Banana Feeds Australia

Equine scoping studies using B-Complete, Nature's Elite Equine Supplement.
Date Commenced: April 2020
Date Completed: October 2021.

Banana Feeds Australia are proud to have completed a collaborative study with the University of Adelaide, to evaluate the benefits of supplementing our dried whole green banana supplement into the diet of equine.

The study was funded in equal contributions by Banana Feeds Australia and the SME Solutions Centre through the Fight Food Waste CRC.

This was a world first study of its type, despite historical evidence of green bananas being included in the diets of range of species, including equine.

The research project bought over $100k of research into the equine industry, and had the support from many highly qualified nutritionists, veterinarians, and equine specialists in delivering the project. We are grateful to the University of Adelaide, The South Australian Research and Development Institute, and the Fight Food Waste CRC for their professionalism, the independence, and their hard work to deliver what was a complex research project.

Through this project, we have investigated the outcomes of supplementing a horse with 100 grams per day, with the primary objectives of observing and recording beneficial outcomes and to confirm that its 100% natural origin is safe, and that it can assist in the management of EGUS. It was compared to the highest standard of medicative care for EGUS (Omeprazole) with the learnings to be adapted to improving better animal well-being overall.

We are delighted with the results and anticipate further R & D to investigate the other strong anecdotal benefits that customers have reported during the use of B-Complete. Although we are a small family business, and our funds are limited, we do look forward to further collaborations with the University of Adelaide, for an even deeper dive into the mode of mechanisms for these benefits.

Although we set our standards high in comparing whole dried green banana's ability to reduce ulcers against the industry standard of care (Omeprazole), it was evident that the medicative treatment was more effective in reducing ulcers than green bananas, however, it was also apparent that it added benefits against our control groups.

We firmly believe that using B-Complete as a gut health supplement, for prevention rather than cure, for overall health rather than symptomatic treatment of ailments, provides the industry with a new, natural option for horse well-being.

This report has enabled us to better understand the beneficial compounds, antioxidants, anti-ulcerogenic, amino acid profiles, calming agents (all naturally present in green bananas and their skins) and how they interact within the equine's overall physiology.

John McArthur, Director, Banana Feeds Australia

The full unedited copy of the study follows:

SME Solutions Centre
Final Report

Effects of a dried green banana crumble on equine gastrointestinal health

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In collaboration with Banana Feeds Australia, directors Robert Borsato and John McArthur

Executive Summary

Highlights:

- Successful laboratory and clinical trials were conducted over 16 months in collaboration with the University of Adelaide and the South Australian Research and Development Institute (SARDI).
- Dried green banana supplement was found to be a rich source of antioxidants including polyphenols and flavonoids.
- Zero negative impacts were reported in trial horses.
- Dried green banana did not negatively influence microbial population of the hind gut of horses after 28 days of feeding at a dose of 100g/horse/day.
- There was an overall reduction of squamous ulcers when dried bananas were compared against untreated controls.
- At current dosages, omeprazole outperformed dried green bananas for preventing development of equine squamous ulcers.
- Neither omeprazole nor the banana supplement was effective for preventing the development of glandular ulcers.
- Studies are planned to further explore the optimum dosage levels of dried green bananas for the maintenance of oxidant-anti-oxidant balance in the blood, the improvement in EGUS results, and will also include investigation of other benefits identified by clients, such as improvements in behaviour, coat health and faecal consistency.
- Studies are planned to further investigate the benefits for multiple other species, including bees and canines.

This project aligns with the Fight Food waste CRC Program 2: Transforming waste resources. Banana Feeds Australia Pty Ltd is an innovative, Queensland based, start-up company that has established a processing facility to re-purpose raw bananas that would otherwise end up as food waste.

Bananas are one of the most cultivated tropical fruits produced globally for human consumption, accounting for approximately 15% of the world's fresh fruit. However, almost a third of all bananas gathered are wasted because bananas are prone to mechanical damage and are perishable during the maturation process. Furthermore, flaws associated with fruit size and cosmetic appearance typically leads to market rejection or significant downgrading of otherwise edible fruit, contributing to these losses. Australian banana farmers incur significant farm gate losses of around 10-30% of the total production, equating to approximately 37,000 tonnes per annum and valued at $26.9 million.

Unripe bananas are recognised as a rich source of nutrients and phytochemicals which are known to have positive effects on human health and well-being. The high antioxidant levels and resistant starch present in banana have been recognised for their associated health benefits including protection against oxidative stress, and improved gastrointestinal health, including gut microbiome stability and anti-ulcerogenic effects.

Against this backdrop, Banana Feeds Australia Pty Ltd has created an alternate market option for banana waste, thereby improving the economic returns to growers and providing natural alternative products for the equine industry. The study aimed to investigate the benefits of feeding horses their commercial product, 'B-Complete-Nature's Green Banana Supplement'.

Outcomes from this study showed the 'B-Complete - Nature's Green Banana Supplement' to be a rich source of antioxidants including polyphenols and flavonoids. This study benchmarked levels of total antioxidants, total polyphenols, total flavonoids and total antioxidant activity in early batches of the supplement. The total antioxidant content was observed to range from ~ 4 – 9 mg GAE g^{-1} on a 'as is' basis, comparable to other research in the published literature. Some variation was identified between batches which could be attributed to environmental variables, processing conditions and the natural elements of the product. Product used for this study, was from some of the first batches produced by Banana Feeds Australia, and since that time, Feed

Safe Accreditation and production improvements have been made to ensure a more consistent and reliable product.

During the clinical trials in horses, the product was found to be palatable for horses when fed as a "top dressing" and was not associated with any adverse effects. Clinical trial 1 investigated the impact of feeding 'B-Complete - Nature's Green Banana Supplement' on the faecal microbiome of horses at a dose of 100g/day. No significant change was observed over a 28-day period indicating that, at this dose, the feeding of a dried green banana product containing resistant starch did not negatively influence the microbial population of the hind gut and could safely be fed to horses without any adverse effects. This study also investigated the effects of feeding the dried green banana supplement on the total antioxidant capacity (TAC) in the blood of horses, which failed to show a significant increase in the TAC at a dose of 100g/day for 28 days.

Clinical trial 2 investigated the effect of feeding 'B-Complete - Nature's Green Banana Supplement' on the development of Equine Gastric Ulcer Syndrome (EGUS), commonly known as "stomach ulcers". Comparisons were made with a preventative dose of the industry standard treatment, omeprazole and with horses receiving no treatment. Although banana showed some benefit at preventing the development or exacerbation of equine squamous gastric disease (ESGD), in comparison with no treatment, it was not as effective as omeprazole. Further studies will investigate whether a higher dose affords increased benefit as has been shown in previous studies of rodents fed green banana products.

Objective(s)	Result(s)
1) Develop a profile of the biologically active compounds in the dried green banana supplement ('B-Complete - Nature's Green Banana Supplement').	The total antioxidant content, flavonoids, polyphenols and antioxidant activity were quantified in batches of 'B-Complete - Nature's Green Banana Supplement'. There was some variation between batches, which could be attributed to environmental variables, processing conditions and the natural elements of the product.
2) Determine the effects of dried green banana on gastrointestinal health of horses by:	Clinical trials were performed in horses receiving the dried green banana supplement at a dose of 100g/day for 28 days.
a) investigating its effect on microbiome and total antioxidant status; and	No significant change was observed in faecal microbiome over 28 days, indicating that, at this dose, the supplement did not negatively influence the microbial population of the hind gut.
	No significant effect was observed on total antioxidant capacity of the blood of horses, after 28 days.
b) determining its effectiveness as an EGUS preventative.	There was some benefit in the prevention of equine squamous gastric disease in horses in work, but this was not as effective as the industry standard, omeprazole.

Next Step(s)	Timing
• Quality control and batch optimisation for product consistency.	Ongoing.
• Dose optimisation to determine whether feeding different dosage rates of the green banana supplement affords increased benefits to horses.	12 months
• Additional studies, including effects on faecal microbiome at higher doses and investigation of other health benefits as identified by clients.	12 months
• Investigate other potential benefits as identified by consumers and expand to other species including dogs and bees.	12 months

Project Impacts

- Validation of an innovative new food product for horses from surplus food.
- Animal health benefits from bioactive and functional foods.
- Reduction in food waste.

Utilisation/Commercialisation Opportunities

- The product has been commercialised for use in horses and is now being extended to other species including dogs and bees.
- The product has commenced being exported to numerous countries, with in-country representation developing further opportunities.
- Additional products have been developed for the commercial market, including natural horse treats.

IP

Trademarks for B-COMPLETE AND BEE-COMPLETE have been granted, in addition to an international patent pending.

Confidentiality

Confidentiality remains regarding the production processes, facility components, customer details. Results from the study will be made available upon request.

Approved By

John McArthur, Director, Banana Feeds Australia

Signed_____ Date__05/11/2021_____

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1. INTRODUCTION

Bananas are one of the most cultivated tropical fruits produced globally for human consumption, accounting for approximately 15% of the world's fresh fruit (Falcomer *et* al., 2019). However, almost a third of all bananas gathered are wasted because ripe bananas are prone to mechanical damage and are perishable during the maturation process. Furthermore, flaws associated with fruit size and cosmetic appearance leads to market rejection of otherwise edible fruit and contributes to these losses (Falcomer *et al.,* 2019)

Banana Feeds Australia Pty Ltd is a Queensland based start-up company that has established a processing facility to re-purpose raw bananas that ends up as food waste. Australian banana farmers incur significant farm gate losses of around 10-30% of the total production, equating to approximately 37,000 tonnes per annum and valued at $26.9 million (White *et al*. 2011).

Bananas are recognised as a rich source of nutrients and phytochemicals which are known to have positive effects on human health and well-being (Yangilar, 2015; Singh *et al*, 2016). The high antioxidant levels and resistant starch present in banana have been recognised for their associated health benefits including protecting the body against various oxidative stresses, improved gastrointestinal health and gut microbiome, and anti-ulcerogenic effects (Best et al., 1984; de Lira Mota *et al*. 2009; Mitsou *et al.,* 2011; Powthoong *et al*., 2020; Wu *et al* 2020). Against this backdrop, Banana Feeds Australia Pty Ltd hopes to create an alternate market option for banana waste, thereby improving the economic returns to growers and providing natural alternative products for the equine and livestock feed industry.

2. LITERATURE REVIEW

Bananas belong to the Musa genus of the *Musacaea* family, with more than 1,000 varieties produced worldwide. Bananas are divided into two predominant varieties; dessert (sweet), of which the Cavendish variety is the most common, and plantains which are larger, more starchy fruit intended for cooking (Periera and Maraschin, 2015; Singh *et al.,* 2016, Falcomer *et al.,* 2019). The primary source is the fruit peel and pulp, generally sourced as either unripe (green) or ripe depending on product specification, but its use as a functional food can also extend to its roots, stem and flowers. Unripe banana compounds and associated by-products (including flour and pulp biomass) have proven health benefits to humans and rodents (Falcomer *et al*, 2019; Sidhu *et al* ,2018).

To our knowledge, studies on the health benefits of banana and/or associated by-products are limited to human and rodent models, with no studies in equine species published. However, as both human and rodent species are recognised as monogastric, like horses, many physiological and biochemical mechanisms may be applicable to equine gastrointestinal health and animal well-being. As such, this review will highlight bioactive properties found in bananas recognised for their health benefits and we believe they may have the potential for beneficial applications as a health supplement in equine nutrition and health.

2.1. Bioactive compounds present in banana
Banana fruit has more recently been recognised as a rich source of both major essential nutrients and "phytonutrients". In particular, the high levels of antioxidants, fibre and resistant starch present in bananas have been associated with beneficial effects in human health and well-being (Almeida-Junior *et al*., 2017; Falcomer *et al.,* 2019; Singh *et al.,* 201; Yangilar, 2015,).

2.1.2. Major nutrients

Banana contains approximately 75% water and 25% carbohydrate, with trace amounts of protein and fat (Powthong *et al.,* 2020). Other major nutrients present in bananas include various vitamins (Vit C, B6, provitamin A) and high levels of minerals including phosphorus, sodium, potassium and calcium, which are all essential to human and animal general health. Green banana also provides bioactive compounds such as phenolic compounds and resistant starch in both the pulp and peel (Lii *et al.*, 1982; Hettiaratchi *et al.*, 2011; Powthong *et al.*, 2020) potentially contributing to health benefits (Bodinham *et al.*, 2010; Basso *et al.*, 2011).

Resistant starch can promote health benefits, acting as a prebiotic since it is not hydrolysed in the digestive tract. Physiologically like fibre, resistant starch reduces glycemia and decreases the risk of developing chronic diseases (Basso *et al.*, 2011). Many other benefits include prevention of intestinal diseases (Basso *et al.*, 2011); improvement of the immune response and the prevention of intestinal cancer (da Silva *et al.*., 2016; Fuentes-Zaragoza *et al.*, 2010). Fibre is also considered an important ingredient in the formulation of functional food. According *to* Cassettari *et al.* (2019) the level of resistant starch and dietary fibre in green banana pulp was 7.8 g/100 g and 4.4 g /100 g, respectively. It is recognised however that banana composition will vary according to the soil, climate, banana variety, maturation stage, local of production, and other factors (Falcomer *et al.*, 2019).

2.1.3. Phytochemicals

Phytonutrients are becoming an area of interest due to their beneficial health effect as a natural alternative to current pharmaceuticals. Bioactive compounds from plant secondary metabolism and are recognised to have clear therapeutic potential, referred to as phytochemicals. In fruits and vegetables, phenolics and carotenoids are the main phytochemicals related to human health (Singh *et al.,* 2016). Bananas are a particularly rich source of antioxidants, with an abundance of bioactive phenolic compounds (primary antioxidants), carotenoids, biogenic amines and phytosterols (Sulamian *et al.,* 2011; Singh *et al*, 2016). The following phytochemicals are recognised for being exceptionally high in unripe bananas: carotenoids, flavonoids and phenolics (Baskar *et al.,* 2011; Pereira and Marashin, 2015; Sidhu *et al.,* 2018). Some phytosterols have also been found at low levels in the banana pulp. The capacity of these antioxidants appears to increase during fruit maturity.

The primary **carotenoids** identified in the Musa species germplasm (point of growth with genetic information; seed, stem, leaf) are a-carotene, trans-B-carotene, lutein, 13-cis-B-carotene and 9-cis-B-carotene, with concentrations in descending order (Bhat *et al.,* 2019). The peel of ripe green banana contains higher levels of the carotenoid lutein than in the pulp, in addition to the presence of non-provitamin A carotenoids such as lycopene and zeaxanthin (Singh *et al.*, 2016). **Flavonoids** such as quercetin, myricetin, kaempferol, and cyanidin (i.e. leucocyanidin), are found in green bananas and act as free radicals - scavenging both reactive oxygen and nitrogen species to limit oxidation (Prabha *et al.,* 2011; Bhat *et al.*, 2019; Falcomer *et al.,* 2019; Sidhu *et al.,* 2018). **Phenolics** in green banana fruit include gallic acid, catechin, epicatechin, tannins, and anthocyanins, with greater concentrations identified in the peel than the pulp (Amah *et al.*, 2019). Green bananas are rich in **phytosterols** including stigmasterol, -sitosterol, campesterol, 24-methylene cycloartenol, cycloeucalenol, and cycloartenol (Sidhu *et al.*, 2018). Phytosterols are naturally occurring plant sterols with nutraceutical benefits.

Many of these active compounds present in bananas are typically higher in unripe banana peels compared to pulp and ripe bananas (Pereira and Marashin 2015). In addition, the concentration and properties of these compounds appear to differ depending on plant species, growing location and plantation environment (Maduwanthi *et al.,* 2021).

