Received: 19 March 2020 | Accepted: 14 December 2020

DOI: 10.1111/jpn.13508

ORIGINAL ARTICLE

Journal of Animal Physiology and Animal Nutrition WILEY

Effects of a nutraceutical supplement on gastrointestinal health in racing standardbreds

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Funding information
Funding provided by G's Organic Solutions Inc. (Duncan BC, Canada) (Grant number 054083).

Abstract

Background: Feed additives that accelerate gastrointestinal transit time may help support normal gastrointestinal function in horses at risk for impaction colic. Previous research has demonstrated significant stimulatory effect of a hemp-based nutraceutical product (Gs Formula; GF) on contractility of gastric smooth muscle and gastrin production *in vitro*.

Objectives: To quantify effects of GF on indicators of GIT transit time and tight junction proteins.

Study Design: Randomized placebo-controlled cross-over study.

Methods: Eight Standardbreds were administered 200 plastic beads by nasogastric tube before (baseline; BL) and after receiving a diet containing GF (CON: 0 g/day, LO: 160 g/day or HI: 480 g/day) for 28 days. Total manure collection occurred every 2 hours for 72 hours after bead administration. Outcome measures included GIT transit time, faecal dry matter (DM), water intake, and complete biochemistry and haematology screens.

Results: There was no effect of GF on GIT transit time. Faecal output was significantly lower in LO and HI horses than CON horses after 28 days on the supplement. HI horses have significantly lower rouleaux formation and lower faecal DM on Day 28 compared with BL. GF also produced changes in electrolytes associated with pH balance, which may indicate a role for GF as an alkalinizing compound in exercising horses. Clinical pathology results support the safety of GF up to 480 g/day for 28 days with no adverse effects being observed in haematology or biochemistry results.

Main Limitations: Future studies on GF should focus on evaluating effect of GF on gastrointestinal transit in horses with naturally or experimentally delayed gastrointestinal motility, and its effect on exercise performance and onset of fatigue.

Conclusions: GF may help support normal gastrointestinal function in horses at risk for impaction colic by reducing faecal DM and rouleaux formation.

1 | INTRODUCTION

Acute abdominal pain (ie 'colic') is among the most common reasons for emergency veterinary treatment in horses and is a premier cause of pre-mature equine death (Curtis et al., 2019). It is generally accepted that stabled horses (Cohen et al., ,,2000, 2006; Hillyer et al., 2002; Hudson et al., 2001; Scantlebury et al., 2015) experiencing periods of daily fasting (Gonçalves et al., 2002) and exercising at high intensity (Cohen et al., 1999; Curtis et al., 2019) have an elevated risk for development of colic compared with horses in pasture-living conditions with unrestricted access to long-stem fibre and social contact with conspecifics. While this information provides guidance

on 'ideal' management practices, these are not always possible to implement in horses participating in high-performance athletic endeavours and/or living in environments in which pasture turnout is impossible. Therefore, there is a need to identify interventions during occasions of increased colic risk for stabled horses exercising at high intensity.

Pro-kinetic compounds are frequently administered to horses as treatment for recurrent colic (Blake et al., 2012; Guschlbauer et al., 2011) . These drugs accelerate gastrointestinal transit and reduce collateral reperfusion injury to the gastrointestinal tract. In humans, dietary pro-kinetics based on insoluble and soluble fibre are usually sufficient to resolve recurrent constipation, and drug interventions are typically not required (Kuczynska et al., 2017). But horses differ in this regard due in part to the fact that their diets are fibre-based, so a meaningful increase in dietary fibre can be difficult to achieve. Thus, feed supplements are an attractive option to prevent intermittent gastrointestinal sluggishness, particularly in horses with elevated colic risk.

GS Formula (GF; G's Organic Solutions, Duncan BC Canada; Table 1) contains hemp, carrot, cabbage and β-glucans from wheat flour. Recent reports of increased contractility of gastric smooth muscle when exposed to an extract of GF *in vitro* (MacNicol et al., 2020), may have been due to pro-kinetic and gastroprotective properties of hemp and β-glucans. CBD from hemp has effectively alleviated consequences of experimentally induced colon motility disorders in rats (Wei et al., 2019), and a recent review that captures almost 10,000 people reports that cannabis use significantly reduces risk of constipation (Adejumo et al., 2019). Similarly, β-glucans protect the epithelial mechanical barrier in the GIT, and relieve symptoms of constipation, in part by regulating expression of neurotransmitters involved in GIT motility (Chen et al., 2019; Ganda Mall et al., 2018). Improved GIT barrier function has also been reported in swine fed β-glucans (Luo et al., 2019).

TABLE 1 Composition of GF

Analysis	
Moisture (%)	12.56
Dry matter (%)	87.44
Protein (crude; %)	13.5
Fat (crude; %)	4.40
Fibre (acid detergent; %)	14.8
Ash (%)	3.22
Total digestible nutrients (%)	73.6
Net energy (maint.; Mcal/lb)	0.80
Digestible energy (Mcal/lb)	1.47
Sulphur (total; %)	0.24
Phosphorus (total; %)	0.47
Potassium (total; %)	0.98
Magnesium (total; %)	0.19
Calcium (total; %)	0.20
Sodium (total; %)	0.18
Iron (total; ppm)	100
Manganese (total; ppm)	56.9
Copper (total; ppm)	8.9
Zinc (total; ppm)	40.9
Glucose (% sugar)	2.9

The purpose of the current study was to determine the effect of GF on GIT motility and barrier function in a group of horses at risk for development of colic due to their management. It was hypothesized that inclusion of GF into the diet accelerates gastrointestinal transit time, and thus reduces colic risk in stabled racehorses.

