Effects of direct-fed microbial supplementation on digestibility and fermentation end-products in horses fed low- and high-starch concentrates

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J Anim Sci 2008.86:2596-2608.
doi: 10.2527/jas.2007-0608 originally published online Apr 11, 2008;

Effects of direct-fed microbial supplementation on digestibility and fermentation end-products in horses fed low- and high-starch concentrates[1]

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ABSTRACT: A study was conducted to determine whether direct-fed microbials (DFM) could be used to increase digestibility and minimize the risk of acidosis associated with feeding an increase in the amount of starch fed to horses. Fifteen mature Thoroughbred geldings were randomly assigned to 1 of 3 treatments in a 3 × 3 Latin square design balanced for carryover effects. Within each 26-d period, horses were offered grass hay + low-starch concentrate (LS; 1.2 g of starch·kg of BW^{-1}·$meal^{-1}$) from d 1 to 13 and then were abruptly changed to hay + high-starch concentrate (HS; 2.4 g of starch·kg of BW^{-1}·$meal^{-1}$) on d 14 continuing through d 26. The DFM treatments were offered in concentrate pellets at a target dosage of 10^8 cfu/(50 kg of BW·d) as follows: no DFM (CON; control), *Lactobacillus acidophilus* (LAC1; single-species DFM), or a mixture of *L. acidophilus, Lactobacillus casei, Bifidobacterium bifidum,* and *Enterococcus faecium* (LAC4; multiple-species DFM). Total feces were collected over 72 h from d 11 to 13 (LS; low dietary starch collection), from d 15 to 17 (AC; abrupt change in dietary starch collection), and at the end of each experimental period, from d 24 to 26 (HS; high dietary starch collection). Data collected consisted of total DM intake and fecal output, fecal pH, fecal acetate and propionate concentrations, and viable numbers of DFM in the feed. With the exception of Fe digestibility, there were no starch × DFM interactions. There was an effect of starch level ($P \leq 0.02$) on most nutrient digestibilities, except for Mg ($P = 0.08$) and CP, K, and Zn ($P > 0.10$). Horses supplemented with LAC4 had increased ether extract ($P < 0.05$) and a tendency for decreased Na ($P < 0.10$) digestibilities compared with CON horses. All DFM-supplemented horses had increased Cu ($P < 0.05$) and Fe and numerically increased Zn digestibilities compared with CON horses. Fecal pH decreased ($P < 0.05$), and fecal propionate concentration increased ($P < 0.05$) as dietary starch content changed from LS to HS. There was a tendency for elevated fecal pH ($P < 0.10$) in LAC1 horses compared with CON horses. These results confirm that increasing starch in the equine diet can enhance nutrient digestibility of the diet. Supplementing equine diets with either a single or mixed strain direct-fed lactic acid bacteria had limited effects on nutrient digestibility or on reducing the risk of acidosis associated with feeding high-starch concentrates to horses. The potential response of DFM supplementation should be evaluated when a more acute acidotic state is induced in horses than in the current study.

Key words: digestibility, direct-fed microbial, horse, *Lactobacillus acidophilus*, probiotic, starch

J. Anim. Sci. 2008. 86:2596–2608
doi:10.2527/jas.2007-0608

INTRODUCTION

It is a common practice among horse owners to supplement roughage diets with starch-based concentrates to meet nutrient requirements (NRC, 1989). When nondegraded starch escapes small intestinal digestion and reaches the hindgut, microbial populations are altered, pH is decreased, propionate concentration is

[1]We thank R. Johnson (Cargill Animal Nutrition, Minneapolis, MN), M. Newcomb and the team at the Cargill Animal Nutrition Innovation Center (Elk River, MN), and M. Young and the team at Star-Labs (Clarksdale, MO) for their collaboration and financial contribution. Thanks are extended to the Maryland Horse Industry Board (Annapolis) and the University of Maryland General Research Board Grant Programs (College Park) for their financial contribution. Counsel from J. S. Weese (University of Guelph, Guelph, Ontario, Canada), L. W. Douglass (University of Maryland, College Park), and J. Sylvester (Mars Horsecare US Inc., Dalton, OH) is gratefully acknowledged. The assistance of the staff and students at the Central Maryland Research and Education Center (Clarksville, MD) is greatly appreciated.

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Received September 25, 2007.
Accepted April 3, 2008.

increased, and fiber digestion is decreased (Kern et al., 1973; Pagan, 1998; Kohnke et al., 1999; Medina et al., 2002), resulting in an increased risk of acidosis, colic, or laminitis in the horse (Bailey et al., 2003; Hoffman, 2003).

In feedlot cattle fed high-concentrate diets, feeding direct-fed microbials (**DFM**) has reduced the risk of acidosis (Huffman et al., 1992; Ghorbani et al., 2002). Direct-fed microbials, also referred to as probiotics, are a source of live, naturally occurring microorganisms (Yoon and Stern, 1995), which may have beneficial effects on the host animal by creating an optimal intestinal microbial balance (Fuller, 1989). Lactic acid-producing bacteria (**LAB**), predominantly from the *Lactobacillus* genus, are the most widely used bacteria in DFM preparations and show promise toward enhancing digestive health in other species (Kung, 1999; Weese, 2001; Krehbiel et al., 2003). Because most DFM preparations have combinations of bacterial species, it is difficult to assess the efficacy of individual species.

Despite the positive nutritional benefits shown using DFM in other species and the availability of DFM supplements on the market intended for horses, no peer-reviewed journal articles have been published on the effects of single vs. multiple strains of direct-fed LAB offered to horses. The objective of this study was to determine whether DFM could be used to increase digestibility and minimize the risk of acidosis associated with abruptly increasing the starch content in the equine diet.

MATERIALS AND METHODS

All experimental procedures were approved by the University of Maryland's Institutional Animal Care and Use Committee.

Animals and Diets

Fifteen mature Thoroughbred geldings at rest (average initial age, 10 ± 3 yr; average initial BW, 557 ± 112 kg) were randomly assigned to 1 of 3 treatments arranged in a 3 × 3 Latin square split-plot (dietary starch level and sampling time) experiment balanced for carryover effects with repeated measures (total fecal collections) within each period. All geldings were vaccinated against *Ehrlichia risticii bacterin* spp. (Potomac horse fever), West Nile virus, Eastern-Western-Venezuelan encephalomyelitis, and tetanus toxoid (Fort Dodge Animal Health, Fort Dodge, IA) and dewormed against large and small strongyles, encysted cyathostomes, ascarids, pin worms, hair worms, large mouth stomach worms, and bots (moxidectin; Fort Dodge Animal Health) before the beginning of the study. Horses were weighed using a livestock platform scale (Digi-Star, Fort Atkinson, WI) and assigned a BCS (1 to 9 scale; Henneke et al., 1983) at the beginning and end of each experimental period. The geldings were housed in individual 3.6-m^2 box stalls with rubber mats and wood shavings (American Wood Fibers, Jessup, MD) at the University of Maryland Equine Research Unit located at the Central Maryland Research and Education Center in Clarksville. Horses received their diets in 2 equal meals twice daily at 0800 and 1700 h. The geldings were taken out of their stalls and walked (1.3 m/s) once daily at 1600 h for 10 to 35 min (depending on heat index, horses were exercised less on days above a 32°C heat index) using an automated 6-horse exerciser (Priefert, Mount Pleasant, TX) and alternating directions each day. The geldings were moved from pasture 7 d before the beginning of the study to allow for acclimation to housing, feeding, and exercise protocol. During acclimation, horses were fed mixed grass hay and given a step-wise introduction to the control diet to meet daily DE requirements for maintenance (NRC, 1989).

