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ORIGINAL ARTICLE

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The influence of a simulated digest of an equine dietary feed additive G's formula on contractile activity of gastric smooth muscle in vitro

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Abstract

G's Formula is a novel equine feed additive formulated to promote optimal GI function. The objective of this study was to determine whether the addition of a simulated digest of the composite feed additive G's Formula (FA) would alter the contractile response of gastric smooth muscle to acetylcholine (Ach). Smooth muscle strips from porcine stomachs were excised and attached to an isometric force transducer. An experiment was run to compare tissue contraction between tissue exposed to FA (FA; n = 8, simulated digest of FA was added to the bath) and control tissue (CO; n = 8, no additions made). Increasing concentrations of Ach were added into the bath such that the concentration increased from 10^{-8}–10^{-3} M. Based on the analysis of these data, a difference between FA and CO was observed. Therefore, another trial was run which included a blank group (BL n = 6) in which the tissue was exposed to the simulated digest without FA. More CO (n = 5) and FA (n = 4) tissue was also run. Force was compared to baseline and between groups. In FA group, mean force for 1-min following all Ach additions was higher than baseline (p < .05) and by 2-min the integral-under-force/time curve (AUC) was higher than baseline from 10^{-7}–10^{-3} M compared to lower concentrations of Ach in both CO (10^{-6} M for both) and BL (10^{-5} M and 10^{-6} M, respectively). By 8-min AUC of all Ach concentrations were higher than baseline in FA compared to an Ach of 10^{-6} M in both CO and BL. A simulated digest of FA appears to sensitize gastric smooth muscle to Ach in vitro. FA may increase GI contractility, and the functional effect of this should be studied further in vivo.

KEYWORDS
contractility, dietary supplement, equine, gastric, in vitro, smooth muscle

1 | INTRODUCTION

Sensitivity of gastrointestinal (GI) tissue to contractile stimuli influences several parameters such as GI transit, nutrient absorption and faecal water content. Smooth muscle, which lines the GI tract, is responsible for the motility and contractile patterns which mix and propel the contents through the various GI compartments. Multiple serious medical complications are related to aberrant GI transit in horses including equine grass sickness (Hudson, Mayhew, & Pearson, 2001) and post-operative ileus (Cohen, Lester, Chris Sanchez, Merritt, & Roussel, 2004). The severity of these conditions highlights the importance of GI motility and proper smooth muscle functioning.

Multiple factors including feed ingredients, particle size (Frape, 2004; Wilfart, Montagne, Simmins, Noblet, & Milgen, 2007) and time of day (Landgraf, Neumann, & Oster, 2017) can act on the GI

tract and impact contractility. The complexity of interacting factors makes understanding and predicting GI motility a challenging task. Long stem fibre, coupled with ample freshwater, is generally believed to be the principal factors necessary to promote optimal GI health in horses, but this is frequently inadequate to prevent potentially life-threatening diseases associated with delayed (Cohen et al., 2004) or accelerated (Kutscha, Sutton, Preston, & Guthrie, 2012) transit time. Multiple pharmaceuticals are available which modulate aberrant motility during emergency situations (Koenig & Cote, 2006), but there is a potential that in-feed supplements may assist in the maintenance of proper GI motility for disease prevention.

G's formula is an equine dietary feed additive (FA) (G's Organic Solutions Inc) formulated to promote optimal GI health in horses. It is composed of a proprietary blend of oat flour, dried cabbage, carrot and hemp meal. G's Formula has Low Risk Veterinary Health Product Certification.

The combination of ingredients is based on their individual gastroprotective properties. Oat flour is a rich source of beta-glucans (Ahmad, Anjum, Zahoor, Nawaz, & Ahmed, 2010). Oat dietary fibre is known to influence gastrointestinal transit. However, the majority of its effects, such as delayed gastric emptying, can be attributed to its physical structure and its influence on the viscosity of gastric contents (Johansen, Bach Knudsen, Sandström, & Skjøth, 1996) (Mälkki & Virtanen, 2001).