2 | MATERIALS AND METHODS

2.1 | Experimental approach

The owners of the horses in this study signed an Informed Consent. Eight (8) horses (Table 2) in active race training at a Standardbred training facility in Campbellville Ontario, Canada were randomized into one of the three treatment groups (CON, LO and HI) in a crossover manner. The experiment was run in three 28-day rounds separated by 14-day wash-out periods. Three horses completed all three phases and participated in all three treatment groups. Five horses in round three were replaced with new horses due to race schedule conflicts. Two horses (one CON, one HI) did not complete the final round due to colic-like clinical signs not related to treatments; their data were not included in the analysis. Following intubation and collection of BL samples (see below), horses were assigned to their treatment groups such that there were 2 or 3 horses per treatment per round. CON horses received their normal diet with no additional supplement; LO and HI horses received GS Formula (160 and 480 g respectively) in 100 g bran mash and 500 g of Evolution Juvenile. Supplementation occurred for 28 days after which time catheterization, blood collection and manure collections were repeated as described for BL above.

Horses were housed individually in 3.0 x 3.0 m box stalls on chopped shavings with no scheduled turnout. They were trained on a half-mile (805 m) crushed stone track 6 days per week and were hand-walked on the 7th day. Water was provided *ad libitum*. All horses received a diet comprised of 6 kg Evolution Juvenile (Agribrands Purina; Tables 3 and 4) split into 2 feedings at 6 am and 4 pm, and 6 kg alfalfa hay cubes soaked in approximately 8 L of water and split into 2 feedings at 11 am and 9 pm.

On Days 0 (baseline; BL) and 28 of each treatment period, jugular venous blood was aspirated into EDTA- or silicone-coated vacutainer for analysis of complete blood count (CBC), and equine complete serum profile (ESP) respectively (see Blood Outcome Measures below for details). On BL (prior to feeding of treatments) and Day 28, a flexible tube was inserted directly into the stomach via the left or right nostril and exactly 200 non-digestible plastic beads (Perler Fuse Beads; 3 x 5 mm) were deposited into the stomach as

TABLE 2 Horse demographics

Horse ID	Phase 1 Treatment	Phase 2 Treatment	Phase 3 Treatment	Initial, Final Mass (kg)	Gender	Age, y
CD	HI	CON	LO	448, 473	F	1.93
ES	LO	HI	–	499, 473	M	2.06
TK	LO	HI	CON	526, 526	M	2.14
A	CON	LO	HI	424, 424	M	1.97
CT	CON	LO	–	499, 503	F	2.01
FVS	HI	LO	–	456, 448	F	2.11
MC	LO	HI	–	424, 448	F	1.87
IB	HI	CON	–	424, 401	F	1.95
EN	–	–	HI[a]	473, 473	M	1.97
SS	–	–	CON	473, 448	M	1.99
ZS	–	–	CON[a]	448, 448	M	1.87
CA	–	–	LO	473, 473	M	2.85
J	–	–	CON	448, 473	M	1.99

[a]Removed during phase 3 day 28 due to colic-like symptoms.

TABLE 3 Ingredients of Evolution Juvenile

Nutrient	Ingredients
Protein / amino acids	DL-Methionine, Soya Bean Meal, L-Lysine, L-Threonine
Fibre	Beet Pulp, Dehydrated Alfalfa Meal
Starch	Extruded corn, ground wheat, ground corn
Fat	Flaxseed, Soya oil
Pre-biotic	Yeast
Pro-biotic	Mixture of live bacteria
Vitamins	Choline Chloride, Vitamin A, Vitamin D3, Vitamin E, Vitamin K, Riboflavin, Niacin, Thiamine, Vitamin B12, Biotin, Pantothenic Acid, Vitamin B6, Folic Acid, Ascorbic Acid
Minerals	Salt (Sodium Chloride), Calcium Carbonate, Mono Dicalcium Phosphate, Calcium Iodate, Copper Sulphate, Ferrous Sulphate, Manganese Oxide, Zinc Oxide, Organic Copper, Organic Manganese, Organic Zinc, Organic Selenium, Ferric Oxide, Cobalt Carbonate
Multi-Attributes	Wheat Shorts
Flavour/Binder	Molasses

TABLE 4 Nutritional analysis of Evolution Juvenile

Protein (%)	15.00
Fat (%)	7.00
Fibre (%)	15.00
Calcium (%)	1.25
Phosphorus (%)	0.65
Sodium (%)	0.45
Vitamin A (IU/kg)	11300
Vitamin D3 (IU/kg)	2330
Vitamin E (IU/kg)	275
Selenium (ppm)	0.45

a solid-phase transit time marker with approximately 4L of warm water (Elfenbein et al., 2011).

2.2 | Handling of samples

Blood samples were cooled on ice immediately following collection. CBC and ESP samples were delivered to a commercial laboratory [Animal Health Laboratory (AHL), University of Guelph, Guelph ON] within 2 hours of collection.

Freshly voided faecal grab samples were immediately sealed into an evacuated freezer bag and frozen at −20°C until dry matter analysis.

2.3 | Outcome measures

2.3.1 | Solid phase-transit time

Beginning 4 hours after administration of beads, manure was completely removed from each stall and weighed every 2 hours for a total of 72 hours, or until 180 of the 200 beads were recovered in the manure (Elfenbein et al., 2011). After weighing, warm water was applied to the sample to create a slurry; beads appearing in the manure floated to the top of the water and were counted. After weighing of manure on days 7, 14, 21 and 28 a grab sample of approximately 40 g of manure was placed into an evacuated freezer bag and frozen at −20°C until analysed for dry matter (DM) content.