2.2. Health benefits of banana supplementation in human and animal models

As mentioned in the previous section, bananas are a rich source of phytochemicals, particularly antioxidants. Phytochemicals are now being widely examined for their ability to provide health benefits as substrates for biochemical reactions, cofactors or inhibitors of enzymatic reactions, scavengers of reactive or toxic chemicals (antioxidant capacity) and influence gastrointestinal function and microbiota. Antioxidants, classified as one such class of phytochemicals, are recognised to support human and animal health. The health benefits are primarily due to their ability to delay, retard or prevent the oxidation or free radical-mediated oxidation of a substrate when present in low concentrations, leading to the formation of stable radicals after scavenging (Singh *et al.,* 2016).

Different types of antioxidants act via different mechanisms. Phenolic acids are a source of antioxidants that reduce inflammation and reduce damage from oxidative stress, whilst carotenoids act to scavenge and deactivate reactive oxygen species (Bhat *et al.,* 2019; Maduwanthi *et al.,* 2021). Due to the high antioxidant levels present in green bananas, they have the potential to increase total antioxidant capacity (TAC) concentrations in blood. Four weeks of banana consumption has been shown to improve TAC in humans (Sae-Taew *et al.,* 2013; Leelarungrayub *et al*, 2017). However, optimal dose rates require further clarification (Hernandez-Carranza *et al,* 2015).

Green banana compounds such as fibre and starch may enhance prebiotic efficacy and faecal microbiome balance in humans and rodents (Falcomer *et al,* 2019; Sidhu *et al,* 2018; Prowthong *et al*., 2020). A prebiotic is defined as fibres that pass through the GI tract undigested and stimulate the growth and activity of certain existing healthy 'good' bacteria (*Bifidobacteria*, also known as probiotics) in the large intestine.

The prebiotic properties in unripe bananas have demonstrated support of gut mucosal integrity, gut mucous production, and protection against harmful bacteria and pathogens (Ahmad and Akbar, 2016). Banana supplementation, in the form of a medium ripe banana or a banana drink daily, was found to limit bloating and bifidogenic effects in healthy women (Mitsou *et al*, 2011). Green banana flour has proven suitable in improving anthropometric parameters, lipid profiles and inflammation, limiting the damage inflicted onto gut microbes (Falcomer *et al,* 2019). In rodents receiving more significant amounts of banana flour, triglyceride levels increased by 20% due to the amount of excess resistant starch consumed - therefore, research into suitable quantities depending on bodyweight and additional factors are to be determined (Escobar *et al,* 2019). Concerning microbial communities in the human intestines, banana powder supplementation altered bacterial diversity by increasing *Bacteroidetes* and *Lactobacillus* while maintaining the abundance of *Bifidobacterium* in faecal matter (Tian *et al,* 2020). Resistant starch also improves immunity, reduces cholesterol (related to phytosterol contents), increases vitamin and mineral synthesis, and improves overall digestion and glucose/insulin metabolism (Falcomer *et al*, 2019). Therefore, green banana extracts and by-products have provided beneficial prebiotic effects in human and rodent models.

Gastrointestinal studies evaluating the use of banana or banana extract in rodents have demonstrated a protective effect on the gastric mucosa against medically induced lesions (Best *et al*, 1984; Lewis *et al.*, 1999; Lewis and Shaw, 2001; Prahba *et al*., 2011; Alese *et al*., 2017; Prasad *et al,* 2020). Most studies utilised unripe plantain bananas (*Musa sapientum)* as a preventative or treatment for induced ulcers. The findings suggest that *Musa sapientum* possesses a significant protective effect against gastric ulcers which is comparable to that of omeprazole (Prahba *et al.,* 2011; Prasad *et al,* 2020). Pharmacological investigation of banana extract suggested that a natural flavonoid (leucocyanidin) may be responsible for the protecting effect on the gastric mucosa (Best *et al*., 1984, Lewis and Shaw, 2001). The exact mechanisms through which flavonoids exert their anti-ulcerative effect are yet to be confirmed but may be related to their antioxidant properties (Goel *et al* 2001) or reduced acid secretion from parietal cells (Biel *et al*., 1995). Leucocyanidin also acts as an anti-inflammatory and

may aid the healing of gastric ulceration by increasing the gastric mucosal lining of the intestines (Sidhu *et al* 2018).

2.3. Potential applications for banana supplementation in equine health and performance
This section focuses on the beneficial health effects found in other animal and human models and the potential application for equine health.

2.3.1. Antioxidants and oxidative stress effects in horses
Oxidative stress, described as the "irreversible modification of cellular components leading to cell dysfunction", occurs due to an imbalance of oxidants and antioxidants, where antioxidants are deficient (Kirschvink *et al.*, 2008). Oxidants are reactive oxygen species (ROS) generated by metabolic enzymes, inflammatory cells, and mitochondrial electron leakage (Kirschvink *et al.*, 2008). Oxidants *in excess* concentrations may damage cellular molecules such as DNA, RNA, proteins, epithelium, and signalling molecules (Milkovic *et al.*, 2019). Oxidants include free radicals in which molecules contain one or more unpaired electrons within molecular and or atomic orbits and other reactive compounds without unpaired reactive electrons (Kirschvink *et al.*, 2008). Ultimately, an excess concentration of oxidants, free radicals, or lipid oxidation products (LOP's) cause oxidative stress, cell damage and inflammation (Kirschvink *et al.*, 2008; Milkovic *et al.*, 2019). The antioxidant enzymes of most importance include superoxide dismutase, catalase, and glutathione peroxidase. The enzymes have the catalytic activity to transform superoxide anions to hydrogen peroxide and water (Figure 1). This process ultimately inactivates significant concentrations of oxidants (Kirschvink *et al.*, 2008; Milkovic *et al.*, 2019).
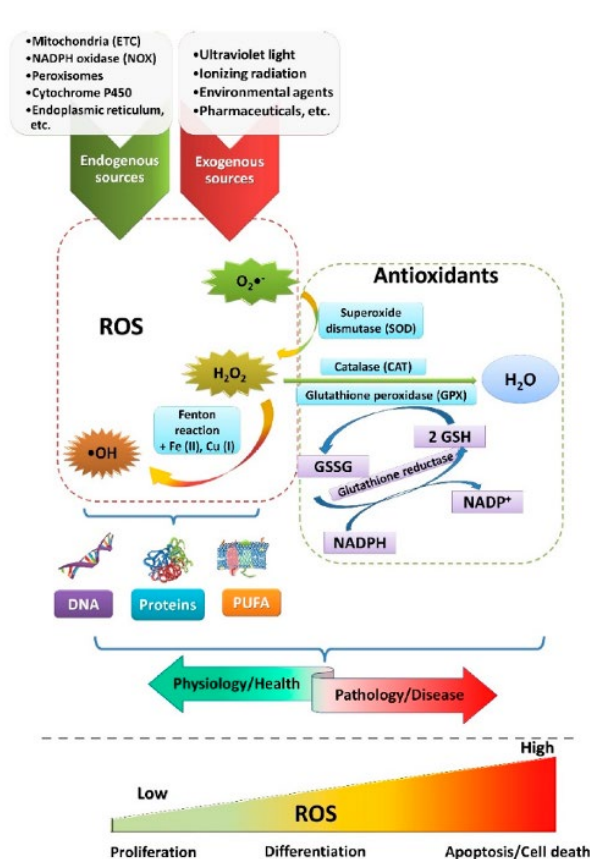
Figure 1. Presentation of the generation of reactive oxygen species (ROS) and their impact on cells; the oxidant: antioxidant equilibrium and the relation to health versus disease (taken from Milkovic et al., 2019).

Stress, exercise and high starch and sugar diets are precursors for oxidative stress in horses (Kirschvink *et al.*, 2008; Williams *et al.,* 2016). Additionally, horses undergoing high-performance exercise or stress have an increased demand for oxygen by the respiratory system and the muscles, leading to reactive oxygen species (ROS) and cell damage (Kirschvink *et al..,* 2008; Williams *et al.*, 2016). In a state of oxidative stress, antioxidant supplementation is required to prevent cell damage and the onset of disease (Kirschvink *et al.,* 2008; Pham-Huy *et al.*, 2008; Williams *et al.,* 2016).

2.3.2. Equine microbiome optimisation

Horses are hindgut fermenters and rely on the microbial interactions within the gastrointestinal tract to maintain a homeostatic environment, structural integrity, and digestive processes. Each compartment maintains its specialised microbial community, which differs in microbe type and diversity, relative abundance and overall function. As herbivorous mammals, the degradation of cellulose, hemicellulose, fibre, and resistant starch is essential for converting indigestible matter to volatile fatty acids such as propionate, butyrate and acetate, used as a fuel source for energy production (Costa and Weese, 2012). Each horse has a unique set of faecal microbiota, but the predominating phyla in all healthy horses mainly are the Firmicutes; furthermore, *Bacteroidetes*, *Verrucomicrobia*, *Actinobacteria*, *Clostridiales* and *Spirochaetes* are present (Schoster *et al.*, 2014; Costa *et al*., 2015, Costa and Weese, 2018). Similarly, a recent study identified *Bacteroidetes* mostly commonly in faecal microbiota followed by Firmicutes in 79 horses at two farms (Theelen *et al.* 2021). High variability in the equine faecal microbiome between horses is dependent on age, housing, pasture habits, diets and genetics; furthermore, contact with humans, veterinary health care and medication can affect the microbiome (Costa *et al*., 2015, Costa and Weese, 2018; Kauter *et al.,* 2019). Theelen *et al.* (2021) recently demonstrated that the alpha-diversity and richness decreased with age, while the beta-diversity was modified by location, age, season, horse type and access to pasture.

When the equine microbiome is in poor condition, the efficacy of fibre fermentation, vitamin absorption, and gut mucosal and intestinal integrity may be affected, further contributing to gut microbes' dysbiosis poor gastrointestinal health. Severe dysbiosis is responsible for several health implications, including colic, colitis and laminitis (Costa and Weese, 2012, 2018; Kauter *et al.,* 2019). A study investigating the effect of Jerusalem artichoke meal supplementation equine gastrointestinal microbiota (by DNA extraction and amplification of the V1-V2 region of the 16S rRNA gene) demonstrated that this prebiotic significantly increased the diversity in nearly all parts of the gastrointestinal tract (sampled post-mortem) (Glatter *et al.,* 2019). Another recent study demonstrated a positive and synergistic effect of aleurone supplementation on the glucose-insulin metabolism and microbiome composition in horses (Boshuizen *et al.,* 2021). Similarly, a small significant change in the microbiome of thoroughbreds was identified following dietary supplementation with amylase-rich malt extract (Proudman *et al.*, 2015).

The bioactive composition of green bananas includes high concentrations of resistant starch, which has proven metabolic health and digestion benefits in humans (Falcomer *et al.,* 2019). In horses, resistance starch is less susceptible to digestion in the small intestines and will reach the large intestines used by the microbiota. However, horses given fermentable starch in excess are at risk of digestive complications. Indeed, Harlow *et al.* (2016) demonstrated the differential effect of starch sources on the equine faecal microbiota. Additionally, it is reported high-starch diets were associated with increased behavioural reactivity in horses, linked to microbiota changes in the gut, via gut-brain axis pathways when compared to a high fibre diet (Bulmer *et al.,* 2019). Similar to a recent study investigating the effect of different doses of an insoluble supplement aleurone (Boshuizen *et al.,* 2021), the benefits of dried green banana feed supplementation likely depend on the concentration of fibre and resistant starch and the associated dose rate of the supplement.

2.3.3. Equine Gastric Ulceration Syndrome (EGUS)

It is estimated that 60 - 100% of horses are affected by equine gastric ulceration syndrome (EGUS) (McClure *et al.,* 1999; Nieto *et al.,* 2004; Jonsson and Egenvall, 2006; Luthersson *et al.,* 2009; Sykes *et al.,* 2015). The syndrome is associated with poor performance, weight loss, colic, and behavioural changes, representing an important welfare issue (Sykes *et al.,* 2015; Sykes, 2019). The negative impacts on health and performance, along with costs related to diagnosis and treatment, also contribute to significant economic losses within the equine industry.

The equine stomach is divided into two regions (squamous and glandular mucosa) (figure 2). Both regions may be affected by EGUS however the risk factors and aetiology differ depending on the region. The squamous region has limited protective mechanisms against gastric acid and hence sensitive to acid exposure, particularly during exercise when the acid can splash against the squamous mucosa leading to Equine Squamous Gastric Disease (ESGD). The glandular region is continuously exposed to stomach acid but has defence mechanisms in the form of a bicarbonate mucus layer. Ulcerations in this region may develop when these defence mechanisms are impaired in the face of stress or the use of certain medications, such as non-steroidal anti-inflammatories (NSAIDs), leading to Equine Glandular Gastric Disease (EGGD). Diet and high-intensity exercise are important risk factors for development of ESGD, whereas stress and repetitive exercise are key factors for EGGD (Sykes *et al.,* 2015).
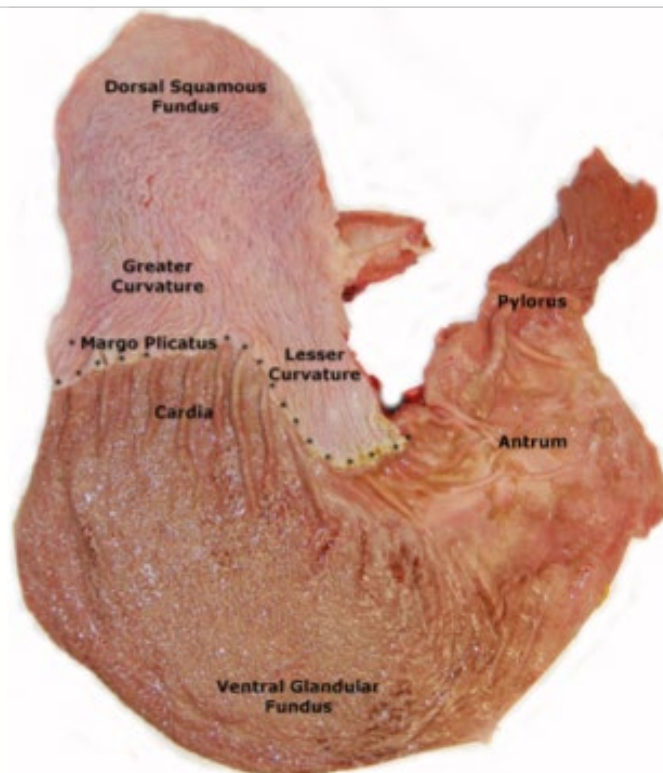
Figure 2. *A post-mortem specimen of the equine stomach depicting the different regions of the stomach. (Taken from Sykes et al., 2015)*

Oral omeprazole is the reference standard therapy for gastric ulceration in humans and is also used as the mainstay therapy for EGUS (Sykes et al., 2015). Omeprazole is a potent inhibitor of gastric acid secretion and works by blocking the H^+/K^+-ATPase pump (hydrochloric acid or proton pump) in the secretory membrane of the parietal cell in the stomach. This is the last step in gastric acid production and secretion, explaining why omeprazole has good efficacy for treating ulcerations in the squamous portion of the stomach which is negatively affected by exposure to stomach acid. Efficacy of treatment with omeprazole for the healing of gastric ulcers varies between horses and differs for squamous and glandular lesions. Healing rates of 67–87% are reported for squamous lesions following 28 days of omeprazole, whilst healing rates for glandular lesions are significantly lower (approximately 25%) (Sykes et al., 2015; Sykes, 2019). The reasons for lower healing rates of EGGD when treating with oral omeprazole are unknown.