Horses were randomly assigned to 1 of 3 treatments for each of the 3 experimental periods: a control diet of grass hay + pelleted concentrate (CON), grass hay + pelleted concentrate with 10^8 cfu/(50 kg of BW·d) of *Lactobacillus acidophilus* (LAC1; custom preparation of single-species DFM provided by Star-Labs, Clarksdale, MO), or grass hay + pelleted concentrate with 10^8 cfu/(50 kg of BW·d) of a commercial DFM mixture of LAB (LAC4; PrimaLac 454; Star-Labs, Clarksdale, MO). The experiment consisted of 3 consecutive 26-d feeding periods (Figure 1) such that all horses received all treatments. The first 10 d of each period were a washout of the previous treatment and an adaptation to the new treatment. Two levels of target starch intake were offered: 1.2 (**LS**; low starch) and 2.4 g of starch·kg of BW^{-1}·$meal^{-1}$ (**HS**; high starch) for the first and last 13 d of each period, respectively, with an abrupt change (**AC**) from LS to HS on d 14. Pelleted concentrates were formulated to contain either 20 (LS) or 38% starch (HS). Starch content was increased in the HS concentrate with an isonitrogenous and isoenergetic substitution of corn meal for wheat middlings. Custom pellets (Nutrena Feeds, Cargill Animal Nutrition, Lebanon, PA) were fed at a rate of 5 g·kg of BW^{-1}·d^{-1}. Feed composition, based on an analysis of feeds by a commercial laboratory (Dairy One Forage Testing Laboratory, Ithaca, NY), is shown in Table 1. Horses were fed hay and concentrate to meet or slightly exceed their individual daily requirements for DE relative to BW (NRC, 1989). Hay was fed using stationary wooden box feeders affixed to each stall, and concentrate was offered in canvas feed bags (Derby Originals, North Canton, OH) to avoid wastage. Hay and concentrate offered and refused were collected, weighed, and recorded daily to determine total DM intake. Horses had ad libitum access to water and salt (sodium chloride) blocks.

The DFM treatments were included in the LS and HS pelleted concentrates, resulting in 6 total formulas for the study: low- and high-starch control (no DFM), low- and high-starch LAC1, and low- and high-starch LAC4. All probiotic preparations were included in the concentrate before pelleting. A 1.5 kg/t inclusion level

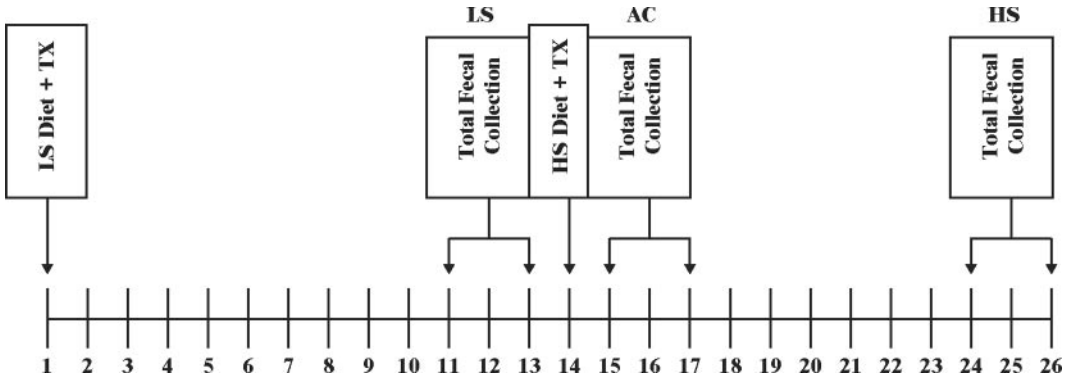


Figure 1. Experimental timeline of each 26-d period. Each horse received a low-starch pelleted concentrate (LS) from d 1 to 13 in addition to their assigned direct-fed microbial treatment (TX). Horses were abruptly changed (AC) to the high-starch concentrate (HS) on d 14 plus their TX and were sustained through d 26. Total feces were collected at 3 time points as follows: low dietary starch (LS, d 10 to 13), abrupt change in dietary starch (AC, d 15 to 17), and high dietary starch (HS, d 24 to 26).

of each DFM preparation into the pelleted horse feed was determined based on results from a companion study conducted earlier at the USDA (Beltsville, MD). All finished pelleted concentrates with and without DFM inclusion were sampled weekly for enumeration and identification of bacterial species.

The 85-d study was conducted from June to September in Clarksville, Maryland. The summer climate around the location of the study is typified by temperate, humid days with average daily precipitation of 150 mm (NOAA, 2007). During this investigation, average ambient temperatures ranged from 5 to 38°C (NOAA, 2007).

Sample Collection

Samples of hay and concentrate offered and total feces were collected on d 11 to 13 (LS), 15 to 17(AC), and 24 to 26 (HS) within each experimental period. Length of the collection period was determined based on the report that 95% of digesta passes by 65 h postfeeding (Van Weyenberg et al., 2005). Samples of concentrate and hay (approximately 200 g) were collected every 7 d throughout the study. Concentrate and hay samples were weighed, dried in a forced-air oven (105°C for 72 h), and reweighed for calculation of DM. Stalls were stripped of all shavings, feedstuffs, and manure at 1500 h on d 11, 15, and 24 and were swept and hosed daily after the 0700 h total collection. Each gelding was equipped with a collection harness (Equisan, Melbourne, Victoria, Australia) for total fecal collection to reduce potential for hay, concentrate, urine, and fecal mixing in the stall. Horses were accustomed to wearing the harnesses before the beginning of the study. The harnesses were fitted to each horse the evening before total fecal collection began. Feces were removed from the harnesses thrice daily (0700, 1200, and 1600 h), and horses were checked and treated for minor abrasions caused by harnesses as needed. Feces were removed from the harnesses into individual plastic tubs with plastic bag liners to reduce moisture loss and were weighed over each 24-h collection period for determination of total fecal output. At each collection, grab samples of fresh feces (approximately 500 g) were removed from the harness bags before emptying so that the most recently defecated feces were sampled, weighed, and immediately analyzed for pH, dried for determination of digestibility, or stored at −20°C for

Table 1. Nutrient composition of grass hay and low- and high-starch concentrates fed to Thoroughbred geldings[1]

	Feedstuff		
Nutrient	Hay[2]	LS[2,3]	HS[2,3]
DM, %	82.4	84.0	84.5
Ash, %	3.9	8.9	8.2
CP, %	7.7	14.4	14.4
Ether extract, %	1.8	7.3	6.8
NDF, %	61.6	28.1	17.1
ADF, %	34.4	12.2	6.3
WSC,[4] %	3.6	3.9	3.1
Starch, %	6.3	21.4	35.8
Ca, %	0.3	1.0	0.9
P, %	0.2	0.7	0.8
Mg, %	0.2	0.4	0.4
K, %	1.0	1.0	0.8
S, %	0.2	0.2	0.2
Cl, %	0.3	0.8	0.7
Na, %	0.0	0.5	0.4
Cu, mg/kg	4	54	51
Zn, mg/kg	15	166	205
Fe, mg/kg	64	829	1,011
Mn, mg/kg	141	148	166

[1]Mean values of weekly samples of feedstuffs taken during 3 periods; reported on a DM basis (Cargill Animal Nutrition, Innovation Center, Elk River, MN; n = 12).

