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Impacts of DigestaWell NRG® supplementation on post exercise muscle soreness in unconditioned horses, a pilot study

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Abstract

Exercising horses are commonly plagued by muscle fatigue and soreness, which can result in reduced performance ability. In the present study, ten unconditioned horses were fed 200g per day DigestaWell® NRG, a commercial dietary supplement containing *Yucca schidigera* and *Trigonella foenum-graecum*, two herbs shown in other species to reduce post-exercise muscle pain and soreness. A control, unsupplemented group contained ten horses of similar age, breed, and gender. Horses completed a 50 min, ridden standardized exercise test of moderate intensity immediately prior to (Period1) and after 28 d of supplementation (Period2). Muscle soreness and tightness were evaluated 24 h prior to and after each exercise test and used to determine the percent increase in post-exercise muscle soreness and tightness. Blood samples were collected before, and at 10 and 30 min, and 1, 4, and 24 h post exercise. Plasma was analyzed for glucose, lactate, non-esterified fatty acid (NEFA), tumor necrosis factor-α (TNFα), and interleukin-1β (IL-1β) concentrations. Data were analyzed by repeated measures ANOVA using SAS Enterprise Guide v. 7.1. No changes in plasma parameters were indicated between periods for unsupplemented horses ($P > 0.1$) during Period2, excepting glucose, which was greater during Period2 ($P = 0.018$). Supplemented horses had lesser concentrations of TNFα ($P = 0.016$) and lactate ($P = 0.058$) during Period2 than during Period1. During Period2, supplemented horses experienced a smaller percent increase in post exercise muscle soreness ($P = 0.031$). DigestaWell® NRG supplementation may benefit unconditioned horses undergoing moderate intensity exercise through reducing lactate production and inflammation.

Keywords: fenugreek; lactate; muscle soreness; NEFA; yucca

Introduction

Muscular soreness is a result of ultrastructural muscle injury. As shown in humans, post exercise muscle soreness is due in part to a cascade of responses initiated by damaged Z bands and loss of contractile proteins, which results in neutrophil infiltration into muscle and production of interleukin 1-β (IL-1β) and tumor necrosis factor-α (TNFα) by immune cells [1-4]. These cytokines act to increase expression of pain-sensing pathways in muscle cells through prostaglandin production [5-7]. Horses that are in pain frequently exhibit behavioral problems such as bucking and rearing [8, 9] that put them at risk for welfare concerns if their owners do not recognize the underlying health problem. This indicates that there could be a high frequency of welfare concerns in the non-racing population, because muscular pain and lameness are common concerns of horse owners, with owners citing concurrent poor performance or misbehaviors in horses diagnosed with lameness [10, 11]. Post-exercise muscle soreness and increased serum creatinine kinase activity were induced in horses carrying 30% of their bodyweight during a moderate-intensity 45 min exercise protocol designed to mimic a riding lesson [12], while lameness was induced in previously-sound horses undertaking a 30 min dressage test, when carrying more than 17.3% of bodyweight [13]. These studies indicate a potential for muscle damage and poor welfare of horses carrying >17% of bodyweight during moderate intensity exercise, a level of exercise that more horses participate in than racing [14].

Use of herbal supplements, which can be used to moderate the physiological responses to a painful stimulus in mice [15], is increasing in human populations and they are more commonly given to horses as well [16-19]. One such plant, yucca *(Yucca schidigera)* exhibits anti-inflammatory activity through one of its active components, resveratrol [20], which reduces eicosanoid synthesis through inhibiting COX enzyme activity [21]. Fenugreek (*Trigonella*

foenum-graecum) also contains anti-inflammatory compounds that reduce muscle pain through reducing cell membrane peroxidation [22] and has pain relieving properties similar to over-the-counter medications, as tested in mice [15].

Fenugreek has metabolism-altering actions which could reduce muscle fatigue through enhancing nutrient availability during exercise. Fenugreek-supplemented mice experienced lower blood lactic acid concentrations following exhaustive exercise than unsupplemented mice [23]. Fenugreek supplementation also lengthened time to exhaustion and increased post-exercise glycogen resynthesis rates in both humans and mice [24, 25]. For these reasons, we modified a ridden exercise protocol that was previously shown to induce muscle soreness [12], in order to evaluate DigestaWell® NRG, a dietary supplement containing yucca and fenugreek. The hypothesis was that DigestaWell® NRG would benefit unconditioned horses undergoing a bout of moderate-intensity exercise by reducing post-exercise muscle soreness. Secondly, we hypothesized that horses receiving the supplement would have reduced concentrations of both circulating inflammatory cytokines and lactic acid following exercise.

Materials and Methods

Horse Management

The Institutional Animal Care and Use Committee of Murray State University approved the use of horses for this study. Twenty mature, healthy horses from Murray State University's equine program were selected for use in this study. Horses were blocked into three groups by age (11 to 15 years, n=11; 16 to 20 years, n=5; >20 years, n=4), and then assigned either to treatment (NRG) or control (CON; Table 1). Horses assigned to the CON group included 9 Quarter Horses and 1 Thoroughbred; while the NRG group included 6 Quarter Horses, 2 Thoroughbreds, and 2