Recurrence of ulceration is common after cessation of treatment in horses that are continually exposed to risk factors including low fibre-high carbohydrate feed or intermittent feeding, exercise and stress, with 73% of horses developing ulcers within 8 days of training (White et al., 2007) and 84-90% within 28-30 days (Andrews et al., 1999; McClure et al., 2005a&b). Horses in active race-training and regular competition therefore commonly receive long-term maintenance therapy with omeprazole to prevent ulcer development and recurrence, as recommended in the EGUS consensus statement (Sykes et al., 2015). Studies suggest that omeprazole at a dose of 1 or 2 mg/kg once daily can prevent the development or recurrence of ulcers in 79-86% of horses (Andrews et al., 1999; McClure et al., 2005 a & b; White et al., 2007). The long-term management of EGUS is expensive (Aranzalez and Alves, 2013) and prolonged treatment with proton pump inhibitors and the subsequent increase in gastric pH may have negative effects on digestion, including reduced calcium digestibility (Pagan et al., 2020, Sykes, 2021). Therefore, there is increasing interest in the development of a natural feed supplement to maintain gastric health and prevent gastric ulcers. Supplements investigated to date have had variable results and include antacids, aloe vera, lecithin-pectin derivatives, and blends of herbs, antioxidants and probiotics (Woodward et al., 2014; Sykes et al, 2014; Andrews et al., 2016; Bush et al., 2018). The efficacy of a banana supplement for the prevention of EGUS has not been investigated.

Rodents and equids have a similar gastric anatomical structure comprised by squamous and glandular mucosa (Matsukura et al., 1985). Therefore, the gastro protective effect of banana extract that was observed in rodents may also occur in horses. A natural product with anti-ulcerogenic properties would be of considerable benefit in this species.

2.4. Gaps in Current Knowledge

Whilst the health benefits of feeding green banana have been widely studied in other species, to our knowledge, studies evaluating the effect of dried green banana as a supplement to enhance total antioxidant capacity and gut microbiota or to prevent gastric ulceration in horses are not yet available. Given the high prevalence and recurrence rate of EGUS in horses that are continually exposed to risk factors including feed, exercise and stress, the investigation of more economical and efficacious supplements for the prevention of EGUS is warranted.

The health benefits identified in human and rodent models, specifically anti-ulcerogenic properties and gastrointestinal microbiome effects, are of particular interest to equine nutrition and health. It is hypothesised that a supplement derived from market reject raw bananas could potentially be utilised as a feed supplement to optimise gastrointestinal health and prevent gastrointestinal diseases such as EGUS in equine species.

Currently, the levels of antioxidants in the Banana Feeds supplement have not been investigated and the effects of processing the unripe banana are unknown. These factors will be investigated in this study.

3. METHODOLOGY

3.1. Study 1: Profiling of bioactive compounds within the dried green banana crumble

Samples of dried banana crumble, an equine feed supplement, were supplied by Banana Feeds Australia and analysed at SARDI Aquatic Sciences Environment and Analytical Laboratories. Samples were blended into powder and stored at -20°C until further analysis.

3.1.1. Moisture content

Samples were oven dried at 60°C in pre-weighed crucibles to a constant weight in an oven. The method was modified from Jansen *et al.* (2001). The results were expressed as percentage moisture on a 'as is' basis (%). A single measurement was performed per sample. Samples were also analysed using a Mettler Toledo HG63 moisture analyser at 105°C. The results were expressed as percentage moisture on a 'as is' basis (% as is). A single measurement was performed per sample.

3.1.2. Total antioxidants

Total antioxidant content was quantified by Folin-Ciocalteu assay involving colorimetric measurement of antioxidants that include both phenolic and non-phenolic compounds (Everette *et al.,* 2010). Analytical grade tannic acid and gallic acid were used as standards (Blainski *et al.,* 2013). Absorbance was measured at 765 nm using a BioTek Synergy 4 multi-plate reader. Sample blanks were run in triplicates. Samples spiked with a known concentration of the standard were used as quality control checks. A recovery rate of <5% deviation from the expected value was accepted. The total antioxidant content was expressed as weight equivalent (mg) of tannic acid (TAE) or gallic acid (GAE) equivalent per unit weight of the sample on an 'as is' basis (mg TAE/GAE. G^{-1}).

3.1.3. Total polyphenols

The Fe(III)/1,10-phenanthroline method used to quantify total polyphenols is based on electron transfer properties. Polyphenols are electron rich substrates that donate electrons to react with Fe(III) to form Fe(II) (Perron and Brumaghim, 2009). Absorbance was measured at 511 nm using a BioTek Synergy 4 multi-plate reader. Sample blanks were run in triplicate. Samples spiked with a known concentration of the standard were used as quality control checks. A recovery rate of <5% deviation from the expected value was accepted. Total polyphenol content was expressed as weight equivalent (mg) of tannic acid equivalent (TAE) per unit weight of sample on an 'as is' basis (mg TAE. G^{-1}).

3.1.4. Total flavonoid content

Total flavonoids were quantified by Aluminium chloride ($AlCl_3$) colourimetric protocols involving the formation of a colour complex with AlCl3 under alkaline conditions (Downes *et al*., 2010, Fernandes *et al.,* 2012). Flavonoids were quantified against quercetin standard on a BioTek Synergy 4 multi-plate reader at wavelength of 420 nm. Sample blanks were run in triplicates. Samples spiked with a known concentration of the standard were used as quality control checks. A recovery rate of <5% deviation from the expected value was accepted. The results were expressed as weight equivalent of quercetin (QE) per unit weight of the sample (mg QE g^{-1}) on an 'as is' basis. The quantifications were performed in triplicate.

3.1.5. Antioxidant activity

The antioxidant activity was quantified by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay. This technique has been optimised from the published techniques of (Ghasemi *et al*., 2009). The antioxidant activity

was measured as the concentration at which a 50% reduction in absorbance was recorded when reacted with DPPH. Colourimetric measurements were undertaken on a BioTek Synergy 4 multi-plate reader at 517 nm. Quercetin was used as a benchmark. Results are expressed as IC50 in mg mL^{-1}. The lower the IC50 value, higher the antioxidant activity of the sample. The assay was run in triplicate.

3.1.6. Statistical analysis
Data were analysed using two-way analysis of variance (ANOVA) using the statistical package Minitab 17.1.0 at a statistical threshold of P≤0.05. Tukey's pairwise comparison was used as a post hoc test to further determine statistically different samples and treatments.

3.2. Study 2: Effects of feeding dried green banana crumble on antioxidant status and faecal microbiome of horses

3.2.1. Horse details and study design
Thirty-two horses comprising 31 Standardbreds and one Thoroughbred, of which 9 were geldings and 23 mares, aged 3-19 years (median = 13 years) were included in the study. Horses were kept in bare, one-acre paddocks and fed *ad libitum* oaten hay, with free access to water. Four horses (ID No.1 to No.4) that were exercised received additional concentrates (1 kg/day Cool+ pellets, Laucke Mills) and one biscuit of lucerne hay / day, throughout the study.

Horses were divided into 2 groups, matched for age and sex: Group 1 received the banana supplement once daily at a dose of 100g/day orally, with a handful of chaff for 28 days and group 2 received no supplement (control) but received an equal amount of chaff. Two of the horses receiving additional feed were included in each group. All animal procedures were approved by The University of Adelaide Animal Ethics Committee before commencement of the study (AECS04/20, 34377) and conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2013).

3.2.2. Sample collection
Blood samples (10mL) were collected at baseline (time point A) and after 28 days (time point B) via jugular venepuncture. For biochemical analysis, blood was centrifuged at 4226 RPM for 10 minutes at 4°C and serum was collected in 2mL sterile vials and snap frozen in liquid nitrogen and stored at -80°C until analysis.

Faecal samples were collected at baseline (time point A) and after 28 days (time point B) from fresh manure piles from each horse, immediately after defaecation. Faecal balls were selected from the top or centre of the pile to ensure that they were uncontaminated from dirt and free from mechanical damage. Samples were collected in a snap lock bag and stored at 4°C before further processing (within 6 hours). A subsample from the centre of each faecal ball was extracted (according to the protocol described by Stewart *et al*., 2018) and placed into a 15mL polypropylene tube under sterile conditions to prevent cross-contamination. Samples were snap frozen with liquid nitrogen and stored in a -80°C freezer until analysis.

3.2.3. Serum biochemical analysis
Serum total antioxidant capacity (TAC) and malondialdehyde (MDA) were measured in duplicate using commercial EIA detection kits (Caymen, USA). The intra- assay coefficient of variation (CV) for both TAC and TBARS was 20%.

3.2.4. 16S rRNA amplicon sequencing and sample quality

A total of 64 faecal samples (32 at time point A and 32 at time point B) were sent for 16S rRNA amplicon sequencing. Samples were sent on dry ice to the Australian Genome Research Facility (AGRF) where DNA was extracted and PCR amplification and sequencing were performed. PCR amplicons targeting the 16S V3-V4 region were generated using the forward primer (341F) CCTAYGGGRBGCASCAG and reverse primer (806R) GGACTACNNGGGTATCTAAT. PCR amplicons sequencing was done on an Illumina MiSeq platform (San Diego, CA, USA) with a V3, 600 cycle kit (2 x 300 base pairs paired-end).

3.2.5. Bioinformatics analysis

The 16S sequenced data was processed using Quantitative Insights into Microbial Ecology II (QIIME2) (Bolyen *et al.,* 2019). DADA2 (Callahan *et al.,* 2016) plug in QIIME2 was run to denoise sequenced reads and the paired end reads of each amplicon was joined and chimeras were filtered. The denoise parameters was "—p-trim-left-f 20 —p-trim-left-r 20 —p-trunc-len-f 280 —p-trunc-len-r 220 –p-chimera-method consensus". The trimming criteria were based on raw reads quality plots and denoised reads were checked again for quality before the next step. To obtain operational taxonomic units (OTUs), sequences were aligned to the SILVA SSU 138 database (Quast *et al.,* 2013) and classified at the genus level with feature-classifier in QIIME2.

Rarefaction curve along with alpha and beta diversity metrics were generated using the diversity plug of "—p-sampling-depth 14785". Alpha diversity metrics were calculated using Faith's Phylogenetic Diversity (PD) (Faith, 1992) and beta diversity metrics was calculated using weighted UniFrac matrix (Lozupone *et al.,* 2007). Paired Wilcoxon rank-sum exact test was applied to detect any difference in alpha diversity between timepoint A and timepoint B. This test was performed for control and banana groups separately. Wilcoxon rank-sum exact test was used to test if control and banana groups at timepoint B had any significant change in alpha diversity. Principal coordinate analysis (PcoA) based on weighted UniFrac was conducted in R with the package qiime2R v0.99.23 (Bisanz, 2018). The aldex2 (Fernandez *et al.,* 2014) in QIIME2 was used to identify differential taxonomies, and the resulted p-values were adjusted and filtered by false discovery rate (Benjamini and Hochberg, 1995).

3.2.6. Statistical analysis (for antioxidant status)

Serum biochemical parameters (TAC and MDA) were analysed using repeated measures analysis of variance (ANOVA) using mixed model with the statistical package SPSS (version 27), including only those horses where the CV was below 20%. The model included ulcer (yes, or no), treatment (banana or control) and time (pre- and post-treatment) as fixed effects and horse was included as a random effect. For the TAC analysis this included 16 horses in the control group and 14 in the banana group and for MDA there were 12 horses in the control group and 15 in the banana treatment group. All data were checked for normality using Shapiro-Wilks and Q-Q plots.

3.3. Study 3: Clinical trial: Efficacy of dried green banana supplement for the prevention of Equine Gastric Ulceration Syndrome.

3.3.1. Animal recruitment

The study was designed to evaluate the noninferiority and superiority of the dried green banana supplement compared with omeprazole and no treatment respectively, by assessing the proportions of horses that did not develop EGUS while on an increasing level of exercise. A power calculation was performed based on previous work (Andrews e*t al.,* 1999; McClure *et al*., 2005a&b; White e*t al.,* 2007). With a one-sided test of proportions with a noninferiority margin of 20% and an assumed prevention rate of 80% in horses treated with omeprazole and 15% in control horses, 28 horses per treatment group would be required to achieve a power of 80%, with a significance level of 5%. A total of 84 horses were required, stratified by discipline: 42 racehorses and 42 performance horses.

Client owned horses were recruited for initial examination from the University of Adelaide's Equine Health and Performance Centre client list, social media, and word-of-mouth. Eligible horses included adult horses of any age, breed, sex or discipline that fulfilled the following animal specific selection criteria:

- Are on an increasing workload for example, from slow to fast work, none to slow work, horses returning to exercise from a spell or are in full work and have recently completed treatment for EGUS.
- Exercised at least 3 times per week.
- Should not be receiving medical treatment for EGUS during the study period
- Are free of other significant disease as reported by the owner/trainer.

3.3.2. Initial examination

Signalment, use, management factors including diet, housing and exercise regimen as well as a clinical history was recorded for each horse prior to a complete physical and gastroscopic examination. Horses were fasted for 12-14 h and water was withheld for 2h prior to gastroscopy. Horses were weighed and sedated with 0.01 mg/kg bwt detomidine intravenously (i.v). The gastroscopic examination was performed using a 300cm flexible video gastroscope (Aohua VET9830 Video-endoscope). The gastroscope was passed through a preplaced tube inserted into the cranial oesophagus. The stomach was insufflated with air and the mucosa flushed with water to remove any food material adhered to the stomach wall. If food remaining in the stomach was too great to allow adequate observation of the squamous and glandular mucosa, gastric emptying was considered prolonged, and the gastroscopic examination was repeated after an extended period of fasting. Horses were either fasted for another 4–6 h if the gastroscopy was repeated on the same day or fasted for 16–20 h if this took place on another occasion. Video-recordings were made of all examinations to allow for blinded, randomised review at a later date.

The gastroscopic examination included observation of the squamous fundus, the greater curvature, the lesser curvature, the glandular mucosa, and the pyloric antrum. The squamous regions were graded on a scale of 0–4, while the glandular regions were assigned subjected severity and graded descriptively as recommended by the 2015 European College of Equine Internal Medicine consensus statement (Sykes *et al.,* 2015). Maximal grades were recorded for all regions of both the squamous and glandular regions based on the consensus of two specialist clinicians. Horses were enrolled into the study if they had a maximal squamous ulcer grade of ≤2 or maximal glandular disease graded as mild.

3.3.3. Group allocation and treatment

This study was a 3-armed, parallel-group randomised clinical trial. Horses were stratified based on use (racehorses vs. performance horses) and randomly allocated to either the experimental group (Banana), the positive control group (Omeprazole) or the negative control group (Control). One of the investigators not conducting the gastroscopic examinations, was responsible for randomisation.

Horses were fed and exercised according to their usual routine as determined by the owner/trainer. Horses in the experimental group (Banana) were fed 100g/day of the dried green banana supplement in a small feed, as per the manufacturers' labelled dose recommendations. Those in the positive control group (Omeprazole) received 1 mg/kg bwt, orally, once a day (s.i.d), of a commercially available buffered omeprazole paste (Omoguard Paste®, Ceva Animal Health). Owners were instructed to administer the omeprazole approximately 30-60 min before the morning meal. For horses with access to *ad libitum* forage, owners were instructed to give the treatments early in the

morning. Horses in the negative control group did not receive any specific medications or supplements. All treatments were administered for 28 days.

3.3.4. Follow-up gastroscopy
Follow-up gastroscopy examinations were done on day 28 wherever possible. When a gastroscopy could not be performed on that day, horses remained on the treatment until it was carried out. The same background information was recorded as for the initial examination, including whether any difficulties were experienced with the treatment, changes in management, additional medications administered during the trial period and the current level and frequency of exercise. The gastroscopy and lesion grading were carried out as described for the initial examination. Treatment of horses with a lesion grade >2 in any area of the stomach was advised at the owner's cost.

