Investigation of the equine glandular gastric microbiota in association with equine glandular gastric disease in Thoroughbred racehorses using 16S sequencing

Sarah J. Voss BVMedSci (Hons) BVM BVS MRCVS

Submitted in fulfilment of the requirements for the Master Degree in Veterinary Medicine

School of Veterinary Medicine

College of Medical, Veterinary and Life Science (MVLS)

University of Glasgow

August 2020

Abstract

Background: Equine glandular gastric disease (EGGD) is a condition common to sports and leisure horses world wide, and is characterised by inflammatory and erosive lesions affecting the glandular gastric mucosa. The aetiopathogenesis of EGGD has not been fully elucidated, and success rates of treatment are poor compared to equine squamous gastric disease (ESGD). The role of pathogenic bacteria has often been proposed, but no single pathogen has been consistently identified in association with EGGD lesions. Despite altered gastrointestinal microbiota community profiles being implicated in many other disease processes in the horse, the normal equine gastric microbiota has not been established. Establishing the normal and EGGD-associated glandular gastric microbiota may help to improve understanding of the aetiopathogenesis of this common but poorly understood syndrome, and may assist in optimising treatment protocols.

Objectives: Investigate the equine gastric glandular microbiota *in vivo* in a population of Thoroughbred racehorses subject to the same management conditions. We aimed to evaluate the microbial population at sites of EGGD lesions compared to normal mucosa, and investigate whether a dysbiosis or specific candidate pathogen may be implicated in the pathogenesis of EGGD. A secondary objective was to explore the use of transendoscopic cytology brushes for acquisition of mucosal samples appropriate for 16S sequencing.

Hypothesis: We hypothesised that the glandular gastric microbiota would show inter-individual variation, but that a dysbiosis would be associated with EGGD lesions in a population of Thoroughbred racehorses.

Materials and Methods: Two different cohorts of Thoroughbred racehorses were examined (Yard One, and Yard Two). At Yard One, eight Thoroughbred racehorses from one racing yard were examined gastroscopically following a report of poor performance from the trainer. Samples were taken from EGGD lesions and adjacent normal mucosa using guarded transendoscopic cytology brushes and frozen at -80 °C. DNA was extracted for 16S rRNA sequencing, and sequences compared against a database to generate taxonomic classification of the microbiota. At Yard Two, five Thoroughbred racehorses from one training centre underwent gastroscopy as part of poor performance investigation The

same horses, and one additional animal, were sampled again six months later. Samples underwent a similar DNA extraction and sequencing process as for Yard One.

Results: In Yard One samples *Proteobacteria* was the predominant phylum present (median abundance of 67% at normal mucosa, and 65% at EGGD lesions). This was followed by *Bacteroidetes* and *Firmicutes*. Alpha diversity analysis demonstrated EGGD lesions to be more diverse compared to normal mucosa; a finding that approached statistical significance ($p = 0.052$). Yard Two normal mucosa samples were characterised by a higher proportion of *Proteobacteria* (46.3 %) compared to lesions (18.9 %) ($p = 0.017$). Relative abundance of *Firmicutes* was lower in samples from normal mucosa (20.0 %) compared to lesions (41.2 %) ($p = 0.006$). Linear discriminant analysis effect size (LEfSe) confirmed that a greater proportion of *Firmicutes* species was characteristic of samples collected from EGGD lesions. This was due to a very high relative abundance of *Sarcina* (up to 92.4 %) associated with EGGD lesions in two horses from Yard Two. Across both data sets weighted and unweighted UniFrac analysis demonstrated that samples were of increased similarity if they were collected from the same horse, or collected from the same yard. This effect was stronger than whether samples were from EGGD lesions or normal mucosa. Finally, samples were pooled and recategorized into three groups according to lesion description. When beta diversity analysis was performed on the pooled sample samples did not appear similar according to which lesion group they represented.

Conclusion: There is evidence to suggest that the gastric microbiome is altered in horses with EGGD, although we are unable to demonstrate a causative effect. *Sarcina* was identified as a potential biomarker of disease and warrants further investigation as a gastric pathogen in the horse. There is an effect of inter-individual variation on the gastric microbiota community, and this effect appears to be of more importance than the difference between normal and diseased glandular mucosa. We have also demonstrated that sheathed cytology brushes can be used as an effective method of sampling the gastrointestinal microbiota transendoscopically in live animals.