Hemp (Smeriglio et al., 2016), cabbage (Cacciola et al., 2016) and carrot (Chandra, Kishore, & Ghosh, 2015) all represent sources of bioflavonoids. In vitro and in vivo experiments have demonstrated spasmolytic effects of several bioflavonoids on intestinal motility (Di Carlo, Mascolo, Izzo, & Capasso, 1999; Escobar-Ramos et al., 2017; Gharzouli & Holzer, 2004; Rao et al., 1997) Amira, Rotondo, and Mulè (2008) (Amira et al., 2008) evaluated the impact of several bioflavonoids on the gastric tone in mouse isolated stomachs. All flavonoids tested demonstrated concentration-dependent relaxation, with the flavonoids apigenin and genistein displaying the highest potencies. It has been postulated that the anti-spasmolytic effects of various flavonoids may be related to nitric oxide and Ca^{2+} regulatory pathways (Wu et al., 2016). Rotondo, Serio, and Mulè (2009) (Rotondo et al., 2009) noted that the gastric relaxant effects of apigenin and quercetin in vitro were reduced in Ca^{2+} free solutions or when a Ca^{2+} voltage-gated channel blocker was added. This indicates that bioflavonoids may mediate their relaxant effects, at least in part, by their activity on voltage-gated Ca^{2+} channels (Gharzouli & Holzer, 2004).

The objective of this study was to investigate the effect of a simulated digest of the feed additive G's Formula on the contractility of gastric smooth muscle.

2 | MATERIALS AND METHODS

All materials were purchased from Sigma-Aldrich (Sigma-Aldrich Canada Co) unless otherwise stated.

2.1 | Simulated digest

The simulated digest of the composite feed additive G's Formula was made as previously described (Pearson, Orth, Karrow, Maclusky, & Lindinger, 2007) with the following changes: the FA was added to simulated gastric fluid (37 mM NaCl, 0.03 N HCl, 3.2 mg/ml pepsin) and incubated at 37°C on a shaker for 2 hr. The suspension was then added to simulated intestinal fluid (30 mM K_2HPO_4, 160 mM NaH_2PO_4, 20 mg/ml pancreatin) and incubated at 37°C on a shaker for a further 2 hr. The suspension was then centrifuged at 4°C for 20 min at 4,696 ×*g*. The supernatant was pre-filtered using a 0.45 µm filter followed by a 0.22 µm filter. Two hundred (200) µl of liver microsomes (male rat) was added to the solution and incubated on a shaker at 37°C for a further 30 min. The suspension was then filtered again (0.45 followed by 0.22 µm) into a 50 kDa ultrafiltration centrifuge unit (Amicon Ultra, Millipore) and centrifuged at 3,500 × *g* for 30 min. The digest was then frozen at −20°C until use. A blank digest was made using the same methodology but without the addition of FA.

2.2 | Experimental protocol

Porcine stomachs were collected from a local abattoir (Reist and Weber). The mucosa was removed and strips of smooth muscle (approx. 2 × 5 mm) from the fundus of the stomach were excised and hung in 25 ml organ baths. Organ baths were filled with Krebs–Henseleit Solution (KHS; in mmol/L: 118 NaCl, 4.75 KCl, 2.54 $CaCl_2$, 1.18 KH_2PO_4, 24.8 $NaHCO_3$, 10 glucose and 10 U/L insulin aerated with 95% O_2 5% CO_2; pH 7.4), and tissue was allowed to equilibrate for 1 hr. Tissue length was increased throughout the hour until the tissue stabilized between 6–8 g of baseline tension. Using a force transducer (FTo3 Grass Force Transducer), tissue contractile force was recorded following increasing concentrations of acetylcholine (Ach; 10^{-8} M–10^{-3} M). A dose response was generated by removing 2.5 ml of KHS and making additions of increasing concentrations of 2.5 ml Ach every 10 min. Ten minutes after the last addition of Ach the experiment was terminated, and tissue wet weight was recorded.

The mean and peak generated force, integral from under the force-time curve and delta force between initial and final selected times were extracted for 1, 2 and 8 min following each Ach addition using AcqKnowledge software (BIOPAC Systems Inc). Baseline (0) measurements were collected for each factor 1, 2 and 8 min prior to the first 2.5 ml of KHS removed from the bath. Baseline measurements were used to standardized force measurements. Force was normalized per g of tissue.