[2]Hay was fed at 1.5% of BW, and concentrates were fed at 0.5% BW on an as-fed basis.

[3]LS = low-starch pelleted concentrate fed for target intake of 1.2 g of starch·kg of BW^{-1}·$meal^{-1}$; HS = high-starch pelleted concentrate fed for target intake of 2.4 g of starch·kg of BW^{-1}·$meal^{-1}$.

[4]WSC = water-soluble carbohydrates (sugar; Dairy One, Ithaca, NY).

later VFA analysis. Ten-gram subsamples obtained from 1200- and 1600-h fecal collections were prepared for analysis of VFA and lactate by adding 40 mL of 1 *N* HCl (modified the methods described by Hussein et al., 2004), after which they were stored in air-tight 120-mL plastic containers (Hi-Profile, Delamar, Newark, DE) at −20°C for later analysis. From the 500-g fecal samples, approximately 200-g subsamples were weighed, dried in a forced air oven (55°C for 72 h), reweighed for determination of DM, and then stored for later nutrient analysis.

Sample Analysis

Dried fecal, hay, and concentrate samples were ground in a Wiley Mill (Thomas Model 4, Swedesboro, NJ) using a 1-mm screen. Fecal samples for each horse over each total collection period were composited, and subsamples were sent to a commercial laboratory (Cargill Animal Nutrition, Innovation Center, Elk River, MN). Samples were analyzed for DM, OM, CP, ADF, NDF, ether extract (**EE**), ash, Ca, P, Mg, K, Cu, S, Cl, Fe, Mn, Na, and Zn using AOAC (2005) methods with modifications (CP, 968.06; NDF, 2002.04; ADF, 973.18; EE, 920.39; ash, 942.05; and minerals, 968.08). Chloride analysis was conducted by titration using the PC-titrate system (ManSci Inc., Tonawanda, NY). Samples were also sent for analysis of water-soluble carbohydrates (**WSC**) and starch (Dairy One). For starch analysis, WSC were preextracted by incubation in a water bath and filtered with Whatman 41 filter paper, followed by starch determination using an analyzer (YSI 2700 SELECT Biochemistry Analyzer, Application Note No. 319, 1994, Yellow Springs Instrument Co. Inc., Yellow Springs, OH). The extracted WSC were determined according to the methods of Hall et al. (1999). Total DMI (kg/d) and DM fecal output (FO, kg/d) were used to calculate apparent DM digestibility (DMD, %) using the equation: DMD = 1 − FO/DMI.

Fecal pH of fresh feces was determined (approximately 100 g) thrice daily (0700, 1200, and 1600 h) on d 11 to 13, 15 to 17, and 24 to 26. The pH was determined within 30 min of collection with a portable pH meter (model 13704, Denver Instruments, Arvada, CO). The probe (Orion semi-micro electrode model 91–16, Thermo Electron Corp, Beverly, MA) was submerged in the solid fecal mixture until the reading stabilized. Triplicate readings were recorded for each sample and averaged for each collection time. The pH meter was calibrated at 0700 h before the beginning of each collection day using pH 4 and 7 stock solutions. A back-up handheld pH meter, calibrated to the first reading, was used when intermittent mechanical failures occurred with the first (model IQ400, Scientific Instruments, Carlsbad, CA).

For VFA analysis, frozen samples of feces suspended in HCl were thawed to room temperature and poured into 30-mL centrifuge tubes and centrifuged at 22,000 × *g* at 4°C for 15 min. A 1-mL supernatant aliquot was transferred to 5-mL centrifuge tubes. One milliliter of constantly stirred 3.6 *M* CaOH was added to each tube and vortexed, after which 0.5 mL of 0.4 *M* $CuS0_4$ was added, and the solution was vortexed again. Samples were then centrifuged at 16,000 × *g* at 4°C for 15 min, after which 1.5 mL of supernatant was transferred to a new 5-mL tube and 20 μL of concentrated H_2SO_4 was added. Samples were subsequently centrifuged again at 16,000 × *g* at 4°C for 15 min. Sample extracts were filtered through a 0.45-μm syringe filter to remove particulate matter. The VFA concentrations were determined using HPLC (Waters, Alliance HPLC System, Milford, MA) with a 300 × 7.8 mm Aminex HPX-874 ion exclusion column (Bio-Rad Laboratories, Hercules, CA) at 6.895×10^6 Pa and 42°C. The VFA concentrations were reported as milligrams per milliliter, and a ratio of acetate to propionate (**A:P**) was calculated.

Enumeration of LAB

Batches of feed were made in the following order: CON, LAC1, and then LAC4. Batches were individually pelleted, bagged, tagged, and shipped to the study location (Clarksville, MD), where the diets were stored on pallets.

Weekly samples (approximately 200 g) were collected by hand directly from feed bags over the 85-d period and stored (4°C) before analysis. Viable LAB were enumerated by a commercial laboratory (Cargill Animal Nutrition, Innovation Center) using AOAC (1995) methods (14.1). Samples were individually ground, and then duplicate 1-g subsamples were weighed and placed into 15-mL centrifuge tubes. Nine milliliters of autoclaved deionized H_2O was added to each tube and then shaken for 1 h to mix. One milliliter of the liquid solution was drawn into 15-mL centrifuge tubes, 9 mL of autoclaved deionized H_2O was added, the solution was vortexed, and then plated by a spiral autoplater (Autoplate 4000, Spiral Biotech, Norwood, MA). Three plates were prepared for each sample to determine growth of (1) *Lactobacillus*, (2) *Enterococcus*, and (3) *Bifidobacterium*. The lactobacilli and bifidobacteria cultures were grown under anaerobic conditions (anaerobic jars with GasPaks; Becton Dickinson Microbiology Systems, Cockeysville, MD) and incubated for 48 h at 37°C on de Man, Rogosa, and Sharpe (**MRS**; de Man et al., 1960) agar and modified MRS (Simpson et al., 2003), respectively. *Enterococcus* cultures were grown aerobically on bile esculin azide agars (Novicki et al., 2004) and then incubated for 48 h at 37°C. Once growth was visualized, colony-forming units were determined by an automated colony counter (Protocol XR, Protocol Systems, Synoptics Ltd., Frederick, MD) and numbers were reported as cfu/mL.

Bacterial Species Identification

To determine if any cross-contamination had occurred in the diets, analysis of ribosomal DNA was performed.

Bacteria were isolated and anaerobically grown on agar at another commercial laboratory (Star-Labs Forage Research Inc., Clearwater, FL) using procedures outlined previously (SL-01; Swyers, 2007). A random selection of colonies was selected and then automatically identified (RiboPrinter, DuPont Qualicon, Wilmington, DE). The identification of bacterial species was determined at an 85% confidence level or greater, in which case a DuPont identification number was assigned and reported in addition to a genus and species name (SL-04 method, Star-Labs Forage Research Inc.).