warmbloods. Eight geldings and 2 mares were assigned to the CON group; while 9 geldings and 1 mare were assigned to the NRG group. All mares were non-pregnant. Although breed and gender numbers differed, the investigators considered age as the primary blocking factor. Body condition score (BCS) was determined on a scale of 1-9 prior to the first SET at the same time bodyweight was measured [26]. Horses were between a (BCS) of 5 to 6.5 (Table 2). Sixteen of these horses were housed on well managed Coastal-Bermudagrass pasture, while 4 of the geldings (CON n=1; NRG n=3) were housed in stalls because they developed anxious behaviors when turned out for long periods of time. Stalled horses were unequally divided amongst treatments because age was considered the primary blocking factor. Stalled horses received several hours of daily turnout onto adjacent pastures and were offered *ad libitum* Coastal Bermudagrass hay (Table 3) when stalled. The hay was from the same batch throughout the study. Horses received concentrate in amounts necessary to maintain condition, with pastured horses receiving a maintenance concentrate (11-Six Pelleted Horse Feed, Southern States Cooperative, Richmond, VA; Table 2) at 0.2 kg per day, with a predominant purpose of carrying the supplement. Stalled horses receiving a higher calorie concentrate, due to individual tendencies to lose condition (Triple 10 Texturized Feed, Southern States Cooperative) at 0.5 to 0.7% of bodyweight per day (Table 3). Horses were fed their total feed divided into two equal feedings, twice daily. Amounts of nutrients consumed were calculated as the sum of concentrate, hay (stalled horses only), and pasture, with hay and pasture intake estimated based on equations [27, 28]. Nutrient intake by treatment and housing are presented in Table 4. First daily feedings took place between 0730 and 0900, as horses were individually fed in order to observe feed and supplement consumption. Second daily feedings occurred at 1500. All horses had *ad libitum* access to water and trace mineralized salt blocks (Southern States Cooperative). Feeds were

analyzed for nutritional content by Equi-Analytical (Ithaca, NY), while vitamin C and E analysis was conducted by NP Analytical Laboratories (St. Louis, MO).

Treatments

Horses assigned to the treatment diet received 200 grams of a nutritional supplement, DigestaWell NRG® (Probiotech International, Saint-Hyacinthe, Quebec, Canada) once a day, during the morning offering of feed, for a total of 4 weeks. The *Yucca schidigera* and *Trigonella foenum-graecum* used to produce the DigestaWell NRG® product were in the form of powdered extracts that were blended into a dry carrier of ground alfalfa, wheat middlings, and grape pomace. Liquid flavors (vanillin and diacetyl) were dried over silica to convert them to dried powders. Ceylon cinnamon was included as a flavor and was included as a dried powder. Yeast culture was included as well as the preservative, calcium propionate. The product is delivered to the horse in a powdered form. Pastured horses were brought one at a time into a small paddock where they had access to the supplement for 10-15 min. Horses remained in the paddock until consumption was complete or the horse showed no interest in the feed for at least 5 min despite encouragement to eat. No horses finished the supplement on d 1. By d 3, all pastured horses consumed the entire supplement and their feed ration within 15 min. Originally, three stall horses were assigned to the NRG treatment. Stalled horses had access to the supplement for 60 min, because one stalled horse regularly refused at least 100 g of supplement, and never consumed all 200 g at any point during the study. Due to lack of compliance, this horse's data was dropped from the statistical analysis. The other two stalled horses assigned to NRG, regularly consumed their entire supplement and feed. Therefore, the CON treatment contained one stalled horse and the NRG treatment contained two.

Standardized Exercise Test

A ridden standardized exercise test (SET, Table 5) similar to that conducted by Powell et al. [12], was conducted prior to the start (Period1) and following the conclusion of the study (Period2). For this SET, horses exercised for a total of 50 min, consisting of 2.5 min of brisk walking, 15 min of trotting, 5 min of canter, 2.5 min of trotting, reversing direction, 2.5 min of brisk walking, 15 min of trotting, 5 min of canter, and 2.5 min of trotting. Horses were to trot at approximately 3 m/s and canter at 5 m/s. Horses were randomly allocated to one of three groups, with each group assigned to a consecutive day for performing the SET. Seven horses were assigned to d 1 (NRG n=4; CON n=3), 6 horses to d 2 (NRG n=2; CON n=4), and 7 horses to d 3 (NRG n=4; CON n=3). Prior to the start of this study (January – May), horses participated in riding classes and equestrian team practices, however they had not received any forced exercise for six weeks before the start of the study. With the exception of the standardized exercise test, horses did not receive forced exercise during this trial.

The SETs were conducted in a 30 x 60-meter indoor arena with horses carrying 20% of their body weight. Prior to the start of the SET, each horse was weighed on a livestock scale while wearing only a halter. Following this, the rider was weighed along with tack, which included a roping-style western saddle, cinch, saddle pad, breast collar, and bridle. Additional weights were added to the scale in order to reach 20% of horse bodyweight. Weights consisted of custom-made nylon bags containing lead pellets at weights of 1, 5, and 10 pounds. Bags had grommets sewn in which allowed attachment to the saddle through use of carbineer clips. Placement of weights were equally distributed on the saddle from side to side and front to back in order to prevent tipping and pulling on the saddle. Horses were walked around to desensitize them to the feeling of these bags, yet no negative reactions were indicated. During the SET, cones were placed every 15 m, with horses needing to trot the distance in 5 s and canter the

distance in 3 s. An assistant on the ground kept time and advised the rider to increase or decrease speed to meet requirements. Riders and assistants had participated in previous exercise research trials and were familiar with the protocol. During the SET's, horses wore heart rate monitors (Equine H7, Polar USA, Bethpage, NY) that transmitted heart rate data to a wrist watch worn by the rider. Heart rate data from watches were recorded after horses were tacked up, after standing still for several minutes, at the end of each gait during the SET, and at 10 and 30 min post exercise (Figure 1). Riders dismounted immediately after the completion of the exercise test and horses were untacked after post-exercise vitals were obtained. Six advanced level riders from the MSU equitation program participated in this project and were blinded to treatment as they only participated in the riding portion. Riders were rotated and allowed breaks between exercise sessions. Riders were matched with horses in order to effectively meet the 20% of bodyweight goal.

