, birds infected with *C. perfringens*, the PB6 supplemented

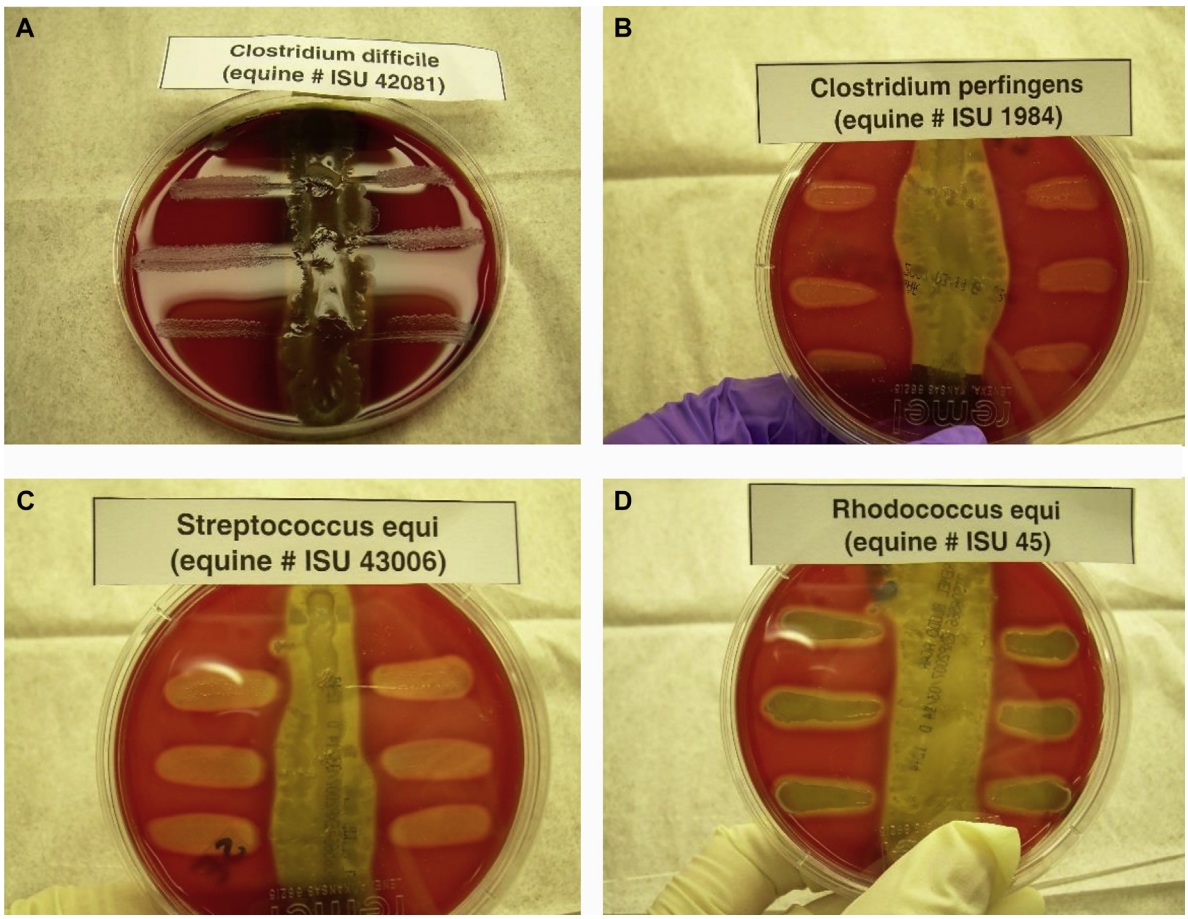

Fig. 1. PB6 inhibited the growth of *Clostridium difficile* (A), *Clostridium perfringens* (B), *Streptococcus equi* (C), and *Rhodococcus equi* (D) as demonstrated with a cross-streak agar diffusion assay. PB6 is the vertical streak with the specific pathogen as the horizontal streak. PB6, *Bacillus subtilis* PB6.

group had an improved feed:gain ratio and reduced *C. perfringens* counts in the intestines. The PB6 group also had an increased villi length to crypt depth ratio indicating improved gut health [16].

Further studies identified immunomodulatory activity in animals treated with PB6. One study with poultry resulted in an enhanced innate immune response as noted by increased bursa weights, decreased *E. coli* populations in the lower intestine, and increased phagocytosis of *E. coli* [17]. In a standardized colitis model, mice treated with PB6 demonstrated an increase in expression of the anti-inflammatory cytokine IL-10 [18]. In a clindamycin-induced *C. difficile*–associated diarrhea model, hamsters treated with PB6 showed reduced weight loss, less diarrhea, and lower mortality than the untreated hamsters. The reduction in mortality on cessation of treatment was similar to that of vancomycin [19]. In an inflammatory bowel disease model, colitis was induced in male rats. The authors theorized that the reduction in inflammation observed in the study could be from the surfactins produced by *B. subtilis* PB6. In different animal models, surfactins have demonstrated anti-inflammatory activity [19]. Therefore, it was of significant interest to identify antagonistic activity of the PB6 strain toward pathogens of equine importance as a first step in identifying its potential use as a probiotic for this species.

Table 1
Mean zones of inhibition (n = 9; ±SEM) measured for each equine isolate in millimeters (mm).

Equine Isolate	Zone of Inhibition, mm
Clostridium perfringens	18.00 ± 0.99
Salmonella Typhimurium	None
Escherichia coli	None
Streptococcus equi	10.89 ± 1.06
Rhodococcus equi	9.11 ± 0.48
Clostridium difficile	13.33 ± 0.65

Abbreviation: SEM, standard error of the mean.

Both the live culture and CFS preparation of PB6 exhibited inhibitory activity toward selected equine pathogens. The broth microdilution assay was conducted under aerobic conditions; therefore, the two *Clostridium* species were not tested in that assay. In both assays, PB6 markedly inhibited the growth of *R. equi*. Using the cross-streak assay, PB6 additionally inhibited *S. equi*, *C. perfringens*, and *C. difficile* although no inhibition was found against *E. coli* or *S.* Typhimurium. These results were consistent with previous

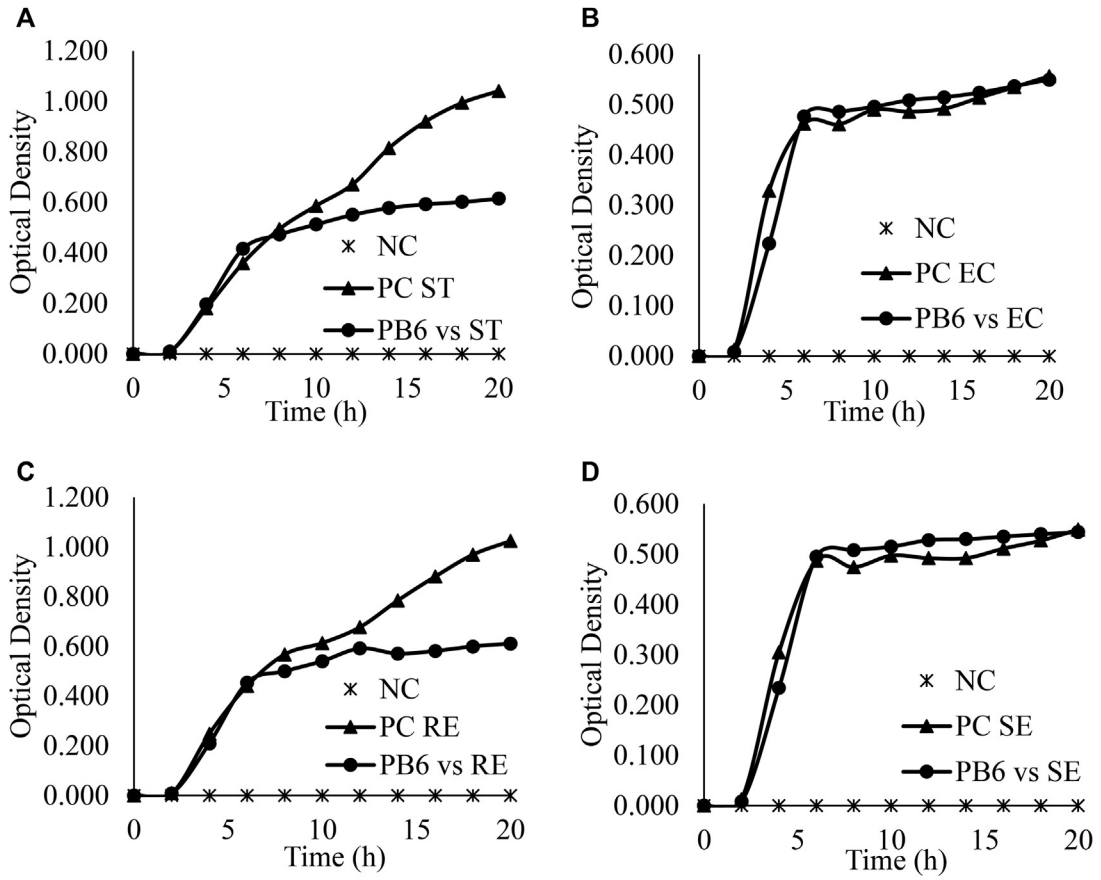

Fig. 2. The inhibitory activity of PB6 toward ST (A), EC (B), RE (C), and SE (D) as determined in a broth microtiter assay. EC, *Escherichia coli*; NC, negative control (broth without bacteria); PB6, *Bacillus subtilis* PB6; PC, positive control (broth with bacteria, without PB6 supernatant); RE, *Rhodococcus equi*; SE, *Streptococcus equi*; ST, *Salmonella* Typhimurium.

findings by other researchers in a similar agar diffusion assay [5]. In the cross-streak assay, zones of β-hemolysis were observed surrounding *B. subtilis* PB6 (Fig. 1) on blood agar plates, further indication of the presence of inhibitory molecules. The correlation between β-hemolysis and antimicrobial activity of secretory lipopeptides has been described in the literature for various *B. subtilis* species [20]. When the broth microdilution assay was used, PB6 was found to be inhibitory against *S.* Typhimurium.

It is not uncommon for in vitro assays to produce conflicting results and is further support for using multiple methodologies when evaluating biological treatments. Production of antimicrobial molecules occurs at different growth phases and has been shown to decrease once growth has entered early stationary phase [21,22]. This may be a possible explanation for some of the differences found between the two assays.

In conclusion, the data collectively indicate potential for *B. subtilis* strain PB6 to have probiotic application in equine species. Although *Bacillus* spp. do not colonize the equine gut, they can reside in that environment. Although the transit time through the equine small intestines is quick (1–3 hours), time through the hind gut can be as long as 2 days [23]. This should be sufficient time for spore germination and production of the antimicrobial molecules; however, it is recognized that in vivo efficacy trials will be needed to confirm any beneficial effect in horses.

Acknowledgments

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References

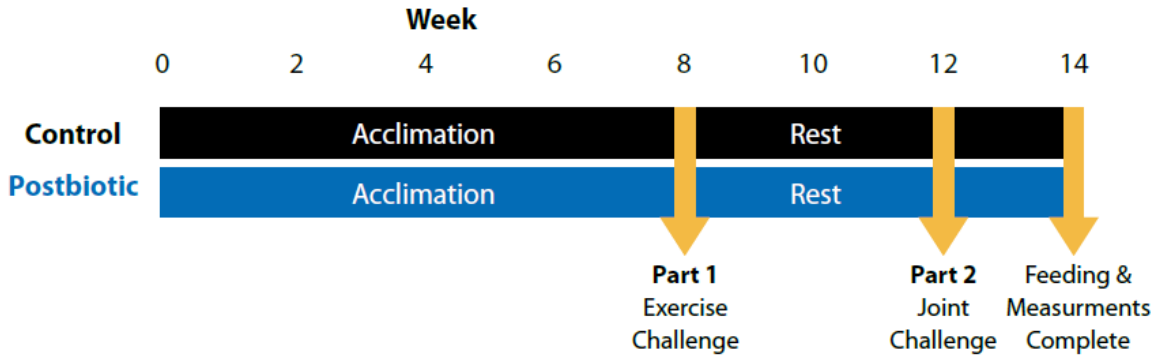
[1] Khachatourians GG. Agricultural use of antibiotics and the evolution and transfer of antibiotic-resistant bacteria. Can Med Assoc J 1998; 159:1129–36.
[2] Yirga H. The use of probiotics in animal nutrition. J Prob Health 2015;3:2.
[3] Guarner F, Schaafsma GJ. Probiotics. Int J Food Microbiol 1998;39: 237–8.
[4] Seah AHL, Teo AYL, Tan HMinventors; Kemin Ind., Singapore, assignee. Antimicrobial compounds from *Bacillus subtilis* for use against animal and human pathogens; 2002. United States Patent US 20040101525.

[5] Teo AYL, Tan HM. Inhibition of *Clostridium perfringens* by a novel strain of *Bacillus subtilis* isolated from the gastrointestinal tracts of healthy chickens. Appl Environ Microbiol 2005;71:4185–90.
[6] Teo AYL, Tan HM. Effect of *Bacillus subtilis* PB6 (CloSTAT) on broilers infected with a pathogenic strain of *Escherichia coli*. J Appl Poultry Res 2006;15:229–35.
[7] Weese SJ, Rousseau J. Evaluation of *Lactobacillus pentosus* WE7 for prevention of diarrhea in neonatal foals. J Am Vet Med Assoc 2005; 226:2031–4.
[8] Kahn CM, editor. Merck veterinary manual. 9th ed. Whitehouse Station, NJ: Merck & Co., Inc; 2005.
[9] Baverud V, Gustafsson A, Franklin A, Aspan A, Gunnarsson A. *Clostridium difficile*: prevalence in horses and environment, and antimicrobial susceptibility. Equine Vet J 2003;35:465–71.
[10] Weese JS, Staemfli HR, Prescott JF. A prospective study of the roles of *Clostridium difficile* and enterotoxigenic *Clostridium perfringens* in equine diarrhoea. Equine Vet J 2001;33:403–9.
[11] Henson S, Barton MH. Bacterial isolates and antibiotic sensitivity patterns from septicemic neonatal foals: a 15-year retrospective study (1986-2000). In Proceedings of the Dorothy R. Havemeyer Foundation Neonatal Septicemia Workshop III, Talloires France 2001: 350–352.
[12] Giguere S, Cohen ND, Chaffin MK, Slovis NM, Hondalus MK, Hines SA, et al. Diagnosis, treatment, control and prevention of infections caused by *Rhodococcus equi* in foals. J Vet Intern Med 2011;25:1209–20.
[13] Takai S, Takeda K, Nakano Y, Karasawa T, Furugoori J, Sasaki Y, et al. Emergence of rifampin-resistant *Rhodococcus equi* in an infected foal. J Clin Microbiol 1997;35:1904–8.
[14] Sweeney CR, Timoney JF, Newton JR, Hines MT. *Streptococcus equi* infections in horses: guidelines for treatment, control and prevention of strangles. J Vet Intern Med 2005;19:123–34.
[15] Sumi CD, Yang BW, Yeo IC, Hahm YT. Antimicrobial peptides of the genus *Bacillus*: a new era for antibiotics. Can J Microbiol 2015;61: 93–103.
[16] Jayaraman S, Thangavel G, Kurian H, Mani R, Mukkalil R, Chirakkal H. *Bacillus subtilis* PB6 improves intestinal health of broiler chickens challenged with *Clostridium perfringens*-induced necrotic enteritis. Poult Sci 2013;92:370–4.
[17] Teo AY, Tan HM. Evaluation of the performance and intestinal gut microflora of broilers fed on corn-soy diets supplemented with *Bacillus subtilis* PB6 (CLOSTAT). J Appl Poultry Res 2007;16: 296–303.
[18] Foligné B, Peys E, Vandenkerckhove J, Van hemel J, Dewulf J, Breton J, et al. Spores from two distinct colony types of the strain *Bacillus subtilis* PB6 substantiate anti-inflammatory probiotic effects in mice. Clin Nutr 2012;3:987–94.
[19] Peys E, Varghese J, Suresh P, Vandenkerckhove J, Van hemel J, Chaniyilparampu RN, et al. Effects of *Bacillus subtilis* 'PB6' (ATCC – PTA6737) on *Clostridium difficile* associated diarrhea (CDAD) and inflammatory bowel disease (IBD) in animal models. Am J Infect Dis 2007;3:254–65.
[20] Monteiro L, de Lima Ramos Mariano R, Souto Maior AM. Antagonism of *Bacillus* spp. against *Xanthomonas campestris* pv. *Campestris*. Braz Arch Biol Technol 2005;48:23–9.
[21] Parente E, Brienza C, Moles M, Ricciardi A. A comparison of methods for the measurement of bacteriocin activity. J Microbiol Methods 1995;22:95–108.
[22] Biswas S, Ray P, Johnson MC, Ray B. Influence of growth conditions on the production of a bacteriocin, pediocin AcH, by *Pediococus acidilactici* H. Appl Environ Microbiol 1991;57:1265–7.
[23] Dicks LMT, Botha M, Dicks E, Botes M. The equine gastro-intestinal tract: an overview of the microbiota, disease and treatment. Livest Sci 2014;160:69–81.

Effects of Postbiotic in Young Horses: Part 2 – LPS Joint Challenge

This study is part two of a two-part study with the same set of horses.

Figure 1: Timeline of experimental conditions



- 19 yearling Quarter Horses (9 ± 1 months; 266 ± 32 kg; 11 fillies, 8 colts) were included in this study. One colt was removed from Part 1 of the study (Exercise Challenge), but recovered and was included in Part 2.

- Yearlings were balanced by age, sex, body weight (BW), and farm of origin and randomly assigned to 1 of 2 treatment groups:

- Control – pelleted grain with no supplementation (CON; n=9)

- Treatment – pelleted grain plus Saccharomyces cerevisiae fermentation product

– 21 g/hd/d [(10.5 g/feeding (n=10)]3

- Horses were individually fed a basal diet (1.25% BW/d; DM basis) of custom-formulated pelleted grain + Coastal bermudagrass hay (fed ad libitum).

- Horses received diets for 14 weeks.

- At 12 weeks, one radiocarpal joint from each horse was randomly selected to receive an injection of 0.5 ng lipopolysaccharide solution (LPS) obtained from E.

coli 055:B5; the remaining carpal joint from each horse was simultaneously injected with sterile lactated Ringer's solution (LRS) and served as a contralateral control.

- Synovial fluid was obtained at hour 0 (pre-injection) and 6, 12, 24, 168, and 336 hours post-injection. Samples were tested for

- Prostaglandin E2 (PGE2; inflammatory biomarker), carboxypeptide of type II collagen (CPII; cartilage synthesis), and collagenase cleavage neopeptide (C2C ; cartilage degradation) by commercial ELISA.