2.3 | In vitro organ bath experiments

An initial experiment was run with a control group (CO; n = 8) and an experimental group (FA; n = 8) to screen whether the simulated

digest of the composite FA G's Formula would alter contraction compared to an unstimulated control. The concentration of FA in the bath was 0.4 mg/ml. This was based on a dosage of 132 g of FA/day in a 330 L total body water in a 500 kg horse (Forro et al., 2000). Nothing was added into the baths of the CO group.

Based on the data collected from this experiment, the experiment was repeated with CO (n = 5), an additional blank group (BL; n = 6) in which only the simulated digest without FA was added into the bath, and FA (FA; n = 4) in which a slight increase in the concentration in the bath was made to 0.48 mg/ml. This was based on revised dosage of 160 g of FA/day in a 500 kg horse.

2.4 | Statistics

Data from both experiments were analysed using PROC GLIMMIX in SAS 9.4 (SAS Institute Inc). Force (y) was subjected to a RM ANOVA according to the following model:

$$y_{hijk} = \mu + expt_h + pig_i + ach_j + txt_k + pig_i \times txt_k + ach_j \times txt_k + \varepsilon_{hijk}$$

where μ = the overall mean, $expt_h$ = the random effect of the experiment (h = 1 or 2), pig = the random effect of the pig (I = 1 to), ach = the repeated measure of ach (j = 10^{-8} – 10^{-3}), txt = the fixed effect of treatment (k = CO, BL, or FA), the model included the interaction between pig and treatment, the interaction between ach dose and treatment, and ε = the experimental error. A lognormal distribution was used, and the residuals of different covariance structures were analysed to identify the most appropriate structure. Least square means were used to analyse the difference between treatments, least square means sliced by Ach were used to analyse the difference between treatments at each dose of Ach, and least mean estimate statements were used to compare each dose of Ach within treatment to 0. $p \leq .05$ was considered significant.

Although a test of the random effect of experiment yielded no significant differences in any of the parameters investigated between the 2 experiments, it was maintained in the model as a conservative measure.

3 | RESULTS

Results are presented as mean ± *SEM* unless otherwise stated. Measurements of Ach represent the concentration of Ach in the bath. For each parameter investigated, the main effect of Ach addition was significant.

3.1 | Mean force

At 1 and 2 min, CO was higher compared to 0 from 10^{-6} to 10^{-3} M Ach ($p \leq .05$). At 1 and 2 min, BL was higher compared to 0 from 10^{-5} to 10^{-3} M Ach ($p \leq .05$). At 1 min, FA was higher compared to 0 from 10^{-8} to 10^{-3} M Ach ($p \leq .05$). By 2 min, FA was higher compared to 0 from only 10^{-6} to 10^{-3} M Ach ($p \leq .05$) and FA was higher than CO at 10^{-3} M Ach (106.0 ± 9 g/g tissue vs. 81.9 ± 7 g/g tissue, p = .03). At 8 min, all treatments were higher compared to 0 from 10^{-6} to 10^{-3} M Ach ($p \leq .05$) (Table 1).

3.2 | Peak force at 1 min

Peak force was generated within 1 min of Ach additions. Hence, the results from 1 min were reflected at 2 and 8 min as well. Therefore, only the results from 1 min are presented herein.

Each treatment was higher compared to 0 from 10^{-6} to 10^{-3} M Ach ($p \leq .05$). FA was higher than CO at 10^{-3} M Ach (132.0 ± 12 g/g tissue vs. 100.2 ± 9 g/g tissue, p = .02) (Figure 1).