Massage testing

A licensed equine massage therapist, blinded to treatments, conducted a muscle soreness and tension exam on each horse 24 h before and 24 h after each SET (Figure 1) [12]. The system used a Likert-type scale to grade the severity of muscle soreness and tightness in horses and ranged from 0 (no soreness/tightness) to 2.5 (extremely tight or sore). The scoring system included 10 muscles: the trapezius, deltoid, rhomboideus, latissimus dorsi, longissimus, triceps, biceps, gluteals, hamstring group, and tensor fascia lata, on the left and right sides of the horse. The massage therapist pressed a blunt plastic evaluation tool into the muscle and moved it caudally (trapezius, rhomboideus, latissimus dorsi, longissimus) or distally (triceps, deltoid, biceps, gluteals, hamstring, tensor fascia lata) along the muscle using consistent pressure. For each muscle and on each side of the horse, a soreness score and a tightness score were separately

recorded, therefore, each muscle on each side ranged from 0 (no soreness or tightness) to 2.5 (extremely sore or extremely tight). Muscle soreness and tightness scores from both sides of the horse and all muscles were summed for each horse during each evaluation, yielding a value that ranged from 0 (no soreness or tightness) to 100 (extremely sore and tight in each muscle on both sides of the horse). These values were used to calculate percent change within each period, which was calculated as (Period2 – Period1)/Period2, yielding two numbers per horse (pre-supplment[Period1] and post-supplement[Period2]).

Blood sampling and sample analysis

Blood samples were obtained via jugular venipuncture prior to the start of the SET (time 0) and at 10 and 30 min and 1, 4, and 24 h post exercise (Figure 1). Samples were collected into evacuated heparin and EDTA coated tubes (Vacutainer, Becton, Dickinson and Company, Franklin Lakes, NJ) and then placed on ice in a cooler until centrifugation (<2 h). Plasma was harvested and stored at -20°C until later analysis. All samples were analyzed in duplicate.

Plasma glucose and L-lactate concentrations were determined using commercially available enzymatic assay kits (2300 Stat Plus, YSI Inc., Yellow Springs, OH) designed for the YSI 2700 Select system (YSI Inc., Yellow Springs, OH). Plasma IL-1β and TNFα were analyzed using enzyme linked immunosorbent assays with methods previously published for use in the horse [29, 30]. Briefly, plasma samples were analyzed for TNFα using Nunc-Immuno 96 MicroWell flat bottom plates (Nalge Nunc International, Rochester, NY, USA) following a 1:4 dilution. The blocking buffer used for all assays consisted of 4% ELISA-grade BSA (Calbiochem, La Jolla, CA, USA), and 5% sucrose (Fisher Scientific, Fair Lawn, NJ, USA), in BuPH phosphate-buffered saline (ThermoFisher Scientific, Waltham, MA). Plates were washed in a solution of 0.05% Tween 20 (Fisher Scientific) in phosphate-buffered saline. The

manufacturer's instructions were followed, except an additional wash step was included after blocking. For IL-1β, plates were coated overnight with 3 µg/mL of capture antibody (prepared in DPBS), blocked for one hour with reagent diluent (4% BSA in DPBS), and then incubated with samples (diluted 1:2 in DPBS) for one hour. Following sample incubation, plates were incubated with detection antibody prepared at 3 µg/mL in DPBS and allowed to react with streptavidin-HRP (Kingfisher Biotech Inc., St. Paul, MN) for 30 minutes prior to incubation with substrate solution (Kingfisher Biotech) for 30 minutes. Reactions were stopped with the addition of stop buffer (Kingfisher Biotech). Rinsing protocols were the same as for the TNFα ELISA. ELISAs were read at 450 nm. Non-esterified fatty acid concentrations were analyzed using a commercially available spectrophotometric assay (Zenbio, Research Triangle Park, NC). Intra and inter-assay CV's were 3.9 and 11.5% for TNFα, 7.6 and 13.2% for IL-1β, and 7.2 and 5.9% for NEFA. Intra-assay CV's for glucose and lactate were 1.3 and 2.5%, respectively.

Statistics

All statistical analyses were performed using the MIXED procedure of SAS (v. 9.4, Cary, NC). For all analyses, normality and homogeneity of variance of residuals was determined through use of influence statistics and visual analysis of residual box and whisker plots. Outliers were determined through evaluation of the Internally Studentized Residual, with values >2.7 or < -2.7 being scrutinized. For all repeated measures analyses, the covariance structure yielding the lowest AICC index was selected for each analysis. Simple effect differences for a main effect of time were detected using a Dunnett test, which compares each time point to time 0, reducing the number of multiple comparisons. For all analyses, significance is considered at $P < 0.05$ and a tendency at $P < 0.09$.

Data for bodyweight and body condition scores were analyzed using repeated measures ANOVA for the main effects and interaction of period and treatment (trt), where the repeated effect was period and horse was a random effect. The statistical model was $\gamma = \mu + horse + period + trt + period*trt + \varepsilon$. Data are presented as the mean ± SEM. Mean nutrient intakes were analyzed for the effect of treatment and data are presented as the mean ± SEM.

Heart rate and plasma glucose, lactate, NEFA, IL-1β, and TNFα data were analyzed using repeated measures ANOVA for the effects and interactions of time and period within treatment. The statistical model was $\gamma = \mu + time(trt) + period(trt) + time*period(trt) + \varepsilon$. Muscle soreness data were analyzed for the main effect of period within treatment, with a statistical model of $\gamma = \mu + period(trt) + \varepsilon$. Day of SET (horses were assigned to one of three consecutive testing days during each period) included as a random effect. For all analyses except IL-1β, a covariate (time 0 value) was found to be significant ($P < 0.001$), and therefore included in the model. All plasma variables required transformation to achieve normality and homogeneity of variance. Therefore, plasma variable means are presented as geometric means bounded by the 95% confidence interval. Heart rate and muscle soreness data are presented as means ± SEM.