3.3.5. Randomised, blinded gastroscopy review
Four of the investigators, three internal medicine specialists and one sports medicine specialist, evaluated the gastroscopy videos in a randomised, blinded manner at completion of the study. All the gastroscopy videos were deidentified and randomised, and the initial and follow-up gastroscopy videos were paired in a randomised manner by investigators not participating in video evaluation. Each pair of gastroscopy videos were independently evaluated by two of the specialist investigators. If a consensus grade for each area of the stomach was not achieved by the two evaluators, the video was re-evaluated by all four investigators as a group and a consensus grade assigned.

The squamous fundus, greater curvature, lesser curvature, glandular mucosa, and pyloric antrum were evaluated and graded. Each region was graded on a scale of 0–4, as recommended by the EGUS council (Andrews *et al.,* 1999b; Wise *et al.,* 2020) (Table 1). The investigators that did not participate in gastroscopy video review, subsequently reidentified the videos to assign initial and follow-up examination grades. These lesion grades were used for data analysis.

Lesion grade	Squamous mucosa	Glandular mucosa
0	The epithelium is intact and there is no visible hyperkeratosis	The epithelium is intact and there is no visible hyperaemia
1	The epithelium is intact but there are areas of hyperkeratosis	The epithelium is intact but there are areas of hyperaemia
2	Small single or small multifocal lesions	Small single or small multifocal lesions
3	Large single, large multifocal or extensive superficial lesions	Large single, large multifocal or extensive superficial lesions
4	Extensive lesions with areas of apparent deep ulceration	Extensive lesions with areas of apparent deep ulceration

Table 1: *The equine gastric ulcer council (EGUC) 5-point ordinal grading system used to grade the squamous and glandular mucosa for data analysis purposes. (Andrews et al., 1999b & Wise et al., 2020)*

3.3.6 Data analysis
The trial was designed to test the noninferiority of the dried green banana crumble with omeprazole and superiority to no administration of preventative medication (control). Population characteristics between groups were compared including age, sex, breed (Thoroughbred, Standardbred, other), body weight and discipline (racehorse or performance horse).

All ulcer data were analysed using SPSS (version 27). Squamous maximum score was defined as the maximum score across the squamous fundus, greater curvature and lesser curvature, glandular maximum was defined as the maximum score across the glandular mucosa and pyloric antrum.

Squamous and glandular maximum data were analysed using a two-way analysis of variance with the fixed effects of discipline and treatment and the two-way interaction. Age, sex, breed and body weight were not statistically significant (P<0.05) and so were not included in the model. The residual distributions were checked in each treatment group using Q-Q plots, frequency distributions and Shapirio-Wilk test for normality. A test for three-armed non-inferiority trials (using the Three-Armed package in R) was also performed on maximum squamous and glandular score data.

Health status was defined as binary variable where the maximum ulcer score increased (=yes) i.e. the ulcer got more severe, or remained the same/got better (=no). Since health status was considered as a binary trait, a logistic regression was fitted to the data with treatment and discipline and the 2-way interaction included in the model. Age, sex, breed and bodyweight were not statistically significant (P<0.05) and were not included in the final model.

4. RESULTS

4.1. Study 1: Profiling of bioactive compounds within the dried green banana crumble
4.1.1. Experiment 1: Preliminary biochemical benchmarking

This preliminary trial involved biochemical benchmarking of three trial samples to benchmark selected parameters. The three samples were analysed for total antioxidants, total polyphenols, total flavonoids and antioxidant activity.

Sample 1 had a total antioxidant content of 9.61 mg TAE g^{-1} on an 'as is' basis in contrast to sample 3 that recorded 4.57 mg TAE g^{-1} on an 'as is' basis (Figure 3).
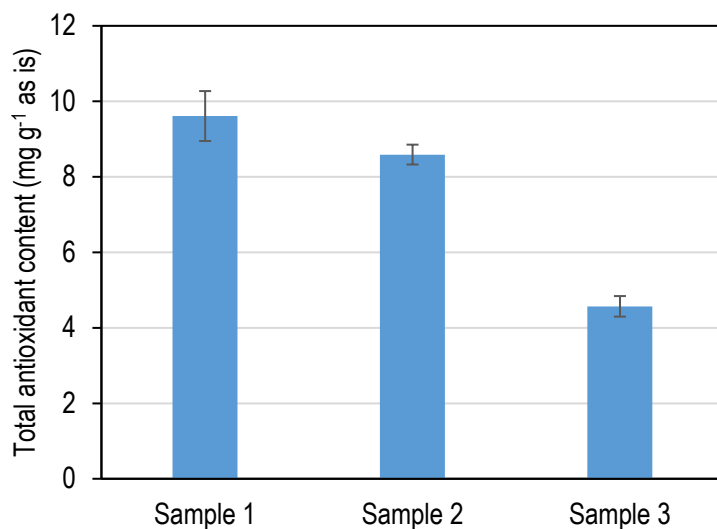
Figure 3: *Total antioxidant content in trial samples using tannic acid as standard. Values depicted (mg TAE g^{-1}) are mean ± SD (n=3).*

Total polyphenol content in sample 1 followed by sample 2 and least in sample 3 with concentrations of 6.76, 6.05 and 5.10 mg TAE g^{-1}, respectively (Figure 4).
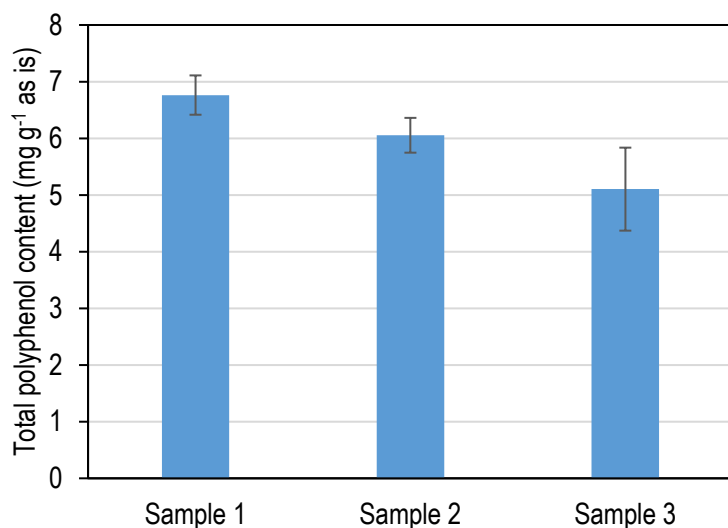
Figure 4: *Total polyphenol content in trial samples using tannic acid as standard. Values depicted (mg TAE g^{-1}) are mean ± SD (n=3).*

Total flavonoid content in the trial samples measured against quercetin standard showed a comparable trend to that for total antioxidants (Figure 5). Sample 1 registered the highest flavonoid content (2.45 mg QE g^{-1} 'as is' basis) and least in Sample 3 (1.66 mg QE g^{-1} 'as is' basis).
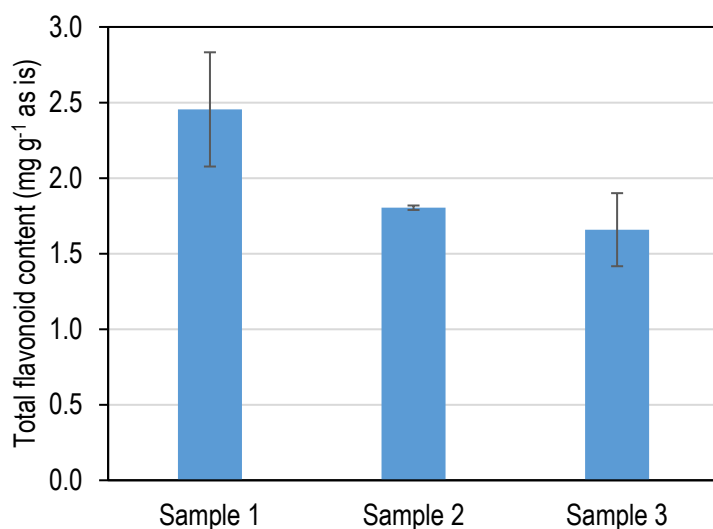
Figure 5: *Total flavonoid content in trial samples using quercetin standard. Values depicted (mg g^{-1}) are mean ± SD (n=3).*

All three samples tested displayed different levels antioxidant activity (Figure 6). Sample 1, with an IC50 value of 41.25 mg mL^{-1}, exhibited the highest antioxidant activity. In contrast, sample 3 registered the least antioxidant activity (IC50 value of 87.09 mg mL^{-1}) among all the samples tested.
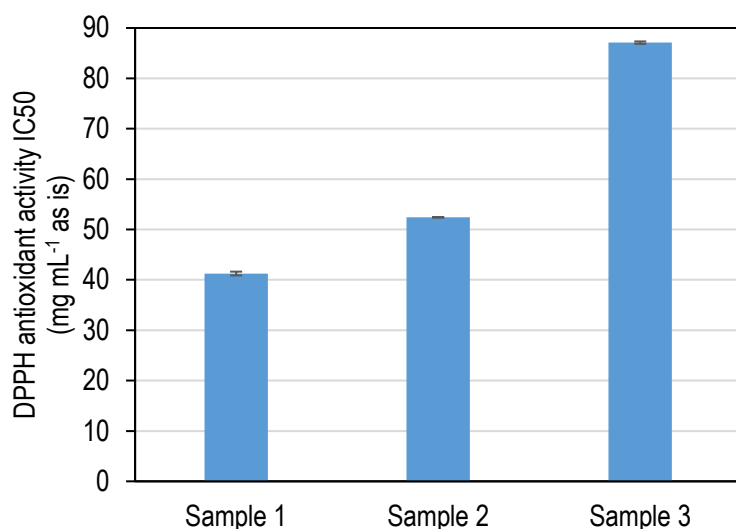
Figure 6: *Antioxidant activity in trial samples tested by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) method using quercetin as benchmark. Values depicted (mg mL^{-1}) are mean ± SD (n=3).*

4.1.2. Experiment 2: Comparing tannic acid and gallic acid as standards for the quantification of total antioxidant content

In Experiment 2, antioxidant content was measured by Folin-Ciocalteu method using both tannic acid and gallic acid as standards. Samples analysed in this experiment were trial samples 1, 2 and 3 as in Experiment 1 in addition to batches 3, 4 and 5. Statistically significant differences were observed between the two standards and the various batches of samples (p≤0.05). Post hoc analysis revealed significantly higher concentrations of antioxidant content measured by tannic acid over levels measured by gallic acid (p≤0.05). Among the various b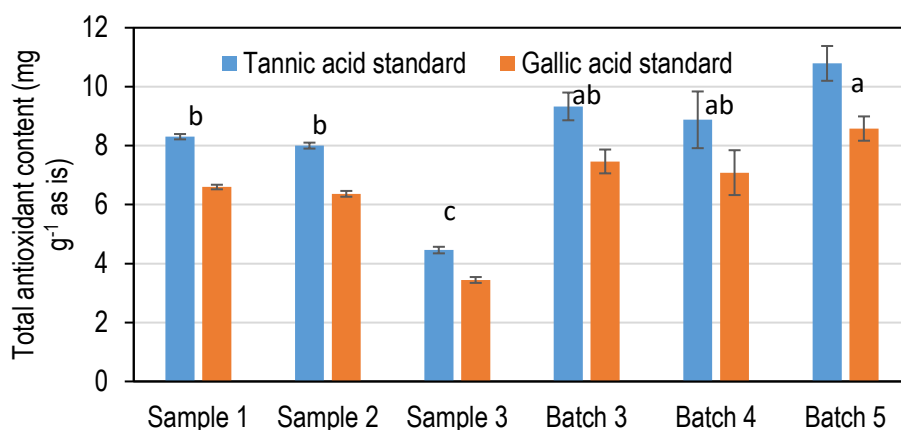atches analysed, batches 5, 3 and 4 registered significantly higher concentrations of antioxidants with mean values of 9.69, 8.40 and 7.98 mg g^{-1} on an as is basis (Figure 7).

Figure 7: *Comparison of antioxidant content in trial samples and batches of banana crumble using tannic acid and gallic acid as standard. Values depicted (mg TAE/GAE g^{-1}) are mean ± SD (n=3). Different letters denote significant differences (P < 0.05) among samples.*

4.1.3. Experiment 3: Quantification of the antioxidant content in all banana crumble samples quantified using gallic acid as a standard

The antioxidant content in banana crumble samples (excluding the three trial samples) were analysed by Folin-Ciocalteu method with gallic acid as a standard (Figure 8). In this experiment, Batch 5 (including the repeat batch 5) was observed to contain the highest amount of antioxidants, equating

to 8.99 and 9.04 mg g^{-1} GAE on an 'as is' basis. Batch 10 was the next highest at 7.65 mg g^{-1} GAE on an 'as is' basis. There was no significant difference in antioxidant content between Batch 5 and Batch 10.

Sample to be used for equine feeding trials (feeding trial tubs 1, 2, 3 and 4) were also tested. Variability in antioxidant content was observed between the tubs with tubs 3, 2 and 4 registering higher concentrations of 5.81, 5.50 and 5.32 mg g^{-1} GAE, respectively on an 'as is' basis. Tub 1 contained 4.31 mg g^{-1} GAE on an 'as is' basis, the least of all the tubs. Among all samples, batch 9 was found to have the least amount of antioxidants at 4.07 mg g^{-1} GAE on an 'as is' basis.
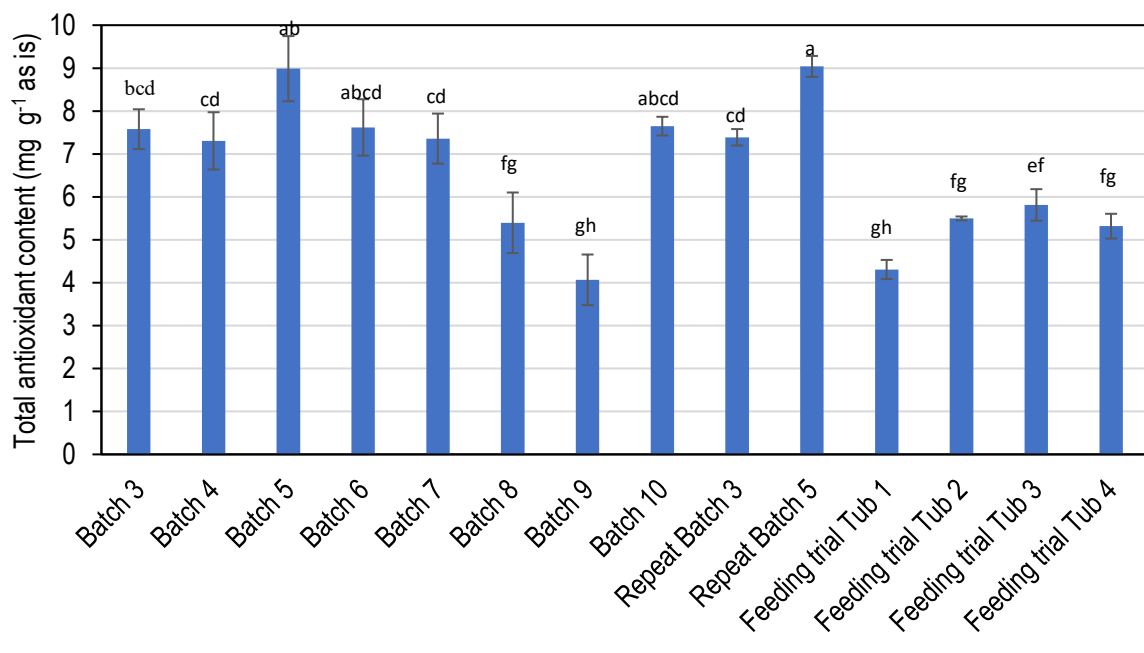
Figure 8: *Total antioxidant content in banana crumble samples. Values depicted (mg GAE g^{-1}) are mean ± SD (n=3). Different letters denote significant differences (P≤0.05) among samples.*

4.2. Study 2: Effects of feeding dried green banana crumble on the antioxidant status and faecal microbiome of horses

4.2.1. Horse details
All horses assigned to the banana treatment group ate the supplement without issues. No side effects of treatment were reported during the trial.