Results

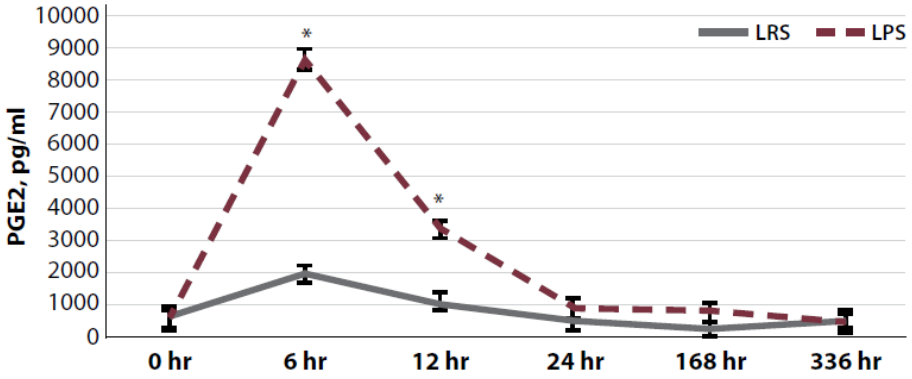
- Intra-articular injection of both LRS and LPS induced mild and moderate inflammatory responses, respectively, as measured by PGE2 in the synovial fluid.

- Overall, synovial PGE2 was lower in horses fed the diet with the Postbiotic versus horses fed the control diet.

- Regardless of injection type, horses fed the Postbiotic demonstrated significantly (P=0.01) lower PGE2 at 6 hours post-injection than horses fed the control diet.

Horses fed the diet with Postbiotic responded more rapidly and demonstrated a higher overall CPII (cartilage synthesis):C2C (cartilage degradation) ratio versus control horses. This suggests that they were able to balance the acute inflammatory insult to the joint with consistent, insult-appropriate levels of cartilage breakdown and synthesis until the response was no longer needed and these functions returned to baseline. In contrast, the responses of control horses appeared to be more erratic and less efficient.

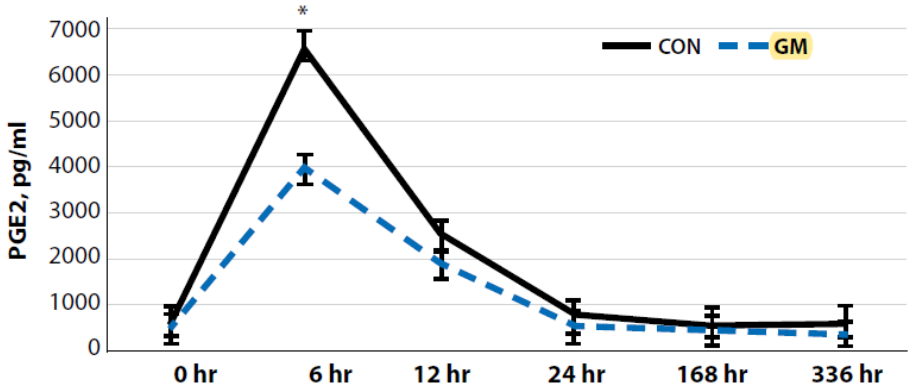
Figure 2: Effects of Time on Synovial Fluid PGE2 Concentrations After Intra-Articular Injection of Lipopolysaccharide (LPS) or Lactated Ringer's Solution (LRS)



Main Effect	*P*-Value
Diet	0.0007
Injection Type (IT)	< 0.0001
Hour (hr)	< 0.0001
Diet x IT	0.165
Diet x hr	0.0001
IT x hr	< 0.0001
Diet x IT x hr	0.428

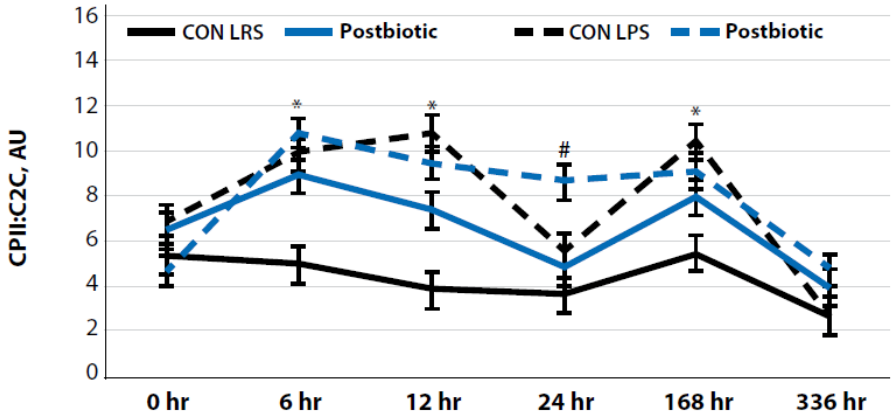
*Within time, LPS differs from LRS ($P < 0.05$)

Figure 3: Effects of Postbiotic on Synovial Fluid PGE2 Concentrations After Intra-Articular Injection of Lipopolysaccharide (LPS) or Lactated Ringer's Solution (LRS)



Main Effect	*P*-Value
Diet	0.0007
Injection Type (IT)	< 0.0001
Hour (hr)	< 0.0001
Diet x IT	0.165
Diet x hr	0.0001
IT x hr	< 0.0001
Diet x IT x hr	0.428

Figure 4: Effects of Postbiotic on Synovial Fluid CPII:C2C Ratio After Intra-Articular Injection of Lipopolysaccharide (LPS) or Lactated Ringer's Solution (LRS)



Main Effect	*P*-Value
Diet	0.003
Injection Type (IT)	< 0.001
Hour (hr)	< 0.001
Diet x IT	0.012
Diet x hr	0.089
IT x hr	< 0.0001
Diet x IT x hr	0.008

*Within time, CON LRS differs from all other treatments ($P < 0.05$)
#Within time, the Postbiotic differs from all other treatments ($P < 0.05$)

Evaluation of Postbiotic Supplementation on Immune Function in Senior Horses

Background

Senior horses are a demographic of increasing importance to equine enthusiasts as numbers continue to rise worldwide. Senior horses are frequently ridden for pleasure or kept as companion animals, while some continue engaging in athletic competitions and breeding into their late teens and twenties. The onset of old age is associated with changes in the immune system. Horses, like humans, exhibit the phenomenon of "inflammaging" characterized by physiologic marker responses reflecting chronic, systemic, low-grade inflammation that is associated with advanced age, in addition to exhibiting immunosenescence, a decreased immune response associated with old age. Furthermore, older horses exhibit changes in their gut microflora compared to younger adult horses. Therefore, supporting the gut and immune system could play a vital role in supporting optimal health and vitality during the aging process. Postbiotic technology has been shown to affect beneficial effects on digestive and immune balance in horses. The objective of this study was to determine changes in several aspects of immune function as the result of dietary supplementation of the postbiotic, a Saccharomyces cerevisiae fermentate, as a part of the total daily ration of senior horses.

Overview

Sixteen senior horses (24.8 ± 3.0 y; BW = 1200 ± 136.1 lb)

Control (CON; n=8; no supplementation) and the postbiotic top-dressed onto a common concentrate (TEC; 21 g/d; n=8).

The total supplementation period was 56 days.

Body weight and body condition score (BCS) readings and blood samples were obtained at baseline (day 0) and post supplementation at days 42, 49, and 56.

Immediately following the day 42 blood sampling all horses were vaccinated with a monovalent influenza vaccine (Fluvacc Innovator®; Zoetis Animal Health, Parsippany, NJ) as an immune system challenge.

Immune function assays included:

- Flow Cytometry: In vitro non-specific cell-mediated immune responses (IFN-γ and TNF-α) to determine the percent of IFN-γ and TNF-α producing lymphocytes.

- Cytokine Gene Expression in whole blood and peripheral blood mononuclear cell (PBMC) fraction expressed as relative quantity (RQ) including IFN-γ, TNF-α, IL-1β, IL-6, IL-10, IL-4, IL-8, IL-13, IL-17, and COX-1 and COX-2.

- H1 titers were measured to evaluate the response to vaccination.

Data was analyzed to evaluate changes before the vaccine challenge (d 0-42) and after (d 42-56).

Results

Pre-Vaccination Challenge Period (d 0-42)

• IL-10 gene expression trended lower (P < 0.090) for TEC vs CON horses (Figure 1). IL-10 plays major role in regulating inflammatory balance. A tendency for lower IL-10 gene expression as a marker may suggest immune function that is less suppressed (immunosenescent) and better able to respond appropriately to challenges.

• TNF-α produced per cell trended lower (P < 0.089) for TEC vs CON horses (Figure 2). Given the role of TNF-α as a physiological marker of inflammation, this observation may suggest a lower state of inflammation.

Table 1: Whole Blood Gene Expression Response, Ln RQ

Figure 1: IL-10 Whole Blood Gene Expression

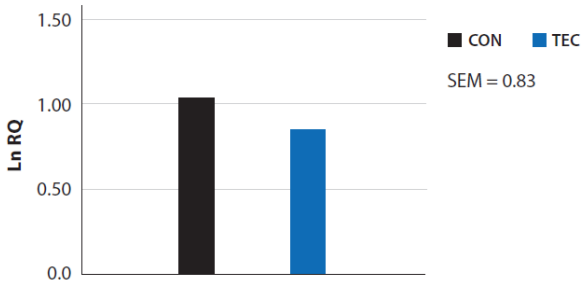
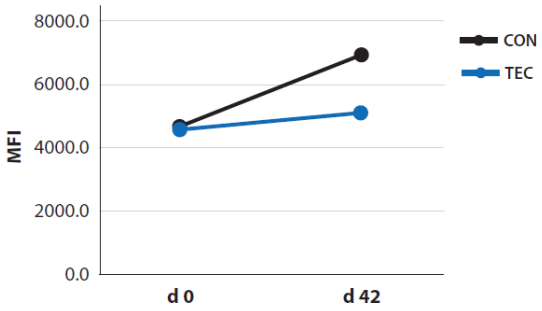


Figure 2: TNF-α MFI



Post-Vaccination Challenge Period (d 42-56)

• The response in IL-13 gene expression tended to be different over time (QUADRATIC, P < 0.080) between treatments (Figure 3).

• Horses supplemented with TEC exhibited lower gene expression for several cytokines involved in inflammation signaling compared to CON horses (Table 1).

• The response in IL-13 gene expression tended to be different over time (QUADRATIC, P < 0.080) between treatments (Figure 3).

- Serum titers in response to vaccine challenge were similar between treatments (Figure 4).

Table 1: Whole Blood Gene Expression Response, Ln RQ

Gene	Whole Blood Gene Expression Response, LnRQ		
	CON	TEC	probF
IFN-γ	1.03	0.79	*P* = 0.054
IL-10	1.04	0.78	*P* = 0.030
IL-6	1.16	0.70	*P* = 0.047

Figure 3: IL-13 Whole Blood Gene Expression

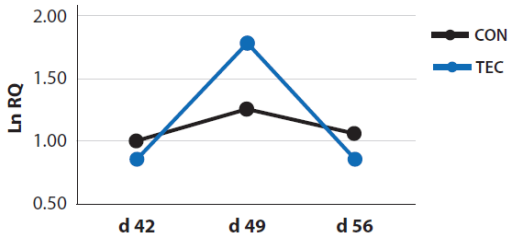
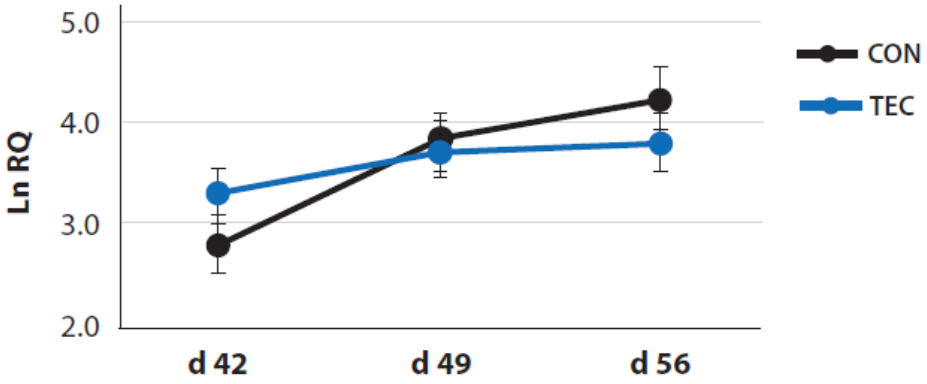


Figure 4: Serum H1 Titers Following Vaccination



Summary

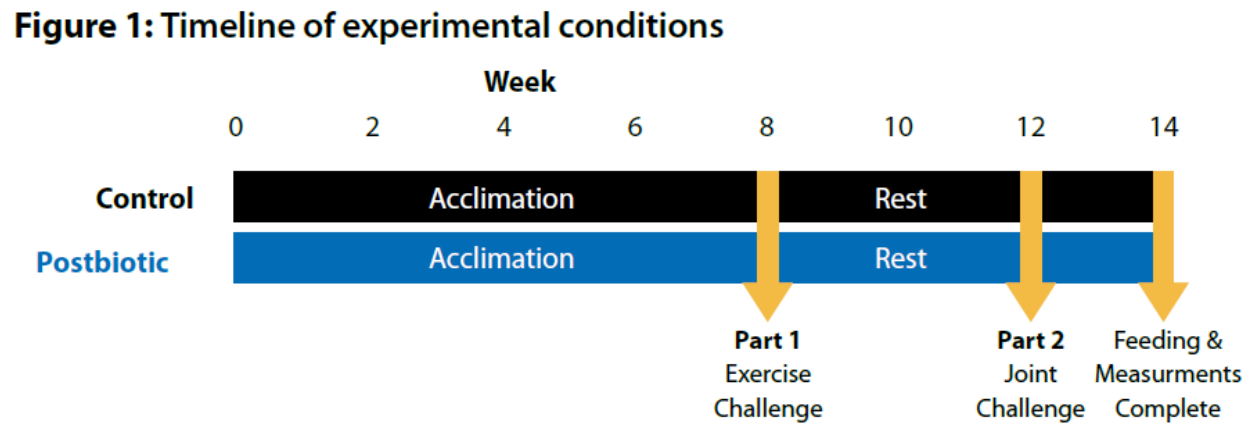
Under the conditions of this study, Postbiotics tended to modulate pro-inflammatory and anti-inflammatory cytokine gene expression in senior horses to differ from non-supplemented controls under both vaccine-challenged and non-challenged states.

Effects of Postbiotic in Young Horses: Part 1 – Exercise Stress

Overview

- This study is part one of a two-part study with the same set of horses.

Figure 1: Timeline of experimental conditions



- 19 yearling Quarter Horses were initially enrolled in the study. One horse was removed during Part One due to lameness unrelated to the study.

- The remaining 18 yearling Quarter Horses (9 ± 1 months; 267 ± 32 kg; 11 fillies, 7 colts) were included:

- Yearlings were balanced by gender, age, body weight (BW), and farm of origin and randomly assigned to 1 of 2 treatment groups:

» Control — pelleted grain with no supplementation (CON; n=8)

» Treatment – pelleted grain plus Saccharomyces cerevisiae fermentation postbiotic

– 21 g/hd/d [(10.5 g/feeding (n=10)]3

- Horses were individually fed a basal diet (1.25% BW/d; DM basis) of custom-formulated pelleted grain + Coastal bermudagrass hay (fed ad libitum).

- Horses received diets for 8 weeks prior to the exercise challenge.

- After 8 weeks, a 2-hour standardized submaximal exercise test (SET) was performed using a free stall mechanical exerciser.

• Blood samples were collected at week 0 (before supplementation), week 8 (pre-SET), and 0, 1, and 6 hours post-SET. Samples were tested for:

- Serum amyloid A (SAA; inflammatory biomarker) and cortisol concentration (stress biomarker) by commercial ELISA.

Results

Serum Cortisol Response to Exercise

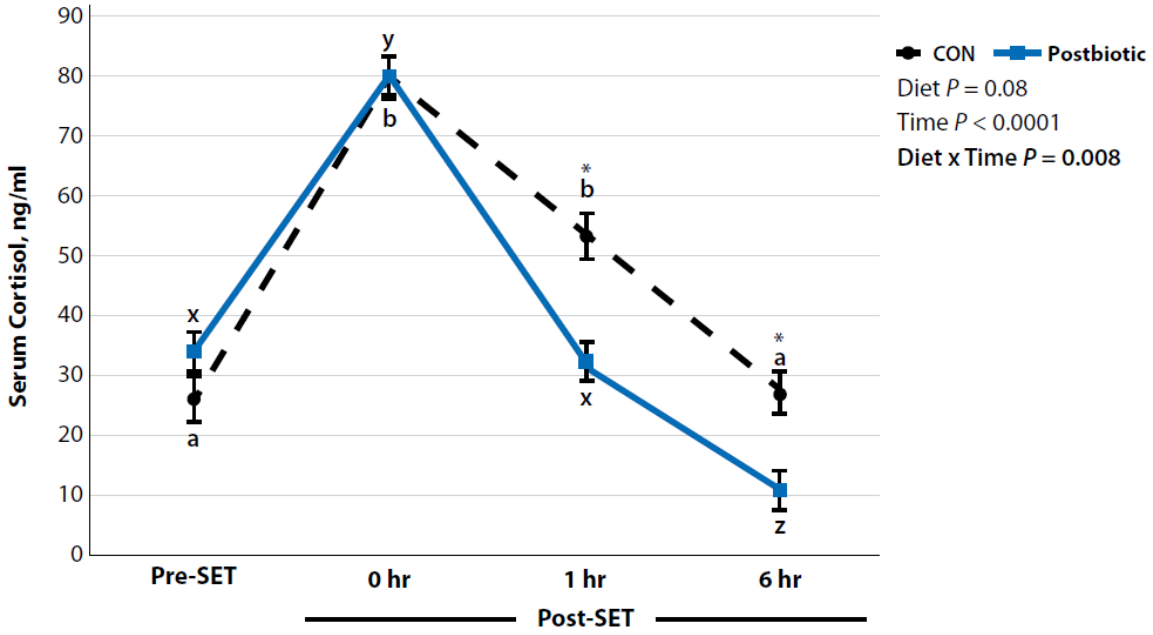
• Serum cortisol increased in both groups immediately after exercise (0 hour post-SET;

• In horses fed the diet supplemented with Postbiotic, serum cortisol returned to pre-SET levels 1 hour post-SET while serum cortisol did not return to pre-SET levels until 6 hours post-SET in unsupplemented control horses.

• Further, at 6 hours post-SET, serum cortisol in treatment horses fed the diet with Postbiotic had decreased to 68% of pre-SET levels.

In response to the SET at week 8, SAA was significantly increased (P < 0.0001) at 6 hours post-SET in CON horses but did not show a significant increase through 6 hours post-SET in treatment horses fed the diet with Postbiotic.