Prevention of Cross-Contamination

Measures to minimize cross-contamination among treatment groups were taken throughout the study. All pelleted concentrates were processed in the expected order of least number of added bacterial species to most (i.e., CON, LAC1, and then LAC4) from mixing to pelleting and bagging at the feed mill. Between runs, feed mill equipment was flushed with batches of feed that were unrelated to the study and not containing DFM (as is standard protocol by the commercial mill). At the research facility, the weighing and handling of feed, handling of horses for grooming, exercising, cleaning of stalls, and collection and analysis of samples were all conducted in the order of CON, LAC1, and then LAC4. Separate tools were designated and labeled for each treatment group. Treatment groups were physically separated and unable to make nose-to-nose contact. If this order needed to be altered, clothing, boots, and hands were disinfected at bleach scrub stations placed throughout the barn and feed preparation areas before handling horses or equipment. Feed preparation area was disinfected at the end of each shift.

Statistical Analysis

The trial was analyzed as a 3 × 3 Latin square design [3 consecutive 26-d periods × 3 DFM treatments (CON, LAC1, and LAC4)] balanced for carryover effects with repeated measures (total collections: LS, AC, and HS) within each period. Fifteen horses (n = 15) were randomly assigned to 1 of 5 squares. Dietary starch level and sampling time were treated as split plots. Data were analyzed using mixed model procedures (SAS Inst. Inc., Cary, NC). The model included the fixed effects of period, DFM, starch, and the interaction of DFM × starch level and random effects of squares, horses within squares, and horse × Latin square × period × DFM treatment interaction.

The base-10 logarithm colony-forming unit bacterial enumeration data were analyzed as a repeated measures (with respect to samples taken from same batches of feed over a 12-wk period) using the MIXED model procedure with batches of feed as the experimental units. A best fit multiple linear regression model was developed using the GLM procedure of SAS with backward elimination of nonsignificant terms. The model included DFM treatment, time (wk), ambient temperature, and the treatment × time interaction. Ambient temperature was recorded and used as a covariate in the model. Average colony-forming units of lactobacilli, enterococci, and bifidobacteria were the response varibles.

Significance was declared at $P \leq 0.05$ and a tendency at $0.05 < P \leq 0.10$. For greater levels of significance, results are reported as $P \leq 0.01$ and $P \leq 0.001$. Tukey's comparison procedure (significance at $P \leq 0.05$) was used to test differences among least squares means if significant (or tendencies of) main effects or interactions were found.

RESULTS

Intake and Digestibility

All geldings maintained their initial BW and BCS (Table 2). One horse from the LAC4 treatment had to be removed at the end of the third period (during HS collection) for health reasons unrelated to treatments. Intake of hay and concentrate (DM basis) was not different among treatments (Table 3). Average intake of starch was greater during AC and HS (0.91 kg/d ± 0.02) compared with LS (0.55 kg/d ± 0.02; $P \leq 0.001$; data not shown).

Although differences ($P \leq 0.10$) were detected within starch levels (LS, AC, and HS) among DFM treatments, with the exception of Fe, there was no interaction between starch and DFM on nutrient digestibilities (Table 4). There was an effect of starch level on the digestibility of most nutrients ($P \leq 0.02$) except for CP, Mg, K, and Zn (Table 5). Feeding the high-starch concentrate at AC and HS was associated with greater digestibilities of DM, OM, NDF, ADF, P, Cu, Mn, and Na ($P < 0.05$) than LS. There was no difference in EE digestibility between the AC and HS, but the AC in starch resulted in a greater digestibility of EE ($P < 0.05$) than when LS was fed. The S digestibility was greatest ($P \leq 0.05$) at HS after the high starch concentrate had been fed for 12 d. The WSC digestibility was lowest ($P < 0.05$) during HS, whereas starch digestibility was lowest during AC ($P < 0.05$). All DFM-supplemented hors-

Table 2. Body weight and BCS of Thoroughbred geldings consuming low- and high-starch concentrates containing no DFM[1] (CON), 1 strain (LAC1), or 4 strains (LAC4) of lactic acid-producing bacteria DFM (n = 15)

	Treatment			
Item	CON	LAC1	LAC4[2]	SE
Initial BW, kg	565	568	566	3
Final BW, kg	570	570	571	2
Initial BCS	5.7	5.8	5.6	0.1
Final BCS	5.7	5.7	5.8	0.1

[1]DFM = direct-fed microbials.
[2]n = 14.

Table 3. Daily intakes of geldings consuming low- and high-starch concentrates containing: no DFM[1] (CON), 1 strain (LAC1), or 4 strains (LAC4) of lactic acid-producing bacteria DFM (n = 15)[2]

	Treatment										
	CON			LAC1			LAC4				
Item	LS	AC	HS	LS	AC	HS	LS	AC	HS[3]	SE	*P*-value[4]
Intake, kg/d (DM basis)											
Hay	6.41	6.38	6.58	6.46	6.42	6.47	6.55	6.64	6.55	0.19	0.31
Concentrate	2.32	2.35	2.35	2.33	2.36	2.36	2.35	2.35	2.34	0.06	0.29
Total	8.73	8.72	8.93	8.79	8.78	8.83	8.90	8.99	8.89	0.24	0.29

[1]DFM = direct-fed microbials.
[2]LS = low starch collection, d 10 to 13; AC = abrupt change to HS collection, d 15 to 17; HS = high starch collection, d 24 to 26.
[3]n = 14.
[4]For DFM × starch interaction.

es had increased Cu ($P < 0.05$) and Fe and numerically increased Zn digestibilities compared with the CON horses. The LAC4-supplemented horses had increased EE ($P < 0.05$) and a tendency for decreased Na ($P < 0.10$) digestibilities compared with CON horses.

Fecal pH

There was no starch × DFM interaction on fecal pH (Table 4); however, there was an effect of starch ($P < 0.001$) and a trend for an effect of DFM ($P = 0.06$) on fecal pH (Table 5). Fecal pH decreased ($P < 0.05$) as the starch content of the diet increased. Horses supplemented with LAC1 had a trend for a greater fecal pH ($P < 0.10$) than the CON group, but there was no difference between the 2 DFM groups or between the CON and LAC4 groups. Fecal pH was lowest ($P < 0.05$) among all treatment groups at the 0700-h collection compared with 1200 and 1600-h collection (data not shown). There was a starch × time of sampling interaction on fecal pH ($P = 0.04$) when averaged among all DFM treatment groups, which was caused by the greater fecal pH for the LS group at 1200 h (Figure 2a).

Fecal VFA

There was no starch × treatment interaction on fecal acetate and propionate concentration or on the A:P molar ratio (Table 4). Fecal concentration of acetate and propionate increased ($P < 0.05$) during the abrupt starch increase in the diet, although acetate concentration decreased to the previous level 12 d after the change (Table 5). The A:P decreased ($P < 0.05$) when measured 12 d after horses were fed the high-starch diet. There were no differences in fecal acetate or propionate when horses were supplemented with either LAC1 or LAC4. There was a starch × sampling time interaction ($P = 0.003$) on fecal propionate concentration when averaged among all DFM treatment groups, whereby fecal propionate concentration increased from 0700 to 1600 h during AC in contrast to a decrease during LS and HS (Figure 2b). Concentrations of fecal lactate and other VFA (isobutyric, isovaleric, valeric, 2-methylbutyric, hexanoic, and heptanoic acids) were below the level of detection and not reported.