Results

Bodyweight, body condition scores, and nutrient consumption

Neither bodyweight nor body condition score were affected by period, treatment or the period by treatment interaction ($P > 0.1$; Table 2). Nutrient intakes were not different between treatments ($P > 0.1$; Table 4).

Heart Rate

Neither the time by period interaction nor period affected heart rates for NRG or CON horses ($P > 0.6$; Table 5). However, heart rates were affected by time for both treatments ($P < 0.001$), whereby heart rates were elevated above baseline at all time points except post 30 minutes ($P < 0.05$).

Plasma Metabolites

Neither the time by period interaction nor period affected plasma glucose concentrations for NRG or CON horses ($P > 0.1$; Figure 2A, B). Period affected plasma glucose concentrations for CON horses only ($P = 0.018$), whereby plasma glucose was higher ($P = 0.018$) during Period2 [5.1 [5.0, 5.2] mmol/L) than Period1 [4.8 [4.7, 4.9] mmol/L). Period did not affect plasma glucose concentrations for NRG ($P > 0.5$).

There was no effect of the time by period interaction on plasma lactate concentrations for NRG or CON horses ($P > 0.4$; Figure 2C, D). For CON horses, there was an effect of time ($P < 0.001$) but not period ($P > 0.3$), whereby lactate concentrations, when averaged across periods, were higher at 10 min (1.27 [1.16, 1.39] mmol/L; $P < 0.001$) and 30 min (0.99 [0.90, 1.08] mmol/L; $P < 0.001$) post exercise than baseline concentrations (0.57 [0.52, 0.63) mmol/L). For NRG horses, there was an effect of time ($P = 0.021$). Similar to CON horses, lactate concentrations, when averaged across periods, were elevated above baseline (0.43 [0.39, 0.47] mmol/L) at 10 min (0.96 [0.88, 1.06] mmol/L; $P < 0.001$), 30 min (0.71 [0.88, 1.06] mmol/L; $P < 0.001$), and also 1 h (0.59 [0.54, 0.65] mmol/L; $P = 0.040$) post exercise. Average lactate concentrations tended to be higher during Period1 (0.65 [0.61, 0.69] mmol/L) than Period2 (0.58 [0.54, 0.61]; $P = 0.058$).

There was no effect of the time by period interaction on plasma NEFA concentrations for NRG or CON horses ($P > 0.1$; Figure 2E, F). For CON horses, there was an effect of time ($P <$

0.001) but not period ($P > 0.4$), whereby NEFA concentrations, when averaged across periods, were greater at 10 min (551 [451, 674] µM; $P < 0.001$), 30 min (310, [253, 381] µM; $P < 0.001$), and 1 h (191 [156, 233] µM; $P < 0.01$) than baseline (98 [81, 120] µM). For NRG horses, there was a main effect of time ($P < 0.001$) but not period ($P > 0.7$), whereby NEFA concentrations were greater at 10 min (702 [599, 824] µM; $P < 0.001$) and 30 min (378, [322, 443] µM; $P < 0.001$) than baseline (164 [140, 193] µM).

Plasma Inflammatory Cytokines

There was no effect of the interaction of time and period for plasma TNFα concentrations for NRG or CON horses ($P > 0.5$; Figure 3 A, B). For CON horses, there was no effect of time or period ($P > 0.4$). For NRG horses, there was no effect of time ($P > 0.7$), but concentrations were lower during Period2 (170, [167, 173] pg/mL) than Period1 (182, [178, 185] pg/mL; $P = 0.012$). There were no effects or interactions of time and period for CON or NRG horses for plasma IL1-β concentrations ($P > 0.2$; Data Not Shown).

Muscle Soreness

The percent increase in muscle soreness and tightness was lower during Period2 (44 ± 16%; $P = 0.031$) than Period1 (95 ± 16%) for NRG treated horses ($P = 0.031$; Figure 3B). The percent increase in muscle soreness and tightness was not affected by period for CON horses ($P > 0.9$; Figure 3A).

Discussion

The primary objective of this experiment was to test the hypothesis that 30 d of dietary supplementation with DigestaWell® NRG would reduce muscle soreness following a bout of moderate-intensity exercise in horses that receive minimal ridden exercise. Secondly, we

hypothesized that horses receiving the supplement would have reduced concentrations of circulating inflammatory cytokines and lactic acid following exercise. During this study, horses carried 20% of their bodyweight. This weight was chosen as an intermediate between that of 25% previously shown to have an effect on heart rates and 17% previously shown to induce lameness in riding horses [12, 13], as our goal was to utilize moderate exercise that induced muscular soreness, but also to have horses complete the 50 minute exercise test without becoming lame. The current study differs from that of Dyson et al., due to the use of lead weights to adjust total weight instead of finding heavier riders, and this could account for differences in post-exercise lameness. The exercise program increased heart rates and plasma lactate concentrations to levels indicating that horses were being exercised at a moderate intensity level [12, 31-33].