Figure 2: Impact of Postbiotic on Cortisol Response Post-Exercise[1]

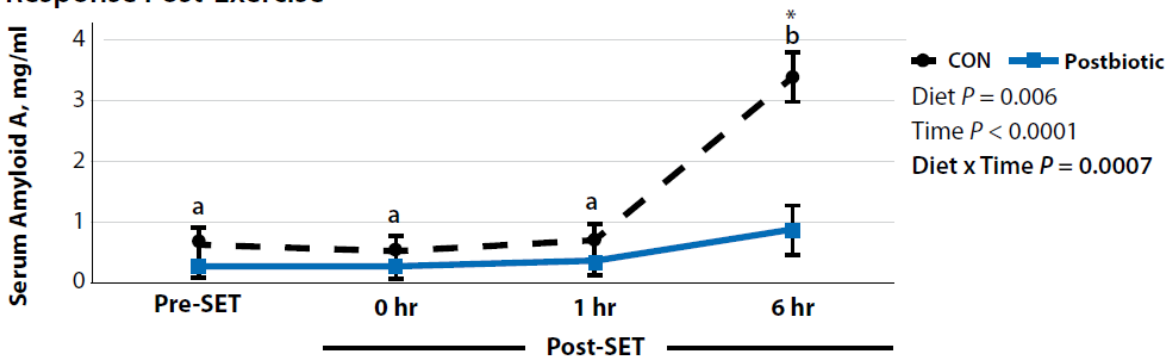


[1] Statistical significance was based on $\log_{10}$ transformed data.

[a,b,x,y,z] Within dietary treatment, time points with different letters differ ($P < 0.05$).

* Within time, CON differs from Postbiotic ($P < 0.05$).

Figure 3: Impact of Postbiotic on Serum Amyloid A (SAA) Response Post-Exercise



[a,b] Within CON, time points with different letters differ ($P < 0.0001$).

* Within time, CON differs from Postbiotic ($P < 0.0001$)

Summary

- Eight weeks of dietary supplementation with 21 g/d of postbiotic may mitigate stress (as indicated by decreased cortisol) and inflammatory responses (as indicated by lack of increased SAA) in young horses following prolonged exercise.

Effect of Coated Probiotic and Butyric Acid on Non-steroidal Anti-Inflammatory Drugs (NSAID)-
Induced Gastrointestinal Inflammation in Horses

Abstract

This study was conducted to determine the effect of Coated Probiotic and Butyric Acid on equine gut health parameters prior to and during NSAID-induced inflammation. Phenylbutazone was used as the NSAID. Thirty horses were randomly assigned to one of 3 treatments: control (no NSAID and no PB and BA), Phenylbutazone (BUTE; 4.4 mg/kg every 24 hrs; no additive), and BUTE + Butyric acid (4 g/hd/d) + Probitotic (4 g/hd/d) on -14 Day of Treatment (DOT). On 1 Day of Treatment (DOT), BUTE was administered using an oral paste as a carrier for BUTE. Gastroscopy for stomach ulcers and circulating rDNA for bacterial abundance were measured prior to and during BUTE administration. butyric Acid and Probiotic decreased squamous and glandular ulcers scores (during challenge) and 16s rDNA (prior to challenge) compared to the control/BUTE. These results indicate that the combination of Butyric Acid + Probiotic can provide a protective effect to the intestinal barrier. Further investigation is needed using a more enterically-challenged model.

Butyric acid and zinc play an important role in key biological processes affecting animal health and performance. Research has shown that butyric acid and zinc positively influence the structural integrity of the intestinal barrier through various mechanisms affecting different processes. Butyric acid is an encapsulated butyric acid and zinc product that is released in a controlled manner along the intestinal tract. In addition, Probiotic contains a unique, patented strain of *Bacillus subtilis* PB6. Although the benefits of Butyric Acid and Probiotic are known, there are no studies looking at the effect of these products combined in an equine *in vivo* model. Thus, the objective was to evaluate the effect of Butyric Acid and Probiotic using a NSAID-induced intestinal inflammation model in horses.[1, 2]

Experimental Design

Thirty-six horses were randomly selected from over 70 available horses at Texas A&M University. Three horses were matched based on age (+/- 2 years), breed, sex and weight (+/- 100 pounds) and randomly assigned to one of 3 treatments:

1. Control (no NSAID and no Butyric Acid + Probiotic)
2. Phenylbutazone (BUTE; 4.4 mg/kg every 24 hrs; no additive)
3. BUTE + Butyric Acid(4 g/hd/d) + Probiotic (4 g/hd/d)

The matching and assignment of treatments was performed 11 more times, so there was a total of 12 horses per treatment. When the horses were moved to the assigned pen pasture, they were put on a basal diet during the acclimation period for 14d (-28d to -14d DOT). On -14d DOT 10 horses were assigned to Butyric Acid + Probiotic by top dressing their basal diet with pellets containing the products (0.5 lb/hd/day). The Control and BUTE groups received the same top dressing of pellets, but the pellets did not contain Butyric Acid and Probiotic.

On 1 DOT, oral paste containing BUTE was given to the BUTE only and Butyric acid + Probiotic treatment groups and oral paste containing no added BUTE was given to the Control treatment group. The BUTE (or just oral paste) was given every 24hr up to 10 DOT. BUTE was given during the feeding time in their individual stalls. Blood and feces were taken on 3, 5, 7 and 10 DOT (during BUTE administration) and at 15 DOT of the experiment (no BUTE).

Metrics Measured

Gastroscopy was performed on all horses on d1 and d10 of the experiment. Squamous scoring was based on a previously published scoring system: 0= intact normal mucosa, 1= intact mucosa with reddening and/or hyperkeratosis, 2= small single or small multifocal ulcers, 3= large single or large multifocal ulcers, 4= extensive (often coalescing) ulcers with areas of deep ulceration.[2] Glandular ulcers were scored using the same method.

Blood was collected on -14d, d1, d3, d5, d7, d10 and d15 from each horse to determine changes in the bacterial 16S rDNA gene. Quantification of the bacterial 16S rDNA gene in blood has been used as a marker for loss of GI barrier function and bacterial translocation in people with inflammatory bowel disease and in animal models of GI diseases.[3-5]

Statistical Analysis

Data were analyzed using JMP® (SAS, Cary, NC) with significance set at P< 0.05. Gastroscopy (10d) scores were compared using ANOVA across treatments, and score proportions was compared with a chi-square test. Relative 16S rDNA concentrations were compared using repeated measures ANOVA for 0-15d data points across treatments (challenge period). In addition, ANOVA comparisons were made between -14d and 0d (pre-treatment). Since BUTE and Control were considered the same (no challenge), they were combined as one group (Control/BUTE vs. Butyric Acid + Probiotic). Contrasts were used to determine treatment differences.

Results

All horses consumed > 95% of the therapeutic and/or placebo with enthusiasm suggesting no concerns with palatability. There were no differences in body weight among groups during the pre-treatment period (-14d and 0d) nor during the challenge period (data not shown).

Gastroscopy

No horses had evidence of squamous ulcers on 0 DOT. On 10 DOT, there was a significant difference in average squamous scores with BUTE compared to Control, with Butyric Acid + Probiotic being the intermediate (Figure 1A, P=0.02). There was an association with treatment to squamous scores using Chi Square test, P=0.04 (Figure 1B).

No horses had evidence of glandular ulcers on day 0. There were treatment differences with average glandular scores (Figure 2A), and no treatment association with scores according to Chi-Square test on 10d (Figure 2B).

Figure 1A

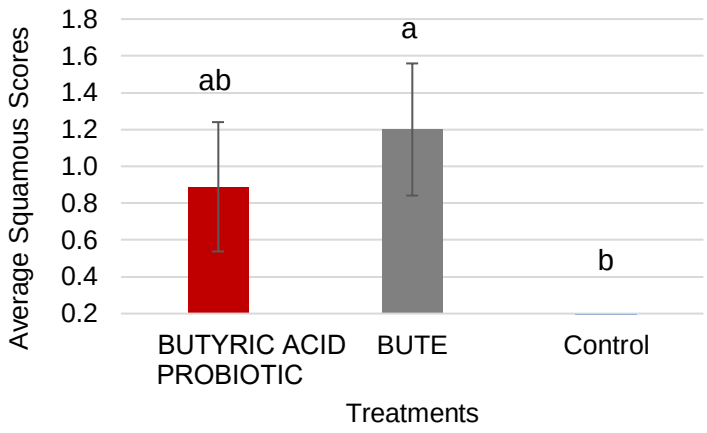


Figure 1B

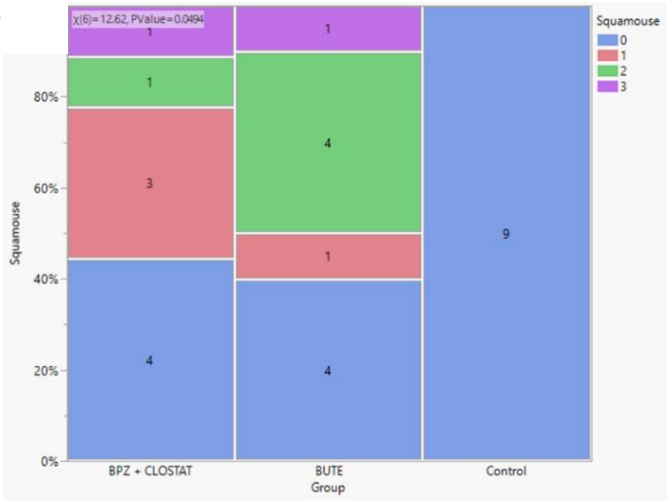


Figure 1. Average (1A) and Chi-Square (1B) Squamous scores at 10 DOT in control horses and horses challenged with either phenyl butazone or challenged and supplemented with Butyric acid and Probiotic for 10 days. Avg ± SE. [a,b]Superscripts indicate significant differences between treatments.

Figure 2A

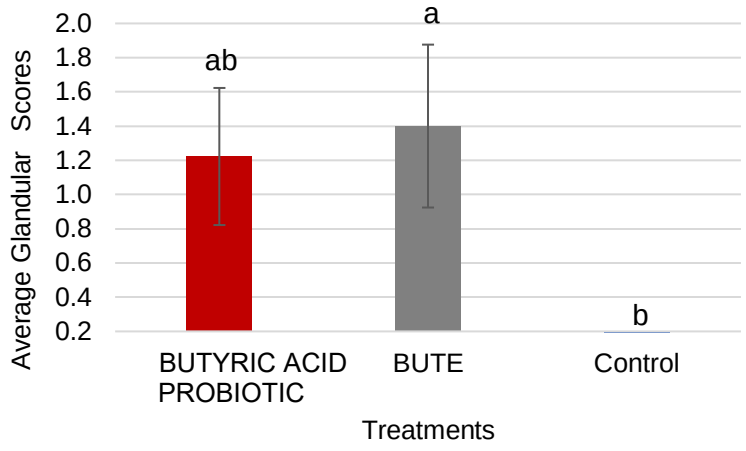


Figure 2B

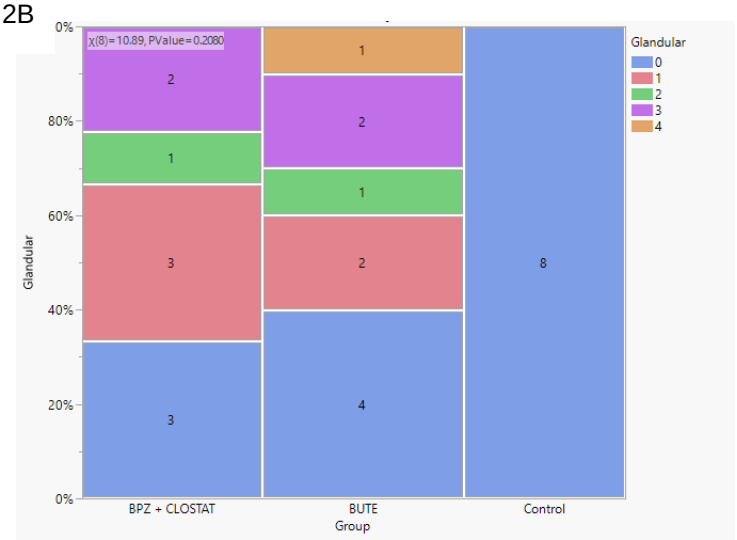


Figure 2. Average (2A) and Chi-Square (2B) Glandular scores at 10 DOT in control horses and horses challenged with either phenyl butazone or challenged and supplemented with Butyric Acid and Probiotic for 10 days. Avg ± SE. [a,b]Superscripts indicate significant differences between treatments. *One control horse was removed from the statistical analysis for non-conformity.

Circulating 16s rDNA:

There was no difference among treatments with 16s rDNA during the challenge period (0 to 15 DOT; Figure 3). During the pretreatment period (-14 to 0 DOT), there was a signficant difference between control (control and BUTE combined) and Butyric acid + Probiotic at 0d (P= 0.0003; Figure 4).

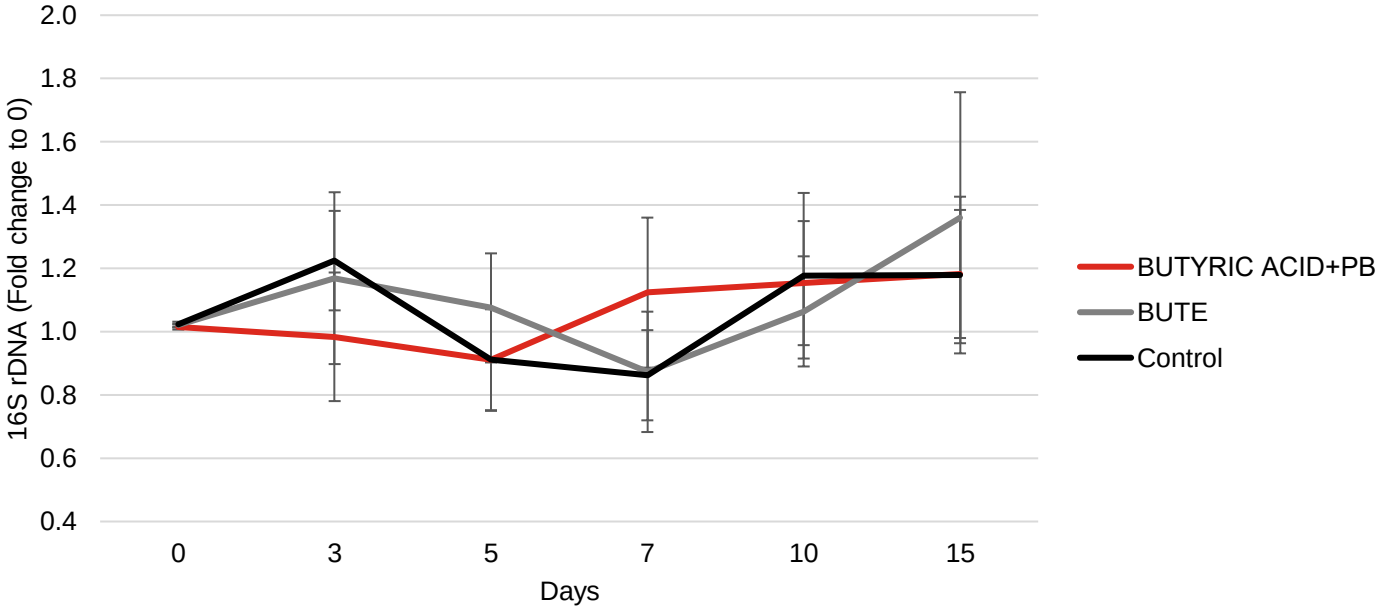


Figure 3. Fold Change of 16S rDNA for each treatment within the challenge period.
Avg ± SE

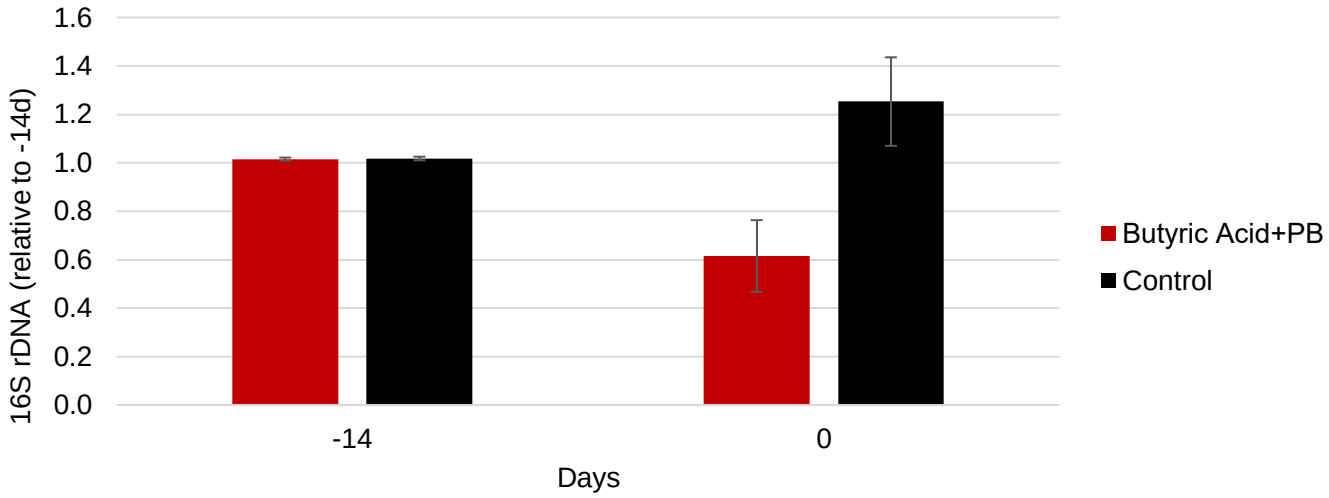


Figure 4. Fold Change of 16S rDNA for each treatment prior to challenge.
Control and BUTE horses were combined for ANOVA analysis. Avg ± SE Contrasts: -14d Control vs. 0d Control, P =0.15; -14d BPZ vs. 0d BPZ EQ, P=0.10; 0d Control vs. 0d BPZ EQ, P=0.003

Conclusions

Butyric acid + Probiotic was palatable based on normal eating behavior of the horses, and no changes in body weight across treatments were observed during the testing period. Overall, Butyric acid + Probiotic showed an effect on squamous and glandular ulcers (during the challenge period) and 16s rDNA (prior to challenge) compared to the control and/or BUTE. These results indicate that the combination of Butyric acid and Probiotic provides a protective effect to the gut barrier under NSAID induced challenge. In order to investigate further on the effect of Butyric acid and Probiotic combination on gut health, enterically-challenged horses need to be used.