Bacterial Enumeration

After repeated plate growth, 4 data points were removed from LAC4 lactobacilli samples due to contamination from a rapidly growing bacterial species that produced spreader colonies on the surface of MRS plates. Enumeration data indicated the presence of bacterial species in the control diet. Average viable lactobacilli counts were not affected by treatment ($P = 0.15$) or time ($P = 0.96$; Figure 3a). Average viable enterococci counts over the 12-wk period did not change in the CON diet, had a tendency to increase in LAC1, and decreased in LAC4 (treatment × time interaction; $P = 0.02$; Figure 3b). Average viable bifidobacteria counts over the 12-wk period increased in CON and LAC4 but decreased in LAC1 (treatment × time interaction; $P < 0.001$; Figure 3c). The *P*-values for the main effects of treatment and time on average viable enterococci and bifidobacteria are not shown due to significant treatment × time interactions ($P = 0.02$ and $P < 0.001$, respectively; Figures 3b and 3c).

Bacterial ribosomal DNA from the treatment diets was identified and is reported in Table 6. No treatment-type bacterial species contamination was found in CON. All of the expected bacterial species were present in LAC1 and LAC4, except no bifidobacteria were identified in LAC4.

DISCUSSION

The main goals of this study were to test the hypotheses that feeding direct-fed LAB to horses would reduce the risk of acidosis associated with feeding high-starch concentrates and that direct-fed LAB could improve nutrient utilization of the equine diet. The risk of feeding high-starch concentrates to horses was previously discussed by Potter et al. (1992), who showed that feeding at least 3.5 g of starch/(kg of BW·meal) exceeds the digestive capacity of the small intestine of the horse,

Table 4. Interaction (DFM[1] × starch level) effects of low- and high-starch concentrates containing no DFM (CON), 1 strain (LAC1), or 4 strains (LAC4) of a lactic acid-producing bacteria DFM on apparent total tract digestibility of nutrients, fecal pH, and fecal VFA concentrations of Thoroughbred geldings (n = 15)[2]

	DFM treatment × starch										
	Control			LAC1			LAC4				
Item	LS	AC	HS	LS	AC	HS	LS	AC	HS[3]	SE	*P*-value[4]
Digestibility, % of intake (DM basis)											
DM	41.9^{a}	45.8^{b}	47.1^{b}	40.3^{a}	46.2^{b}	46.4^{b}	41.3^{a}	47.0^{b}	47.0^{b}	1.0	0.64
OM	43.0^{a}	47.2^{b}	48.3^{b}	41.3^{a}	47.6^{b}	47.7^{b}	42.3^{a}	48.1^{b}	48.2^{b}	1.0	0.67
CP	54.8	53.6	53.3	52.0	42.9	51.3	53.9	52.5	52.6	3.5	0.61
Ether extract	55.3	60.4	53.5	57.0	64.8	60.3	57.0	64.9	62.7	2.4	0.62
NDF	24.4^{a}	28.1^{ab}	31.3^{b}	22.4^{a}	28.6^{b}	30.1^{b}	23.9^{a}	29.4^{b}	30.1^{ab}	1.4	0.69
ADF	20.0^{a}	24.5^{ab}	27.4^{b}	17.8^{a}	24.7^{b}	26.0^{b}	19.3^{a}	25.7^{b}	26.1^{b}	1.6	0.82
WSC[5]	91.2	91.8	90.0	92.0^{a}	91.1^{ac}	88.7^{bd}	92.4^{a}	91.6^{ab}	89.8^{b}	0.8	0.34
Starch	95.7^{a}	92.0^{b}	95.3^{a}	95.2^{a}	92.2^{b}	94.6^{a}	96.3^{a}	91.3^{b}	94.8^{a}	1.0	0.64
Ca	16.4	13.0	24.5	19.2	19.3	24.6	15.1^{c}	20.0^{cd}	28.5^{d}	4.2	0.59
P	−13.2	−8.8	−2.1	−14.2^{a}	−4.7^{ab}	−0.2^{b}	−11.1	−6.8	−2.4	3.4	0.83
Mg	18.6	15.0	20.8	16.2	17.6	20.5	17.0	18.8	24	3.2	0.50
K	49.4	49.4	50.6	50.7	48.5	48.9	54.0	48.8	50.5	2.8	0.40
Cu	−16.4	−1.3	0.0	4.0	13.0	10.1	9.30	12.1	−3.8	4.8	0.84
S	46.4	49.9	52.5	48.7^{c}	50.4^{cd}	55.0^{d}	47.7^{a}	51.8^{ab}	56.8^{b}	1.8	0.79
Cl	87.9	89.8	92.7	88.5	91.5	92.3	88.0	88.2	92.2	2.1	0.91
Fe	−33.2^{a}	−0.6^{b}	−1.0^{b}	−1.0	3.3	−2.6	4.1	7.7	3.8	4.2	<0.001
Mn	−12.0	−0.1	4.0	−8.5	8.2	10.8	−10.9^{a}	18.9^{b}	6.4^{ab}	6.7	0.64
Na	8.0^{a}	22.6^{ab}	31.8^{b}	−7.7^{a}	20.5^{b}	26.6^{b}	3.2	16.0	16	5.4	0.23
Zn	−0.8	2.6	10.2	9.1	17.8	19.4	7.9	23.3	15.2	6.5	0.79
pH	6.61^{b}	6.54^{ab}	6.50^{a}	6.63	6.60	6.55	6.60	6.56	6.52	0.03	0.84
VFA,[6] mmol/g											
Acetate (A)	80.5^{c}	92.0^{d}	83.0^{cd}	80.2	89.2	80.6	80.0^{a}	92.3^{b}	80.6^{a}	2.80	0.96
Propionate (P)	6.8^{a}	8.9^{b}	8.0^{ab}	6.9	7.7	8.1	7.3	8.6	8.2	0.41	0.81
A:P	12.4	10.9	11.1	12.2^{a}	12.1^{a}	10.2^{b}	11.3	11.1	10.2	0.50	0.15

[a,b]Within a row and treatment, means that do not have a common superscript differ ($P < 0.05$).
[c,d]Within a row and treatment, means that do not have a common superscript differ ($P < 0.10$).
[1]DFM = direct-fed microbials.
[2]LS = low starch collection, d 10 to 13; AC = abrupt change to HS collection, d 15 to 17; HS = high starch collection, d 24 to 26.
[3]n = 14.
[4]For DFM × starch interaction.
[5]WSC = water-soluble carbohydrates (sugar; Dairy One, Ithaca, NY).
[6]VFA data were confirmed by a second laboratory using gas chromatography (University of Maryland, College Park).

allowing nondegraded starch particles to be rapidly fermented in the hindgut. Rapid fermentation of starch in the hindgut alters the microbial ecology (Julliand et al., 2001) whereby lactobacilli and streptococci bacteria proliferate (Goodson et al., 1988; Bailey et al., 2003), creating an accumulation of lactic acid and other organic acids in the cecum (Nordlund et al., 1995; Owens et al., 1998). This unbalanced microbial population associated with the production of lactic acid causes 2 problems: first, a decrease in pH that depresses fiber digestion (Pagan, 1998; Kohnke et al., 1999; Julliand et al., 2001) and, second, an increase in the potential of certain microbes to release endotoxins (Sprouse et al., 1987; Clarke et al., 1990). Ultimately, these disturbances can perpetuate acidosis, which can lead to ulcers (Andrews et al., 2005), colic (de Fombelle et al., 2001), endotoxemia (Sprouse et al., 1987; Clarke et al., 1990), or laminitis in the horse (Garner et al., 1977; Sprouse et al., 1987; Bailey et al., 2003).