TABLE 1 Mean contractile force of gastric tissue (g/g tissue wet weight) treated with a simulated digest of the equine feed additive G's Formula (FA; n = 12) added to the organ bath, a simulated digest without any added nutraceutical (BL; n = 6) added to the organ bath, and nothing added to the organ bath (CO; n = 13) for 1, 2 and 8 min following a dose response of acetylcholine (Ach)

[Ach] M								
Txt	**Min**	**0**	**10^{-8}**	**10^{-7}**	**10^{-6}**	**10^{-5}**	**10^{-4}**	**10^{-3}**
FA	1	−0.2 ± 5	3.7 ± 5*	6.4 ± 5*	14.2 ± 6*	36.2 ± 7*	60.5 ± 8*	85.6 ± 9*
	2	−0.2 ± 5	4.5 ± 5	6.8 ± 5	18.1 ± 5*	43.4 ± 7*	69.0 ± 8*	106.0 ± 9*,**
	8	0.0 ± 4	5.3 ± 5	7.0 ± 5	21.7 ± 5*	40.0 ± 6*	56.6 ± 7*	93.4 ± 8*
CO	1	−0.1 ± 4	−0.4 ± 4	2.3 ± 4	8.9 ± 5*	26.4 ± 6*	47.4 ± 6*	68.6 ± 7*
	2	−0.1 ± 4	−0.1 ± 4	2.4 ± 4	11.1 ± 4*	31.0 ± 5*	54.4 ± 6*	81.9 ± 7*,**
	8	−0.2 ± 4	0.7 ± 4	2.8 ± 4	12.8 ± 4*	29.1 ± 5*	46.0 ± 6*	75.1 ± 7*
BL	1	−1.6 ± 6	−0.3 ± 6	2.8 ± 6	9.0 ± 6	27.7 ± 7*	52.7 ± 9*	77.3 ± 10*
	2	−0.5 ± 5	0.9 ± 5	3.9 ± 6	11.5 ± 6	30.0 ± 7*	53.9 ± 8*	85.0 ± 10*
	8	−0.6 ± 5	1.7 ± 5	4.7 ± 5	13.5 ± 6*	27.9 ± 7*	43.3 ± 7*	73.3 ± 9*

*Represents a significant difference from 0 within row ($p \leq .05$).

**Represents a significant difference between groups at [Ach] ($p \leq .05$).

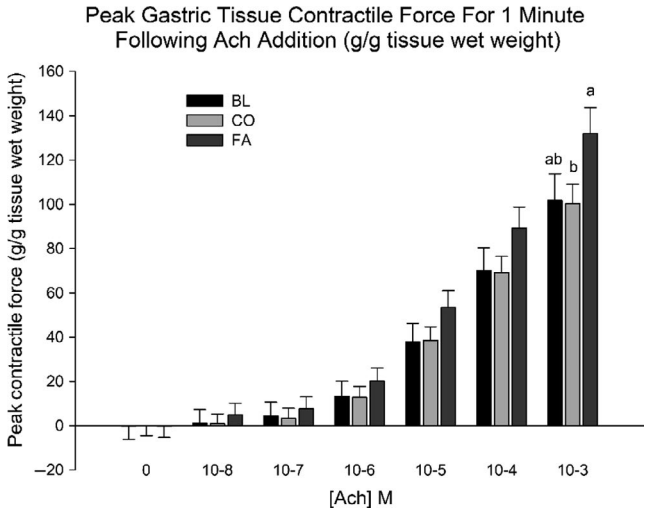

FIGURE 1 Peak contractile force in the fundus of the pig stomach 1 min following the addition of increasing concentrations of Ach into an organ bath (10^{-8} to 10^{-3} M Ach). Comparison of peak contractile force between groups: FA (n = 12; tissue was exposed to a simulated digest of the feed additive G's Formula), BL (n = 6; tissue was exposed to a simulated digest that had no nutraceutical added), CO (n = 13; no additions were made to the organ bath). Different superscripts represent a significant difference between groups at the [Ach] ($p \leq .05$)