A principal finding of this study was that NRG supplemented horses experienced reduced post exercise muscle soreness following the 30 d supplementation period. Yucca and fenugreek possibly reduce muscle soreness through their protective effects on cell membrane lipids, which when damaged during exercise [34], induce the sensing of pain through an increase in local inflammation. Derivatives of yucca contain antioxidant activities that reduce cell membrane peroxidation [35-38] while fenugreek inhibits the activity of the lipid peroxidase enzyme [22]. Fenugreek also downregulates pain sensing through inhibiting the activity of cyclooxygenase (COX)-1, and COX-2, the enzymes that convert arachidonic acid to prostaglandins [22, 39]. Fenugreek has similar pain reduction levels to ibuprofen when administered to mice [15]. Although extracts of both yucca and fenugreek have been evaluated for their pain-relieving activity, neither appears to have been previously tested in a model of exercise induced muscle soreness despite widely accessible over-the-counter herbal supplements for humans and horses.

A relationship exists between post-exercise muscle soreness and inflammation in humans [40]; with production of pro-inflammatory cytokines such as IL-1β and TNFα increasing in response to tissue damage [41]. These cytokines have a purpose of initiating clearance of damaged tissue, peak 1-2 days post exercise, and are then down regulated by anti-inflammatory cytokines following tissue cleanup [1, 42, 43]. Therefore, we were interested in evaluating the inflammatory protein response to exercise. Unfortunately, our exercise protocol did not influence IL-1β or TNFα protein in either CON or NRG treated horses. These findings are inconsistent to the findings of Liburt et al. [44], who reported increases in blood IL-1β mRNA at 2 hours and muscle and blood TNFα mRNA at 6 hours. However, many differences exist between the methods of these two studies. The former research group measured mRNA expression in white blood cells of blood and muscle, whereas we measured circulating protein concentrations. It is now known that IL1β is regulated at the level of protein secretion and a measurement of increased mRNA expression without an increase in secreted protein, does not reflect the activity of IL1β protein [45]. We also captured a slightly shorter window at 4 h post exercise instead of 6. The former study also included greater exercise intensity, type, and duration and it is most likely that the horses on the Liburt et al. study experienced more soreness than horses used for the present study. However, the purpose of this study was to investigate the potential benefits of DigestaWell® NRG in moderately exercised horses, and therefore, the exercise protocol employed in this study was of lower intensity.

An interesting finding of this study was the reduction in average TNFα concentrations in NRG treated horses after 30 d of supplementation. One possible explanation for this finding is that the extracts of *Yucca schidigera* and *Trigonella foenum-graecum* contain anti-inflammatory activity. For instance, resveratrol is an extract of *yucca schidigera* that reduced TNFα protein

production in cultured equine lymphocytes [46]. This is similar to results in mouse models, where resveratrol reduced TNFα protein in mouse spleen [47] and inhibits the TNFα response to lipopolysaccharide stimulation in a mouse cell line [48, 49]. This is possibly through the effects of yucca extracts to reduce LPS-induced binding of NFκB to the promoter of target genes [37], such as TNFα [50, 51]. Resveratrol also down regulates JAK1-STAT3 transcription factor mRNA levels [47]. These two transcription factors are important for mediating the inflammatory effects of TNFα in target cells [52]. Eight weeks of fenugreek seed powder supplementation also reduced TNFα protein concentrations in human blood [53], which could be due to one or more of the bioactive compounds contained in fenugreek: diosgenin, 4-OH-Ile, and galactomannan, all of which purportedly contain anti-inflammatory activity.

Horses supplemented with DigestaWell NRG® had altered metabolic responses to the moderate intensity exercise employed in this study. Unconditioned horses use a combination of fats, blood glucose, and muscle glycogen as energy sources during low and moderate intensity exercise (35% of VO_{2max}) [54], with muscle glycogen contributing 81% of energy at the start of exercise and 44% by one hour into the test. Despite using multiple sources of energy, unconditioned horses utilize aerobic metabolism until they reach speeds of about 4 m/s, at which point plasma lactate concentrations begin to accumulate, indicating that unconditioned horses increase their reliance on anaerobic mechanisms above this speed [55]. In that study, 11 weeks of conditioning increased the breakpoint to nearly 6 m/s, suggesting that fitter horses could exercise at the speeds used in our study without requiring anaerobic metabolism in contrast to unconditioned horses. The capacity to utilize aerobic metabolism can be increased through conditioning [56, 57], but it may also be possible to achieve increased aerobic capacity without conditioning, as fenugreek-treated mice exhibited increased capacities for aerobic metabolism

[24]. In these mice, both muscle and liver glycogen contents were higher immediately post-exercise than muscle and liver contents of untreated mice, supporting that fenugreek supplementation could possibly alter metabolic responses to exercise. The tendency for lower plasma lactate concentrations in NRG horses following exercise suggests that the dietary supplement increased capacity for aerobic metabolism. Future research should investigate the potential for reduced glycogen depletion following DigestaWell NRG® supplementation and in horses undergoing a regular exercise program.

Limitations of this study include that we used only one licensed massage therapist to perform muscle soreness and tightness scores. Unfortunately, we were unable to locate a second licensed massage therapist within the geographical region. Similarly, Powell et al. [12] used one licensed massage therapist to perform post-exercise muscles soreness and tightness scoring. When visually evaluating behaviors indicating equine musculoskeletal pain, agreement among trained veterinarians and behaviorists was 92% [8] and a second study evaluating lameness found that agreement increased with experience level [58]. Physical therapists evaluating human subjects' muscle tenderness to palpation reported an average of 72% agreement, with agreement being highest (95%) for lumbar muscles [59]. The therapist utilized for this study had several years of experience in the field and was blinded to treatments.