Reports of a reduced risk of acidosis in beef feedlot cattle fed high-concentrate diets supplemented with direct-fed LAB (Ware et al., 1988; Huffman et al., 1992; Van Koevering et al., 1994; Ghorbani et al., 2002) prompted our interest in the potential of direct-fed LAB to reduce the negative effects on digestion associated with feeding high-starch concentrates to horses. Although the digestive physiologies of cattle and horses are distinctly different, Kern et al. (1973, 1974) reported that bacterial populations in the rumen and equine cecum are similar.

It is acknowledged that the HS concentrate offered in the current study did not reach the level of intake, indicated by Potter et al. (1992), which would induce the aforementioned metabolic disturbances. The intent of the current study was to offer 2 levels of starch through an AC and to observe the effects of that change on digestion and fermentation end-products. Consequently, the reference to the HS concentrate in the cur-

Table 5. Effects of low- and high-starch concentrates containing no DFM[1] (CON), 1 strain (LAC1), or 4 strains (LAC4) of a lactic acid-producing bacteria DFM on apparent total tract digestibility of nutrients, fecal pH, and fecal VFA concentrations of Thoroughbred geldings (n = 15)[2]

	Treatment								
	Starch			DFM				*P*-value	
Item	LS	AC	HS[3]	CON	LAC1	LAC4[3]	SE	Starch	DFM
Digestibility, % of intake (DM basis)									
DM	41.2[a]	46.3[b]	46.9[b]	44.9	44.3	45.1	0.8	<0.001	0.36
OM	42.2[a]	47.6[b]	48.1[b]	46.2	45.5	46.2	0.9	<0.001	0.46
CP	53.6	49.7	52.4	53.9	48.7	53.0	2.3	0.34	0.15
Ether extract	56.5[a]	63.4[b]	58.9[ab]	56.4[a]	60.7[ab]	61.5[b]	1.5	0.002	0.03
NDF	23.6[a]	28.7[b]	30.5[b]	27.9	27.0	27.8	1.1	<0.001	0.54
ADF	19.0[a]	25.0[b]	26.5[b]	24.0	22.8	23.7	1.2	<0.001	0.50
WSC[4]	91.9[a]	91.5[a]	89.6[b]	91.0	90.6	91.3	0.7	<0.001	0.42
Starch	95.7[a]	91.9[b]	94.9[a]	94.4	94.0	94.1	0.8	<0.001	0.78
Ca	16.4[a]	13.0[a]	24.5[b]	18.0	21.0	21.1	3.4	0.001	0.39
P	−12.8[a]	−6.8[b]	−1.6[b]	−8.0	−6.4	−6.75	2.4	<0.001	0.77
Mg	17.3	17.1	20.8	17.2	18.1	19.9	2.6	0.08	0.32
K	51.4	48.9	50.0	49.8	49.4	51.1	2.6	0.11	0.31
Cu	−5.4[a]	7.9[b]	6.5[b]	−5.9[a]	9.0[b]	5.9[b]	2.9	<0.001	0.002
S	47.6[a]	50.7[b]	54.8[c]	49.6	51.4	52.1	1.3	<0.001	0.14
Cl	88.1[a]	89.9[ab]	92.4[b]	90.2	90.7	89.5	1.5	0.02	0.71
Fe	−10.0[a]	3.4[b]	0.1[b]	−11.6[a]	−0.1[b]	5.2[b]	2.5	<0.001	<0.001
Mn	−10.5[a]	9.0[b]	7.1[b]	−2.7	3.5	4.8	3.9	0.001	0.37
Na	1.1[a]	19.7[b]	24.8[b]	20.8[c]	13.1[cd]	11.7[d]	3.6	<0.001	0.07
Zn	5.4	14.6	14.9	3.9	15.4	15.5	4.0	0.12	0.08
pH	6.61[a]	6.57[b]	6.53[b]	6.55[c]	6.60[d]	6.56[cd]	0.03	<0.001	0.06
VFA,[5] mmol/g									
Acetate (A)	80.2[a]	91.2[b]	81.4[a]	85.2	83.3	84.3	1.8	<0.001	0.70
Propionate (P)	7.0[a]	8.4[b]	8.1[b]	7.9	7.6	8.0	0.3	<0.001	0.33
A:P	11.9[a]	11.4[a]	10.5[b]	11.5	11.5	10.9	0.3	<0.001	0.18

[a,b]Within a row and treatment, means that do not have a common superscript differ ($P < 0.05$).
[c,d]Within a row and treatment, means that do not have a common superscript differ ($P < 0.10$).
[1]DFM = direct-fed microbials.
[2]LS = low starch collection, d 10 to 13; AC = abrupt change to HS collection, d 15 to 17; HS = high starch collection, d 24 to 26.
[3]n = 14.
[4]WSC = water-soluble carbohydrates (sugar; Dairy One, Ithaca, NY).
[5]VFA data were confirmed by a second laboratory using gas chromatography (University of Maryland, College Park).

rent study is reflective of the greater of the 2 levels of starch offered to the horses.

Digestibility

In agreement with others (Karlsson et al., 2000; Drogoul et al., 2001; Hussein et al., 2004), DM and OM digestibilities increased when dietary starch increased. This increase could be attributed to the observed increase in fiber digestion or that our high-starch concentrate had a lower fiber concentration (17 vs. 28%), which occurred as a result of diet formulation.

Starch was nearly 100% digested, which is in agreement with others (de Fombelle et al., 2004; Hussein et al., 2004). Although we showed no difference in starch digestion between the LS and HS time points, starch digestion was lowest during the abrupt change, indicating that the digestive function was reduced due to an abrupt increase in starch. However, horses seemed to have adjusted to the greater starch level after the HS concentrate had been offered for 12 d.

The LAC4 increased the apparent digestibility of the lipid portion of the diet by 5.1%, regardless of starch level. Previously, it has been shown in other species that certain microbes express lipase enzymes capable of breaking down dietary fat (Grunewald, 1982; Danielson et al., 1989; de Rodas et al., 1996; Hobson and Stewart, 1997). Whether a similar mechanism was responsible for increasing fat digestion in horses fed LAC4 is not known. Nevertheless, the current study indicates that, perhaps, supplementation with LAC4 improved the digestibility of the lipid portion of the diet.