References

1. Richardson LM, Whitfield-Cargile CM, Cohen ND, Chamoun-Emanuelli AM, Dockery HJ. Effect of selective versus nonselective cyclooxygenase inhibitors on gastric ulceration scores and intestinal inflammation in horses. Veterinary surgery : VS. 2018;47(6):784-91. Epub 2018/08/11. doi: 10.1111/vsu.12941. PubMed PMID: 30094858.

2. Whitfield-Cargile CM, Chamoun-Emanuelli AM, Cohen ND, Richardson LM, Ajami NJ, Dockery HJ. Differential effects of selective and non-selective cyclooxygenase inhibitors on fecal microbiota in adult horses. PloS one. 2018;13(8):e0202527. Epub 2018/08/24. doi: 10.1371/journal.pone.0202527. PubMed PMID: 30138339; PubMed Central PMCID: PMCPMC6107168.

3. Vrakas S, Mountzouris KC, Michalopoulos G, Karamanolis G, Papatheodoridis G, Tzathas C, et al. Intestinal Bacteria Composition and Translocation of Bacteria in Inflammatory Bowel Disease. PloS one. 2017;12(1):e0170034. doi: 10.1371/journal.pone.0170034.

4. Kramski M, Gaeguta AJ, Lichtfuss GF, Rajasuriar R, Crowe SM, French MA, et al. Novel sensitive real-time PCR for quantification of bacterial 16S rRNA genes in plasma of HIV-infected patients as a marker for microbial translocation. Journal of clinical microbiology. 2011;49(10):3691-3. Epub 2011/08/05. doi: 10.1128/jcm.01018-11. PubMed PMID: 21813723; PubMed Central PMCID: PMCPMC3187295.

5. Potgieter M, Bester J, Kell DB, Pretorius E. The dormant blood microbiome in chronic, inflammatory diseases. FEMS microbiology reviews. 2015;39(4):567-91. Epub 2015/05/06. doi: 10.1093/femsre/fuv013. PubMed PMID: 25940667; PubMed Central PMCID: PMCPMC448740

6. Effect of CLOSTAT and ButiPEARL Z EQ on Non-steroidal Anti-Inflammatory Drugs (NSAID)-Induced gastrointestinal Inflammation in Horses -TD-22-8083

Protective Effects of Butyric Acid and Zinc on Equine Intestinal Cell Function Under Hypoxic Conditions

Abstract

Spheroids were isolated from small intestinal crypts to evaluate the effect of butyric acid and zinc on dye leakage under hypoxic conditions. Spheroids were luminally injected with butyric acid and zinc-FD4 or FD4 only and cultured in normoxic or hypoxic conditions for 17 hours. Images were taken at the start and end of the experiment and corrected total cell fluorescence (CTCF) was calculated to determine FD4 dye loss. Treatment with butyric acid and zinc decreased dye loss in both normoxic (p=0.09) and hypoxic conditions (p=0.039) compared to control spheroids. Results support that supplementing equine intestinal spheroids prior to cellular injury with butyric acid and zinc may benefit the intestinal epithelium in horses undergoing gastrointestinal stressors.

Introduction

Intestinal epithelial cells are essential in absorbing nutrients needed by the host while simultaneously creating a barrier that excludes harmful toxins and pathogens from entering the host. Environmental and disease stressors can play a major role in compromising these functions which can lead to intestinal and systemic inflammatory challenges. Butyric acid[1-3] and zinc[4,5] have been shown to improve intestinal barrier function via tight junction expression, a key component in maintaining intestinal integrity. Previous *in vitro* studies in swine and poultry have demonstrated a protective effect with the combination of butyric acid and zinc on barrier function in heat stress and inflammatory conditions.[6,7]

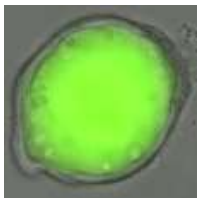
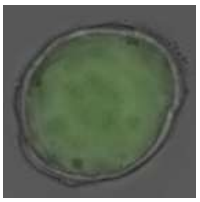
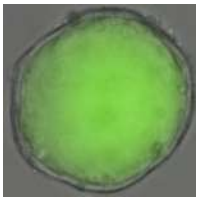
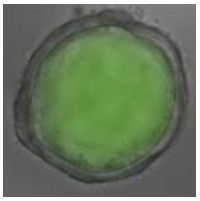
The current study evaluated the protective effects of butyric acid and zinc on intestinal barrier function under normoxic and hypoxic conditions in primary equine spheroids.

Materials and Methods

Equine spheroids were isolated from small intestinal crypts and cultured in a three-dimensional extracellular matrix (Matrigel® Matrix, Corning Inc., Corning, NY) supplemented with growth factors and media. FITC-dextran 4 kDa (FD4), a fluorescent marker, was used to quantify permeability of the spheroids. Spheroids were luminally injected with butyric acid and zinc-FD4 or FD4 only (3-5 spheroids/ treatment/group). Successful injection criteria included: the entire lumen needed to be filled with dye, no dye could be seen outside of the spheroids and initial exposure time on the microscope needed to be low. After injection, spheroids were placed in normoxic (1% O2) or hypoxic (1% O2) conditions for 17 hours. Fluorescence images were taken at the start of the experiment (0 hour) and 17 hours after and corrected total cell fluorescence (CTCF) was calculated to determine FD4 dye loss. Multiple t-tests were performed with corrections for multiple comparisons using the Holm-Sidak method to compare percentage of dye loss of the treatment (butyric acid and zinc) to control in normoxic and hypoxic conditions.

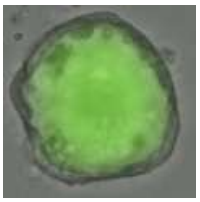
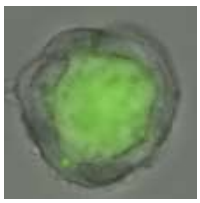
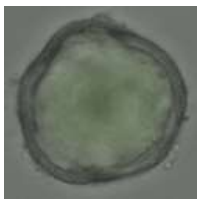
Results

Figure 1 and 2 are representative images of the spheroids in normoxic and hypoxic conditions, respectively. In normoxic conditions, the spheroids injected with butyric acid and zinc showed a reduction (15% difference) in dye loss compared to the control (Figure 3). There was a significant reduction (13% difference) in dye loss with butyric acid and zinc spheroids compared to control in hypoxic conditions.

Treatment	0 hours	17 hours
Control*		
Butyric acid and zinc*		

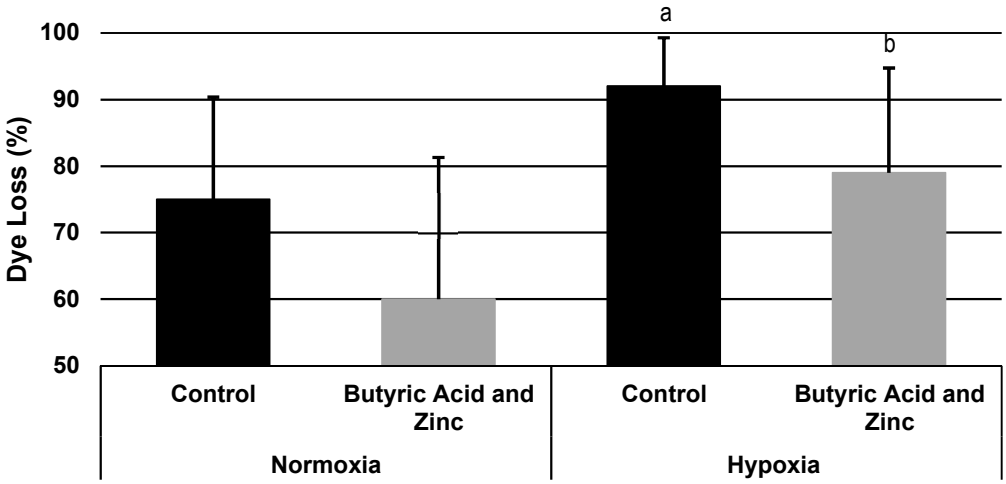
*Dye loss for control was 70%; butyric acid and zinc was 44%

Figure 1. Representation of percent dye loss of spheroids in normoxic conditions.

Treatment	0 hours	17 hours
Control*		
Butyric acid and zinc*		

*Dye loss for control was 92%; butyric acid and zinc was 55%

Figure 2. Representation of percent dye loss of spheroids in hypoxic conditions.



Differing superscripts indicate statistical significance within culturing conditions P ≤ 0.05.
Error bars represent standard deviation.
Treatments were replicated N=11 control, normoxia; N=10 butyric acid and zinc, normoxia; N=control, hypoxia; N=18 butyric acid and zinc, hypoxia.

Figure 3. Percent dye loss of spheroids in normoxic and hypoxic conditions

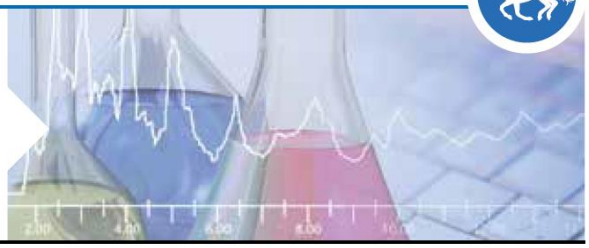
Conclusions

Results of this study demonstrate that supplementing equine intestinal spheroids with butyric acid and zinc may have a protective effect on intestinal barrier function under hypoxic conditions. Further research using this novel model will provide an avenue to develop and investigate additional solutions to protect against intestinal injury.

References

1. Peng, L., et al., Butyrate enhances the intestinal barrier by facilitating tight junction assembly via activation of AMP-activated protein kinase in Caco-2 cell monolayers. J Nutr. 2009. 139(9): p. 1619-25.
2. Ma, X., et al., Butyrate promotes the recovering of intestinal wound healing through its positive effect on the tight junctions. J Anim Sci, 2012. 90 Suppl 4: p. 266-8.
3. Wang, H.B., et al., Butyrate enhances intestinal epithelial barrier function via up-regulation of tight junction protein Claudin-1 transcription. Dig Dis Sci, 2012. 57(12): p. 3126-35.
4. Hu, C., et al., Diosmectite-zinc oxide composite improves intestinal barrier function, modulates expression of pro-inflammatory cytokines and tight junction protein in early weaned pigs. Br J Nutr, 2013. 110(4): p. 681-8.
5. Roselli, M., et al., Zinc Oxide Protects Cultured Enterocytes from the Damage Induced by *Escherichia coli*. J. Nutr., 2003. 133: p. 4077–4082.
6. Vignale, K, Koltes D., Weil J., West S., Weimer S.L., Iseri V. and Christensen K.D 2017. The effect of encapsulated butyric acid and zinc on performance and gut integrity in heat stressed male broiler chickens. 2017 International Poultry Scientific Forum. Atlanta, GA. Abstract: T180, page 53.
7. Mani, V., Rubach, J.K., Koltes, D.A., Gabler, N.K., and Poss, M.J. 2016. The protective effects of ButiPEARL™ Z during heat stress as measured through in vitro studies with swine intestinal epithelial cells and an in vivo swine trial. 2016 Midwest animal science meeting. Journal of Animal Science, Volume 94, Issue suppl. 2, Page 150.
8. Kemin Internal Document, 18-00652.

FOCUS ON RESEARCH



Effects of feeding Postbiotic on exercise performance of Arabian horses

Overview

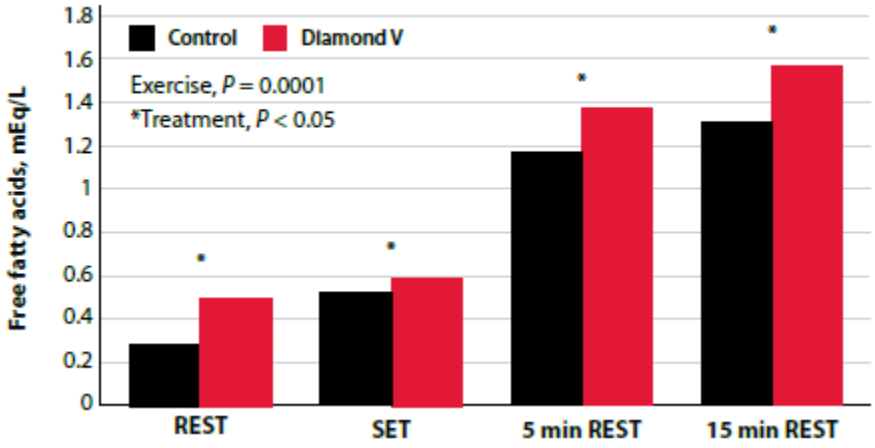
- 6 Arabian horses (4 males and 2 females) that were previously not exercised were used in a crossover design
- Average body weight of 445 kg
- Treatments
  1. Control
  2. Postbiotic (60g per day)
- Following a 3-week dietary treatment adjustment period, a Standard Exercise Test (SET) was conducted on a treadmill (Table 1)
- SET was designed to produce a heart rate up to 190 beats/min and blood lactate levels of at least 4mM to observe the change to anaerobic catabolism
- One REST period was included before and two (5 and 15 min) after the exercise test
- Blood was collected and heart rate measured for each period.

Table 1: Standard exercise test (SET) parameters.

Interval	Speed, m/s	Time, min	Incline, %
Warm-up	1.6	5	0
STEP1	3.5	3	0
STEP2	4.5	3	6
STEP3	5.0	3	6
STEP4	5.5	3	6
STEP5	6.0	3	6

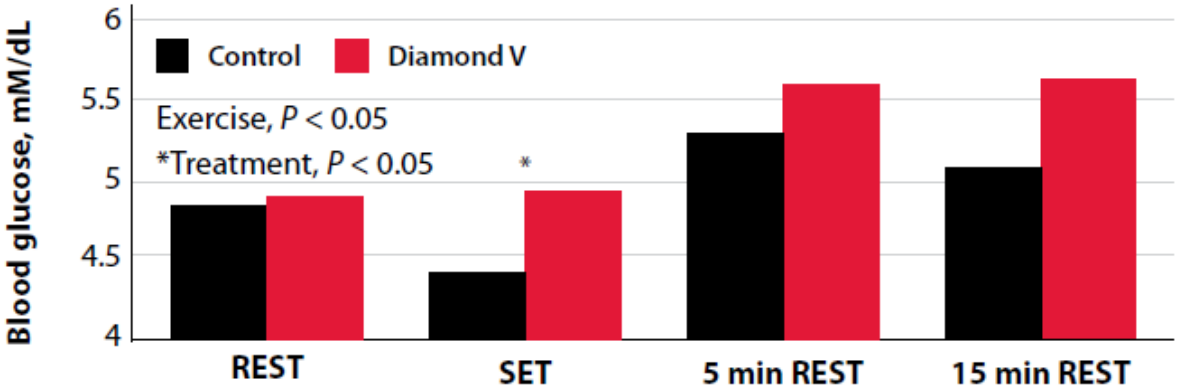
Results

Figure 1: Free fatty acid (FFA) levels before, during, and following exercise.



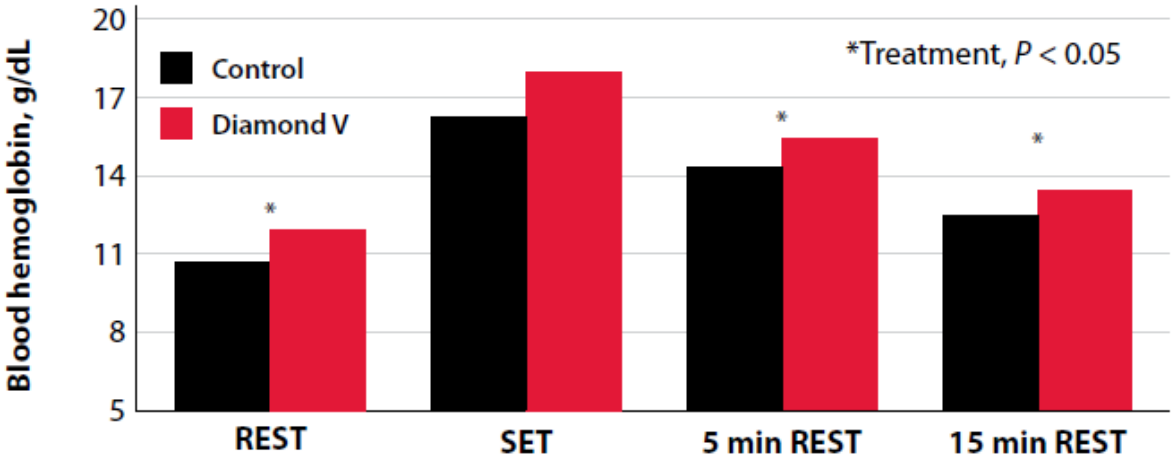
- Supplementing horses with Postbiotic increased FFA during both all REST and SET exercise periods ($P < 0.05$; Figure 1).
- FFA levels in the blood increased with the increase in exercise intensity ($P = 0.0001$).

Figure 2: Blood glucose levels before, during, and following exercise.



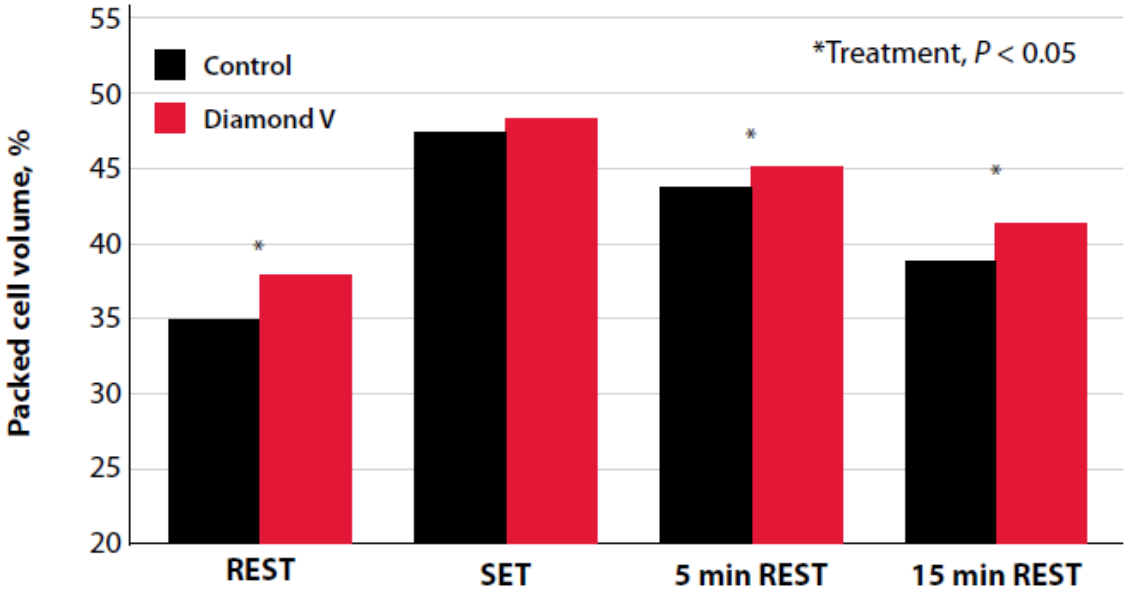
- Horses fed Postbiotic had greater blood glucose during the SET periods ($P < 0.05$; Figure 2).
- Blood glucose initially decreased, but then increased with the exercise intensity ($P < 0.05$).