4.2.2. Antioxidant Status
There was a significant difference between the 2 treatment groups in the baseline TAC levels P=0.031. The banana treated group started 46% higher (1.747 vs. 1.200 mM) than the control horses. There was no statistically significant change in TAC concentration between the control and banana treatment groups (P=0.181). There was a slight increase in TAC in the banana treated horses (mean difference = +0.08mM) relative to a small decrease in the control horses (mean difference = -0.107mM) (Figure 9).
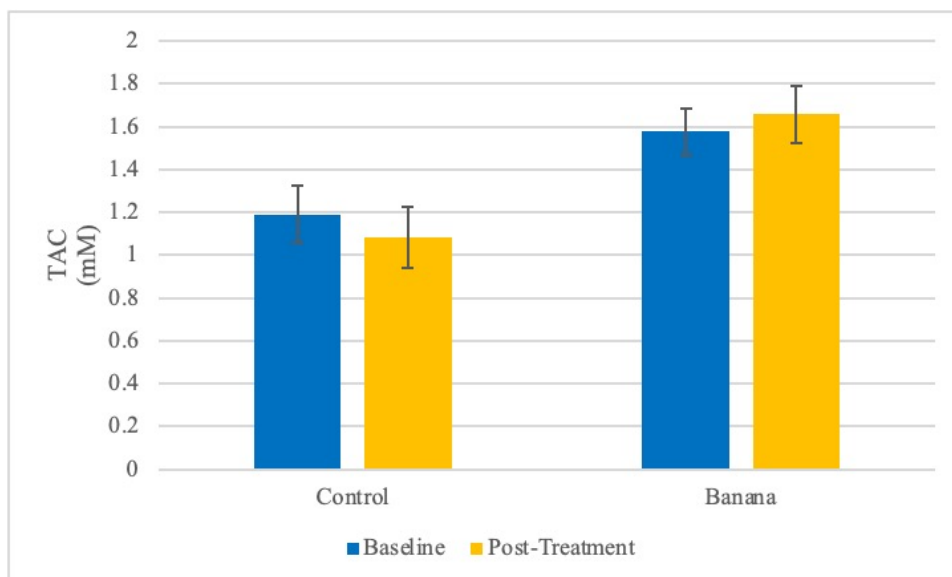
Figure 9. *TAC concentration for each treatment and the baseline and post-treatment time points.*

There was no significant change in MDA concentration between the control and banana treatment groups and time interaction (Mean +/-SE for control, baseline = 9.720 +/- 1.454; control, post-treatment = 8.512 +/-1.330; banana, baseline =7.340 +/- 1.295; banana post-treatment = 7.202 +/- 1.242) (P=0.658) indicating that the banana and control groups responded similarly over time. Both treatment groups tended to have reduced MDA levels at the post -treatment time point, however this was not significant.

4.2.3. Faecal microbiome

4.2.3.1. Alpha and beta diversity analysis
There was no significant difference in alpha diversity between timepoint A and timepoint B, and between control and banana groups (Figure 10). The PCoA plot for beta diversity metric revealed no clustering of control and banana groups (Figure 11A, 11B). This suggests there was unlikely any major changes in microbiota after banana supplementation using the current dose over a period of 28 days.
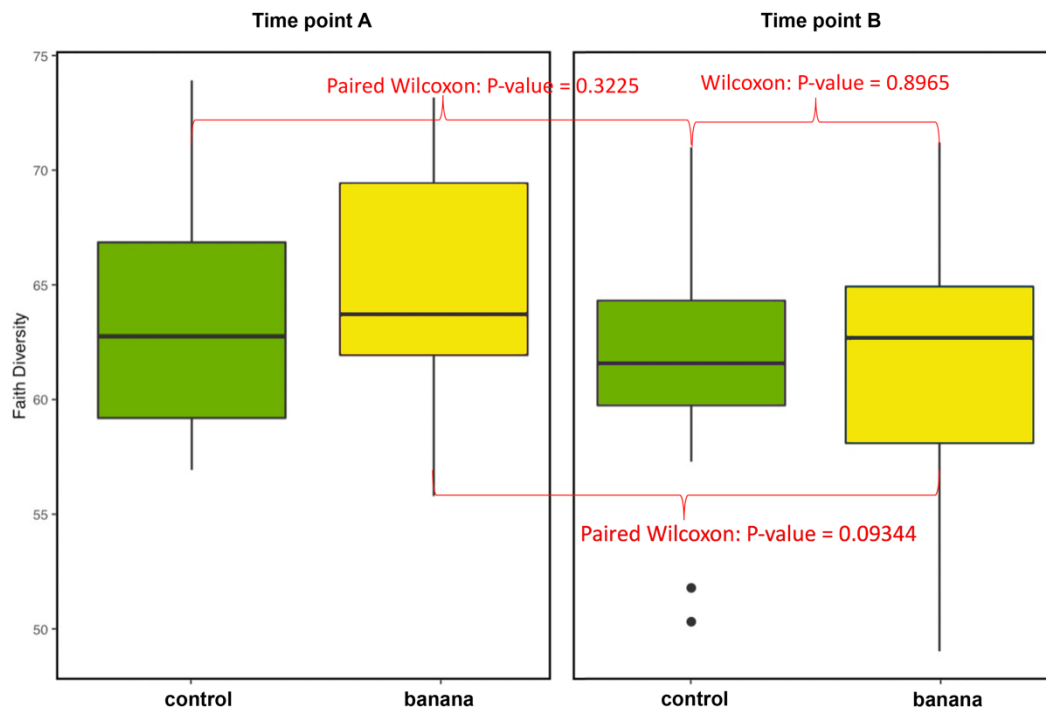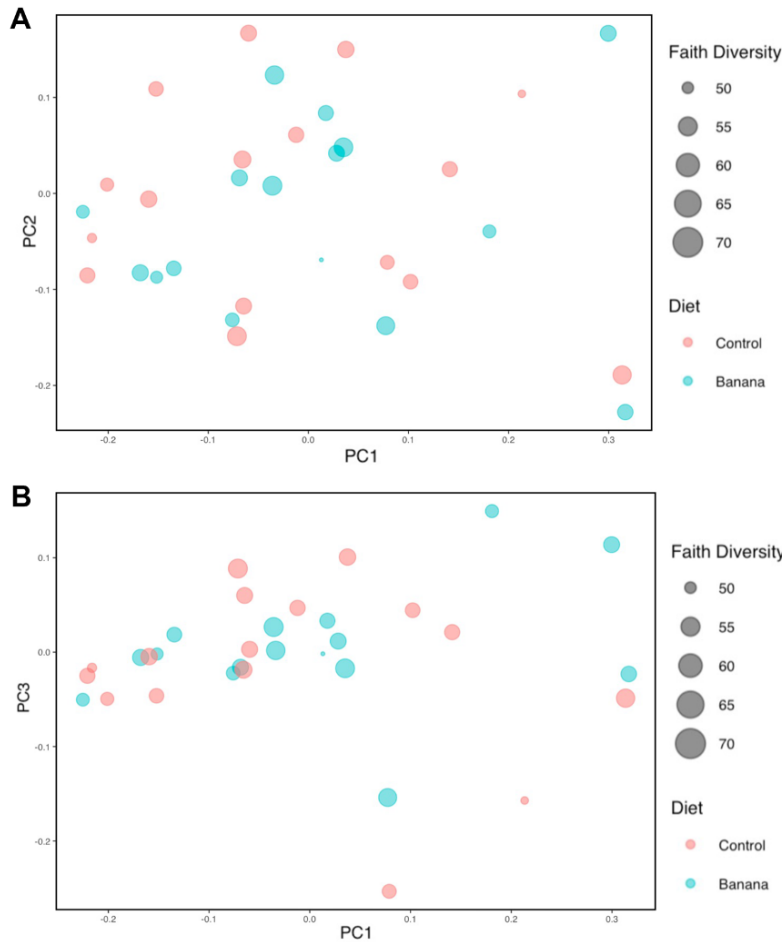
Figure 11A & B. *Beta diversity PCoA plot of weighted UniFrac values between samples.*

4.2.3.2. Taxonomy identification

No significant differential OTU was identified at the genus or phylum level between control and banana groups at either timepoint. The communities were dominated by members of the Firmicutes and Bacteroidetes phyla (figure 12).

The top 30 average abundant taxonomies were ranked at the genus level across all samples that grouped into four categories (control time point A, treatment time point A, control time point B, treatment time point B) to create a heat map (Figure 13). There was no clear difference between the groups. The most abundant genus was *p-251-o5* (mean abundance 7.66%), followed by genus *Phascolarctobacterium* (mean abundance 6.80%), *Rikenellaceae RC9 gut group* (mean abundance 5.94%), *Treponema* (mean abundance 5.00%) and *Christensenellaceae R-7 group* (mean abundance 4.14%).
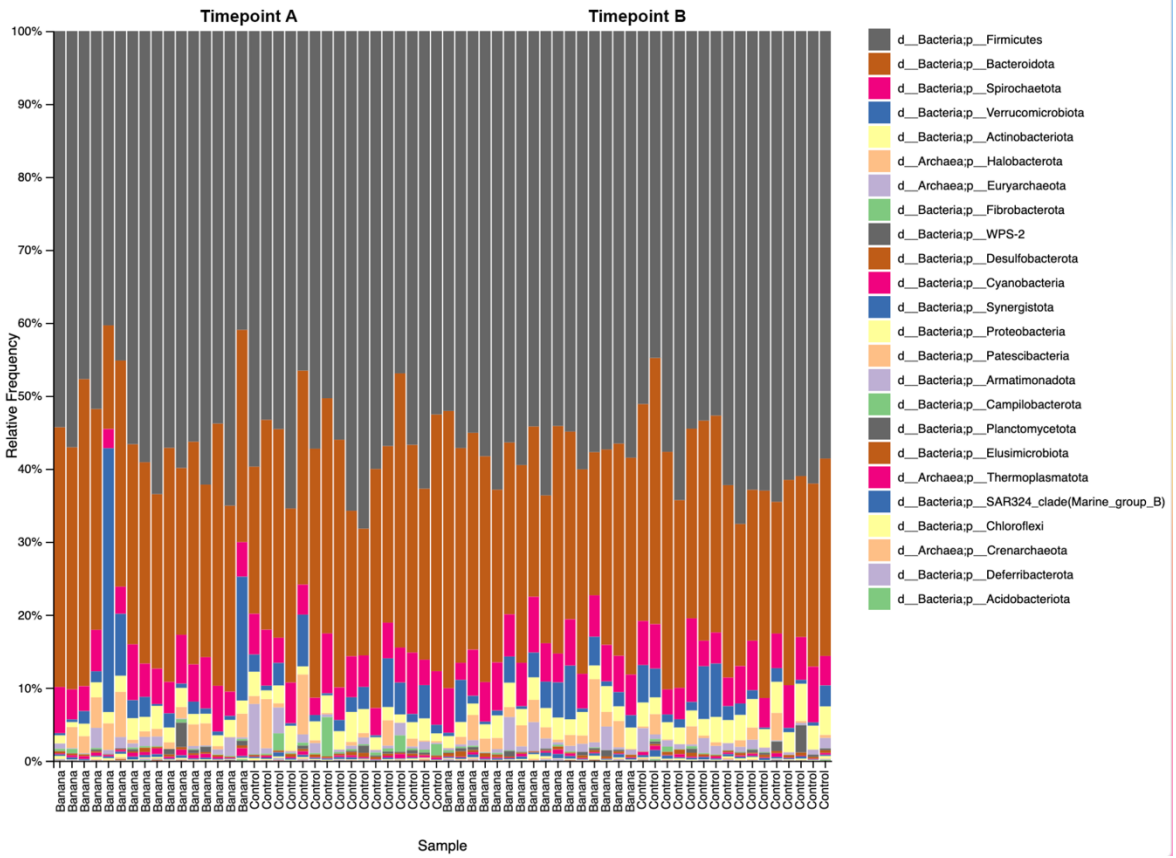
Figure 12. *Plot of relative abundance of different bacterial phyla identified in the study.*
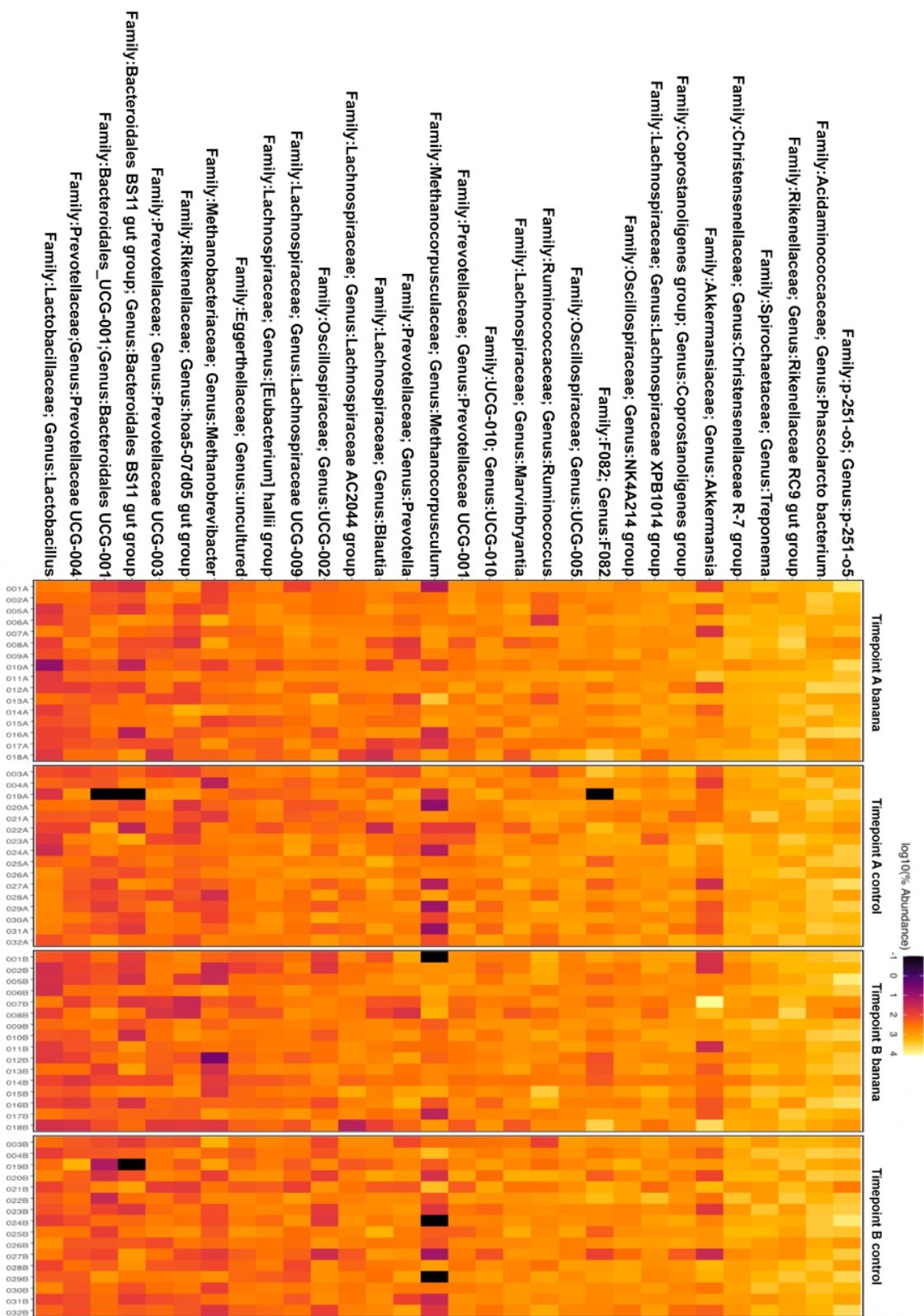
Figure 13. *The heatmap of top 30 most abundant taxonomies at the genus level.*

4.3. Study 3: Clinical trial: Efficacy of dried green banana supplement for the prevention of Equine Gastric Ulceration Syndrome.