Future research using herbal supplements should include a flavonoid analysis. Flavonoids are the active ingredients in herbs, therefore determining their presence and concentration enables insights into the mechanism of a supplement's actions. Findings of this study can only be related to the product in entirety and not to the components of the ingredients. Furthermore, intakes of antioxidant-related minerals, such as selenium, zinc, and copper, and vitamins, such as E and C, would potentially influence inflammatory responses post exercise. All horses met or

exceeded their estimated requirements for vitamin E, selenium, and copper, while zinc was slightly low in pastured horses. However, all horses had *ad libitum* access to trace mineralized salt blocks, and intake of micronutrients from salt blocks was not included in the calculations. Therefore, it is highly likely that all horses met their copper and zinc requirements. Regretfully, we were only able to obtain a vitamin C analysis on the NRG supplement, with findings that vitamin C was undetectable and that the supplement provided NRG horses with an additional 3.7 IU of α-tocopherol per day. This is a small percentage of the average daily requirement of 500 IU (NRC, 2007), which was met by the other components of their diet, including fresh pasture, a rich source of vitamin E [60]. While the stalled horses consumed several hours of pasture daily, they also received a greater quantity of commercial concentrate, which was formulated to meet vitamin E requirements when fed at rates between 0.5 and 0.7% of bodyweight (Southern States Cooperative, Richmond, VA). Therefore, it is less likely that the observed differences were due to the increased antioxidant intakes of NRG horses. In order to further address the effects of a treatment on reducing inflammatory responses to exercise, plasma concentrations of TBARs and PGE2α could have been analyzed. Unfortunately, we lacked the funds necessary to complete these analyses.

Finally, this study was conducted during the months of June and July, with similarly warm and humid weather conditions during each of the SET's. June was slightly cooler (25.6°C, 73.8% humidity) than July (27.6°C, 77.6% humidity); however, both of these months exceeded the thermoneutral zone of the horse, yielding a heat index of 152-159. This high heat index would have required increased reliance on evaporative cooling mechanisms as compared to exercise in cooler and drier conditions [61, 62]. Others have indicated the additional stress placed on equine athletes to perform as the heat index increases above 150 [63, 64], with higher post

exercise plasma lactate concentrations and a more rapid time to fatigue in higher heat index conditions. Notably, our horses did not experience higher lactate concentrations during Period2 (July), despite the higher heat index.

In conclusion, horses experienced altered metabolic responses to a moderate intensity exercise trial following four weeks of DigestaWell® NRG supplementation. DigestaWell® NRG supplementation may benefit exercising horses through reducing muscle soreness and tightness as identified by massage and a tendency for reduced lactate production following exercise. DigestaWell® NRG supplementation also reduced circulating TNFα concentrations.

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570 **Tables**

571 **Table 1.**

Table 1. Number of horses within each age block that were assigned to the control (CON) or treatment (NRG).

Treatment	11-15 years	16-20 years	>20 years
NRG	6	2	2
CON	5	3	2

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Table 2. Bodyweight and body condition scores of horses prior to (Period 1) and after (Period 2) a 4-week supplementation period with 200 g DigestaWell® NRG per day.

	CON	NRG	SEM	*P* Values		
	Bodyweight, kg			Treatment	Period	Treatment x Period
Period 1	547	574	20	0.26	0.65	0.11
Period 2	543	581				
	Body Condition Score					
Period 1	6.0	5.5	0.3	0.28	0.27	0.71
Period 2	6.2	5.8				

Table 3. Dry matter nutritional content of forages and concentrates provided to horses prior to and during the 4-week supplementation period with 200 g DigestaWell® NRG per day.

Nutrient	Hay	Pasture	Feed1[a]	Feed2[b]	Supplement[c]
DE, Mcal/kg	1.81	2.29	3.04	3.28	3.34
CP, %	9.6	22.4	22.4	14.6	13.0
ADF, %	42.6	27.9	19.3	11.3	18.0
NDF, %	68.1	50.3	30.8	25.3	21.6
ESC, %	2.5	8.6	6.1	5.1	3.7
WSC, %	6.8	12.2	6.0	12.0	3.7
Starch, %	0.4	1.0	18.4	24.6	3.3
NSC,%	2.9	9.6	24.7	29.7	7.0
Fat, %	2.6	4.5	5.78	10.0	7.0
Vitamin C, ppm	NA	NA	NA	NA	<4
α-tocopherol acetate IU/g	61	162	150	100	0.02
Zinc	30	26	100	166	6
Copper	7	9	9	47	0.6
Selenium	0.06	0.16	0.35	0.6	-
Yucca, mg/g	-	-	-	-	8.7
Fenugreek, mg/g	-	-	-	-	36.0

[a]Feed provided to horses housed on pasture (n=16), horses provided with 0.2 kg per day.
[b]Feed provided to horses housed in stalls (n=3), horses provided with 2.3 kg per day.
[c]Nutrients contained in composited supplement.
NA= not available.

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Table 4. Average (±SD) daily nutrient intakes in control horses (CON) and horses supplemented with 200 g DigestaWell® NRG (NRG) per day for 4 weeks.

Nutrient	CON		NRG	
	Stall (n=1)	Pasture (n=9)	Stall (n=2)	Pasture (n=7)
DE, Mcal	31.2	23.9 ± 2.3	35.9 ± 1.3	26.2 ± 4.0
CP, g	1934	2350 ± 228	1959 ± 74	2533 ± 392
ESC, g	547	902 ± 88	588 ± 22	969 ± 150
WSC, g	1095	1280 ± 124	1374 ± 52	1370 ± 213
Starch, g	480	105 ± 10	628 ± 24	134 ± 18
NSC, g	1576	1385 ± 134	2001 ± 76	1504 ± 231
Fat, g	496	472 ± 46	693 ± 26	518 ± 79
α-tocopherol acetate, IU	1682	1710 ±166	1803 ± 269	1633 ± 62
Zinc, mg	614	280 ± 27	902 ± 34	303 ± 46
Copper, mg	114	95 ± 9	102 ± 16	238 ± 9
Selenium, mg	2.3	1.7 ± 0.2	2.7 ± 0.1	2.0 ± 0.4

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Table 5. Characteristics of the standardized exercise test and average heart rates (beats per minute ± SEM) of horses prior to (Period 1) and after (Period 2) a 4-week supplementation period with 200 g DigestaWell® NRG per day (NRG) and unsupplemented controls (CON).