Negative P, Cu, Fe, Mn, Na, and Zn digestibilities were detected, indicating that endogenous losses were greater for those minerals than what was consumed from the diet. A similar finding was reported in horses fed 2 grass hay varieties (Ordakowski-Burk et al., 2006). There was an effect of both DFM supplements on Cu and Fe digestibilities, in addition to having a tendency for increased Zn digestibility. Interestingly, Na digestion decreased with LAC4. Similarly, Huaynate et al. (2006) showed that offering direct-fed LAB

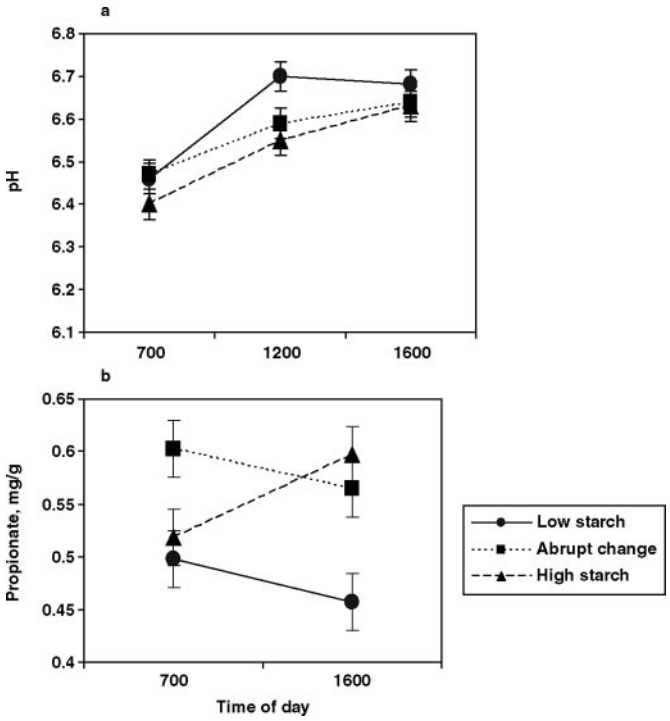


Figure 2. Relationship between collection time of the day and (a) pH and (b) fecal propionate concentration of geldings consuming low- and high-starch concentrates with and without direct-fed lactic acid-producing bacteria. There were starch × time interactions for fecal pH ($P = 0.04$) and fecal propionate concentration ($P = 0.003$). There was no starch × time interaction on fecal acetate concentration ($P = 0.61$; not shown).

to growing pigs numerically decreased fecal content of all micro- and macrominerals with the exception of Na. The effect of the same DFM preparation on Ca digestibility previously reported on broiler chickens (Angel et al., 2005) was not observed in the current study. Our study indicates that supplementing with direct-fed LAB may have the potential to improve mineral balance, specifically Cu, Fe, and Zn.

Fecal pH

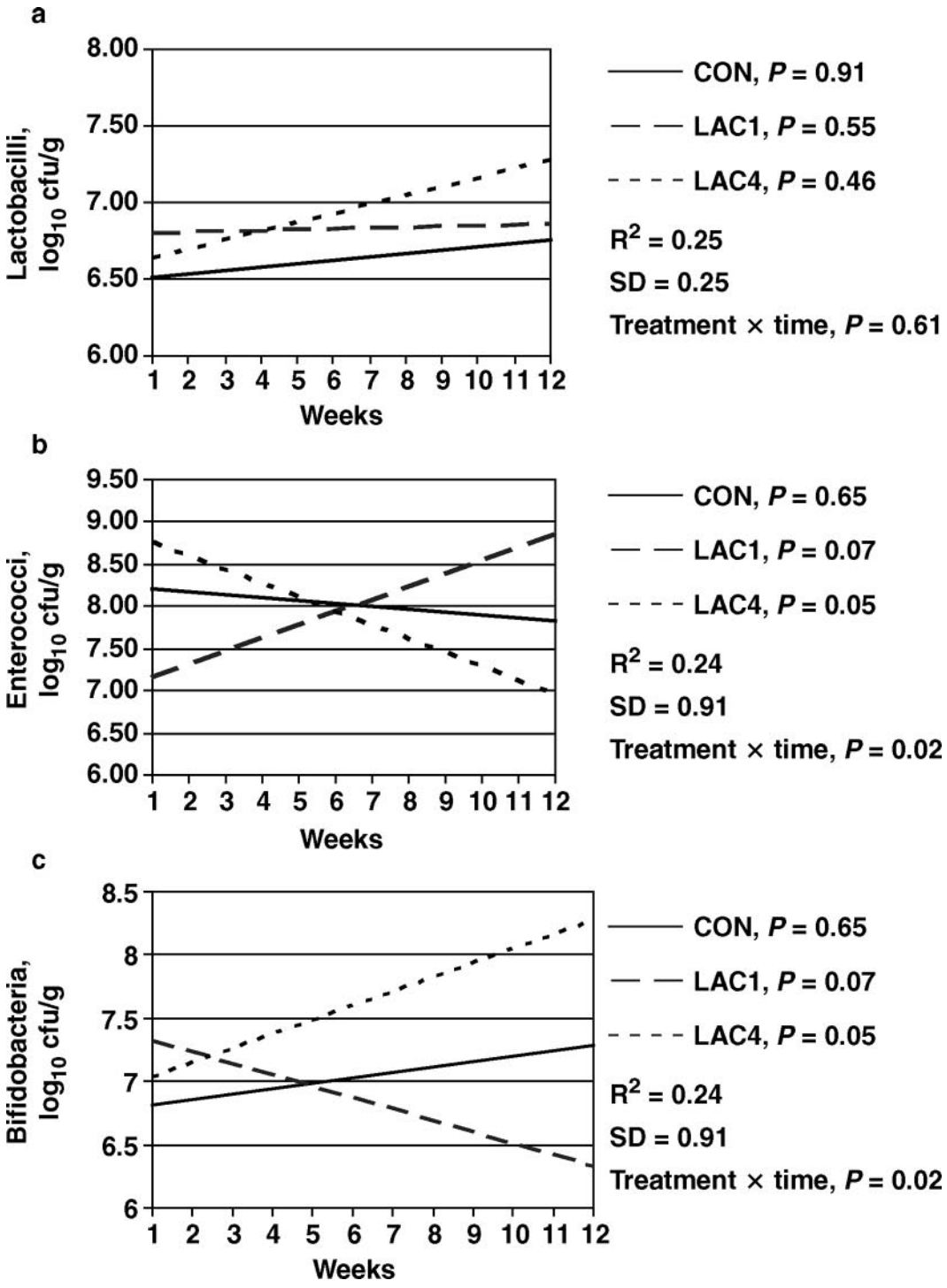
Fecal pH values observed when starch was increased were within ranges reported by Julliand et al. (2001), but somewhat lower than those reported by Hussein et al. (2004). Fecal pH was not affected by an abrupt increase of dietary starch when DFM were supplemented. Although the single-strain DFM showed a tendency toward increased fecal pH as collection times progressed throughout the day, the observed changes in pH were small and may not have practical consequences regarding minimizing hindgut acidosis in horses. However, a small decline in cecal pH from 6.7 to 6.4 was previously associated with altered fermentation patterns and microbial ecology in the hindgut of the horse (de Fombelle et al., 2001; Julliand et al., 2001; Medina et al., 2002). In the current study, we did not determine cecal pH; however, a correlation between cecal and fecal pH has been reported in rats, with fecal pH being consistently greater than cecal pH (Campbell et al., 1997).

Medina et al. (2002) reported that hindgut pH decreased rapidly after feeding mature geldings a high-starch diet, reaching a minimum of 6.43 at 5 h postmeal. Goodson et al. (1988) showed that cecal pH decreased to a low of 5.9 at 7 h postmeal when ponies were abruptly changed from hay to concentrate. In the current study, fecal pH was lowest at 0700 h, 14 h after the last daily meal was offered. However, because this was the first collection after the 1700-h meal, fecal pH could have been lower before 0700 h (hourly samples were not taken). The difference observed among studies could be attributed to differences in gut transit time, because our data were from feces, whereas Goodson et al. (1988) and Medina et al. (2002) reported minimum pH data corresponding to cecal samples.