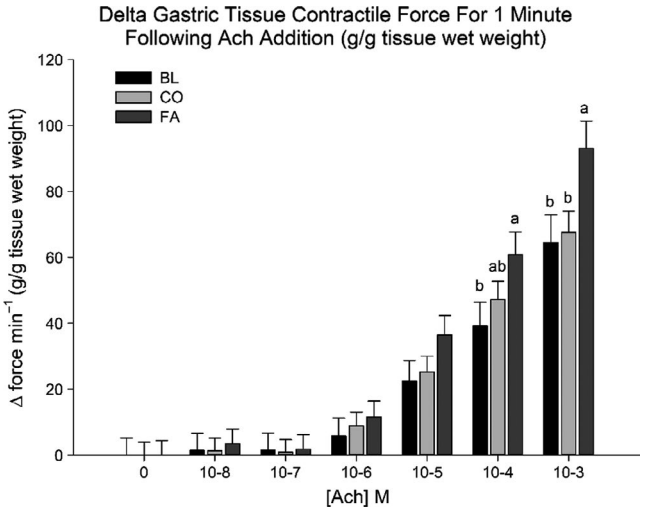

FIGURE 2 Delta force in the fundus of the pig stomach 1 min following the addition of increasing concentrations of Ach into an organ bath (10^{-8} to 10^{-3} M Ach). Comparison of delta contractile force between groups: FA (n = 12; tissue was exposed to a simulated digest of the feed additive G's Formula), BL (n = 6; tissue was exposed to a simulated digest that had no nutraceutical added), CO (n = 13; no additions were made to the organ bath). Different superscripts represent a significant difference between groups at the [Ach] ($p \leq .05$)

3.3 | Delta force at 1 min

Since the steepest increase to peak force occurred within the first min after each Ach addition into the bath, the increase in force during only that first min was investigated.

Both CO and FA were higher compared to 0 from 10^{-6} to 10^{-3} M Ach ($p \leq .05$). BL was higher compared to 0 from 10^{-5} to 10^{-3} M Ach ($p \leq .001$). At 10^{-4} M Ach FA was higher than BL (60.9 ± 7 g/g tissue vs. 39.3 ± 7 g/g tissue, p = .03). At 10^{-3} M Ach FA (93.1 ± 8 g/g tissue) was higher than both BL (64.5 ± 8 g/g tissue, p = .02) and CO (67.7 ± 6 g/g tissue, p = .01) (Figure 2).

3.4 | Integral under the force-time curve

At 1, 2 and 8 min CO and BL were higher compared to 0 from 10^{-6} to 10^{-3} M Ach ($p \leq .05$). At 1 and 2 min, FA was higher compared to 0 at 10^{-7} to 10^{-3} M Ach ($p \leq .05$). By 8 min, FA was higher compared to 0 at 10^{-8} to 10^{-3} M Ach ($p \leq .05$) (Figure 3).

4 | DISCUSSION

A variety of risk factors including electrolyte imbalances, hypoalbuminemia, endotoxemia, peritonitis and certain pharmaceutical agents have been linked to a reduction of GI motility in horses (Koenig & Cote, 2006). Ileus in the horse can result in caecal impaction, a severe form of colic often necessitating euthanasia for humane reasons or GI rupture resulting in fatality (Koenig & Cote, 2006). Endotoxin has also been associated with abnormal gastrointestinal motility and reduced gastric contractions (King & Gerring, 1991). Drugs such as phenylbutazone, flunixin and cisapride have been investigated as methods to counter gastric stasis and delayed gastric emptying (King & Gerring, 1989; Valk, Doherty, Blackford, Abraha, & Frazier, 1998, 1998). It is evident that the maintenance of gastrointestinal motility, throughout the entire GIT, is required for optimal health. Additionally, there is an interest in interventions which are relevant to maintain motility during times of abnormal GIT activity.

Various studies have used in vitro force transduction to investigate the impact of prokinetic pharmaceuticals on specific areas of interest within the equine GIT (Lefebvre, Callens, Colen, & Delesalle, 2017; Nieto, Maher, Maher, Stanley, Larson, & Snyder, 2013; Nieto, Morales, et al., 2013; Nieto, Rakestraw, Snyder, & Vatistas, 2000). In vitro techniques using smooth muscle from different regions of the GIT to examine the impact of different dietary components and bioactive compounds have been performed for application in species of commercial relevance (Jalilzadeh-Amin, Maham, Dalir-Naghadeh, & Kheiri, 2012, 2012b) or those more typically used as human models such as murines and Guinea pigs (Amira et al., 2008; Badary, Awad, Sherief, & Hamada, 2006; Budriesi et al., 2010; Gharzouli & Holzer, 2004). Equine feed additives are becoming increasingly popular as a means to maintain the general health and well-being of companion and sport horses. Due to the prevalence and severity of equine GI disease, many feed additives are focused on optimizing GI health. However, to the authors' knowledge no work has been done examining in vitro smooth muscle responses as a mechanism to screen products of potential relevance to equine GI health.