Figure 3: Blood hemoglobin levels before, during, and following exercise.



- Horses fed Postbiotic had greater blood hemoglobin before and after SET periods ($P < 0.05$; Figure 3).

Figure 4: Packed cell volume before, during, and following exercise.



- Supplementing Postbiotic increased packed cell volume during REST ($P < 0.05$; Figure 4).

Summary

- The SET program led to an increase in FFA ($P = 0.0001$) and blood glucose ($P = 0.05$).
- Supplementing Postbiotics to the horse helped support greater levels of FFA, hemoglobin and packed cell volume before and after exercise ($P < 0.05$).

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Saccharomyces cerevisiae fermentation product improves robustness of equine gut microbiome upon stress

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Introduction: Nutritional and environmental stressors can disturb the gut microbiome of horses which may ultimately decrease their health and performance. We hypothesized that supplementation with a yeast-derived postbiotic (*Saccharomyces cerevisiae* fermentation product-SCFP) would benefit horses undergoing an established model of stress due to prolonged transportation.

Methods: Quarter horses ($n = 20$) were blocked based on sex, age (22 ± 3 mo) and body weight (439 ± 3 kg) and randomized to receive either a basal diet of 60% hay and 40% concentrate (CON) or the basal diet supplemented with 21 g/d a Postbiotic for 60 days. On day 57, horses were tethered with their heads elevated 35cm above wither height for 12 h to induce mild upper respiratory tract inflammation. Fecal samples were collected at days 0, 28, and 56 before induction of stress, and at 0, 12, 24, and 72 h post-stress and subjected to DNA extraction and Nanopore shotgun metagenomics. Within sample (alpha) diversity was evaluated by fitting a linear model and between sample (beta) diversity was tested with permutational ANOVA.

Results: The SCFP stabilized alpha diversity across all time points, whereas CON horses had more fluctuation ($P < 0.05$) at 12, 24, and 72 h post-challenge compared to d 56. A significant difference between CON and SCFP was observed at 0 and 12 h. There was no difference in beta-diversity between SCFP and CON on d 56.

Discussion: Taken together, these observations led us to conclude that treatment with SCFP resulted in more robust and stable microbial profiles in horses after stress challenge.

KEYWORDS

equine, horse, *Saccharomyces cerevisiae* fermentation product (SCFP), postbiotic, stress, microbiome

Introduction

The role of the microbiome and its importance has been well-established in several systems over the past two decades, including in environmental (1, 2), biomedical (3–5), and agricultural (6–8) contexts. Herbivores are particularly impacted by the gastrointestinal microbiome, given their interdependency with metabolic pathways only present in microbes that are necessary for digestion of complex carbohydrates present in forages that typify the equine daily diet.

In horses relatively fewer research studies investigating the microbiome are available, however the number of such studies is increasing rapidly (8, 9). Among the many factors that can impact the equine microbiome, stress is one of the most preeminent ones. Both diet- (10) and exercise- (11, 12) induced stress have been associated with microbiome changes in horses. Unstable microbiomes represent an open niche for opportunistic pathogen establishment and are associated with worse health outcomes. In fact, colonization resistance is one of the biggest roles played by the microbiome in maintaining host health (13–15). Thus, maintaining a robust microbiome upon stressful events would be beneficial for horse health.

Several techniques can be applied to intentionally manipulate the diversity and composition of the gut microbiome in the quest to maintain an optimal microbial community. Diet modification, pre-, pro-, and postbiotic administration, and more drastic therapeutics such as antibiotic therapy and fecal microbiota transplantation are also used for microbiome modulation (16). Postbiotics are defined as a "preparation of inanimate microorganisms and/or their components that confers a health benefit on the host" (17, 18). These preparations do not necessarily originate from probiotic microorganisms and must contain an unpurified mixture of inanimate organisms and their metabolites. Because the mode of action of postbiotics does not rely on presence of live organisms in the final product, they represent an attractive alternative for feed supplementation given their better stability during feed processing (19). Several studies evaluating the efficacy of postbiotic supplementation of *Saccharomyces cerevisiae* fermentation products (SCFP) in bovine (20, 21), avian (22), and equine (23) species have been performed. While the mechanisms by which postbiotics confer benefits to the host have not yet been completely elucidated, much of the literature indicates that postbiotic supplementation is associated with microbiome optimization (24) and improvement of immune function (20, 22, 25). However, less is known about the effects of SCFP on horses. A few recent studies have indicated improvement in immune parameters in a vaccine challenge model (23, 26) while no difference was observed in the microbiota of racehorses fed a yeast supplement (27). Taking the wealth of evidence of the beneficial effects of postbiotic administration in many species, it is reasonable to hypothesize that postbiotic administration would benefit horses under stress.

Horses are exposed to stressful situations daily, including transportation, exercise, and diet changes. Although several studies have demonstrated the impact of stressful events on the equine fecal microbiome (10, 28) little evidence is available on how postbiotic administration can impact the robustness of microbiome in horses under stress. Thus, the objective of this study was to determine if supplementation with SCFP would result in more robust microbiome in an established equine model to simulate stress due to prolonged transportation. We hypothesized that SCFP supplementation would result in a more robust microbiome that would be less impacted by experimental stress.

Abbreviations: CAZy, Carbohydrate Active enZyme; CLR, Centralized Log-Ratio; FDR, False Discovery Rate; SCFP, *Saccharomyces cerevisiae* fermentation product.

Materials and methods

Experimental design, animals, and sample collection

The animal experiment for present microbiome study was described by Tench et al. (29). The protocol for the use of experimental animals was approved by the Institutional Animal Care and Use Committee at the University of Florida in Gainesville, FL (#201810324) under the Guide for the Care and Use of Agricultural Animals in Research and Teaching (30).

Briefly, 20 young and clinically healthy horses in training (mean ± SEM; initial age 22 ± 0.3 mo and BW 439 ± 3 kg) were paired by age and sex and randomly assigned to one of the two experimental treatments for 60 days. Treatments included supplementation with 0 g/d (Control; no treatment Control) or 21 g/d Postbiotic. A basal diet of 60% Coastal bermudagrass hay and 40% concentrate formulated to meet the nutrient requirements of horses at a moderate rate of growth (31) was offered to all horses. Treatment administration was done by top dressing SCFP on the concentrate ration. Horses were exercised 4 days per week for 30–45 min/d at light to moderate intensity. On day 57, horses were placed in individual stalls and tethered with their heads elevated 35 cm above wither height for 12 h to induce mild upper respiratory tract inflammation according to a previously established protocol to mimic long-distance transport stress (32, 33). Induction of inflammation was confirmed by significantly elevated serum cortisol and blood leukocyte measurements performed after stress induction compared to pre-stress (34, 35). The stress period was relieved after the 12 h timepoint by untethering of the horse heads. Fecal samples were collected into sterile containers at seven time points: days 0, 28, and 56 before induction of stress, and at 0, 12, 24, and 72 h post-stress, where 0 h is the time at which the horses were untethered. Samples were immediately placed on ice and transported to the laboratory where they were kept in a −80°C freezer until DNA extraction. A schematic of the experimental design and sample collection is given in Figure 1.

DNA extraction and shotgun metagenomic sequencing

DNA extraction

Fecal samples were removed from the −80°C freezer 1 day prior to DNA extraction and thawed in a 4°C refrigerator overnight. The ZymoBIOMICS 96 MagBead DNA kit (Zymo Research Corporation, Irvine, CA) was used in a Biomek i7 (Beckman Coulter, Indianapolis, IN) workstation for DNA extraction according to manufacturer's instructions. Four extraction blanks were included in each 96 well plate to confirm that cross contamination did not occur.

Nanopore sequencing

Libraries were constructed using the SQK-RPB004 Rapid PCR Barcoding kit (ONT, Oxford, UK). Library preparation included

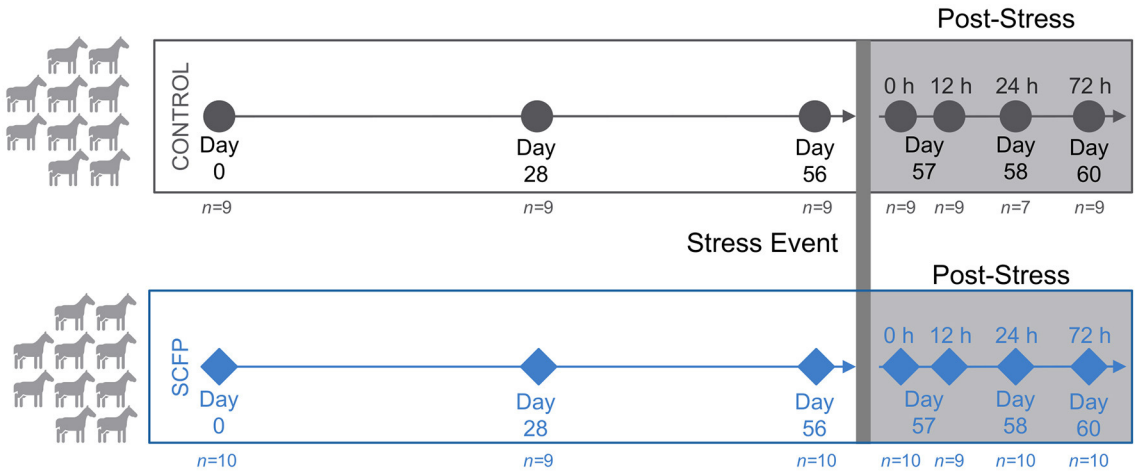

FIGURE 1
Study overview. Young and clinically healthy horses were paired by age and sex and randomly assigned into Control ($n = 10$) or *Saccharomyces cerevisiae* fermentation product (SCFP; $n = 10$). Horses received diets for 60 days. On day 57, horses were subjected to a previously established stress protocol to induce mild upper respiratory tract inflammation that mimicked long-distance transportation. Samples were collected on days 0, 28, and 56 before stress and at 0, 12, 24, and 72 h post-stress.

DNA extraction blanks for quality control. Shotgun metagenomic sequencing was performed using R9.4.1 FLO-MIN 106 flow cells on the GridION platform (ONT, Oxford, UK), multiplexing 12 samples in each flow cell. Each sequencing run lasted 70 h. The MinKNOW ONT software (v 3.6.5) with Guppy basecaller was used for sequencing using the high-accuracy basecalling setting, followed by de-multiplexing, adapter trimming, and quality control using default settings.

Bioinformatics and statistical analyses

Taxonomic assignment and microbial diversity

Fastq files obtained from the MinKNOW ONT workflow were used for microbial taxonomic classification. First, host DNA was removed by mapping fastq files to the horse genome (assembly EquCab3.0) using Minimap2 (36) followed by the removal of any reads matching the horse genome using SAMtools (37). The remaining reads were assumed to be from microbial origin and used for taxonomic assignment. To improve microbial classification, a custom database was made, which contained high quality genomes from the RefSeq database (38) and published metagenome-assembled genomes (38–40). The Kraken2 pipeline (41) was used for species identification and Bracken was used to estimate species abundances (42). Diversity metrics were calculated in R (43) using the Phyloseq package (44, 45) with the rarefied species count table from Bracken as input. Species tables were center-log transformed using the microbiome package (46) after imputation of zeros using a Bayesian multiplicative replacement method from the zCompositions package (47). Species with non-zero presence in at least 75% of samples and relative abundance >0.001% were identified separately in the pre-stress and post-stress periods and the superset containing all species was used for differential abundance analysis.

Functional potential

To have a better understanding of the microbiome functional potential, the Carbohydrate-Active enZymes (CAZy) (48) present in the microbiome communities for each sample were identified. First, genomes from microbial species identified with Kraken2 were annotated using PROKKA (45), followed by additional assessment of gene function using EggNOG-mapper v2 (49). After the annotation process was completed, a custom python script was used to compile the CAZy for each genome, generating a table with the accumulated CAZy potential for all the microbes identified for each sample. Results were compiled into a final table containing numbers of annotated features for each sample.

Statistical analyses

Diversity metrics

Within sample (alpha) diversity was evaluated by fitting a linear model with the lmer function of the lme4 package (50) in R. The model included Shannon diversity index as the dependent variable, horse as a random effect, treatment, timepoint, and their interactions as independent variables. Because stress is nested within timepoint, the effect of stress only is evaluated in a separate model. Between sample (beta) diversity was tested with permutational ANOVA using the adonis function in the vegan package (51) in R. The model included Aitchison distances (52) calculated based on CLR transformed values as the dependent variable, and treatment, timepoint, and their interactions as independent variables. Data was visualized with PCA using the Phyloseq package (44, 45).

Differential abundance

A modified version of the linear discriminant analysis from the LinDA package (53) was used to fit linear models that included relative abundances as the dependent variable, treatment,

timepoint, and their interaction as independent variables, and horse as a random effect. The output from each model was then analyzed with the emmeans package (54) to calculate fold changes of centralized log ratio (CLR) transformed data of each measurement (species) for each animal with respect to their initial sample collected at day 0. False discovery rate (FDR) correction (55, 56) was used to identify species within each timepoint that significantly differed between treatment and Control.

Correlation networks

An adaptation of the CoNet framework (57), which includes generation of a combination of diverse measures of correlation (including Pearson's, Spearman's, and Kendall's correlation coefficients) using CLR transformed data was used for correlation network analyses. Distributions of all pair-wise scores between the nodes were computed for each timepoint. Only edges (correlations) with p-values < 0.05 after FDR correction (55, 56) were taken into further consideration, and edges not supported by at least two measures were discarded.

Clustering

Identification of the optimal number of clusters and clustering was calculated and performed using gap statistics (58) in MATLAB R2019b (59) using the spearman correlation for species and CAZy identifiers, and Aitchison distance for samples. The difference of the CLR transformed values at any time point and its corresponding value at day 0 were used as the input. The data was sorted based on experimental variables or clusters and visualized.

Results

Sequencing parameters

A total of 140 samples were sequenced. On average, 389,680 reads were obtained per sample (mean 389,680, median 377,834, SD 118,596). Read N50 lengths averaged 4,043 bp (mean 4,043 median 4,052, SD 318). Reads had an average quality score of 12 (mean 12, median 12, SD 0.6). On average, 1,429,212,272 total bases were obtained per sample, with a standard deviation of 413,891,226 bases per sample. Four samples had low sequencing throughput and were removed from further analysis.

Taxonomic assignment

On average, 67% of reads were assigned at the species level (mean 66.9%, median 67.4%, SD 4.4%). A total of 119 taxa were identified (Supplementary Table 5). Of those, 27 taxa fit the criteria of being present in at least 75% of samples and relative abundance $>0.001\%$ in the pre-stress period and 18 taxa fit the criteria in the post-stress period. The final superset that was used for differential abundance analysis contained 27 taxa.

Stress significantly impacts microbial diversity and SCFP treatment leads to a more robust microbiome after stress

Alpha diversity was similar between Control and SCFP groups in the pre-stress period (Figure 2), indicating treatment with SCFP did not significantly alter Shannon microbial diversity index values. Stress impacted ($P < 0.0001$) diversity levels both in the Control and SCFP groups. However, stress had a lower impact in changing the SCFP group's diversity levels when compared to Controls. Overall, horses treated with SCFP exhibited robust microbial diversity after stress, with less variation and overall lower stress-induced drop in diversity when compared to the Control group (Supplementary Table 1). When within-group comparisons were made, statistical differences were observed in the Control group between several timepoints (Figure 2, gray dotted lines; Supplementary Table 2). On the other hand, fewer timepoints were significantly different from one another when within-group comparisons were made in the SCFP group (Figure 2, blue dotted lines; Supplementary Table 3), indicating that SCFP treatment might have contributed to more stable diversity levels post-stress.

Beta diversity was variable in the pre-stress period (Figure 3). At time 0 h (time at which the horses were untethered), horses assigned to the SCFP treatment formed two subclusters, whereas horses assigned to the Control treatment clustered in the same overall region (Figure 3, panel 1). On day 28, treated and untreated horses clustered in two overlapping groups (Figure 3, panel 2), and became homogeneous over time, with no clear difference between Control and SCFP-treated horses on day 56 (Figure 3, panel 3). However, Control and SCFP-treated horses had two completely different clustering trajectories after stress, with SCFP and Control horses clustering separately at 0 and 12 h post-stress (Figure 3, panels 4 and 5) and culminating again in a homogenous group at 72 h post-stress (Figure 3, panel 7).