Fecal VFA

Feeding the high-starch concentrate increased fecal acetate. This is in agreement with Hussein et al. (2004), who observed that fecal acetate concentration increased when grains were added to an alfalfa cube diet of geldings. In contrast, Medina et al. (2002) reported decreased cecal acetate concentration when horses were switched from a high-fiber to a high-starch diet. However, they found no change in acetate concentration in samples taken from the colon. The difference observed among studies could be due to differences in the diet or fecal versus cecal VFA concentrations. In agreement with Medina et al. (2002) and Hussein et al. (2004), an abrupt increase in starch elevated fecal propionate concentration in the present study. The observed increase in acetate and temporary increase in propionate fecal concentration with added concentrates agrees with the increase in ruminal total VFA concentration that occurred when concentrates are added to diets of ruminants (Wallace, 1994). Fecal acetate concentration was not affected by the direct-fed LAB in our study, which is in contrast to a decrease in fecal acetate observed in Thoroughbred foals during weaning when administering a direct-fed LAB (Swanson et al., 2003).

The fecal A:P in the present study ranged from 10.9 to 12.4 and was very high compared with previous studies in horses (Medina et al., 2002; Hussein et al., 2004) and ruminants (Bergman, 1990). A preliminary study in our laboratory also showed high fecal A:P molar ratios ranging from 17 to 20 in horses consuming timothy or reed canarygrass hay (A. O. Burk, University of Maryland, College Park, personal communication). The high molar ratios in the current study could perhaps be attributed to differences between acetate and propionate losses through volatilization postdefecation (Merritt and Smith, 1980). Alternatively, a high A:P molar ratio could perhaps be due to the formation of additional acetate through reductive acetogenesis, as



Figures 3. Effect of time (wk) on number of viable colony-forming units (log_{10}) of a) lactobacilli, b) enterococci, and c) bifidobacteria in separate batches of animal feed stored for 12 wk from June to September. Average viable lactobacilli counts: treatment, $P = 0.15$; time, $P = 0.96$ (P-values not shown in panel a). The P-values for the main effects of treatment and time on average viable enterococci and bifidobacteria are not shown due to significant treatment × time interactions. CON = no inclusion of lactic acid bacteria; LAC1 = 1.50 kg/metric ton of single strain *Lactobacillus acidophilus*; LAC4 = containing 1.50 kg/metric ton of a mixture of *L. acidophilus, Lactobacillus casei, Bifidobacterium bifidum,* and *Enterococcus faecium* (LAC4; PrimaLac 454 Feed grade; Star-Labs, Clarksdale, MO). Regression lines were corrected for effects of ambient temperature, which were recorded during the study and used as a covariate in the model. The P-values given in the legend represent the significance of slope for the regression line as different from zero.

Table 6. Bacteria species identification[1] of inoculated concentrates[2]

Diet	Bacteria identification	DuPont no.[3]
CON	*Bacillus licheniformis*	13235, 16694
	Bacillus thuringiensis	16676
	Bacillus cereus	6003
	Leuconostoc mesenteroides	5407, 5408
	Paenibacillus polymyxa	11066
LAC1	*Lactobacillus acidophilus*	18161, 13157
	B. cereus	6082
LAC4	*L. acidophilus*	18161, 13157
	Lactobacillus casei	13084
	Enterococcus faecium	14013
	B. cereus	14747

[1]Analysis of ribosomal DNA was performed by isolating and anaerobically growing bacteria on agar at a commercial laboratory (Star-Labs Forage Research Inc., Clearwater, FL) using procedures outlined previously (SL-01; Swyers, 2007). A random selection of bacterial colonies was selected and then automatically identified (RiboPrinter, DuPont Qualicon, Wilmington, DE).

[2]CON = no inclusion of direct-fed lactic acid bacteria; LAC1 = single strain, *L. acidophilus* (custom preparation provided by Star-Labs, Clarksdale, MO); LAC4 = mixture of *L. acidophilus, L. casei, Bifidobacterium bifidum,* and *E. faecium* (PrimaLac 454 Feed grade; Star-Labs).

[3]DuPont identification numbers are automatically assigned by the RiboPrinter and are reported at 85% confidence.

seen in the hindguts of termites (Breznak, 1994) and humans (Wolin and Miller, 1994). Although fecal VFA concentrations may not represent actual hindgut VFA concentrations, they can reflect the difference between production and absorption (Hussein et al., 2004; Berg et al., 2005).

DFM Viability

As indicated by our enumeration data, the added *Lactobacillus* spp. remained viable, with colony-forming units unchanged over the 12-wk period. *Enterococcus* spp. and *Bifidobacterium* spp. remained viable in the feeds, although, over time, there was an increase in enterococci and a decrease in bifidobacteria in the LAC4 concentrate. In a previous study conducted with commercial pharmaceutical products intended for humans, it was reported that preparations with a single species showed better survivability than those composed of multiple strains (Canganella et al., 1997). The authors suggested inhibitory effects among strains within the multiple-strain DFM preparations. Inhibitory interactions in LAC4 may explain differences between the viability of enterococci and bifidobacteria colony-forming units over time. Bifidobacteria in LAC4 were no longer detectable through ribosomal DNA identification after the experiment.

The CON concentrate was shown to contain naturally occurring bacteria but no contamination with DFM-added organisms. These naturally occurring nonlactobacilli bacteria were able to grow anaerobically on MRS agar, rendering unreliable lactobacilli counting data. This represents a quality control problem, because the methods available for enumerating lactobacilli may not be specific enough.

There have not been DFM dose titration studies with equine. From human dosages, Weese (2001) extrapolated that an average horse (approximately 450 kg) would likely need to ingest at least 10^9 to 10^{11} cfu of a DFM organism/(50 kg of BW·d) for it to be able to colonize in the intestine. The DFM used in the current study were guaranteed by the manufacturer to contain a minimum of 10^8 cfu/g at the inclusion rate recommended and used. In a companion study, where LAC4 was added to feed while mixing, it was shown that lactobacilli remained viable and incurred minimal loss during feed processing (Swyers, 2007). The dosage levels chosen for the current study may not have been appropriate for ameliorating hindgut acidosis in horses and could have affected the results obtained.

Other issues like viable bacterial species selected or survivability of the DFM during gut transit may have resulted in the DFM not having a greater effect on hindgut acidosis. Also, the increase in dietary starch may have been insufficient for the DFM to elicit benefits on the hindgut environment. Although the intent of the current study was not to exceed the digestive capacity of starch in the small intestine, a more dramatic effect of DFM may have been detected if starch intake was at least 3.5 g of starch/(kg of BW·meal) as suggested by Potter et al. (1992).

The results of this study may lend support for the use of greater starch content in the diet of horses to improve digestibility but did not demonstrate that supplementation with direct-fed LAB substantially reduces the risk of acidosis in horses whose dietary starch content is abruptly increased. Although the DFM used in this study showed potential to increase the digestibility of fat and certain minerals in the diet, additional research is needed that more clearly identifies the species and dose of DFM needed to effectively reduce digestive disturbances caused by feeding high-starch concentrates to horses.

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