Transit time measures included: bead number (beads/2 h collection and total for entire collection period), water consumption (g/2 h collection and total for entire collection period), faecal weight (g/2 h

collection and total for entire collection period), beads/kg faeces (average for entire collection period and beads/kg/2 h collection), time to appearance of first bead ('Transit Time'; TT), time to appearance of 25% of beads (T25), time to appearance of 75% of beads (T75) and time to completion of sampling (TCOM) (Elfenbein et al., 2011).

2.3.2 | Faecal dry matter

Faecal samples were analysed for dry matter content using a drying oven (Acosta & Kothmann, 1978). Briefly, thawed samples were thoroughly mixed and homogenized prior to subsampling. A subsample of approximately 20 g was weighed (wet weight) before placing into a drying oven at 70°C. Weight was recorded every 24 hours until such time as no further change in weight was observed (total drying time 72 h) (dry weight). Dry weight was obtained after cooling on the laboratory benchtop for approximately 1 h at room temperature without dessicant and was compared with wet weight (%).

2.3.3 | Clinical pathology

Blood samples were analysed for complete blood count (CBC) and equine complete serum profile (ESP) at AHL. These samples were conducted for the purpose of monitoring systemic effects of treatments.

2.4 | Data analysis

Data analysis was conducted using SigmaPlot (Version 12). Sample size was determined using a power of analysis test, using T75 as the primary outcome measure, with a standard deviation of 19.1 and minimum detectable difference of 20 min (Elfenbein, Robertson, Corser, Urion,& Sanchez, 2011).

Data are presented as mean ±SEM unless otherwise indicated. Normality of data was determined using the Shapiro–Wilk test. One-way RM ANOVA was used to determine effects of time within individual treatments. Two-way RM ANOVA was used to identify interactions between treatment and time. When a significant F-ratio was obtained, the Holm Sidak post-hoc test was used to identify significantly different means. Significance was accepted at $p < 0.05$.

3 | RESULTS

3.1 | Solid-phase transit time

Excretion curves for CON, LO and HI horses are presented in Figure 1. There were no significant differences in overall rates of excretion of solid-phase markers between treatment groups.

Changes in TT, T25, T50, T75 and TCOM are provided in Table 5. There were no significant changes within treatment groups between BL and Day 28, though there was a trend to accelerated TT in the HI group between BL and Day 28 ($p = 0.1$). There were no significant differences between groups at either time point.

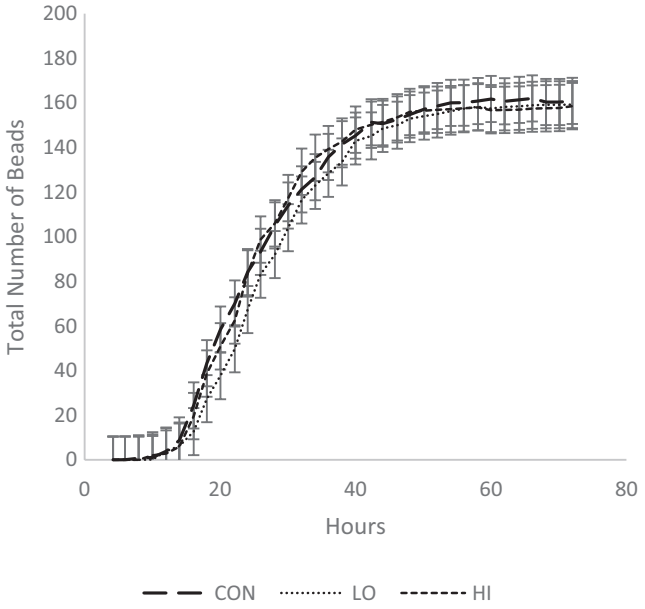

FIGURE 1 Solid-phase markers recovered from faeces every 2 hours of horses receiving a control diet (CON) or a diet containing 160 (LO) or 480 (HI) g/day of Gs Formula for 28 days

3.1.1 | Faecal output (wet weight)

During the 72 h collection periods, CON horses showed a trend to increased faecal bulk between BL (1.20 ± 0.05 kg) and Day 28 (1.36 ± 0.08 kg) ($p = 0.07$). Conversely, the trend in LO horses was to reduced faecal output between BL (1.23 ± 0.07 kg) and Day 28 (1.08 ± 0.05 kg) ($p = 0.1$). There was a slight but non-significant decline in faecal output in HI horses between BL (1.20 ± 0.06 kg) and Day 28 (1.10 ± 0.05 kg) ($p = 0.3$). On Day 28 faecal output was significantly higher in CON horses than in both LO and HI horses ($p = 0.005$ and 0.007 respectively) (Figure 2).

3.1.2 | Faecal dry matter

There was a significant decrease in faecal dry matter in HI horses between BL (33.5 ± 1.6%) and Day 28 (29.9 ± 1.1%) ($p = 0.02$) (Figure 3). There were no significant changes in faecal dry matter in either CON or LO horses between BL and Day 28, and there were no significant differences between groups at either time point.

3.1.3 | Water consumption

Water consumption was not different between groups at any time point (Figure 4). Water consumption in the HI horses tended

TABLE 5 Time (mean hours ±SEM) required after provision of solid-phase markers for appearance of first marker (TT), 25% of markers (T25), 50% of markers (T50), 75% of markers and time to appearance of last marker (TCOM) for control horses (CON) and horses receiving GS Formula at 160 (LO) or 480 (HI) g/day at baseline (BL) and Day 28