The stress impact was greater for Control horses

Stress challenge and SCFP treatment significantly influenced microbial composition at the species level (PERMANOVA of Aitchison distances: Treatment, $P = 0.01$; Timepoint, $P = 0.01$; Treatment $\times$ Timepoint, $P = 0.01$). Two species clusters were identified (Figure 4A, vertical clusters A and B). The larger cluster (cluster A—18 species) comprised mainly species that increased in abundance after stress challenge. The smaller cluster (cluster B—nine species) comprised species that decreased in abundance after stress challenge (Figure 4A, vertical clusters A and B, Supplementary Table 5). Notably, Control horses had a much more marked reduction in species belonging to cluster B after stress when compared to those treated with SCFP. When total microbial composition was used as a basis for clustering analysis of the samples, five major sample clusters were identified (Figure 4A, clusters I, II, III, IV, and V). Very different trajectories were observed between the SCFP and Control treatments after stress (Figure 4B), with microbial composition of Control horses

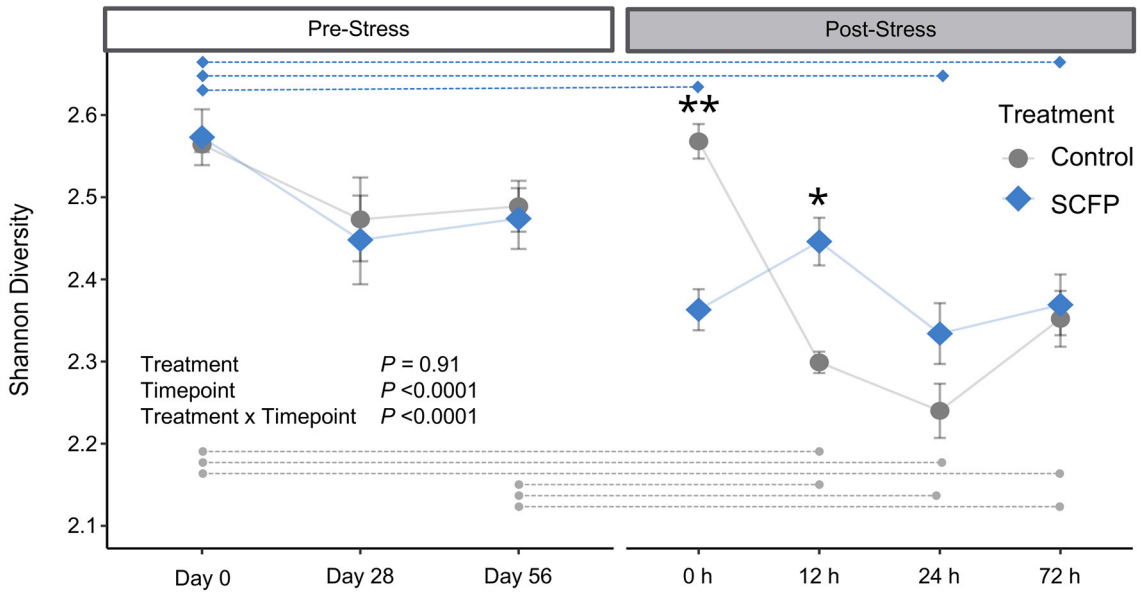

FIGURE 2
Alpha diversity comparisons. Shannon diversity metrics were analyzed with a linear model which included horse as a random effect, treatment, timepoint, and their interactions. Raw means and standard deviations are shown in the plot. Blue diamonds represent SCFP, and gray circles represent Control. Transparent lines represent the hypothetical trajectory of diversity. Dashed horizontal lines represent within-group pairwise significant differences at the 0.05 level after Bonferroni multiple comparison adjustment. Asterisks indicate timepoints in which SCFP significantly differs from Control $^{**}P < 0.01$, $^{*}P < 0.05$.

mostly belonging to cluster V, while SCFP treated horses exhibited microbiome compositions representatives of all clusters.

Stress challenge resulted in significant differential abundances in a time-dependent manner

Treatment with SCFP significantly increased the abundances of *Erysipelotrichaceae* before stress challenge. In fact, this was the only significantly different taxa between Control and SCFP in the pre-challenge period (Figure 5A, panels 1 and 2), and SCFP treated animals had an overall positive log ratios throughout the entire study (Supplementary Figures).

Many more species were significantly differentially abundant after stress challenge particularly at times 0 and 12 h post-stress (Figure 5B). At time 0 h, eight species were significantly increased in the SCFP group compared to Control, and three species were significantly decreased (Figure 5B, panel 1). Statistically different species were observed between groups up to 24 h after stress (Figure 5B, panels 2 and 3), with no significantly different species observed at 72 h after stress (Figure 5B, panel 4).

SCFP treated horses demonstrated more robust microbial functionality post-stress as compared to Control horses

Clustering analysis of the functional potential of the samples, measured by CAZy families, identified two major functional sample clusters (Figure 6A, clusters I and II). The CAZy families identified were Auxiliary Activity Family (AA), Carbohydrate-Binding Module Family (CBM), Carbohydrate Esterase Family (CE), Glycoside Hydrolase Family (GH), Glycosyl Transferase Family (GT), and Polysaccharide Lyase Family (PL). A more pronounced increase in CAZy families was observed after stress in Control horses compared to SCFP horses (Figure 6A). Similar to compositional clustering outcomes, Control and SCFP groups exhibited markedly different functional profiles following imposition of the stressor (Figure 6B), with Control horses demonstrating a switch to cluster I immediately after stress challenge, and again completely switching to cluster II from 12 to 72 h post-stress. Conversely SCFP treated horses displayed microbiome functional potential representatives of both clusters throughout the entire study period.

Correlation networks reveal that post-stress microbial communities are more stable in SCFP treated horses

Overall, a smaller number of significant interactions were observed in the SCFP group compared to Control, particularly following stress (SCFP = 198 positive interactions and 30 negative interactions: Control = 304 positive interactions and 210 negative interactions; Supplementary Table 4). Treatment with SCFP resulted in a smaller number of significant species interactions overall (maximum of 310 interactions before challenge) while the Control group had a total of 520 interactions before challenge. Horses that received SCFP had fewer interactions in total compared

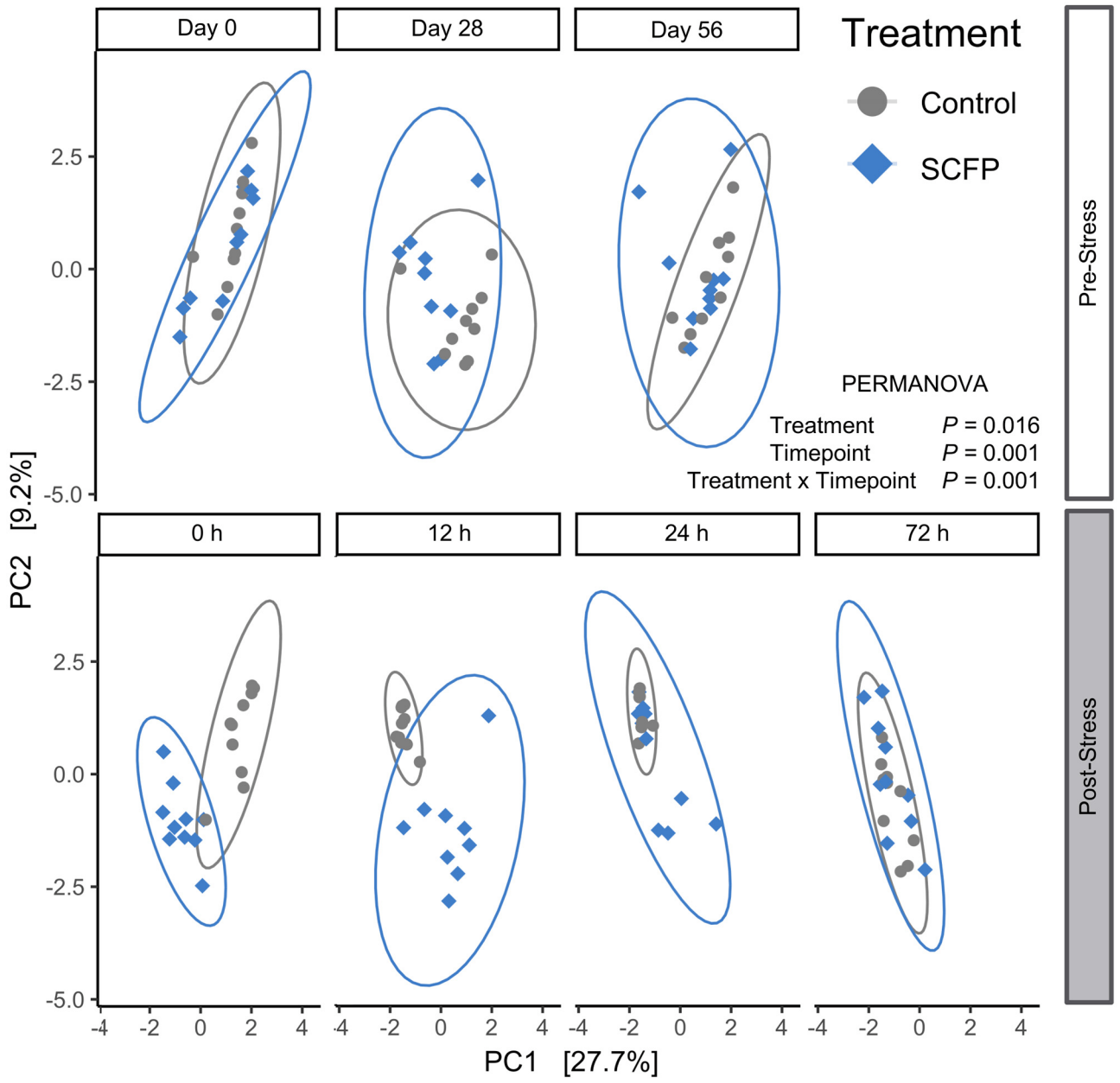

FIGURE 3
Beta diversity comparisons. Aitchison distances were analyzed with permutational ANOVA (PERMANOVA) model which included treatment, timepoint, and their interactions. Plots depict principal component analysis of Aitchison distances with CLR transformed data.

to Control, both pre- and post-stress. While no difference was observed in the percentage of positive interactions before stress, SCFP treated horses had a substantially higher number of positive interactions after stress when compared to untreated Control horses (87 vs. 59%).

Discussion

To evaluate the potential effect of supplementing horses under stressful conditions with a postbiotic, we sequenced the fecal metagenomes of 20 horses undergoing a previously established stress model that mimics prolonged transportation. The rationale that SCFP supplementation could lead to improved microbiome stability is based on recent reports of SCFP having a positive impact in other species undergoing stressful conditions (22, 24, 60–62). Here, we observed that untreated Control horses and treated (SCFP) horses presented very different microbiome trajectories upon stress, both in within- and between-sample diversity measurements. Moreover, a lower magnitude of changes was observed in the functional potential and microbial profile of SCFP horses vs. Control. Taken together, these observations led us to conclude that treatment with SCFP resulted in more robust and stable microbial profiles in horses after stress challenge.

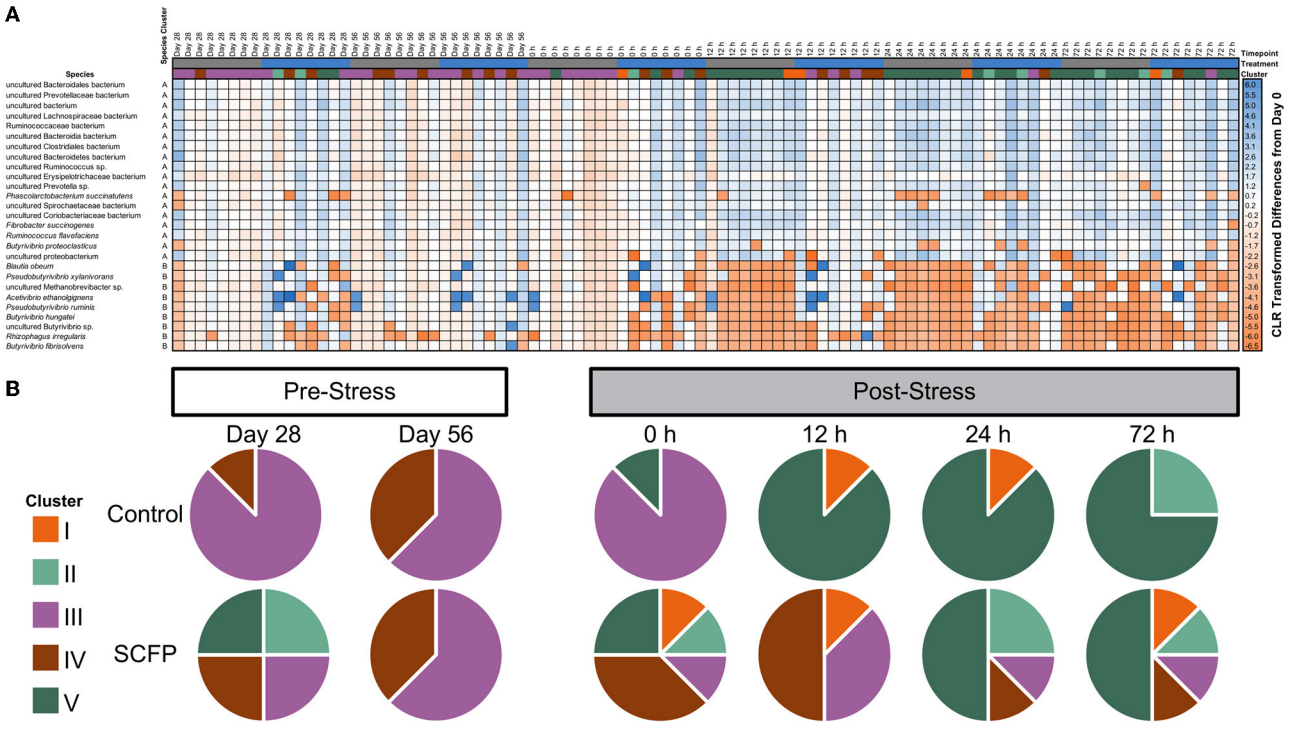

FIGURE 4
Stress challenge results in different microbial profiles in Control and SCFP treated horses. **(A)** Heatmap of centered log-ratio relative abundance of bacterial species detected in fecal samples of horses. **(B)** Pie charts represent the presence of samples across different clusters for Control and SCFP groups across time. Species cluster A contains species that drastically increase in relative abundance upon stress challenge, whereas species cluster B is comprised of species which decrease after stress challenge.

Less variation in microbial and functional profiles were observed for SCFP compared to Control horses. This was noted in several of our analyses including Shannon diversity index, total number of microbial network interactions, percentage of positive network interactions, and microbial and functional clustering profiles, which was illustrated in heatmaps with Control horses having a higher degree of change than SCFP treated horses. These data led us to conclude that dietary SCFP supplementation results in a more stable and robust community that is less impacted by stress. Our findings are in agreement with Tun et al. (24) who observed that postbiotic treatment tends to stabilize the microbiota of cows in a subclinical acidosis challenge. In that study authors concluded that SCFP supplementation attenuated the impacts of subacute ruminal acidosis on the composition and functionality of the rumen microbiome. Taken together, our results and those from others leads us to hypothesize that SCFP treatment results in a microbial community that is more robust (defined as resistance against change) in responding to stress. This hypothesis is corroborated by observations in our correlation network analyses, where the number of connections generally decreased with SCFP, but the percentage of positive interactions increased, indicating that a leaner (defined as a community with fewer connections among members but with a larger number of positive connections) and more connected microbial community in SCFP horses.

Our results are also in agreement with other studies that demonstrated that postbiotic supplementation is associated with improved microbiome balance which has been translated into host health in many species (23, 60, 62, 63). In horses, postbiotic supplementation resulted in increased relative abundances of fibrolytic bacteria (64) and attenuated exercise-induced stress markers (28). Additionally, Lucassen et al. showed that postbiotic-treated horses have more efficient response to vaccination (23). In contrast, a recent study by the same group evaluating SCFP supplementation did not identify significant alterations in the fecal microbiota of thoroughbred racehorses (27). Possible explanations for this disparity are the use of a lower resolution technique (16S rRNA gene sequencing) and the smaller dataset (11 horses) in that study when compared to our study, which used shotgun metagenomics to analyze the fecal microbiome of 20 horses. Additionally, those authors reported a high degree of horse-dependent effects of treatment, which can be attributed to high horse-to-horse variability.

The potential effects of stress and SCFP treatment on CAZy families was evaluated due to the importance and dependence of the horse on the degradation of structural carbohydrates of forages by gut microbiome for health and wellbeing. We observed that SCFP stabilized the composition and functionality of the hindgut microbial community. This was observed particularly immediately after stress relief (0 h) where lower CAZy abundance was observed in Control horses (light green in most cases) while abundances remained relatively unchanged or increased in SCFP (with the exception of one horse). At 12 h post-stress, Control horses displayed a dramatic switch in functional profile, with most

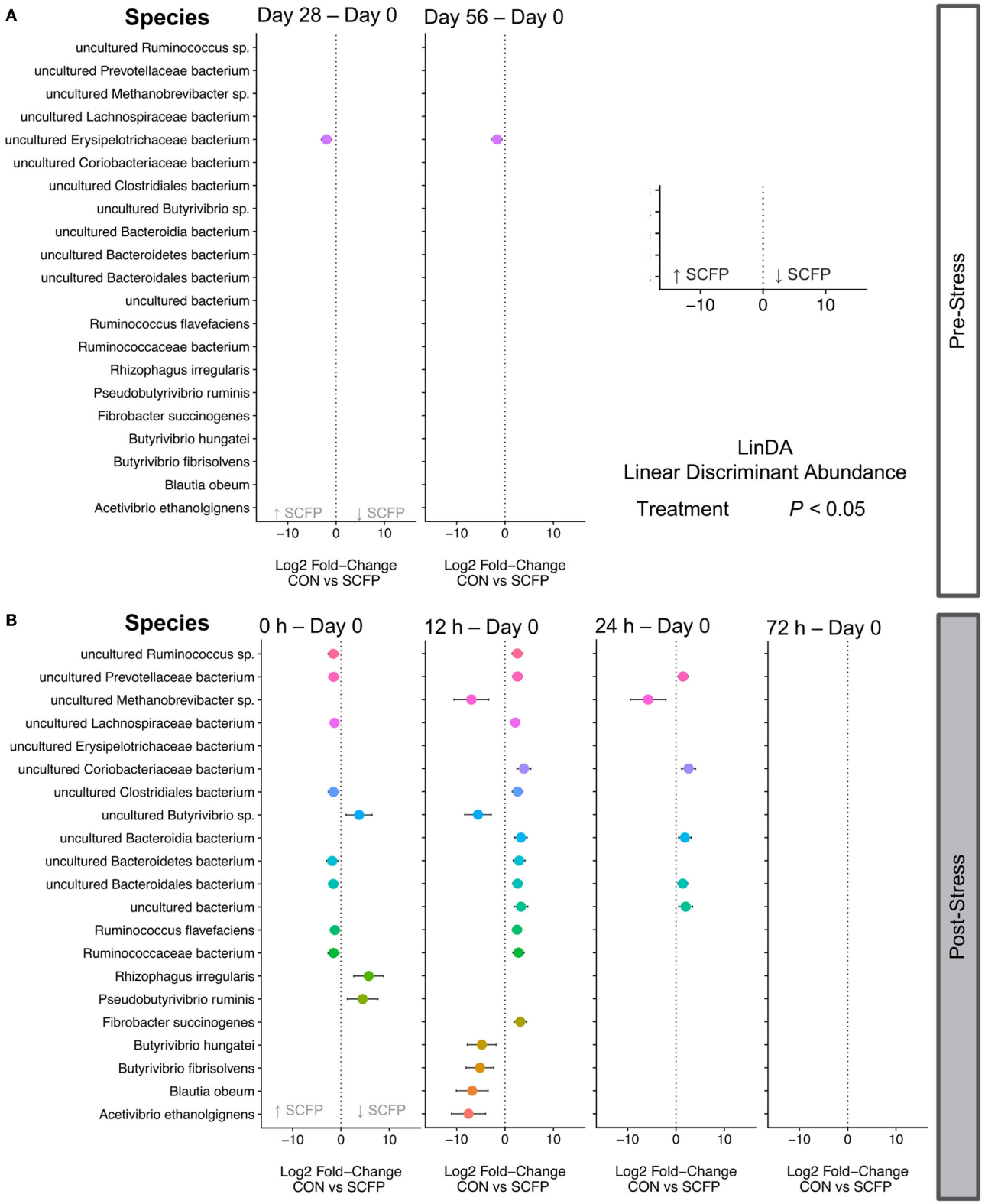

FIGURE 5
Differential abundances. The effect size (log2-fold change) is shown for each species, and only significantly different species are shown in each plot with their correspondent confidence interval. Statistical models included relative abundance as the dependent variable, horse as a random effect, treatment, timepoint, and their interactions as independent variables. Multiple hypothesis testing correction was performed with Benjamini Hochberg False Discovery Rate method. A negative fold change indicates an increase in relative abundance in SCFP compared to Control, and a positive fold indicates a decrease in relative abundance in SCFP compared to Control. **(A)** Differentially abundant species before stress. **(B)** Differentially abundant species after stress.