4.3.1. Horses

A total of 153 underwent initial gastroscopic examination for inclusion in the trial. Of these, 97 horses met all the inclusion criteria and were enrolled in the study; 12 horses were withdrawn from the study because of factors unrelated to the study (retired, spelled, colic, lost to follow-up). A total of 85 horses were retained in the study; 29, 29 and 27 horses were assigned to the banana, omeprazole and control groups respectively (Figure 14). Blinded randomised review of the gastroscopic videos resulted in exclusion of 2 and 4 horses from the ESGD and EGGD analysis respectively. These horses were excluded as the grades assigned during blinded review were >2 for either ESGD or EGGD or both. Two horses that were originally assigned to the banana group refused to eat the supplement and were reassigned to the control group. No adverse effects of treatment with banana or omeprazole were reported.
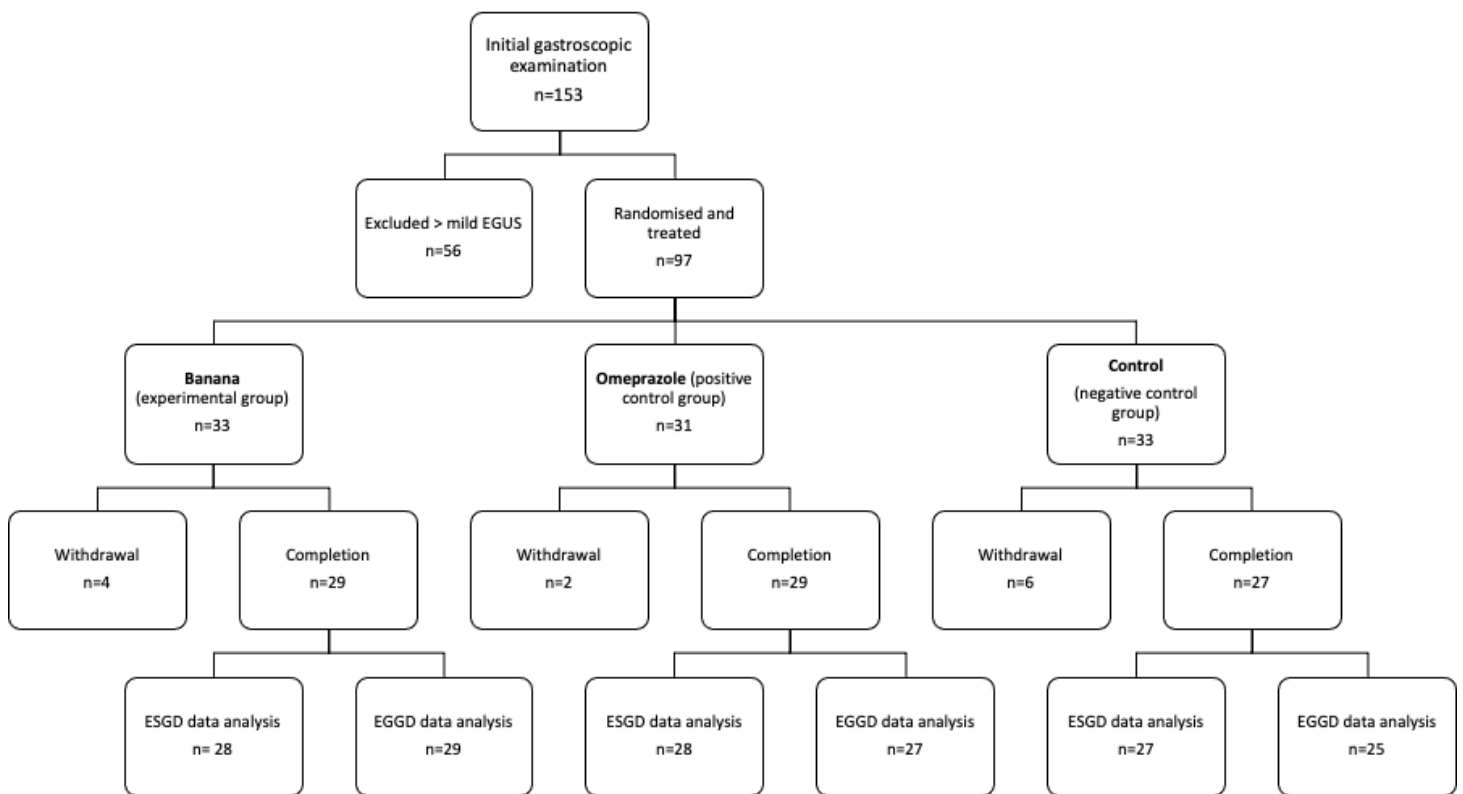
Figure 14: *Patient recruitment flowchart.*

4.3.2. The efficacy of dried green banana supplement for the prevention of Equine Squamous Gastric Disease

A total of 83 horses were included in the equine squamous gastric (ESGD) disease analysis; control group (n=27), banana group (n=28) and omeprazole group (n=28).

4.3.2.1. Comparison of maximum squamous scores

The mean, median and distribution of maximum squamous scores of the population and for each treatment group at initial examination (pre-treatment) and after treatment (post-treatment) can be seen in table 2.

	Number of horses (n)	Squamous Maximum Score		
		Pre-treatment	Post-treatment	P-value
Population mean (SEM)	83	1.31 (0.08)	1.54 (0.1)	
Population median (range)		1 (0 - 2)	2 (0 - 3)	
Control mean (SEM)	27	1.07 (0.13)	1.6 (0.17)	0.016
Control median (range)		1 (0 – 2)	2 (0 – 3)	
Banana mean (SEM)	28	1.39 (0.15)	1.79 (0.16)	0.080
Banana median (range)		2 (0 – 2)	2 (1 – 3)	
Omeprazole mean (SEM)	28	1.46 (0.14)	1.21 (0.18)	0.230
Omeprazole median (range)		2 (0 – 2)	1 (0 – 2)	

Table 2: *Mean ± standard error of the mean (SEM) and median and range of maximum squamous scores of the population and for each treatment group at initial examination (pre-treatment) and after treatment (post-treatment).*

Squamous maximum score frequency was continuous and normally distributed. The mean and median squamous maximum grades (Table 1) were not significantly different between treatment groups both pre- and post-treatment (Kruskal-Wallis test x^2(2) = 5.407 p=0.067 [pre-treatment] and x^2(2) = 4.76, p=0.092 [post-treatment]). There was no significant effect of breed, sex, age or body weight on squamous maximum scores. Pairwise comparison (using student T-test) of before and after treatment maximum squamous scores within groups, revealed a significant increase in the control group (p=0.016) but not in the banana (p=0.080) or omeprazole (p=0.230) groups (Table 2 and Figure 15).

The two-way analysis of variance of the post-treatment squamous maximum scores, revealed a significant difference between racehorse and performance horses (p=0.016), where the mean squamous score for racehorses was 1.79 and performance horses was 1.32. There was no interaction effect between the horses' discipline and treatment groups (p=0.844). The main effect of treatment (difference between treatment groups) had a significant influence on post-treatment squamous maximum scores (p=0.044). Pairwise comparisons revealed that the estimated post-treatment

maximum squamous scores in the banana group (1.80 ± 0.17) was significantly higher than the omeprazole group (1.23 ± 0.17; p=0.016), but not the control group (1.64 ± 0.17; p=0.487). The control group was not significantly higher than the omeprazole group (p=0.083; Figure 15).
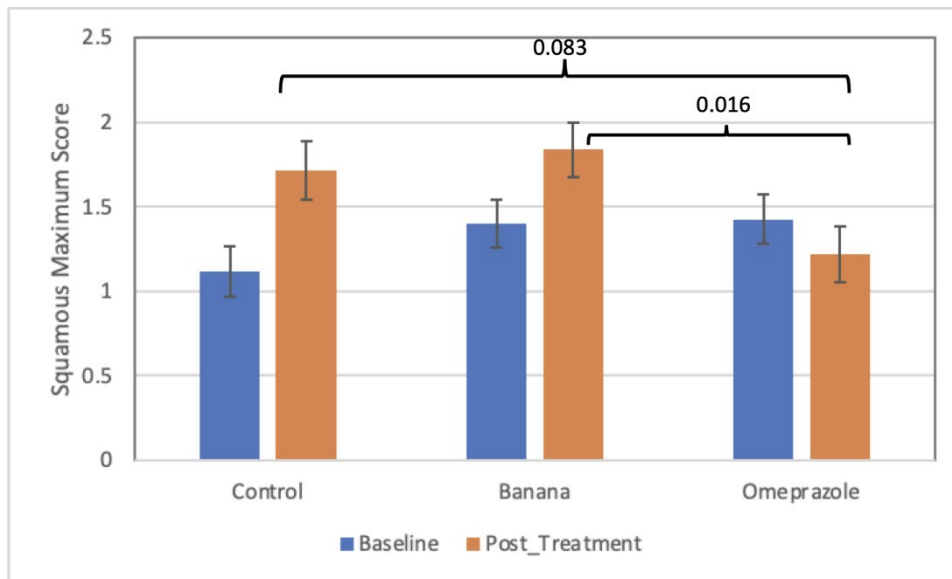
Figure 15. *Mean (± standard error) of squamous maximum score before treatment (Baseline) and after treatment (Post_Treatment) for each treatment group. Overall differences between treatment groups post-treatment were statistically significant (p=0.044). The post-treatment maximum squamous scores in the banana group was significantly higher than the omeprazole group (p=0.016), but not the control group (p=0.487) and the control group was not significantly higher than the omeprazole group (p=0.083).*

4.3.2.2. Comparison of the change in maximum squamous scores

The change in maximum squamous score is defined as the difference when the after treatment maximum squamous scores are subtracted from the before treatment scores. When evaluating the average change in maximum squamous score the two-way analysis of variance revealed no significant difference between racehorse and performance horses (p=0.070) however racehorses increased their squamous maximum score on average 0.487 compared with only 0.003 in performance horses. The interaction effect between the horses' discipline and treatment groups was not significant (p=0.571). Treatment had a significant effect (p=0.032) on the change in maximum scores when comparing all three groups. Subsequent pairwise comparisons revealed that the estimated change in maximum squamous scores in the banana group (0.41 ± 0.23) was significantly higher than the omeprazole group (-0.24 ± 0.23; p=0.046), but not the control group (0.57 ± 0.23; p=0.614). The control group was significantly higher than the omeprazole group (p=0.014; Figure 16).
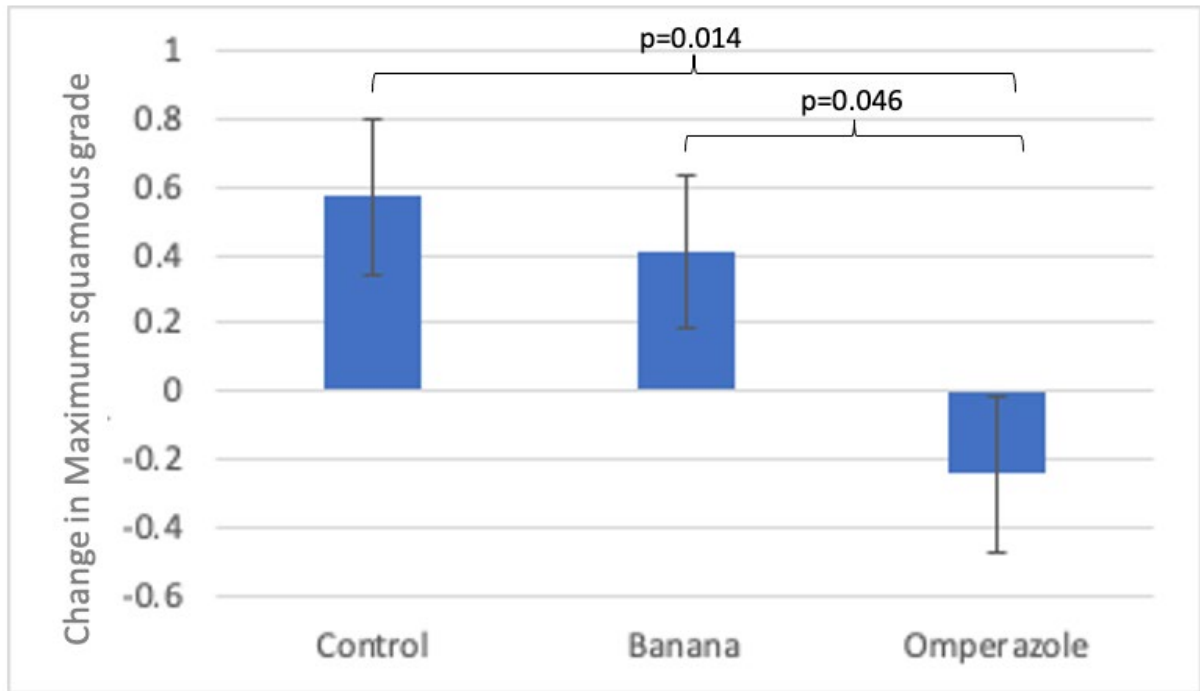
Figure 16. *Mean ± standard error of the mean (SEM) of the change in maximum squamous score for each treatment group. The change in maximum squamous score is defined as the difference when the after treatment maximum squamous scores are subtracted from the before treatment scores. Overall differences between groups were statistically significant (p=0.032). The change in maximum squamous scores in the banana group was significantly higher than the omeprazole group (p=0.046), but not the control group (p=0.614) and the control group was significantly higher than the omeprazole group (p=0.014)*

4.3.2.3. Comparison of binary trait of maximum squamous scores
A binary trait was created to define the health status of the horse, where health status was defined as either got worse (squamous maximum score change >0) or stayed the same/improved (squamous maximum score change ≤0).

Binary logistic regression analysis revealed no significant difference between racehorse and performance horses (p=0.109) and there was no interaction effect between the horses' discipline and treatment groups (p=0.916). The main effect of treatment was not statistically significant (p=0.071) overall. However, pairwise comparison revealed that 52%, 40% and 21% of horses in the control, banana and omeprazole groups, respectively, got worse. Significantly more horses in the control group got worse when compared to the omeprazole group (p=0.012) but not when compared to the banana group (p=0.347). The difference in proportion of horses that got worse in the banana group was not significantly different to the omeprazole group (p=0.125). Horses in the banana and control groups were 2.43 and 4.07 times more likely, respectively, to demonstrate worsening of the maximum squamous score when compared to horses in the omeprazole group (table 3).