	TT		T25		T50		T75		TCOM	
Sample Day	***BL***	***28***	***BL***	***28***	***BL***	***28***	***BL***	***28***	***BL***	***28***
CON	12.0 ± 1.1	13.1 ± 1.5	20.2 ± 1.4	23.7 ± 2.7	25.8 ± 2.0	29.1 ± 2.8	36.0 ± 2.2	38.3 ± 4.5	69.5 ± 3.2	66.0 ± 3.4
LO	15.4 ± 1.0	14.7 ± 1.5	22.5 ± 1.8	20.6 ± 1.4	29.3 ± 1.2	31.3 ± 2.4	38.3 ± 3.6	42.9 ± 3.9	63.8 ± 3.2	68.5 ± 3.2
HI	**14.5 ± 1.2**	**13.4 ± 1.2***	20.5 ± 1.2	20.9 ± 1.8	27.0 ± 3.0	28.0 ± 2.2	36.9 ± 4.1	38.7 ± 5.4	69.8 ± 3.2	72.0 ± 3.4
P-value between treatments	0.11	0.74	0.62	0.58	0.64	0.41	0.87	0.73	0.37	0.46

*p = 0.1 within treatment

to increase between BL (41.7 ± 5.7 L) and Day 28 (46.9 ± 7.5 L) (p = 0.06). (Figure 4).

3.2 | Clinical pathology

3.2.1 | Complete Blood Count

Monocytes were significantly increased on Day 28 (0.403 ± 0.03 x 10^9/L) compared with BL (0.279 ± 0.03), but there were no significant differences between treatment groups (Table 6). There was also a significant decline in **Rouleaux** score between BL (1.54 ± 0.11) and Day 28 (1.10 ± 0.12), but again there were no differences between treatment groups. HI horses had a significant decline in rouleaux score between BL (1.63 ± 0.20) and Day 28 (0.86 ± 0.20) (p = 0.008).

3.2.2 | Biochemistry Profile

Sodium (Na) was significantly higher overall in HI horses (139.11 ± 0.41 mmol/L) compared with CON horses (137.26 mmol/L) (p = 0.01), but there was no effect of treatment at individual time points (Table 7). Serum **carbon dioxide** (CO_2) was higher overall in HI horses (28.69 ± 0.44 mmol/L) compared with CON (26.37 ± 0.47 mmol/L) horses (p = 0.03). At Day 28, HI horses (28.38 ± 0.64 mmol/L) had significantly higher CO_2 than CON (25.86 ± 0.69 mmol/L) horses (p = 0.03). For all horses combined, there was a significant increase in **glucose** between BL (5.00 ± 0.19 mmol/L) and Day 28 (5.82 ± 0.20 mmol/L) (p = 0.005), but there were no differences between treatment groups. Overall, HI horses had a higher **osmolality** (277.89 ± 0.73 mmol/L) than CON horses (274.41 ± 0.78 mmol/L) (p = 0.007), but there were no significant differences between treatments at any individual time point.

It is noteworthy that, while there were no significant differences between treatments or time point for creatine kinase (CK), HI horses on Day 28 had a mean CK (573.50 ± 178.58 U/L) which exceeded the normal reference interval (108–430 U/L). Similarly, serum amyloid A (SAA) exceeded the normal reference interval (0–20 mg/L) in all treatment groups at BL; all groups showed a significant decline in amyloid A but values remained above the normal reference limit in LO horses (31.07 ± 100.04 mg/L) at Day 28.

4 | DISCUSSION

The current study was conducted in order to detect effects of GF on indicators of gastrointestinal transit time, and systemic safety. The main findings are that GF does not influence overall solid-phase gastrointestinal transit time in actively racing Standardbred horses, though there was a trend to accelerated solid-phase TT in horses receiving the HI dose. Faecal output was significantly lower in LO and HI horses during the Day 28 collection period than CON horses. HI horses have significantly decreased rouleaux formation on Day 28

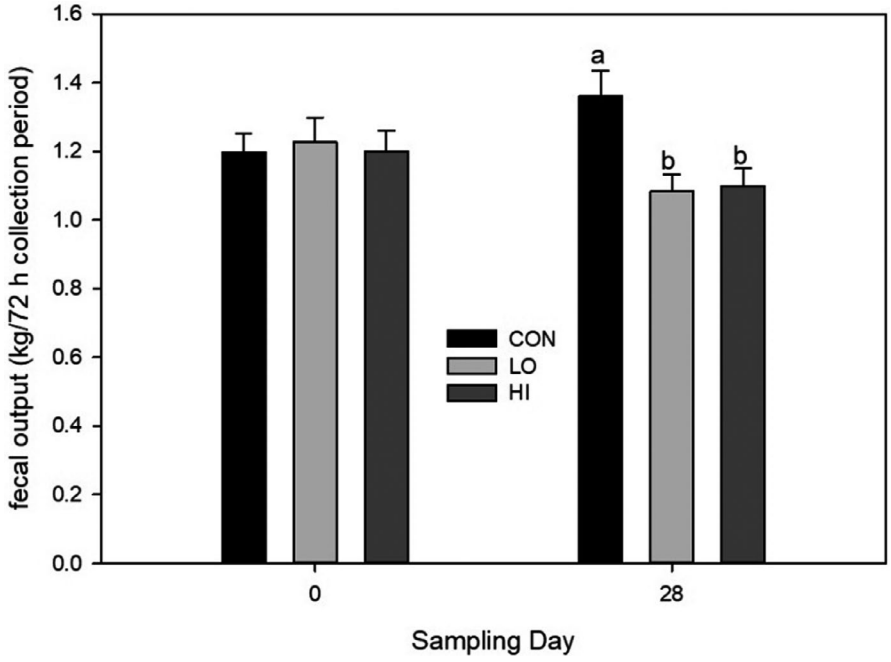

FIGURE 2 Total amount of faeces (kg) collected during the 72 hr collection periods immediately following provision of a solid-phase transit time marker prior to (0) and 28 days (28) following supplementation with GS Formula (CON: 0 g/day; LO: 160 g/day; HI: 480 g/day). Different letters denote significant differences between treatments ($p < 0.05$)