			Heart Rate, beats per min, CON			Heart rate, beats per min, NRG		
Gait	Pace, m/s	Time, min	Period 1 ± 3.0	Period 2 ± 3.2	AVE ± 2.2	Period 1 ± 3.5	Period 2 ± 3.5	AVE ± 2.5
Baseline	---	---	44	42	43	36	39	37
PreEx	---	---	53	50	51***	50	47	49*
Walk	Brisk	2.5	70	67	69***	72	66	69***
Trot	3	15	105	106	106***	110	107	109***
Canter	5	5	132	130	131***	137	136	136***
Trot	3	2.5	112	112	112***	118	114	116***
Reverse	---	---	---	---	---	---	---	---
Walk	Brisk		87	87	90***	90	89	90***
Trot	3	2.5	113	114	113***	118	118	118***
Canter	5	15	128	134	131***	139	137	138***
Trot	3	5	116	116	116***	123	124	123***
Post-10		2.5	60	58	59***	54	59	57***
Post-30			53	49	51	39	48	43
Total		50						

*Within rows $P < 0.05$ for values compared to baseline.
***Within rows $P < 0.001$ for values compared to baseline.

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Table 6. The percent increase in muscle soreness and tightness following a 50 minute standardized exercise test in which control (CON) horses and horses supplemented daily with DigestaWell® NRG (NRG) carried 20% of their body weight.[a]

Treatment	Period1[b]	Period2[c]	*P*-value
CON	59.1 ± 11.1	58.1 ± 11.4	0.9
NRG	94.9 ± 16.3	43.8 ± 16.3	0.031

[a]Percent increase calculated as (post-exercise muscle soreness – pre-exercise muscle soreness)/pre-exercise muscle soreness.
[b]Period1 reflects values obtained prior to the study period.
[c]Period2 values were obtained after 4 wk.

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Figures

Fig. 1

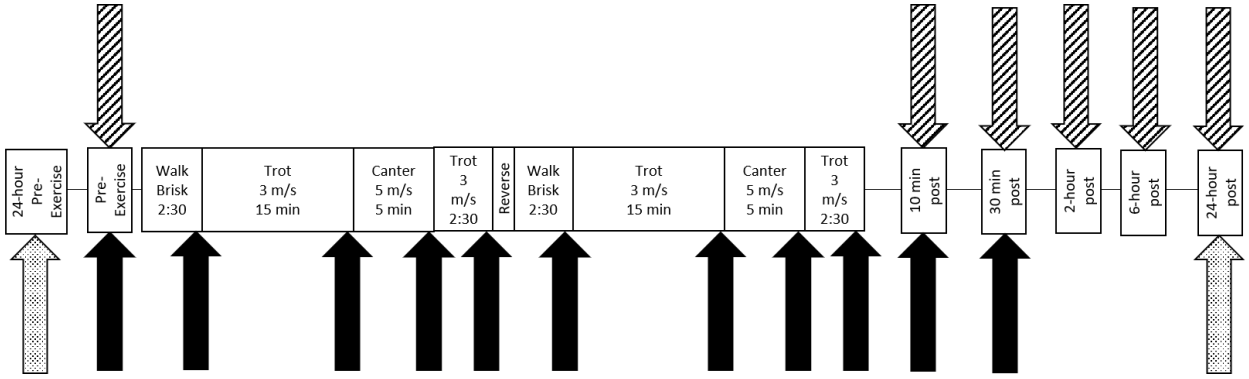


Figure 1. Timeline of data and sample collection from horses completing a standardized exercise test, where arrows with diagonal stripes indicate blood sampling time points, black arrows indicate when heart rate was obtained, and arrows with dots indicate massage testing for muscle soreness and tightness. Speeds during exercise test are approximate. Heart rates during exercise test were obtained at the end of the speed, prior to switching gaits.

Fig 2.