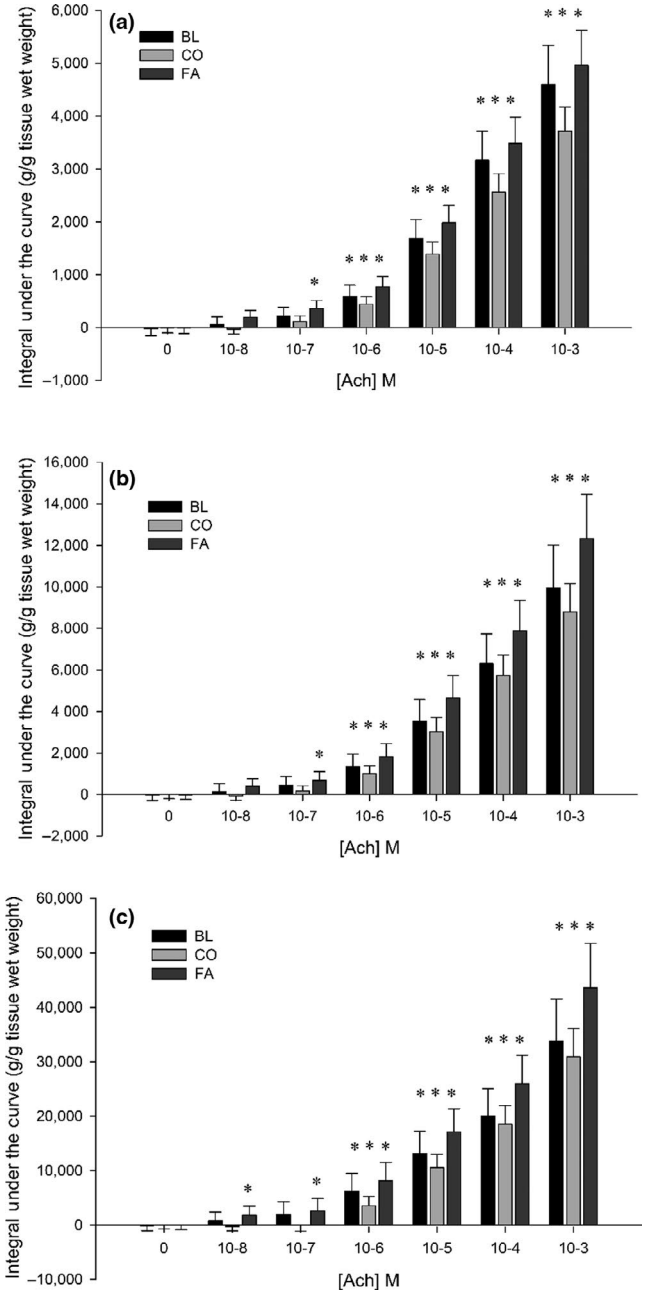

FIGURE 3 Integral under the force-time curve of gastric tissue (g/g tissue wet weight) from the fundus of the pig stomach following the addition of increasing concentrations of Ach into an organ bath (10^{-8} to 10^{-3} M Ach). Comparison of IUC to baseline (0). FA (n = 12; tissue was exposed to a simulated digest of the feed additive G's Formula), BL (n = 6; tissue was exposed to a simulated digest that had no nutraceutical added), CO (n = 13; no additions were made to the organ bath). (a) 1 min following Ach additions; (b) 2 min following Ach additions; (c) 8 min following Ach additions. *Represents a significant difference from 0 ($p \leq .05$)

Following peak contraction, which occurred within the first minute subsequent to Ach stimulation, gradual muscle relaxation was apparent. At higher doses of Ach, relaxation did not return to baseline and often elicited rhythmical contractions. Therefore, in order to provide a complete representation of the contractile response contractile force was measured over 1, 2 and 8 min following muscle stimulation. Additionally, not only mean and peak forces were evaluated. Area under the curve provides an assessment of the pattern of tissue contractility. Higher doses of Ach often elicited rhythmical contractions. Therefore, AUC was also an important measure to fully represent the tissue response to Ach stimulation.