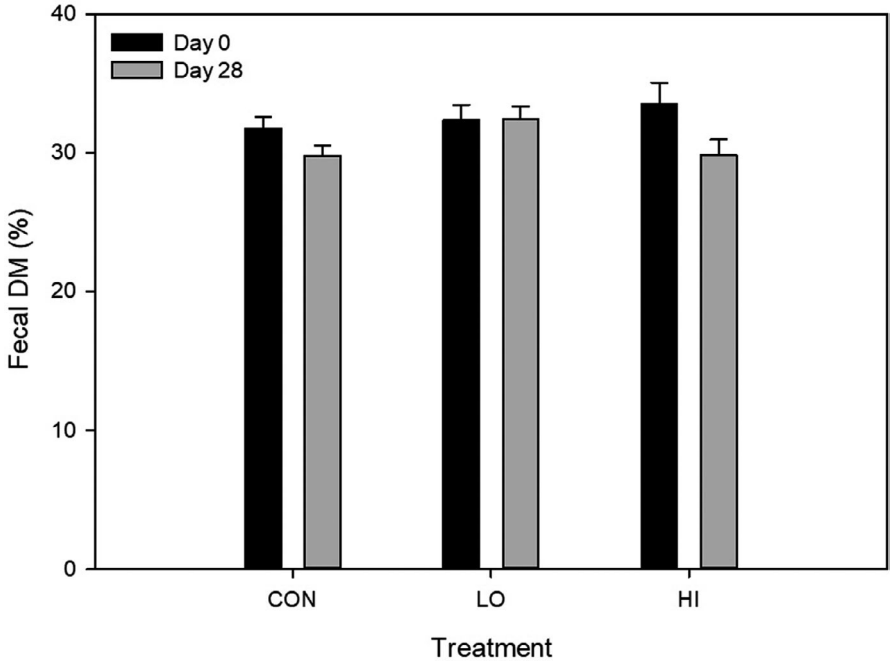

FIGURE 3 Faecal dry matter (%) collected during the 72 h collection periods immediately following provision of a solid-phase transit time marker prior to (Day 0) and 28 days after (Day 28) supplementation with GS Formula (CON: 0 g/day; LO: 160 g/day; HI: 480 g/day). Different letters denote significant differences within a treatment between Day 0 and 28 ($p < 0.05$)

compared with BL. HI horses also had significantly lower faecal dry matter and a trend to increased water consumption on Day 28 compared with BL. Clinical pathology results support the safety of GF up to 480 g/day for 28 days with no adverse effects being observed in haematology or biochemistry results.

The acceleration of gastrointestinal transit time that was predicted to be associated with dietary provision of GF was not observed in the current study. This prediction was based on the sensitizing effect of GF on contractility of gastric smooth muscle *in vitro* (MacNicol et al., 2020). Mean TT, T25, T50 and T75 determined in the current study are accelerated by approximately 21% compared with those reported by others using sedentary horses (Elfenbein et al., 2011), likely due (at least in part) to their high-intensity exercise training (Pagan et al., 1998; Van Weyenberg et al., 2006) and short fibre length of their hay-cube based diet (Van Weyenberg et al., 2006). Furthermore, horses exercise training at high intensity have increased serum levels of gastrin, a hormone that is known to accelerate contractility of the antral stomach (Furr et al., 1994; Sandin et al., 1998). As augmentation of this hormone may have played a role in GF-associated increase in contractility of gastric smooth muscle (MacNicol et al., 2020), it is possible that GF-independent factors already contributing to accelerated transit time in these horses masked any net effect GF may have had on oro-faecal transit time. Future studies to understand the net effect of GF on gastrointestinal transit time should use horses with experimentally delayed transit time with

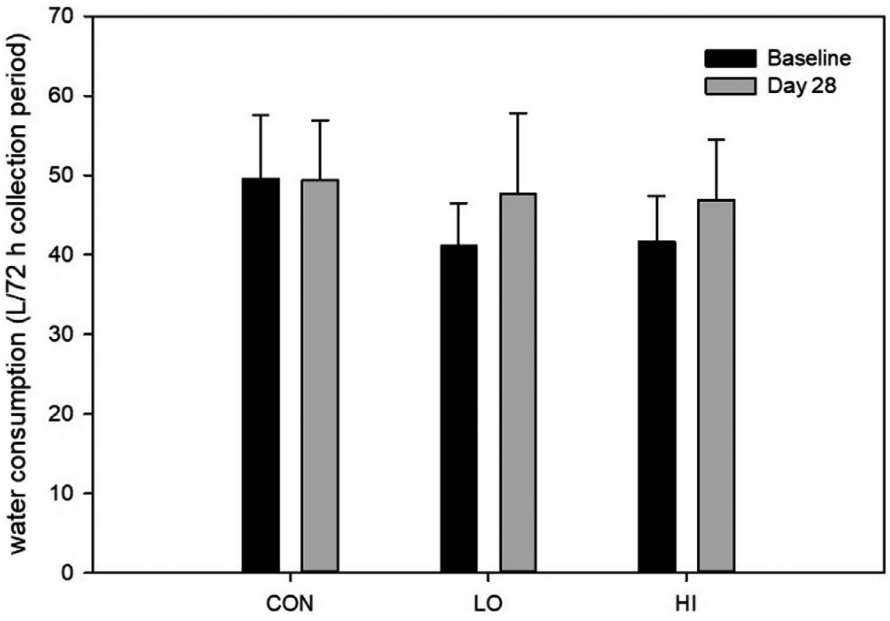

FIGURE 4 Total amount of water (L) consumed during the 72 hr collection periods immediately following provision of a solid-phase transit time marker prior to (baseline) and 28 days (Day 28) following supplementation with GS Formula (CON: 0 g/day; LO: 160 g/day; HI: 480 g/day). A trend to increased water consumption in HI horses was observed between baseline and Day 28 ($p = 0.06$)