Status	Stayed the same/ improved (n/%)	Worse (n/%)	OR	CI	P-value
Control (n=27)	13 (48.15)	14 (51.85)	4.07	1.23 – 13.5	0.012
Banana (n=28)	17 (60.71)	11 (39.29)	2.43	0.74 – 8.1	0.125
Omeprazole (n=28	22 (78.47)	6 (21.43)			

Table 3: *Cross tabulation of binary trait for health status and treatment for maximum squamous scores. Odds ratios are presented relative to Omeprazole. Proportions presented as number (percentage) relative to the group size. OR, odds ratio; CI, confidence interval; n, number; %, percentage*

4.3.2.4. Non-inferiority analysis

Three-armed non-inferiority analysis comparing banana and control groups to omeprazole could not demonstrate non-inferiority of the banana treatment (p=0.284), because of the wide variation of data (seen as the wide CI bars; Figure 17). Whilst the banana treatment was not different from the omeprazole based on the pre-defined delta value of 0.2 it was also not different from the control. The control was inferior to omeprazole (p=0.012).
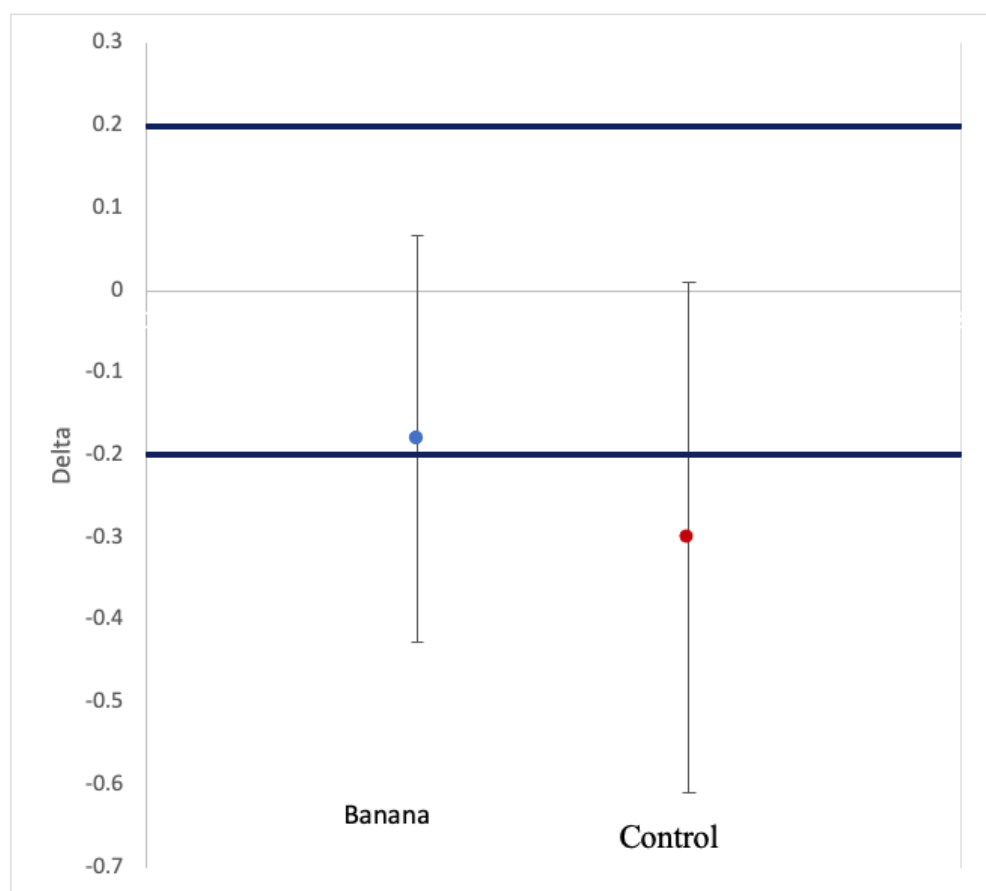
Figure 17. *Non-inferiority margin (delta = 0.2) banana and control relative omeprazole. Mean (dot), 95% confidence intervals (CI, bars). Banana was not non-inferior to omeprazole; control was inferior to omeprazole (p=0.012).*

4.3.2.5. The influence of dried green banana supplement batch on maximum squamous scores

Binary logistic regression analysis (Table 4) comparing the previously described binary traits (got worse or stayed the same/improved) of maximum squamous scores, revealed that 71% of horses that received batch-15 improved or stayed the same, compared with only 55% that received batch 5, although this difference was not statistically significant (p=0.383). Batches with less than 10 horses were excluded from analysis.

Batch	Got worse	Stayed the same/improved	Total
5	5 (45.5%)	6 (54.5%)	11
15	4 (28.6%)	10 (71.4%)	14

Table 4: *Cross tabulation of binary trait for health status and dried green banana supplement batch, for maximum squamous scores. Batches with less than 10 horses were excluded from analysis. Data presented as number (percentage).*

4.3.3. The efficacy of dried green banana supplement for the prevention of Equine Glandular Gastric Disease

A total of 81 horses were included in the equine glandular gastric disease (EGGD) analysis; control group (n=25), banana group (n=29) and omeprazole group (n=27) as previously for analysis of squamous gastric disease.

4.3.3.1 Comparison of maximum glandular scores

The mean, median and distribution of maximum squamous scores of the population and for each treatment group at initial examination (pre-treatment) and after treatment (post-treatment) can be seen in table 5.

Glandular maximum score frequency was continuous and normally distributed. There were no significant differences between the treatments in pre- or post- treatment glandular maximum score (p=0.490 & p=0.820 respectively). Overall, an increase in average glandular maximum scores from baseline to post- treatment was observed which was statistically significant (p=0.007). The average change in glandular maximum score across all treatments was 0.272 meaning that on average the glandular maximum scores increased during the treatment period for all three treatments. Breed, sex, weight and age were not significantly associated with the change in glandular maximum change. Although this increase in glandular maximum scores was observed from baseline to post- treatment, no significant interaction between treatment and time was seen (p=0.852) indicating that all 3 treatments increased the glandular maximum score by a similar margin pre- and post-treatment (Figure 18).

	Number of horses (n)	Glandular Maximum Score		
		Pre-treatment	Post-treatment	P-value
Population mean (SEM)	81	1.10 (0.08)	1.37 (0.1)	0.007
Population median (range)		1 (0 - 2)	2 (0 - 3)	
Control mean (SEM)	25	0.96 (0.15)	1.28 (0.18)	0.148
Control median (range)		1 (0 – 2)	1 (0 – 3)	
Banana mean (SEM)	29	1.14 (0.12)	1.45 (0.16)	0.131
Banana median (range)		2 (0 – 2)	2 (0 – 3)	
Omeprazole mean (SEM)	27	1.19 (0.14)	1.37 (0.16)	0.383
Omeprazole median (range)		2 (0 – 2)	1 (0 – 3)	

Table 5: *Mean ± standard error of the mean (SEM) and median and range of maximum glandular scores of the population and for each treatment group at initial examination (pre-treatment) and after treatment (post-treatment).*
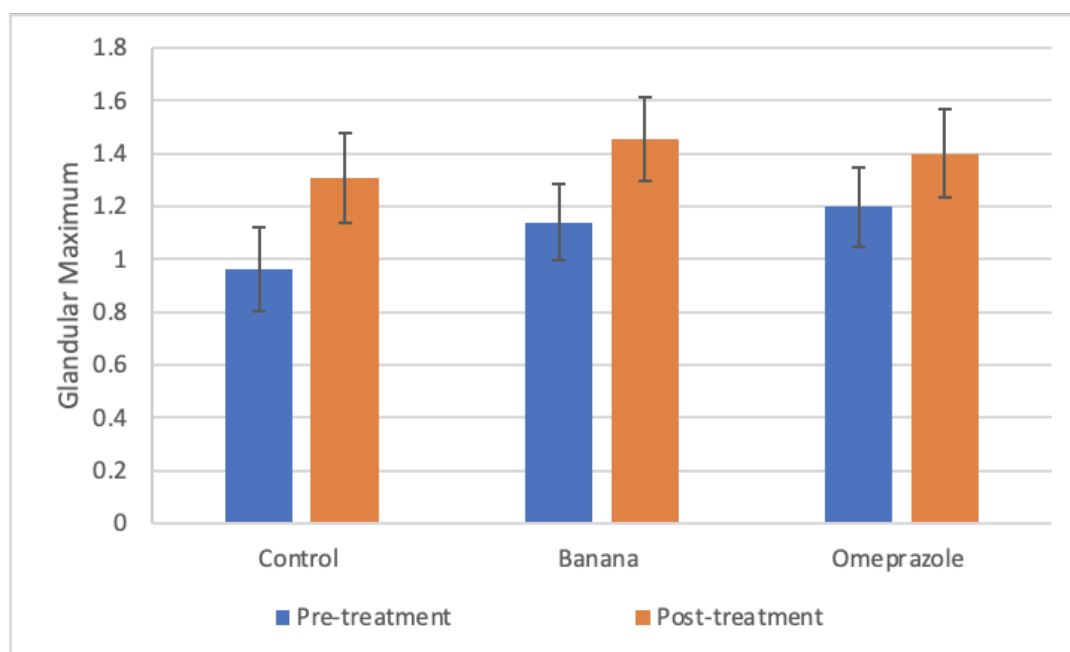
Figure 18. *Mean Glandular Maximum Score (± standard error) data before treatment (Baseline) and after treatment (Post-Treatment) for each treatment group. Differences between groups were not statistically significant (p=0.935)*

4.3.3.2 Comparison of binary trait of maximum glandular scores

When evaluating the glandular maximum score improvement as a binary trait defined as either got worse (glandular maximum score change >0) or stayed the same / improved (glandular maximum score change ≤ 0), no association between glandular maximum score improvement and treatment was observed ($x^2(2)$ = 0.285, p = 0.867). Overall, omeprazole and banana treated horses demonstrated less chances of worsening when compared to control although the differences were not statistically significant. A total of 33% of omeprazole treated horses got worse compared with 34% of the banana treated horses and 41% of the control horses. Omeprazole horses were 0.78 times less likely to demonstrate worsening of the glandular maximum score when compared to control horses which was followed by the banana group which was 0.89 times less likely to worsen the glandular maximum score when compared to controls (Table 6).

Status	Stayed the same/ improved (n/%)	Worse (n/%)	OR	CI	P-value
Omeprazole (n=27)	18 (66.66)	9 (33.34)	0.78	0.25 – 2.39	0.810
Banana (n=29)	19 (65.51)	10 (34.49)	0.89	0.29 – 2.70	0.661
Control (n=25)	15 (60.0)	10 (40.0)			

Table 6: *Cross tabulation of binary trait for health status and treatment for maximum glandular scores. Odds ratios are presented relative to Control. Proportions presented as number (percentage) relative to the group size. (OR, odds ratio; CI, confidence interval; n, number; %, percentage)*

4.3.3.3 The influence of dried green banana supplement batch on maximum glandular scores

Binary logistic regression analysis (Table 7) comparing the previously described binary traits (got worse or stayed the same/improved) of maximum glandular scores revealed that 71% and 73% of horses that received batch-15 and 5 respectively, improved or stayed the same (p=0.618).

Batch	Got worse	Stayed the same/improved	Total
5	3 (27.3%)	8 (72.7%)	11
15	4 (28.6%)	10 (71.0%)	14

Table 7: *Cross tabulation of binary trait for health status and dried green banana supplement batch, for maximum glandular scores. Batches with less than 10 horses were excluded from analysis. Data presented as number (percentage).*

5. DISCUSSION

5.1. Profiling of biologic compounds in the dried green banana supplement

A review of the published literature revealed that Folin-Ciocalteu assay is commonly used to quantify either total antioxidant content or in some cases polyphenol content. Based on the published literature, we opted to use Folin-Ciocalteu assay to quantify total antioxidants. This was also based on the fact that the reagent used in the assay did not exhibit specificity and reacted with a wide range of antioxidants that included non-phenolic compounds as well as polyphenolic compounds (Everette *et al.,* 2010). However, as polyphenols were the most dominant antioxidant in most plant tissues, this assay provided a reasonable estimate of the total phenolic content for most plant based samples. Similarily, Fe(III)/1,10-phenanthroline assay is based on electron transfer process. Polyphenols are electron donors that react with Fe(III) to form Fe(II) (Perron and Brumaghim 2009). This assay was therefore noted to be more specific to polyphenols. Both tannic acid and gallic acid have been used as standards for the quantification of antioxidants (Blainski *et al*., 2013).

From the trials comparing tannic acid and gallic acid as standards to quantify antioxidant content, it was observed that the concentrations quantified using tannic acid was 1.3 times higher than gallic acid. It is assumed that the use of tannic acid as a standard may lead to overestimation of the total antioxidant content. However, more studies are required to establish this observation. As several publications on quantification of antioxidants in banana used gallic acid as a standard (Fatemeh *et al.,* 2012; Rebello *et al*., 2014; Pico *et al*., 2019), gallic acid was therefore chosen for quantification of total antioxidants in this study.

Various studies on antioxidant (polyphenol) content in bananas have reported values as low as 0.1 mg GAE 100 g^{-1} DW (González-Montelongo, *et al*., 2010) to concentrations as high as 2000 mg GAE 100 g^{-1} FW (Shian *et al.,* 2012) equating to 100 mg GAE g^{-1} DW based on a moisture content at ~80 % FW (Rajkumar *et al*., 2012). However, the concentrations reported by most studies (Someya *et al*., 2002; Fatemeh *et al*., 2012; Amri and Hossain 2018) was comparable to our results (~ 4 – 9 mg GAE g^{-1} on a 'as is' basis). Overall, samples containing higher antioxidant content were also reported to have higher total flavenoid content and antioxidant activity.

It is well established that the antioxidant content, flavonoids, polyphenols and antioxidant activity are dependent on environmental variables, biotic variables and processing conditions. Factors such as nutritional state, spatio-temporal distribution of the farms, exposure to various environmental conditions, in addition to factors such as differences in varieties, stage of ripeness and tissue type are known to have a significant bearing on the antioxidant content. As an example, banana peels are known to be a richer source of antioxidants than pulp. Equally important are the processing methods adopted to manufacture the final product. Parameters such as exposure to air, moisture, duration of processing and exposure to heat, are also known to influence antioxidant content in the final product. There is an opportunity for further research to optimise variables under a range of processing and storage conditions to maximise the antioxidant capacity of the product, to continue delivering a high quality product. In testing the product, there is evidence of variation due to the natural elements of the product, namely, green dried whole bananas.

5.2. Effect of feeding the green banana supplement for 28 days on total antioxidant capacity in the blood of horses

No significant difference in TAC of blood was observed after feeding the supplement for 28 days, although there was a very small increase in TAC of banana-fed horses and slight decrease in TAC of control horses. The values for TAC measured in this study were similar to those previously reported in horses in the literature using the same methodology (Brummer *et al*., 2013; Shawaf *et al.,* 2020). Similarly, there was no change in MDA (a marker of oxidative stress) over time in either group. The values of MDA recorded in this study were higher than those previously reported in control horses but

similar to those reported in horses with EGUS (Shawaf *et al*., 2020). Many of the horses in the study had evidence of EGUS, which may explain the higher MDA values observed.

Previous studies have shown that human plasma antioxidant defences can be increased in response to consumption of certain types of fruit including bilberries, red grapes, cherries, strawberries and kiwi fruit, as evidenced by an increase in the TAC 1 – 2 hours after consumption of 300g of fruit (Harasym and Oledski, 2014). Other studies have investigated the effects of banana consumption on antioxidant status in humans. Leelarungrayub *et al.,* (2017) investigated the effects of banana consumption in 38 healthy, sedentary males. They found that consumption of 400g ripe banana pulp daily for 4 weeks resulted in a significant increase in TAC (1.59+/-0.11 mMol/L) compared with the control period (1.01+/-0.13mMol/L). At the same time, MDA was significantly reduced (2.11+/- 0.34 uMol/L versus 3.11 +/-0.16uMol/L). Yin *et al.,* 2008 found that the consumption of a single banana meal (containing 400g of banana blended in 300mL of water) reduced plasma oxidative stress and enhanced the resistance to oxidative modification of low-density lipoprotein in healthy individuals. Sae-Teaw *et al*., 2012 also investigated the effects of consumption of the juice of 1kg oranges, pineapple or 2 bananas (190g) on serum antioxidant capacity by ferric reducing antioxidant power (FRAP) analysis and oxygen radical antioxidant capacity (ORAC) assay in 12 healthy males. Serum antioxidant capacity was increased following consumption of all 3 fruits.