Gastric smooth muscle pre-treated with a simulated digest of FA appears to sensitize the tissue to Ach, resulting in greater contractile force generation. The maximum force generation occurred within the first minute of Ach addition and the slope of this increase provides insight into the rate of force generation. At the highest concentration of Ach, FA increased the rate of force generation compared to both CO and BL, which also points towards a sensitizing effect of the digest on the muscle tissue. Integral under the force curve is a useful characteristic of muscle contraction to measure (Harlow & Weekley, 1986; Lefebvre et al., 2017; Lu et al., 2018) and accounts for both the frequency and amplitude of phasic type contractions. By using AUC to compare the reaction of the smooth muscle CO and BL treated tissue, particularly for the full 8 min following Ach stimulation, pre-treatment with the simulated digest of FA appears to have increased the rate and magnitude of tissue contraction as well as reduced muscle relaxation following stimulation with Ach.

The majority of the literature on the influence of bioflavonoids on gastrointestinal smooth muscle demonstrates an antispasmodic effects (Amira et al., 2008; Gharzouli & Holzer, 2004; Rotondo et al., 2009; Wu et al., 2016). However, these studies used isolated bioflavonoids. FA is a composite product composed of several different ingredients. Although individual bioactive compounds may be present in this supplement their interactions cannot reliably be predicted. Mendel, Chłopecka, Dziekan, and Karlik (2018) demonstrated that certain plant secondary metabolites could enhance the action of prokinetic drugs in isolated bovine abomasal and duodenal smooth muscle. However, these effects were dose-dependent and the flavonoids tested, quercetin and apigenin, abolished the contractile properties of all the prokinetic drugs at concentrations of 100 μM. Therefore, the bioflavonoids present may have acted synergistically with another active component in FA to facilitate the observed effects on gastric smooth muscle. The purpose of this study was to investigate the impact of the combined ingredients of FA. As such, a simulated digest of the entire product was evaluated so as to best mimic the in vivo conditions of feeding. However, the use of the aggregate product represents a limitation of this study. Looking at the response of cholinergic contractile activity to the individual whole ingredients of FA would provide insight into which component of this supplement might be principally involved in facilitating its effect.

Ach acts on smooth muscle through nicotinic receptors directly on ligand-gated ion channels and through muscarinic receptors (primarily M_2 and M_3) via G-protein coupled signalling pathways (Olsson & Holmgren, 2001). Identification of the major pathway concerned could have been revealed through the use of a nicotinic channel blocker. However, GI smooth muscle contraction is mediated to a greater degree by M_2 and M_3 receptors, and it seems more likely any significant impacts to contractile responses to Ach are more likely

modified through these receptor subtypes. Ca^{2+} sensitivity is an important factor impacting the strength of smooth muscle contraction (Sanders, Koh, Ro, & Ward, 2012). The use of a Ca^{2+} free KHS solution could have been helpful to identify whether the simulated digest of FA was acting through extra- or intra-cellular Ca^{2+} channels. Future research should include measures to more thoroughly explore the mechanism behind the increased Ach sensitivity resultant from exposure of smooth muscle to a simulated digest of FA.