TABLE 6 Clinical pathology (Complete Blood Count) mean ± SEM

	CON		LO		HI		
Haematology variable	**BL**	**Day 28**	**BL**	**Day 28**	**BL**	**Day 28**	***P***
WBC ($x10^9$/L)	8.45 ± 0.46	8.73 ± 0.49	8.38 ± 0.46	8.87 ± 0.49	8.36 ± 0.46	9.03 ± 0.49	0.92
RBC ($x10^{12}$/L)	9.66 ± 0.28	9.41 ± 0.28	9.03 ± 0.26	8.86 ± 0.28	9.21 ± 0.26	9.21 ± 0.28	0.90
Hb (g/L)	143.86 ± 3.76	140.00 ± 3.76	134.50 ± 3.52	134.00 ± 3.76	135.00 ± 3.52	135.29 ± 3.76	0.84
HCT (L/L)	0.40 ± 0.01	0.40 ± 0.01	0.38 ± 0.01	0.38 ± 0.01	0.38 ± 0.01	0.38 ± 0.01	0.96
MCV (fL)	41.86 ± 0.83	42.43 ± 0.83	42.50 ± 0.78	43.14 ± 0.83	41.38 ± 0.78	41.86 ± 0.83	0.99
MCH (pg)	15.14 ± 0.32	15.00 ± 0.32	15.00 ± 0.30	15.29 ± 0.32	14.50 ± 0.30	14.71 ± 0.32	0.77
MCHC (g/L)	357.71 ± 2.50	352.00 ± 2.50	354.75 ± 2.34	351.57 ± 2.50	355.25 ± 2.34	352.14 ± 2.50	0.84
RDW (%)	19.54 ± 0.32	19.24 ± 0.32	18.86 ± 0.30	18.76 ± 0.32	19.06 ± 0.30	18.96 ± 0.32	0.94
Platelets ($x10^9$/L)	98.13 ± 7.73	96.86 ± 8.26	110.50 ± 7.73	115.86 ± 8.26	111.25 ± 7.73	104.57 ± 8.26	0.75
MPV (fL)	8.37 ± 0.78	6.73 ± 0.78	6.91 ± 0.73	6.49 ± 0.78	7.00 ± 0.73	6.86 ± 0.78	0.59
T. S. Protein (g/L)	65.88 ± 1.37	64.00 ± 1.46	63.88 ± 1.37	62.57 ± 1.46	64.38 ± 1.37	64.71 ± 1.46	0.72
Seg Neut ($x10^9$/L)	4.23 ± 0.50	4.73 ± 0.54	4.43 ± 0.50	4.67 ± 0.54	4.48 ± 0.50	4.87 ± 0.54	0.97
Lymphocytes ($x10^9$/L)	3.61 ± 0.31	3.36 ± 0.33	3.43 ± 0.31	3.60 ± 0.33	3.40 ± 0.31	3.60 ± 0.33	0.74
Monocytes ($x10^9$/L)	0.276 ± 0.06	0.44 ± 0.06	0.31 ± 0.06	0.36 ± 0.06	0.25 ± 0.06	0.41 ± 0.06	0.53
Eosinophils ($x10^9$/L)	0.28 ± 0.07	0.18 ± 0.08	0.18 ± 0.07	0.24 ± 0.08	0.19 ± 0.07	0.12 ± 0.08	0.53
Basophils ($x10^9$/L)	0.07 ± 0.02	0.04 ± 0.02	0.03 ± 0.02	0.01 ± 0.02	0.01 ± 0.02	0.03 ± 0.02	0.55
Rouleaux (score)	1.50 ± 0.19	1.14 ± 0.20	1.50 ± 0.19	1.29 ± 0.20	1.63 ± 0.19	0.86 ± 0.20*	0.35

Abbreviations: Hb, haemoglobin; HCT, haematocrit; Lymph, lymphocyte count.; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; MCV, mean corpuscular volume; MPV, mean plasma volume; RBC, red blood cell count; RDW, red cell distribution width; Seg Neut, segmented neutrophil count; T. S. Protein, total serum protein; WBC, white blood cell count.

*Significant change from BL within treatment group.

drugs such as ketamine to determine GF effects on accelerating transit time (Elfenbein et al., 2011).

The reason for the significantly higher faecal weight of CON horses at Day 28 compared with horses receiving either dose of GF is not known. It was not related to differences in feed intake, as all horses received identical feed rations. Nor was it related to a difference in Tmax between groups, since CON horses actually had a slightly (non-significantly) lower Tmax than either of LO or HI groups, meaning the duration of time during which faeces were collected was slighter lower for CON horses. A negative association is reported between digestibility of feed and faecal weight (Brambillasca et al., 2010), raising the possibility that GF may have improved digestibility of the diet in the LO and HI groups. This possibility should be explored in the future studies.