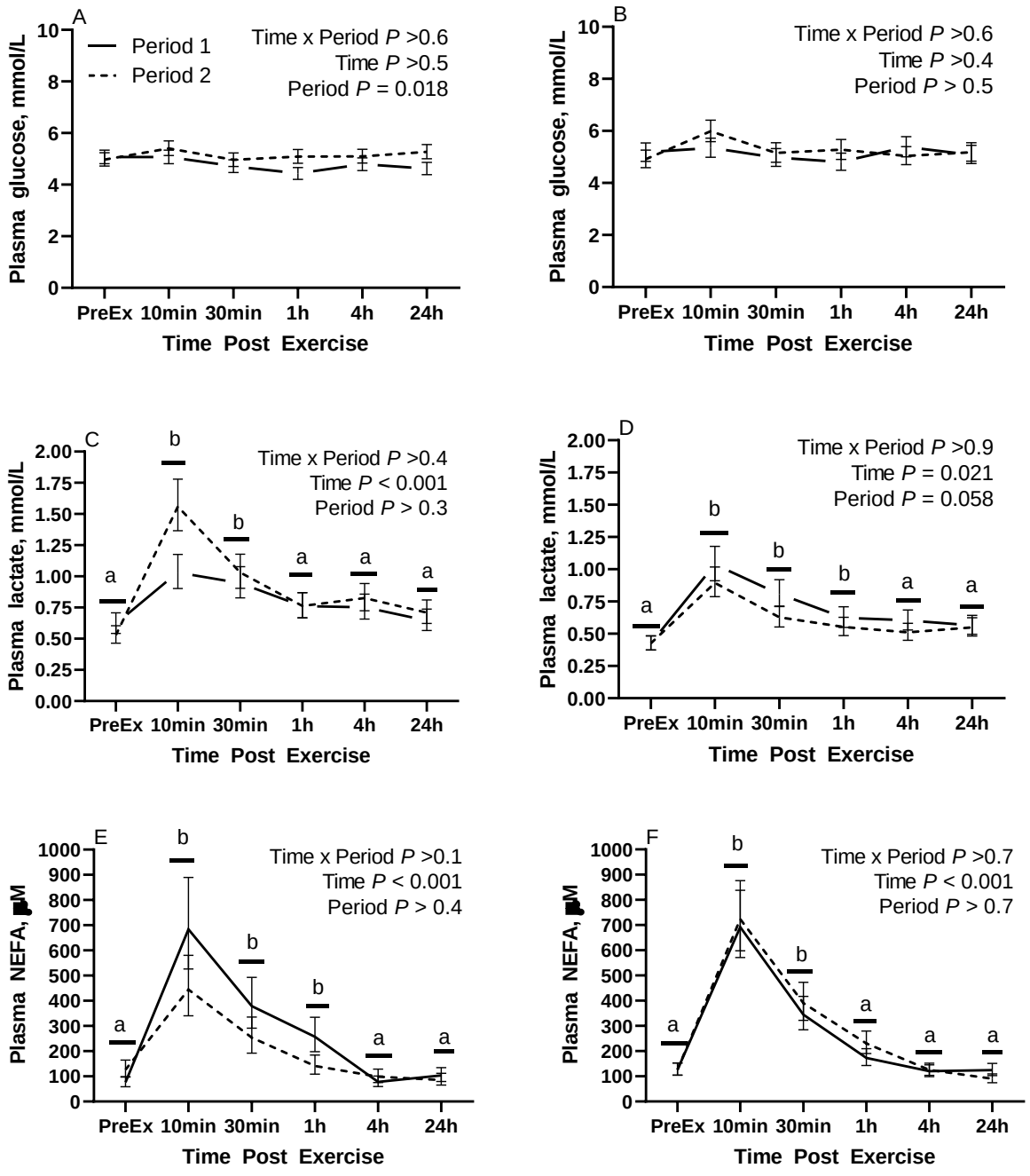
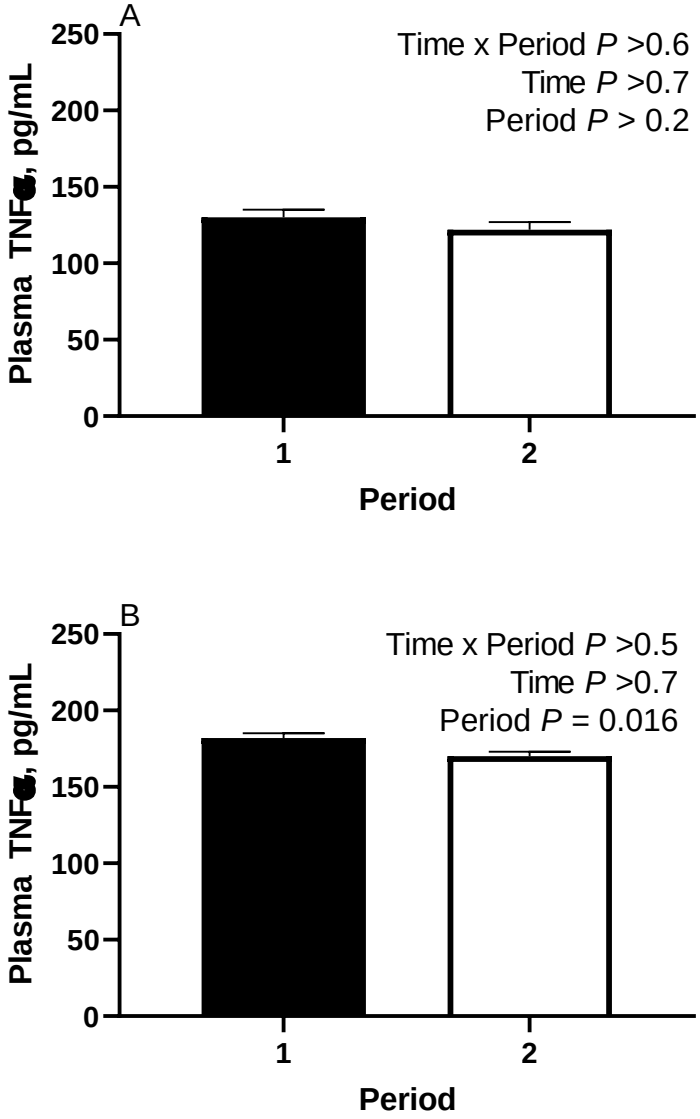


Figure 2. Plasma glucose (A, B), lactate (C, D), and non-esterified fatty acids (NEFA; E, F) concentrations prior to (PreEx) and following a 50 min standardized exercise test. Samples were collected from unsupplemented controls (A, C, E) and horses supplemented daily with DigestaWell® NRG (B, D, F) prior to the study (Period 1, black bars) and after 4 wk (Period 2, white bars). [ab]Means with unlike superscripts differ from PreEx $P < 0.05$.

604 **Fig. 3**



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608 **Figure 3.** Mean and 95% confidence interval plasma tumor necrosis factor-α (TNFα)
609 concentrations during a 50 min standardized exercise test. Samples were collected from
610 unsupplemented controls (A) and horses supplemented daily with DigestaWell® NRG (B) prior
611 to the study (Period 1) and after 4 wk (Period 2).

612