Although FA is intended as a dietary supplement to be given to horses, we chose to use porcine tissue as opposed to equine. The use of porcine tissue is preferable as it is considerably easier to obtain than equine tissue. Abattoir tissue is more consistent as pigs are slaughtered at the same age, and slaughter conditions are the same between animals. Additionally, euthanasia drugs used on horses, such as barbiturates, are known to alter smooth muscle contractility (Edney & Downes, 1975; Nishiwada, Nakamura, Hatano, & Morl, 1991) and enteric nervous system receptors (Taniyama, Hashimoto, Hanada, & Tanaka, 1988). Both horses and pigs are monogastric mammals and share a unique structure of the stomach with a distinct glandular proximal section and non-glandular distal section (Colville & Joanna, 2015). Gastric smooth muscle is similar between the majority of vertebrate species and mechanical activity and stimulation are generally most similar in proximal regions (Mandrek & Kreis, 1992). Acetylcholine is a biologically relevant contractile stimulus of smooth muscle (Bolton, 1979a, 1979b) and one of the major excitatory neurotransmitters in the GIT (Hall & Guyton, 1946). Acetylcholine has the same molecular structure in all animal species and is conserved over time (Olsson & Holmgren, 2001). Therefore, for this preliminary study porcine tissue was considered to provide a reasonable approximation of the response which would be generated in equine tissue. Nevertheless, further studies should clarify whether pig and horse gastric smooth muscle is comparable with respect to the magnitude of the contractile response to Ach. The sensitizing effect of FA on cholinergic receptors in porcine tissue observed in the current study provides evidence for its effect in pig tissue, and future studies are necessary to confirm that this effect can also be observed in equine tissue. Based on the similar structure and mechanism of action of Ach on GI smooth muscle, it is possible the action of FA is conserved between species. However, due to species differences in the GIT between horses, strict herbivores, and pigs, an omnivorous species, the magnitude of effect may significantly differ.

The rate of mortality in cases of equine ileus is extremely high, and although prokinetic pharmaceuticals are commonly used in veterinary medicine, there remains a critical lack of evidence concerning dosing and efficacy (Van Hoogmoed, Nieto, Snyder, & Harmon, 2004). Thus, a simple dietary method to maintain GI transit could be of interest for performance horses. This is particularly relevant as strenuous activity and showing have both been identified as risk factors associated with colic (Gongalves, Julliand, & Leblond, 2002; Kaneene et al., 1997). The sensitizing effect FA appears to have on gastric smooth muscle, if present throughout the GIT, could be useful as a dietary aid to help in the maintenance of proper GI motility. However, several neurotransmitters and hormones regulate contractility along the GIT (Hall & Guyton, 1946; Berner, 2003a; Hansen, 2003b). Furthermore, differential regulation and response to stimulatory, inhibitory and pharmaceutical agents can be observed in different regions and muscle layers within the stomach (Kuriyama, Mishima, & Suzuki, 1975; Milenov & Golenhofen, 1982, 1983; Muramatsu, Itoh, Lederis, & Hollenberg, 1988). Indeed, regional differences in the responsiveness to drugs including erythromycin, lidocaine and metoclopramide can be observed along the equine GIT (Nieto et al., 2000). Similarly, Hoogmoed, Snyder, and Harmon (2000) noted prostaglandins demonstrated variable effects on smooth muscle dependent upon GI region. It is therefore important to examine the influence of FA in other segments of the GIT to obtain a full picture of its potential effect. Of particular interest are the jejunum, caecum and pelvic flexure as these regions are particularly prone to ileus in the horse.

It must be noted that although actions were taken in this study to better reflect in vivo conditions, including the use of a simulated digest and a biologically relevant stimulus, there are inherent limitations when using in vitro studies. Despite best efforts, it is not known whether in vitro results are reproducible in vivo due to the multitude of interacting factors, many of which are unknown, that occur within the live animal. These limitations necessitate a conservative interpretation of any in vitro results. Although in vitro studies represent an important first step when investigating potential effects of novel nutraceuticals, efficacy demonstrated through in vivo research is crucial. The data from this study indicate that the dietary feed additive Gs Formula may impact GI smooth muscle, but implications of this in the live animal and on GI transit require further research.

5 | CONCLUSION

This study provides evidence that a simulated digest of the equine feed additive G's Formula can sensitize gastric smooth muscle and enhance the contractile response to Ach. Future research should focus on whether this sensitizing property is evident in the live animal and the functional consequences on digestion and GI transit.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

J.L. MacNicol involved in experimental design, running of experiments, data extraction and processing, statistical analysis, manuscript writing. C. Murrant involved in experimental design and oversight, and manuscript editing. W. Pearson: involved in experimental design, and manuscript editing.

ANIMAL WELFARE STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was necessary for as abattoir tissue from a Provincially Inspected abattoir only was utilized for this study.

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