TABLE 7 Clinical pathology (Biochemistry Profile) mean ± SEM

	CON		LO		HI		
Biochemistry variable	**BL**	**Day 28**	**BL**	**Day 28**	**BL**	**Day 28**	***P***
Calcium (mmol/L)	2.98 ± 0.04	2.91 ± 0.04	2.98 ± 0.04	2.96 ± 0.04	3.00 ± 0.04	3.01 ± 0.04	0.62
Phosphorus (mmol/L)	1.36 ± 0.08	1.26 ± 0.09	1.41 ± 0.09	1.24 ± 0.09	1.49 ± 0.08	1.36 ± 0.08	0.93
Magnesium (mmol/L)	0.76 ± 0.03	0.76 ± 0.03	0.73 ± 0.03	0.74 ± 0.03	0.73 ± 0.02	0.71 ± 0.03	0.80
Sodium (mmol/L)	137.38 ± 0.60	137.14 ± 0.64	137.71 ± 0.64	138.71 ± 0.64	139.22 ± 0.56	139.00 ± 0.60	0.53
Potassium (mmol/L)	4.34 ± 0.20	4.29 ± 0.22	4.21 ± 0.22	4.17 ± 0.22	3.93 ± 0.19	3.91 ± 0.20	0.99
Chloride (mmol/L)	97.50 ± 0.50	98.43 ± 0.53	97.43 ± 0.53	98.71 ± 0.53*	97.78 ± 0.47	98.00 ± 0.50	0.56
Carbon dioxide (mmol/L)	26.88 ± 0.64	25.86 ± 0.69[b]	28.00 ± 0.69	27.43 ± 0.69*	29.00 ± 0.60	28.39 ± 0.64[a]	**0.03**
Anion gap (mmol/L)	17.63 ± 0.63	17.29 ± 0.67	16.43 ± 0.67	16.57 ± 0.67	16.44 ± 0.59	16.38 ± 0.63	0.94
Na:K ratio	32.38 ± 2.90	32.14 ± 3.10	32.71 ± 3.10	33.86 ± 3.10	36.11 ± 2.73	39.50 ± 2.90	0.82
Total protein (g/L)	57.25 ± 1.43	55.71 ± 1.53	56.00 ± 1.53	56.43 ± 1.53	58.11 ± 1.35	58.50 ± 1.43	0.75
Albumin (g/L)	30.38 ± 1.15	29.71 ± 1.23	30.14 ± 1.23	29.86 ± 1.23	32.11 ± 1.10	32.38 ± 1.15	0.92
Globulin (g/L)	26.88 ± 0.75	26.00 ± 0.80	25.86 ± 0.80	26.57 ± 0.80	26.00 ± 0.71	26.13 ± 0.75	0.60
A:G ratio	1.14 ± 0.06	1.14 ± 0.06	1.17 ± 0.06	1.13 ± 0.06	1.24 ± 0.06	1.25 ± 0.06	0.87
Urea (mmol/L)	6.01 ± 0.33	6.03 ± 0.35	6.44 ± 0.35	6.43 ± 0.35	6.38 ± 0.31	6.35 ± 0.33	0.99
Creatinine (umol/L)	98.88 ± 4.13	96.00 ± 4.42	89.14 ± 4.42	94.14 ± 4.42	93.00 ± 3.90	89.38 ± 4.13	0.55
Glucose (mmol/L)	4.66 ± 0.33	5.59 ± 0.31	5.14 ± 0.36	6.00 ± 0.36	5.21 ± 0.31	5.89 ± 0.33	0.93
Cholesterol (mmol/L)	2.30 ± 0.14	2.21 ± 0.15	2.44 ± 0.15	2.29 ± 0.15	2.24 ± 0.13	2.24 ± 0.14	0.88
Total bilirubin (umol/L)	36.00 ± 3.83	27.57 ± 4.10	28.43 ± 4.09	30.14 ± 4.09	28.78 ± 3.61	28.50 ± 3.83	0.41
Conj bilirubin (umol/L)	5.00 ± 0.55	7.00 ± 0.58	6.43 ± 0.58	6.29 ± 0.58	7.00 ± 0.51	7.38 ± 0.55	0.16
Free bilirubin (umol/L)	31.00 ± 3.95	20.57 ± 4.22	22.00 ± 4.22	23.86 ± 4.22	21.78 ± 3.72	21.13 ± 3.95	0.30
Alk phosp (U/L)	178.00 ± 9.15	164.43 ± 9.78	172.43 ± 9.78	176.57 ± 9.78	171.67 ± 8.63	177.38 ± 9.15	0.53
GGT (U/L)	23.25 ± 4.36	18.71 ± 4.66	25.00 ± 4.66	27.29 ± 4.66	22.33 ± 4.11	28.63 ± 4.36	0.47
AST (U/L)	321.63 ± 119.96	489.71 ± 128.24	297.29 ± 128.24	314.86 ± 128.24	361.56 ± 113.10	525.25 ± 119.96	0.79
CK (U/L)	244.25 ± 178.58	307.43 ± 190.91	242.29 ± 190.91	221.00 ± 190.91	423.00 ± 168.36	573.50 ± 178.58[a]	0.90
GLDH (U/L)	6.00 ± 0.92	4.71 ± 0.98	5.00 ± 0.98	5.86 ± 0.98	4.44 ± 0.87	5.50 ± 0.92	0.40
Osmolality (mmol/L)	274.25 ± 1.06	274.57 ± 1.14	275.86 ± 1.14	278.00 ± 1.14*	277.78 ± 1.00	278.00 ± 1.06	0.63
Amyloid A (mg/L)	24.41 ± 93.58[a]	1.51 ± 100.04	358.76 ± 100.04[a]	31.07 ± 100.04[a]	61.18 ± 88.23[a]	0.04 ± 93.58	0.25

Note: Different letters in the same row indicate significantly different means between treatment groups.

[a]Exceeding normal reference interval; Conj bilirubin, conjugated bilirubin; Alk Phos, alkaline phosphatase; GGT, gamma-glutamyl transferase; AST, aspartate aminotransferase; CK, creatine kinase; GLDH, glutamate dehydrogenase.

*Significant change over time within treatment groups.