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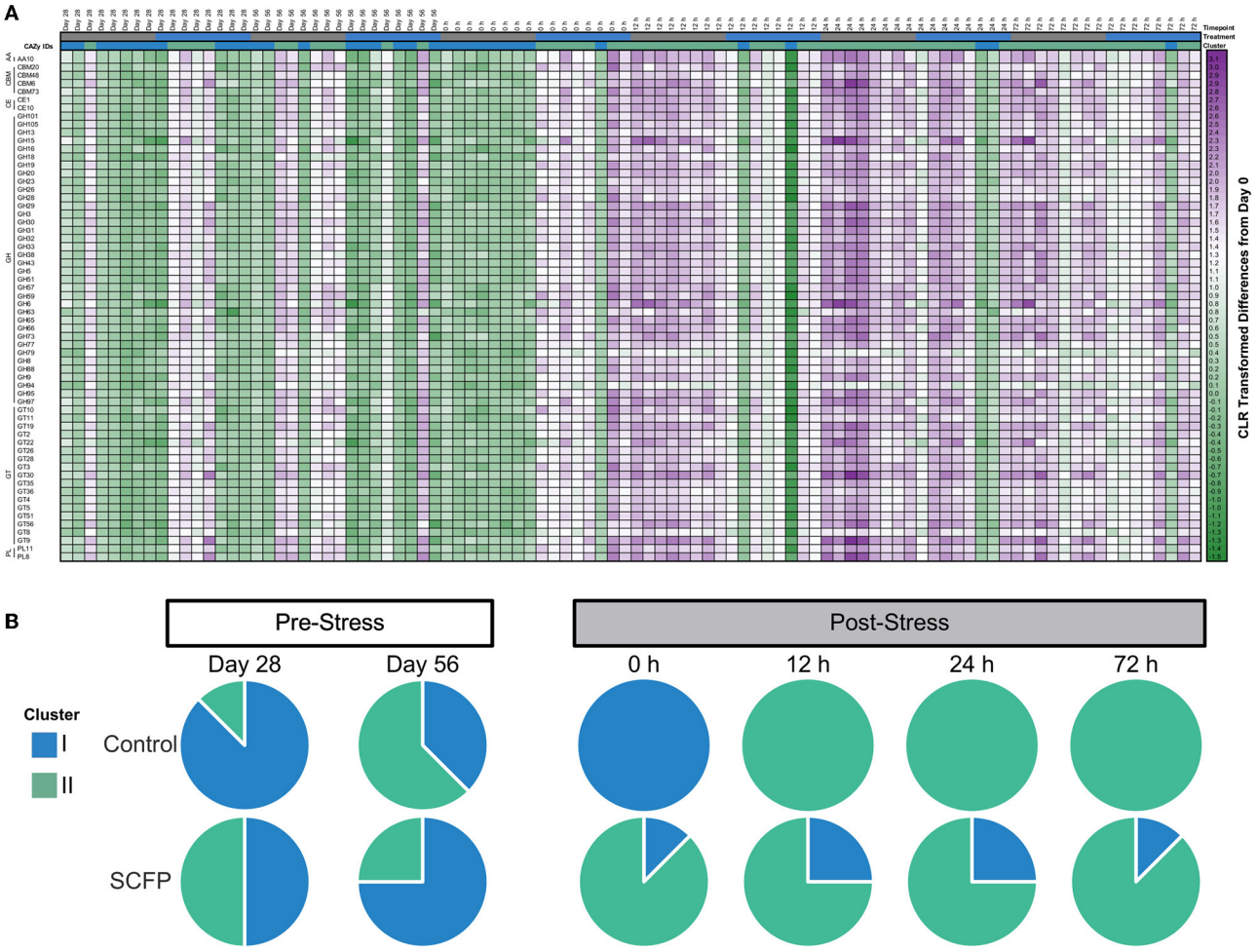

FIGURE 6
Functional potential is differentially impacted by treatment and stress in SCFP and Control horses. **(A)** Heatmap of centered log-ratio relative abundance of carbohydrate active enzymes (CAZy) families detected in fecal samples of horses. **(B)** Pie charts represent the presence of samples across different clusters for Control and SCFP groups across time. AA, Auxiliary Activity Family; CBM, Carbohydrate-Binding Module Family; CE, Carbohydrate Esterase Family; GH, Glycoside Hydrolase Family; GT, Glycosyl Transferase Family; PL, Polysaccharide Lyase Family.

showing increased relative abundances as illustrated in the heat maps in Figure 6. The small sample size of this study precludes us from making further statements regarding the functional potential, but what is evident from this study is that larger swings in relative abundances of CAZy families were associated with Control horses when compared to treated horses throughout the entire post-stress period. Additionally, a strong horse-to-horse effect was observed, indicating that treatment effect is highly dependent on the animal. These findings are similar to those of Lucassen et al. (27) who observed a high degree of horse-to-horse variability in their study of the equine microbiome of horses fed a postbiotic.

The bacteria identified in our study are in agreement with previous reports of healthy equine gut microbiomes, with a composition that is mainly dominated by fibrolytic bacteria (8, 9, 65). It is important to highlight that we chose a very strict threshold for taxa selection for statistical comparisons between treatment groups. Specifically, to be included in the statistical analysis, a microbial species had to be present in at least 75% of all samples. This was a deliberate choice to decrease the chances for spurious findings due to multiple hypothesis testing.

Here, we identified that SCFP treatment significantly impacted the relative abundance of *Erysipelotrichaceae* before stress, with a small, but significant increase in SCFP treated horses compared to Control horses. Biddle and colleagues also observed significant temporal changes in *Erysipelotrichaceae* in obese horses (66), and this family had previously been identified as part of the core microbial community of horse feces (67). However, little is known about the role of this species in the horse gut and diverging evidence has been presented about the role of *Erysipelotrichaceae* in other organisms, with varying levels of *Erysipelotrichaceae* reported in murine and human studies of disease (12, 68).

Immediately after stress 11 bacteria were identified to be significantly different albeit with very small effect sizes. From those, eight were increased in the SCFP group and three were increased in the Control group, with relatively higher effect sizes when compared to species increased in SCFP horses. Microorganisms significantly increased in Control horses included one uncultured *Butyrivibrio* species, *Pseudobutirivibrio ruminis*, and *Ryzophagus irregularis*. We identified *Ryzophagus irregularis*, an arbuscular mycorrhizal fungus that is common in plants, and which (69) has

not been previously reported in the horse gastrointestinal tract. Given the presence of this organism in many plant species, and the plant-based diet of horses, this finding is not completely surprising.

A larger number of significantly different species were observed at 12 h post stress. Out of 18 significantly different species, six had relatively high effect sizes and were increased in SCFP horses. These included three *Butyrivibrio* species in addition to *Blautia*, *Acetivibrio*, and *Methanobrevibacter*, which were found to have significantly higher relative abundances in SCFP treated horses 12 h after stress. *Butyrivibrio* are very versatile bacteria and encode a variety of enzymes to hydrolyze complex carbohydrates (70, 71). They have been reported to carry many genes encoding glycoside hydrolases (GH) that are involved in carbohydrate fermentation and butyrate production. Likewise, *Blautia* and *Acetivibrio* are also fiber fermenters (8). In agreement with increases with *Butyrivibrio* species, in our functional annotation analyses, 36 out of 62 enzymes found to be significantly different in the present study encode for glycoside hydrolases. Lastly, *Methanobrevibacter* was also identified to be increased in the SCFP group at 12 and 24 h post stress. The presence of methanogenic archaea in the horse gut has been previously reported, and the diversity of methane producers in the horse gut is believed to be high (8, 72).

Despite the wealth of data collected as part of this project we acknowledge that a sample size of 20 horses is relatively small. It was further substantiated that horse intrinsic factors impacted the response to stress or treatment, as it could be observed by single animals behaving differently than the remainder of the group at a given timepoint. In fact, horse-to-horse variability has been well-documented in immune parameters in horses subjected to this model of stress (33). Raidal et al. observed varying degrees of change in white cell count, neutrophil count, and total bacterial numbers in six horses subjected to prolonged head elevation (33). This added variability might have confounded our analyses and precluded us from identifying strong signals. Nevertheless, animals are different and thus further research should account for animal-to-animal changes and perhaps quantify the effect in terms of microbiome changes within an animal. Even with the relatively high heterogeneity of this dataset we were able to identify a clear overall signal that treatment with SCFP tends to promote microbiome robustness and stability after stress, as we observed in measures of alpha and beta diversity, as well as bacterial and functional profiles, and bacteria interaction dynamics.

This study adds to the body of knowledge regarding the beneficial impacts of postbiotic administration to horses undergoing stressful situations. While the specific mechanisms by which this robustness and stability are imparted in an equine's gut microbiome by postbiotic administration are not fully elucidated, studies in other species suggested that potential underlying mechanisms by which postbiotic supplementation led to improved health include effects in immunomodulatory pathways (73) and improved microbiome composition and functionality (24). From an immune perspective, animals receiving SCFP seem to be primed to respond with elevated (magnitude of response) and accelerated (speed of response) cytokine production when a threat is detected (61, 62). Additionally, at the site of challenge, increased phagocytic activity and killing ability of white blood cells and reduced activation of inflammatory system leads to a reduction in localized inflammation, and potentially immunopathology in SCFP supplemented animals (62, 74). From the microbiome perspective, ruminant studies have shown that SCFP supplementation boosts the abundances of influential members of the microbiome which promote richness and diversity, and hence, functionality of the microbiome resulting in increased VFA production and improved energetic efficiency of rumen fermentation (24, 75). Therefore, it can be speculated that the dual action of SCFP postbiotic *via* immunomodulatory pathways and optimized microbiome functionality increases robustness of animals against a wide range of infectious and metabolic stressors.

Our results indicate that prophylactic supplementation with a yeast-derived postbiotic might be a beneficial strategy for horses prior to exposure to stress. This exploratory study is limited in the ability to draw mechanistic conclusions on the effects of SCFP in horses subjected to a stress model. We observed a lower degree of change both in microbial diversity and functional profile of horses fed SCFP when compared to Control. Mechanistically, having a more robust and stable microbiome plausibly results in less opportunity for pathogen colonization and better health maintenance. Postbiotics have been demonstrated to have positive impacts in several species, and further research into the mechanisms by which these beneficial effects occur is warranted.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/, bioproject PRJNA788958.

Ethics statement

The animal study was reviewed and approved by Institutional Animal Care and Use Committee at the University of Florida in Gainesville, FL (#201810324).

Author contributions

LW designed the animal experiment. EK and BK designed the microbiome study. MT performed the animal experiment and collected samples. MS performed sequencing and bioinformatics. AC performed statistical analyses. EG, EK, and SN interpreted the data. EG and EK prepared the first draft. All authors read and approved the final manuscript.

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Conflict of interest

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1134092/full#supplementary-material

References

1. Dini-Andreote F, van Elsas JD, Olff H, Salles JF. Dispersal-competition tradeoff in microbiomes in the quest for land colonization. *Sci Rep*. (2018) 8:6. doi: 10.1038/s41598-018-27783-6

2. Forsberg KJ, Reyes A, Wang B, Selleck EM, Sommer MOA, Dantas G. The shared antibiotic resistome of soil bacteria and human pathogens. *Science.* (2012) 337:1107–11. doi: 10.1126/science.1220761

3. Leung H, Long X, Ni Y, Qian L, Nychas E, Siliceo SL, et al. Risk assessment with gut microbiome and metabolite markers in NAFLD development. *Sci Transl Med.* (2022) 14:855. doi: 10.1126/scitranslmed.abk0855

4. Bäckhed F, Ding H, Wang T, Hooper L, Gou YK, Nagy A, et al. The gut microbiota as an environmental factor that regulates fat storage. *Proc Natl Acad Sci USA*. (2004) 101:15718–23. doi: 10.1073/pnas.0407076101

5. Jacobson A, Lam L, Rajendram M, Tamburini F, Honeycutt J, Pham T, et al. A gut commensal-produced metabolite mediates colonization resistance to salmonella infection. *Cell Host Microbe*. (2018) 24:296–307 e7. doi: 10.1016/j.chom.2018.07.002

6. Borda-Molina D, Vital M, Sommerfeld V, Rodehutscord M, Camarinha-Silva A. Insights into broilers' gut microbiota fed with phosphorus, calcium, and phytase supplemented diets. *Front Microbiol.* (2016) 2016:2033. doi: 10.3389/fmicb.2016.02033

7. Cangiano LR, Yohe TT, Steele MA, Renaud DL. Invited review: Strategic use of microbial-based probiotics and prebiotics in dairy calf rearing. *Appl Anim Sci*. (2020) 36:630–51. doi: 10.15232/aas.2020-02049

8. Kauter A, Epping L, Semmler T, Antao EM, Kannapin D, Stoeckle SD, et al. The gut microbiome of horses: Current research on equine enteral microbiota and future perspectives. *Anim Microbiome.* (2019) 1:14. doi: 10.1186/s42523-019-0013-3

9. Morrison PK, Newbold CJ, Jones E, Worgan HJ, Grove-White DH, Dugdale AH, et al. The equine gastrointestinal microbiome: Impacts of weight-loss. *BMC Vet Res.* (2020) 16:1–18. doi: 10.1186/s12917-020-02295-6

10. Destrez A, Grimm P, Julliand V. Dietary-induced modulation of the hindgut microbiota is related to behavioral responses during stressful events in horses. *Physiol Behav*. (2019) 202:94–100. doi: 10.1016/j.physbeh.2019.02.003

11. Plancade S, Clark A, Philippe C, Helbling JC, Moisan MP, Esquerré D, et al. Unraveling the effects of the gut microbiota composition and function on horse endurance physiology. *Sci Rep*. (2019) 9:7. doi: 10.1038/s41598-019-46118-7

12. Mach N, Ruet A, Clark A, Bars-Cortina D, Ramayo-Caldas Y, Crisci E, et al. Priming for welfare: Gut microbiota is associated with equitation conditions and behavior in horse athletes. *Sci Rep*. (2020) 10:1–19. doi: 10.1038/s41598-020-65444-9

13. Litvak Y, Bäumler AJ. The founder hypothesis: A basis for microbiota resistance, diversity in taxa carriage, and colonization resistance against pathogens. *PLoS Pathog.* (2019) 15:e1007563. doi: 10.1371/journal.ppat.1007563

14. Ranallo RT, Mcdonald LC, Halpin AL, Hiltke T, Young VB. The state of microbiome science at the intersection of infectious diseases and antimicrobial resistance. *J Infect Dis.* (2021) 223(Suppl.3):S187–93. doi: 10.1093/infdis/jiab020

15. McLaren MR, Callahan BJ. Pathogen resistance may be the principal evolutionary advantage provided by the microbiome. *Philos Trans Royal Soc B.* (2020) 375:592. doi: 10.1098/rstb.2019.0592

16. Lozupone CA, Stombaugh JI, Gordon JI, Jansson JK, Knight R. Diversity, stability and resilience of the human gut microbiota. *Nature.* (2012) 489:220. doi: 10.1038/nature11550

17. Salminen S, Collado MC, Endo A, Hill C, Lebeer S, Quigley EMM, et al. The International Scientific Association of Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of postbiotics. *Nat Rev Gastroenterol Hepatol.* (2021) 18:649–67. doi: 10.1038/s41575-021-00440-6

18. Gibson GR, Hutkins R, Sanders ME, Prescott SL, Reimer RA, Salminen SJ, et al. Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. *Nat Rev Gastroenterol Hepatol.* (2017) 14:491–502. doi: 10.1038/nrgastro.2017.75

19. Zółkiewicz J, Marzec A, Ruszczyński M, Feleszko W. Postbiotics-A step beyond pre- and probiotics. *Nutrients.* (2020) 12:1–17. doi: 10.3390/nu12082189

20. Magalhães VJA, Susca F, Lima FS, Branco AF, Yoon I, Santos JEP. Effect of feeding yeast culture on performance, health, and immunocompetence of dairy calves. *J Dairy Sci.* (2008) 91:1497–509. doi: 10.3168/jds.2007-0582

21. Guo J, Xu L, Khalouei H, Fehr K, Senaratne V, Ghia JE, et al. Saccharomyces cerevisiae fermentation products reduce bacterial endotoxin concentrations and inflammation during grain-based subacute ruminal acidosis in lactating dairy cows. *J Dairy Sci.* (2022) 105:2354–68. doi: 10.3168/jds.2021-20572

22. Chou WK, Park J, Carey JB, McIntyre DR, Berghman LR. Immunomodulatory effects of *Saccharomyces cerevisiae* fermentation product supplementation on immune gene expression and lymphocyte distribution in immune organs in broilers. *Front Vet Sci*. (2017) 4:37. doi: 10.3389/fvets.2017.00037

23. Lucassen A, Finkler-Schade C, Schuberth HJ. A *Saccharomyces cerevisiae* fermentation product (Olimond BB) alters the early response after influenza vaccination in racehorses. *Animals*. (2021) 11:92726. doi: 10.3390/ani11092726

24. Tun HM, Li S, Yoon I, Meale SJ, Azevedo PA, Khafipour E, et al. *Saccharomyces cerevisiae* fermentation products (SCFP) stabilize the ruminal microbiota of lactating dairy cows during periods of a depressed rumen pH. *BMC Vet Res.* (2020) 16:1–17. doi: 10.1186/s12917-020-02437-w

25. Moyad MA, Robinson LE, Kittelsrud JM, Reeves SG, Weaver SE, Guzman AI, et al. Immunogenic yeast-based fermentation product reduces allergic rhinitis-induced nasal congestion: A randomized, double-blind, placebo-controlled trial. *Adv Ther.* (2009) 26:795–804. doi: 10.1007/s12325-009-0057-y

26. van Dorland HA, Zanoni R, Gerber V, Jeannerat E, Wiederkehr D, Burger D. Antibody response to influenza booster vaccination in franches-montagnes stallions supplemented with equi-strath®: A randomized trial. *Vet Med Sci.* (2018) 4:133–9. doi: 10.1002/vms3.95

27. Lucassen A, Hankel J, Finkler-Schade C, Osbelt L, Strowig T, Visscher C, et al. Feeding a *Saccharomyces cerevisiae* fermentation product (Olimond BB) does not alter the fecal microbiota of thoroughbred racehorses. *Animals.* (2022) 12:1496. doi: 10.3390/ani12121496

28. Valigura HC, Leatherwood JL, Martinez RE, Norton SA, White-Springer SH. Dietary supplementation of a *Saccharomyces cerevisiae* fermentation product attenuates exercise-induced stress markers in young horses. *J Anim Sci.* (2021) 99:skab199. doi: 10.1093/jas/skab199

29. Tench. *Effect of Saccharomyces Cerevisiae Fermentate on Mucosal Immunity in Young Stress-Challenged Horse.* UF Digital Collections. (2022). Available online at: https://ufdc.ufl.edu/UFE0056169/00001/pdf (accessed December 24, 2022).