The lack of a significant change in TAC in the horses examined in our study may be related to a number of factors. The bioactive molecules in the feed supplement may not have been bioavailable in the horse and may not have reached the bloodstream, the dose of antioxidants may have been insufficient to elicit a significant increase in the blood TAC or the length of treatment time may have been too short.

The gastrointestinal system of the horse is different to that of other monogastrics, including humans and rodents, and it is uncertain if the bioactive components in the supplement will enter the blood stream or target organs (McKeever, 2017). The bioavailability of flavenols in cranberry powder have been investigated in horses (Liburt *et al.,* 2008), with the key finding that uptake was rapid, with metabolites appearing in the blood within 15 minutes and excretion in urine occurring at hours.

Natural supplements considered to be high in antioxidants, including orange peel, black tea extract, cranberry extract and ginger failed to produce an effect on oxidative stress or antioxidant status in horses after a single dose (Smarsh *et al.,* 2010). It was concluded that longer term supplementation might be required to elicit changes. Most antioxidant supplementation trials in horses over a period of 4-8 weeks before potential effects are evaluated (Kirschvink *et al.*, 2008). One field study of oral antioxidant supplementation in horses was performed over 3 months, with some parameters unchanged after 6 weeks and only showing significant differences after 12 weeks (DeMoffarts *et al.,* 2005).

Finally, it is suggested that in some studies horses may have insufficient level of oxidative stress for antioxidant supplementation to have a beneficial effect and that nutraceuticals may have most benefit in times of severe oxidative stress such as a disease state or intense endurance type exercise (Smarsh *et al.,* 2010). The horses in this study were largely sedentary although they did appear to exhibit some evidence of oxidative stress, as indicated by the values of MDA which were higher than previously reported in other studies of control animals (Brunner *et al*., 2013; Shawaf *et al.,* 2020).

5.3. Effect of feeding the green banana supplement to horses for 28 days on faecal microbiome
This study found no change in faecal microbiome of horses after 28 days of green banana supplementation at a dose of 100g once daily. There was no significant difference in alpha and beta diversity, and OTU between horses fed the banana supplement and the control group. Previously,

green banana has been shown to modulate gut microbiota in other species, increasing the relative abundance of Bacteroides (Wu *et al.,* 2020; Tian *et al.,* 2020).

Recently, Boshuzien *et al.* (2021) investigated the effect of aleurone supplementation on equine faecal microbiome in healthy horses receiving a forage diet and found a dose-dependent effect. Although there was no change in alpha or beta diversity in that study, there was a change in the relative abundance when the supplement was fed at increasing doses from 100-400g. It is possible that the dose of 100g/day in this study did not promote any changes in faecal microbiome because it is quite low in comparison with the total amount of fed ingested daily. Inter-individual variation in response to dietary change is also reported (Proudman *et al.*, 2015; Bosuzien *et al.,* 2021) and there may have been insufficient subjects in this study to identify a significant change between treatment groups.

The horses in this study were kept on a diet of *ad libitum* forage. In the current trial, the most abundant genus observed in faecal samples of horses were *p-251-o5* phylum Bacteroidetes (7.66%), followed by genus *Phascolarctobacterium* phylum Firmicutes (6.80%)*, Rikenellaceae RC9 gut group* phylum Bacteroidetes (5.94%), *Treponema* phylum Spirochaetes (5.00%) and *Christensenellaceae R-7 group* phylum Firmicutes (4.14%; Figure 11). *Salem et al*. (2018) identified Bacteroidetes, Firmicutes, Fibrobacteres, Spirochaetes, Verrucomicrobia and Proteobacteria phyla at a relative abundance greater than 1% in horses kept at pasture over a 12-month period. However, the dominant phyla were Bacteroidetes and Firmicutes, similar to the findings of this study. Bulmer *et al*. (2019) also noticed that, independent of the diet, the bacterial profile was dominated by Firmicutes and Bacteroidetes phyla. These authors also showed that 18 OTUs from Firmicutes, 1 OTU from Bacteroidetes and 1 OTU from Proteobacteria phylum were significantly different when horses were fed different diets (high-fibre vs. high-starch diets). From the 18 OTUs of the Firmicutes phylum, 17 belong to the Clostridia class (order Clostridiales) and 1 OTU was from the Bacilli class (Lactobacilli order).

It has been observed that forage-only diets promote greater microbial stability, evidenced by lower microbial counts and relative abundances of specific lactic acid producing bacteria (Willing *et al.*, 2009). High-starch diets can alter the hindgut microbiota of horses (Julliand and Grimm*,* 2017; Bulmer *et al.*, 2019). Previous research showed that even small addition of starch to the diet is enough to alter bacterial populations in the hindgut. However, the extent of the changes to faecal microbiota can be influenced by the source of starch fed to horses (Harlow *et al.,* 2016). In the present study no deleterious effect on microbiome was observed for horses fed the green banana supplement. More investigation is needed to determine if green banana can modulate the equine gut microbiome.

5.4. Efficacy of feeding the green banana supplement to horses for prevention of equine gastric ulcer syndrome
In this study we identified some beneficial effect of feeding dried green banana supplement for the prevention of squamous ulcers but not glandular disease. For squamous disease, there appears to be some benefit of feeding banana over control, but the supplement was not as efficacious as omeprazole.

Over the 28-day trial period, banana supplementation prevented the development or worsening of squamous ulcers in 61% of horses compared with 48% of control horses and 79% of those receiving omeprazole. Significantly more control horses experienced worsening of ulcers than the omeprazole group. However, there was no significant difference when comparing the banana group with either those treated with omeprazole or the control horses.

Horses fed the banana supplement or omeprazole did not have a significant difference in the mean grade of ulceration before and after 28 days of treatment, whilst control horses showed a significant increase in mean ulcer grade. However, when the difference in severity was examined over time, the change in ulcer grade was significantly higher in both control horses and those fed the banana

supplement than horses treated with omeprazole. Put differently, the average ulcer grades of horses in both the banana and control groups increased whereas the average ulcer grades in the omeprazole group decreased.

The same potential benefit of feeding dried green banana for the prevention of squamous ulceration was not observed for the prevention on glandular gastric disease. Neither banana nor omeprazole showed a significant benefit for the prevention of glandular disease in comparison with control horses.

These findings differ from studies of the anti-ulcerogenic effects of plantain banana and banana extracts in rodent studies, which have demonstrated a protective effect against experimentally-induced lesions of the glandular stomach (Best *et al.,* 1984; Lewis *et al.,* 1999, Lewis and Shaw, 2001) which was equivalent to omeprazole (Prabha *et al.,* 2011, Alesse *et al.,* 2017, Prasaad *et al.,* 2020). The effects on the squamous portion of the stomach have not been investigated in other species. However, antioxidants have been shown to attenuate the severity of reflux oesophagitis, and prevent oesophageal mucosal damage in rats (Rao *et al.*, 2008).

Most studies investigating the anti-ulcerogenic effects of banana in rodents have been performed using plantain bananas rather than dessert varietals, such as those used in the banana product investigated in this study. Best *et al*., 1984 found ripe dessert varieties, including cavendish to be ineffective in prevention of ulcers. However, another study using unripe Cavendish bananas (Dunji *et al.,* 1983) did show a protective effect against acute lesions, although only incomplete, temporary protection was observed in a chronic model of disease.

The active anti-ulcerogenic ingredient in plantains has been identified as leucocyanidin, a natural flavonoid (Best *et al.,* 1984; Lewis *et al.,* 1999, Lewis and Shaw, 2001, Prahba *et al*., 2011). Variations in study results may be explained by the different protocols used. Total flavonoid content of banana varies widely between cultivars, between the pulp and the peel and stage of ripeness, with higher concentrations in green versus ripe fruit and in peel compared with pulp (Fatemah *et al.,* 2012; Morais *et al.,* 2015). Other factors may also influence the activity of phytochemicals such as flavonoids. It has been reported that the antiulcerogenic activity of banana is lost when heated above 50C (Best *et al.,* 1984). In that study, the Cavendish bananas were dried at 180C, which may have explained the lack of activity.

The exact mechanism of action for the anti-ulcerogenic effects of banana remains uncertain. Flavenoids are known to exhibit anti-inflammatory activity (Rathee *et al.,* 2009) and have also been shown to stimulate gastric mucosal prostaglandin E2 production and reduce acid secretion from gastric parietal cells (Beil *et al.,* 1995). Prasad *et al*., (2020) found that although acidity was reduced following treatment with banana extract, this was less than observed with omeprazole. Increased mucus and bicarbonate production subsequent to growth of mucosal cells also likely play a role in strengthening mucosal defences (Best *et al.,* 1984; Lewis and Shaw, 2001; Alese *et al*., 2017). Alese *et al.,* 2017 reported a progressive, dose-dependent increase in mucus secreting cells. Other studies also found a dose-dependent effect on prevention of ulcers (Lewis and Shaw, 2001).

The dose of banana is also likely to be important for horses. When the effect of batch was investigated for prevention of squamous ulcers there appeared to be some variation between batches which may influence the efficacy of the dried green banana supplement. This warrants further investigation.

Dunji *et al.,* (1983), suggested that other components of banana may also contribute to the anti-ulcerogenic effect, including the pectin content. Several studies have investigated the effect of pectin-lecithin complexes for the prevention or treatment of EGUS, with varying results. Venner *et al.,* (1999) found a significant reduction in both squamous and glandular lesions after 10 days of feeding a pectin-lecithin complex to horses with EGUS. Similarly, Ferrucci *et al.,* 2008 reported that feeding of a pectin-

lecithin complex at a dose of 50mg/100kg per day resulted in improvement of EGUS (predominantly squamous lesions) in 9/10 horses after 30 days. However, others found that feeding horses a pectin-lecithin feed supplement did not prevent or minimise the risk for gastric ulceration of the squamous mucosa, using an experimental ulceration model (Murray and Grady, 2002; Sanz *et al.,* 2014). It has been suggested that pectin-lecithin complexes may be more efficacious when fed in combination with other compounds. Woodward *et al.* (2014) found a pectin-lecithin mixture supplemented with antacid, reduced the severity of gastric ulceration over a 35d period. Sykes *et al.* (2014) also found some benefit of feeding a supplement containing a combination of a pectin-lecithin complex (Apolectol), a live yeast and magnesium hydroxide, for preventing the development or exacerbation of existing EGUS.

The efficacy of several other studies evaluating natural feed supplements for the prevention or treatment of EGUS have also been reported in recent years. These include Sea Buckthorn berries (Huff *et al.,* 2012), aloe vera (Bush *et al.,* 2018), and other products containing combinations of these botanical nutraceuticals (Andrews *et al.,* 2016); (Kerbyson *et al.,* 2016). Similar to green banana, Sea Buckthorn berries are reported to be high in antioxidants, including flavonoids. When fed to horses, no beneficial effect was observed for prevention of squamous ulcers, however, glandular scores were lower in treated horses compared with controls, suggesting that there may be some efficacy for prevention of glandular ulcers (Huff *et al.,* 2012). In contrast, a subsequent study to investigate a pelleted supplement containing a blend of sea buckthorn, glutamine, aloe vera, pectin and lecithin as well as other herbs, amino acids, soluble fibres and probiotics, showed some benefit for the prevention of worsening of squamous lesions (Andrews *et al.,* 2016). This was suggested to be related to the synergistic actions of a combination of ingredients found in the product and not due to a single ingredient. Aloe vera, has also been used for prevention and treatment of gastric ulcers in humans and animal models, with its anti-ulcerogenic effect attributed to a variety of possible mechanisms including it anti-oxidant activity, anti-inflammatory properties, cytoprotective and mucus stimulatory effects, and its ability to regulate gastric acid production (Borelli and Izzo, 2000; Yusuf *et al.,* 2004; Bora *et al.,* 2011). When fed to horses with EGUS, although 56% showed improvement of ulcer scores, aloe vera was found to be inferior to omeprazole for treatment of squamous lesions (Bush *et al*., 2018). However, no control group was included. Furthermore, there were insufficient animals with glandular lesions to be able to determine any effect.

The results from our study are similar to previous studies investigating the benefits of various nutraceuticals for the prevention and treatment of EGUS, where a potential benefit was observed when compared to controls. However, none appear to be as effective as omeprazole. Further optimisation of the dose or combination with other compounds may improve the efficacy.

6. CONCLUSIONS AND RECOMMENDATIONS

The studies performed on the dried green banana supplement found some benefit for preventing the development of or exacerbation of squamous gastric ulcers in horses. The mechanism for this remains uncertain but may be related to the antioxidant capacity or the pectin content of the product, as in other species. We were unable to show a significant effect of banana supplementation on total antioxidant capacity or faecal microbiome in the small number of horses studied. Variation in antioxidant status of different batches were identified and an insufficient dose may explain our findings.

The report analysis performed identified 4 potential areas for further investigation seeking product optimisation:
    1) Improvement of total antioxidant content consistency between batches

2) Dose-response effect on ulcer desired effect
3) Effect of supplementation time to desired effect
4) Pharmacometric modeling for optimal dose response and duration to effectiveness

Based on results discussed within this report, a high variability of total antioxidant content (mg g^{-1} as is) between different raw banana batches was noted. Therefore, standardisation of processing is recommended in order to avoid disparity between total antioxidant content between batches. This could provide improved reliability and consistency of the product in regards to antioxidant activity when administered to horses.

The effect of storage temperature and time have not been investigated and could have an effect on total antioxidant content given the high variability observed between the different batches within this report. Furthermore, normal biological variability within the raw product could also be a possibility for the differences between batches observed within this report. Further investigation in order to determine the source for total antioxidant content variability between batches is recommended. Factors that could be influencing this include the source of raw product (geographical location), storage of raw product prior to processing (temperature and time), the processing itself, as well storage after processing (temperature, time and storage container properties).

The dried green banana was provided as a small feed at a dose of 100 grams per day as per manufacturer's labelled dose. Consequently, horses with different body weights received a different total dosage of dried green banana per day which could also affect not only the total antioxidant capacity measured (weights varied between 420-574 kg for the horses enrolled in the total antioxidant portion of the study), but also the potential benefit of this product in the prevention of EGUS. Previous studies using powdered Indian plantain banana as an anti-ulcerogenic compound in rats, found a dose-response relation with higher doses providing the best ulcer index scores. Therefore, further investigation of the effect of different dosages on total antioxidant capacity as well as ulcer prevention is recommended. Also, all horses allocated to the banana treatment group received the supplement for only 28 days. It is unknown at this stage if the treatment duration influences total antioxidant capacity as well as prevention of EGUS. Further studies evaluating the optimal duration and dosage of this dried green banana supplement for the antioxidant benefits as well as prevention of gastric ulceration in horses is warranted.

Population pharmacometric modelling studies may allow for quantitation of the impact of horse-specific demographic (e.g. weight, age, sex, level of training), total antioxidant content and total antioxidant capacity on beneficial effects of dried green banana for the prevention of EGUS. Dose regimens and treatment duration likely to achieve best outcomes (e.g. higher total antioxidant capacity and improved EGUS scores) and phenotype identification that predicts an individual horse's ideal dosage (race horse versus performance horse dosage) may be able to be determined using Monte-Carlo simulations.

7. ACKNOWLEDGEMENTS

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