The significant decline in faecal dry matter observed in HI horses between BL and Day 28 was not associated with any visible faecal abnormalities (i.e. loose or diarrheic manure). Free faecal water was not observed in any horses for the duration of the trial. The decline in faecal dry matter may have resulted from the trend to increased water consumption observed in HI horses between BL and Day 28. It is possible that this result was also at least partially reflective of the increased fibre intake of horses receiving the HI dose of GF. Our faecal dry matter values are slightly higher for all groups at all-time points than others reported for stabled horses with light exercise (mean 27.2 ± 1.9%) (Williams et al., 2015) and stabled horses with undetermined turnout and exercise (20.6 1.6%) (Gerstner & Liesegang, 2018), perhaps owing to the more intense exercise regimen in our study horses, possibly indicating an increased risk of horses in the current study to intestinal impactions. High faecal dry matter values are not desirable, and dehydrated faeces is considered an important risk factor for development of intestinal impactions in horses (Williams et al., 2015). Our finding of a significant decline in faecal dry matter in HI horses may represent a possible protective mechanism of GF for horses at risk for intestinal impactions associated with dehydrated faeces.

'Rouleaux' is a term given to a stacked formation of red blood cells, and is an indicator of blood viscosity. Increased rouleaux in healthy individuals is generally undesirable, as it reduces red cell surface area available for gas exchange (Panyarachun et al., 2009). In horses, elevated rouleaux formation has been positively associated with fatal outcomes in colic, whereas, colic patients with lower rouleaux formation have improved clinical outcomes (Andrews et al., 1990). Rouleaux is markedly more prevalent in horses than in humans, pigs, cows or sheep (Weng et al., 1996); it is increased in response to a variety of factors, including acute exercise bouts in horses (Coyne et al., 1990) and humans (Kilic-Toprak et al., 2018), likely due, at least in part, to increased formation of reactive oxygen species (Panyarachun et al., 2009). Rouleaux tends to decline in response to exercise training in humans (Filar-Mierzwa et al., 2019; Nader et al., 2018; Sandor et al., 2014). The overall decline in rouleaux observed in all horses between BL and Day 28 in the current study may be explained by a possible increase in their level of fitness, independent of diet, though this was not specifically measured. However, there was a significant time-dependent decline in rouleaux in HI horses that was not observed in the other two groups. Since horses in this group did not differ in their training programme from horses in the other groups, nor did they differ in their initial fitness level, this decline may be associated with their supplementation with GF. Reports of the antioxidant effects of the GF ingredients cabbage (Kapusta-Duch et al., 2012; Rokayya et al., 2014; Wiczkowski et al., 2016), hemp (Pellati et al., 2018; Smeriglio et al., 2016) and carrot (Jiang et al., 2015; Xavier & Pérez-Gálvez, 2016) provide support for an antioxidant role of GF in the HI horses, which may have contributed to the decline in rouleaux formation. Future studies to explore this possibility should measure production of reactive oxygen species in actively racing horses receiving a diet containing GF or a known antioxidant supplement such as vitamin E (Mélo et al., 2016), and compare rouleaux formation in these horses.

The higher levels of Na+, CO_2 and osmolality in HI horses are likely interconnected. Elevated plasma Na+without concomitant decreases in other solutes likely contributed to the increased osmolality observed in the HI horses of the current study. Na+is an important component of HCO_3^- transport which functions to maintain pH balance in the muscle and blood. Na+-coupled bicarbonate transporters are active transport mechanisms on cell membranes, which couple acid extrusion with Na+influx, thus maintaining intracellular and plasma pH. High activity of Na+-facilitated transport of H+ would effectively buffer plasma pH during exercise (a physiological event that typically lowers pH), thereby supporting higher plasma CO_2. Increased plasma CO_2 is a physiologically desirable occurrence for animals exercising at high intensity, as this will buffer (to some extent) accumulation of H+, which contributes to onset of fatigue (Raymer et al., 1985). An alkalinizing substance may thus delay onset of fatigue in exercising horses (Waller et al., 2010).

All treatment groups demonstrated high mean serum amyloid A at the start of the trial, resulting from 9 horses (3 CON, 2 LO, and 4 HI). Serum amyloid A refers to a group of proteins that are associated with the acute phase stress response (Sack, 2018). One of the LO horses started with a level more than double that of any other horse (1700.5 mg/L) which, similar to all other affected horses, declined significantly over the course of the trial. But the very high-starting value of this single horse caused the mean of the LO horses to persist above the normal reference interval at the end of the study. Similarly, the mean CK of HI horses which exceeded the normal reference interval arose from a single horse in this group with very high CK levels both at the beginning (1889 U/L) and end (3142 U/L) of the trial. CK is a myogenic enzyme, which is increased following high-intensity exercise, in part due to exercise-induced muscle damage [47]. Like SAA, the results do not appear to be treatment-related, but do present evidence for acute physiological stress of these horses.

In conclusion, GF supplementation to actively racing Standardbred horses did not result in significant changes in gastrointestinal transit time. However, there was a significant decline in rouleux formation, faecal dry matter and faecal weight in GF-fed horses, which may indicate a protective mechanism for horses with colic. GF also produced changes in electrolytes associated with pH balance, which may indicate a role for GF to delay onset of fatigue in exercising horses.

Future studies on GF should focus on evaluating its effect on gastrointestinal transit in horses with naturally or experimentally delayed gastrointestinal motility, and its effect on exercise performance and onset of fatigue.

ACKNOWLEDGEMENTS

The authors thank Amy and Anthony MacDonald for the use of their horses for this trial. The authors also gratefully acknowledge

the invaluable assistance of undergraduate volunteers for collection of samples.

ANIMAL WELFARE STATEMENT

Use of all animals in this study was reviewed and approved by the University of Guelph Animal Care Committee (approval number 4095), under the guidelines of the Canadian Council on Animal Care.

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How to cite this article: Wellard L, Medyk K, Pearson W. Effects of a nutraceutical supplement on gastrointestinal health in racing standardbreds. *J Anim Physiol Anim Nutr.* 2021;00:1–11. https://doi.org/10.1111/jpn.13508