30. ADSA. *Guide for the Care and Use of Agricultural Animals in Research and Teaching.* 4th ed. (2020). Available online at: www.adsa.org (accessed December 28, 2022).

31. National Research Council. *Nutrient Requirements of Horses: Sixth Revised Edition.* Washington, DC: The National Academies Press (2007). doi: 10.17226/11653

32. Bobel JM, Long MT, Warren LK. 119 Use of a head elevation and nasopharyngeal flush model for short-term immune distress in horses. *J Equine Vet Sci.* (2015) 35:433–4. doi: 10.1016/j.jevs.2015.03.130

33. Raidal SL, Love DN, Bailey GD. Effects of posture and accumulated airway secretions on tracheal mucociliary transport in the horse. *Aust Vet J.* (1996) 73:45–9. doi: 10.1111/j.1751-0813.1996.tb09963.x

34. Johnson AC, Biddle AS, Tench M, Bobel JM, Bazurto C, Hansen TL, et al. 198 effect of *Saccharomyces cerevisiae* fermentate on immune cell function following prolonged head elevation in 2-year-old horses in training. *J Anim Sci.* (2021) 99(Suppl.3):106–7. doi: 10.1093/jas/skab235.193

35. Tench M, Bobel JM, Bazurto C, Dolly J, Hansen TL, Kirk N, et al. 40 dietary *Saccharomyces cerevisiae* fermentate affects mucosal immunity in young stress-challenged horses in training. *J Equine Vet Sci.* (2021) 100:103503. doi: 10.1016/j.jevs.2021.103503

36. Li H. Minimap2: Pairwise alignment for nucleotide sequences. *Bioinformatics.* (2018) 34:3094–100. doi: 10.1093/bioinformatics/bty191

37. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The sequence alignment/map format and SAMtools. *Bioinformatics.* (2009) 25:2078–9. doi: 10.1093/bioinformatics/btp352

38. Tatusova T, Ciufo S, Fedorov B, O'Neill K, Tolstoy I. RefSeq microbial genomes database: New representation and annotation strategy. *Nucleic Acids Res.* (2015) 43:3872. doi: 10.1093/nar/gkv278

39. Almeida A, Nayfach S, Boland M, Strozzi F, Beracochea M, Shi ZJ, et al. A unified catalog of 204,938 reference genomes from the human gut microbiome. *Nat Biotechnol.* (2021) 39:105–14. doi: 10.1038/s41587-020-0603-3

40. Stewart RD, Auffret MD, Warr A, Walker AW, Roehe R, Watson M. Compendium of 4,941 rumen metagenome-assembled genomes for rumen microbiome biology and enzyme discovery. *Nat Biotechnol.* (2019) 37:953–61. doi: 10.1038/s41587-019-0202-3

41. Wood DE, Lu J, Langmead B. Improved metagenomic analysis with Kraken 2. *Genome Biol.* (2019) 20:1891. doi: 10.1186/s13059-019-1891-0

42. Lu J, Breitwieser FP, Thielen P, Salzberg SL. Bracken: Estimating species abundance in metagenomics data. *PeerJ Comput Sci.* (2017). doi: 10.1101/051813

43. R Core Development Team. *R: A Language and Environment for Statistical Computing, 3.2.1.* (2015). Available online at: http://www r-project org (accessed November 22, 2021).

44. McMurdie PJ, Holmes S. phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE.* (2013) 8:e61217. doi: 10.1371/journal.pone.0061217

45. Seemann T. Prokka: Rapid prokaryotic genome annotation. *Bioinformatics.* (2014) 30:2068–9. doi: 10.1093/bioinformatics/btu153

46. Lahti L. *Tools for Microbiome Analysis in R.* (2023). Available online at: http://microbiome.github.io/microbiome (accessed June 1, 2022).

47. Palarea-Albaladejo J, Martín-Fernández JA. ZCompositions—R package for multivariate imputation of left-censored data under a compositional approach. *Chemometr Intell Lab Syst.* (2015) 143:85–96. doi: 10.1016/j.chemolab.2015.02.019

48. Lombard V, Golaconda Ramulu H, Drula E, Coutinho PM, Henrissat B. The carbohydrate-active enzymes database (CAZy) in 2013. *Nucleic Acids Res.* (2014) 42:gkt1178. doi: 10.1093/nar/gkt1178

49. Cantalapiedra CP, Hernández-Plaza A, Letunic I, Bork P, Huerta-Cepas J. eggNOG-mapper v2: Functional annotation, orthology assignments, and domain prediction at the metagenomic scale. *Mol Biol Evol.* (2021) 6:3. doi: 10.1101/2021.06.03.446934

50. Bates D, Mächler M, Bolker BM, Walker SC. Fitting linear mixed-effects models using lme4. *J Stat Softw.* (2015) 67:1–48. doi: 10.18637/jss.v067.i01

51. Oksanen J, Kindt R, Legendre P, O'Hara B, Simpson GL, Solymos P, et al. *vegan: Community Ecology Package.* (2008). Available online at: http://cran.r-project.org/ (accessed November 1, 2021).

52. Aitchison J, Barceló-Vidal C, Martín-Fernández JA, Pawlowsky-Glahn V. Logratio analysis and compositional distance. *Math Geol.* (2000) 32:271–5. doi: 10.1023/A:1007529726302

53. Zhou H, He K, Chen J, Zhang X. LinDA: Linear models for differential abundance analysis of microbiome compositional data. *Genome Biol.* (2022) 23:1–23. doi: 10.1186/s13059-022-02655-5

54. Russell L, Singmann H, Love J, Buerkner PMH. *Emmeans: Estimated Marginal Means.* (2020). Available online at: https://cran.r-project.org/web/packages/emmeans/readme/README.html (accessed November 1, 2021).

55. Benjamini Y, Yekutieli D. The control of the false discovery rate in multiple testing under dependency. *Ann Statist.* (2001) 29:1165–88. doi: 10.1214/aos/1013699998

56. Benjamini Y, Hochberg Y. Controlling the false discovery rate: A practical and powerful approach to multiple testing. *J Roy Stat Soc B.* (1995) 57:289–300. doi: 10.1111/j.2517-6161.1995.tb02031.x

57. Faust K, Raes J. CoNet app: Inference of biological association networks using Cytoscape. *F1000Res.* (2016) 5:1519. doi: 10.12688/f1000research.9050.2

58. Tibshirani R, Walther G, Hastie T. Estimating the number of clusters in a data set *via* the gap statistic. *J R Stat Soc Series B Stat Methodol.* (2001) 63:411–23. doi: 10.1111/1467-9868.00293

59. MATLAB. *Version R2019B.* Natick, MA: The MathWorks Inc. (2019).

60. Al-Qaisi M, Horst EA, Mayorga EJ, Goetz BM, Abeyta MA, Yoon I, et al. Effects of a *Saccharomyces cerevisiae* fermentation product on heat-stressed dairy cows. *J Dairy Sci.* (2020) 103:9634–45. doi: 10.3168/jds.2020-18721

61. Klopp RN, Yoon I, Eicher S, Boerman JP. Effects of feeding Saccharomyces cerevisiae fermentation products on the health of Holstein dairy calves following a lipopolysaccharide challenge. *J Dairy Sci.* (2022) 105:1469–79. doi: 10.3168/jds.2021-20341

62. Mahmoud AHA, Slate JR, Hong S, Yoon I, McGill JL. Supplementing a *Saccharomyces cerevisiae* fermentation product modulates innate immune function and ameliorates bovine respiratory syncytial virus infection in neonatal calves. *J Anim Sci.* (2020) 98:skaa252. doi: 10.1093/jas/skaa252

63. Gingerich E, Frana T, Logue CM, Smith DP, Pavlidis HO, Chaney WE. Effect of feeding a postbiotic derived from saccharomyces cerevisiae fermentation as a preharvest food safety hurdle for reducing *Salmonella enteritidis* in the ceca of layer pullets. *J Food Prot.* (2021) 84:275–80. doi: 10.4315/JFP-20-330

64. Garber A, Hastie PM, Farci V, McGuinness D, Bulmer L, Alzahal O, et al. The effect of supplementing pony diets with yeast on 2. The faecal microbiome. *Animal.* (2020) 14:2493–502. doi: 10.1017/S1751731120001512

65. Gilroy R, Leng J, Ravi A, Adriaenssens EM, Oren A, Baker D, et al. Metagenomic investigation of the equine faecal microbiome reveals extensive taxonomic diversity. *PeerJ.* (2022) 10:13084. doi: 10.7717/peerj.13084

66. Biddle AS, Tomb JF, Fan Z. Microbiome and blood analyte differences point to community and metabolic signatures in lean and obese horses. *Front Vet Sci.* (2018) 5:225. doi: 10.3389/fvets.2018.00225

67. Dougal K, de la Fuente G, Harris PA, Girdwood SE, Pinloche E, Newbold CJ. Identification of a core bacterial community within the large intestine of the horse. *PLoS ONE.* (2013) 8:e77660. doi: 10.1371/journal.pone.0077660

68. Kaakoush NO. Insights into the role of *Erysipelotrichaceae* in the human host. *Front Cell Infect Microbiol.* (2015) 5:84. doi: 10.3389/fcimb.2015.00084

69. Krüger M, Krüger C, Walker C, Stockinger H, Schüßler A. Phylogenetic reference data for systematics and phylotaxonomy of arbuscular mycorrhizal fungi from phylum to species level. *New Phytol.* (2012) 193:970–84. doi: 10.1111/j.1469-8137.2011.03962.x

70. Kopečný J, Zorec M, Mrázek J, Kobayashi Y, Marinšek-Logar R. *Butyrivibrio hungatei* sp. nov and *Pseudobutyrivibrio xylanivorans* sp. nov, butyrate-producing bacteria from the rumen. *Int J Syst Evol Microbiol.* (2003) 53:201–9. doi: 10.1099/ijs.0.02345-0

71. Bryant MP, Small N. The anaerobic monotrichous butyric acid-producing curved rod-shaped bacteria of the rumen. *J Bacteriol.* (1956) 72:16–21. doi: 10.1128/jb.72.1.16-21.1956

72. Elghandour MMMY, Adegbeye MJ, Barbabosa-Pilego A, Perez NR, Hernández SR, Zaragoza-Bastida A, et al. Equine contribution in methane emission and its mitigation strategies. *J Equine Vet Sci.* (2019) 72:56–63. doi: 10.1016/j.jevs.2018.10.020

73. Jastrzab R, Graczyk D, Siedlecki P. Molecular and cellular mechanisms influenced by postbiotics. *Int J Mol Sci.* (2021) 22:2413475. doi: 10.3390/ijms222413475

74. Vailati-Riboni M, Coleman DN, Lopreiato V, Alharthi A, Bucktrout RE, Abdel-Hamied E, et al. Feeding a *Saccharomyces cerevisiae* fermentation product improves udder health and immune response to a *Streptococcus uberis* mastitis challenge in mid-lactation dairy cows. *J Anim Sci Biotechnol.* (2021) 12:1–19. doi: 10.1186/s40104-021-00560-8

75. Guo J, Zhang T, Yoon I, Khafipour E, Plaizier JC. 236 effects of *Saccharomyces cerevisiae* fermentation products (SCFP) and subacute ruminal acidosis (SARA) on co-occurrence patterns and hub taxa of rumen liquid microbiota in lactating dairy cows. *J Anim Sci.* (2021) 99(Suppl.3):175–6. doi: 10.1093/jas/skab235.321

A Postbiotic from *Saccharomyces cerevisiae* Fermentation Improves Microbiome Robustness in Young Stress-Challenged Horses in Training

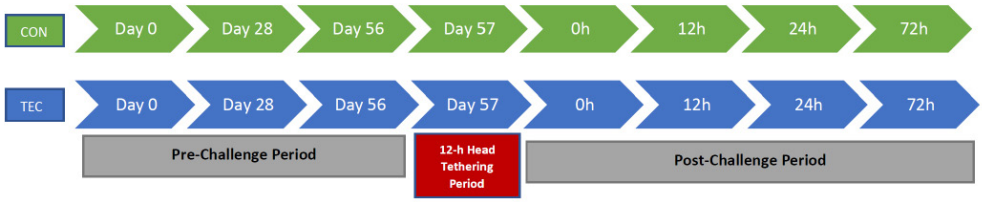
Study Background

Horses are subjected to various stressors on a daily basis. Nutritional and environmental stressors can disturb the gut microbiome of horses which may ultimately decrease their health and performance. A *Saccharomyces cerevisiae* derived postbiotic, has been shown to help balance indicators of immunity and inflammation in horses. The objective of this study was to evaluate changes in the gut microbiome of horses subjected to a stressor that horses commonly encounter during trailering and transport.

Experimental Overview

- Twenty Quarter Horses (22 months old; BW 438.17 kg) were randomly allotted to two daily dietary treatments consisting of 60% forage and 40% a standard non-postbiotic supplemented concentrate (CON; n=10) or supplemented with TEC; 47.8 mg/kg BW; n=10.
- Both treatment regimens were fed throughout the 60-d study.
- From Day 1-56 (pre-stress challenge), horses were exercised four days/week for 30-45 minutes at a light to moderate level of intensity.
- On Day 57, horses were placed in individual stalls and tethered with their heads restrictively elevated 14 inches above wither height for 12 hours to simulate a safety practice commonly imposed during trailering and transport.
- Gut metagenomic changes were characterized from fecal samples collected at Days 0, 28, 56 (pre-stress challenge, and at 0, 12, 24 and 72 hours once untethered (Figure 1).
- DNA extraction and Nanopore shotgun metagenomics were applied to all fecal samples.
- Alpha-diversity and microbial abundances were compared using non-parametric Wilcoxon-Rank-Sum test with Benjamini-Hochberg FDR correction.

Figure 1: Experimental Design and Fecal Sampling Timepoints



Results

- The gut microbiome of CON horses displayed larger and persistent changes in alpha-diversity compared to the TEC group which was more stabilized alpha-diversity across all time points. Significant differences were noted between CON and TEC at 0 and 12 h ($P < 0.05$; Figure 2).

[1]Khafipour, E., Ganda, E., Chakrabarti, A., Sardi, M., Bobel, J.M., Kozlowicz, B., Norton, S.A., Warren, L.K. A Postbiotic from *Saccharomyces cerevisiae* Fermentation Improves Microbiome Robustness in Young Stress-Challenged Horses in Training. 2022. ASAS Annual Meeting. Poster Session XV, Poster, PSXV-11, 2022 Annual ASAS Meeting

Results - continued

- During the post-challenge period compositional clusters in the microbiome of CON horses were characterized by low abundances ($P < 0.05$) of several beneficial commensal species versus TEC (Figure 3).
- No difference in beta-diversity were observed between CON and TEC on d 56 prior to head-tying, however, at 12 h post-challenge TEC horses maintained higher abundances of fibrolytic taxa ($P < 0.05$).
- From this analysis, two distinct clusters of carbohydrase enzyme function (CAZy) were identified, both of which were retained in TEC horses following head-tying while only one was retained at any time from 0-72 h post-stress in CON horses (Figure 4).

Figure 2: Alpha-Diversity Changes Over Time in Response to Treatment and Stress

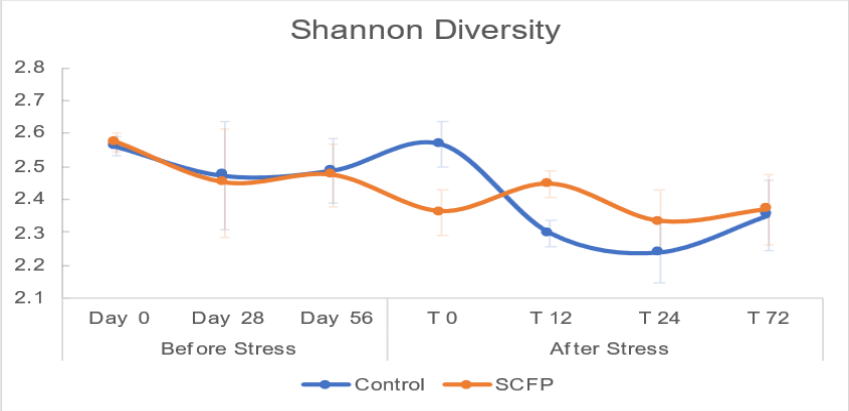


Figure 3: Community Compositional Clusters

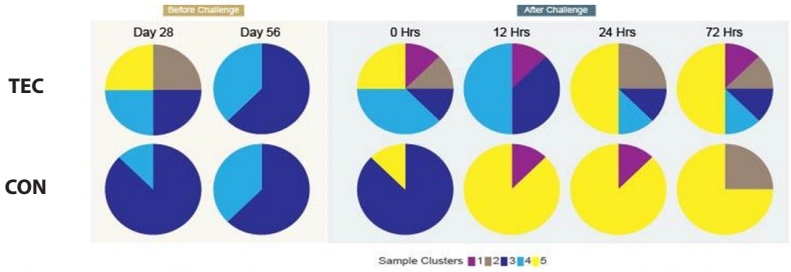
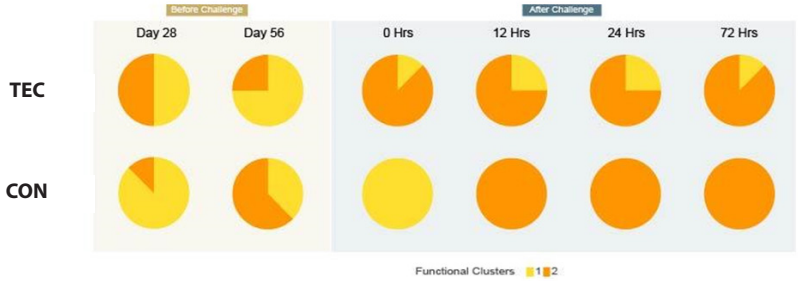


Figure 4: Community Functional Clusters



Summary

These data demonstrate that dietary inclusion of the postbiotics improves microbiome robustness in horses indicating an ability to resist change due to stress.