Contents

List of tables

List of figures

Normal mucosa
EGGD lesions

- **Fig 4.1: PD Whole Tree metric rarefaction curve comparing sample richness of EGGD lesion samples and normal mucosa, which are comparable. Rarefaction curve indicates samples were sequenced in adequate depth.**

- **Fig 4.2: PD Whole Tree alpha diversity metric boxplots comparing normal mucosa and EGGD lesion samples. There was no significant difference between the two groups.**

- **Fig. 4.3: Stacked bar charts comparing bacterial community composition at phylum level between pooled normal mucosa samples and pooled EGGD lesion samples. *Firmicutes* dominate in EGGD lesion samples, whereas *Proteobacteria* are the predominant phylum in normal glandular mucosa samples.**

- **Fig 4.4: Stacked bar charts comparing normal mucosa to EGGD lesions at a genus level. Coloured horizontal bars represent different genera, with each bar representing an individual genus. Due to the extremely large number of different genera identified, a colour coded key is impractical. The tan bar (indicated) denotes the proportion of *Sarcina* in EGGD lesion samples.**

- **Fig. 4.5: 100% stacked bar charts displaying individual samples demonstrating the increased abundance of *Sarcina* in TB02.1, TB03.4, and TB03.5 samples, which represent EGGD lesions.**

- **Fig. 4.6: (A) weighted and (B) unweighted PCoA plots indicating sample similarity in three-dimensional space. There is no clustering or separation of samples according to whether they are from normal mucosa or EGGD lesions.**

- **Fig. 4.7: Cladogram of LEfSe analysis results showing taxa significantly associated with normal mucosa and EGGD lesions. Each concentric ring represents a level of taxonomic rank, moving from kingdom most centrally, to genus most peripherally. Each small circle represents a**

single taxon, and circle size is proportional to relative abundance of that taxon.

- **Fig 4.8: LEfSe analysis bar chart indicating effect size on bacterial community composition for different taxa for normal mucosa and EGGD lesion samples.**

- **Fig. 4.9: Rarefaction analysis of operational taxonomic units (OTUs) generated using the PD Whole Tree metric from all glandular gastric mucosa samples pooled, comparing the first (S1) and second (S2) sample dates. Overall richness between the two sampling occasions is similar, and samples were sequenced to an adequate depth.**

- **Fig.4.10: 100% stacked bar chart comparing normal mucosa and EGGD lesion samples acquired at S1 and S2 sampling dates for all horses with longitudinal data available. Samples are named according to individual horse ID (e.g. TB01), followed by sequential sample numbers, and correspond to data given in Table 1. Samples are grouped by individual animal. Samples prefixed with a '2' were collected at S2. † denotes an EGGD lesion sample.**

- **Fig 4.11: Unweighted PCoA plot displaying samples by horses for which longitudinal data were available. There is evidence of clustering according to horse regardless of sampling occasion.**

- **Fig 5.1: rarefaction curve comparing Yard One and Yard Two samples, showing the increased richness of Yard Two compared to Yard One.**

- **Fig 5.2: PD Whole Tree alpha diversity boxplot comparing yard one and two, showing increased alpha diversity of Yard Two compared to Yard One ($p = 0.001$)**

- **Fig 5.3: alpha diversity boxplot by lesion group. Group 0, normal mucosa; Group 1, erythematous mucosa and pinprick haemorrhagic lesions; Group 2, haemorrhagic and fibrinosuppurative lesions.**

- **Fig 5.4: weighted (A) and unweighted (B) PCoA plots comparing similarity in terms of phylogenetic distance indicated that Yard One samples are more similar to one another than Yard Two samples when considering phylogenetic distance, this was found to be statistically significant (both weighted and unweighted UniFrac distances p = 0.01).**

- **Fig 5.5: Weighted PCoA plot comparing lesion groups - red = 0 (normal), blue = 1 (erythema/mild), orange = 2 (fibrinosuppurative/haemorrhagic). There is no emerging pattern for samples to group according to which sample type they represent.**

Accompanying material

Appendix 1: Abstract presentation at the 2019 World Equine Veterinary Association conference, Verona, Italy.

Title: The role of the gastric microflora in the aetiology of equine glandular gastric disease: a pilot investigation using 16S rRNA sequencing

Authors: S.J. Voss, W. Weir, D.G.M. Sutton

Appendix 2: Consent form signed by owners/trainers of horses enrolled in the studies presented.

Acknowledgements

I gratefully acknowledge the help of a number of people for their support during the planning and execution of the studies included in this thesis. I would like to thank Professor Willie Weir for his time and patience in guiding study design, and for sharing his experience to guide interpretation of the data acquired. I also thank Dr David McGuinness for his skills in sequencing the samples, and his use of the Qiime pipeline to perform the data analysis. I also thank my supervisor Professor David Sutton for his contributions to this project, as well as for his guidance during my residency.

I am extremely grateful to Alex Raftery and Dr Rob Coultous for their advice when I was beginning DNA extraction, and for their valuable suggestions in troubleshooting technical difficulties in the lab. I also wish to acknowledge Claire Dixon and Andee Frei for being so generous with their time in order to help me collect data.

I would like to thank the Weipers Centre Equine Hospital staff; I learnt a great deal from every one of you, and it has been a pleasure to be a part of the team for the last four years.

I have no doubt that without the emotional support, encouragement, and friendship of Hana McNicholas, Dr Phil Webb, Lauren Gummery, Alex Raftery, Anna Davidson, Christian Byrne and Yasmin Aborida, the added challenges of the last year in particular would have seemed impossible to overcome. Thank you.

Lastly, I am grateful for the love and support I have always received from my Mum, Josie; Dad, Malcolm; and brother, Jonathan Voss. I am forever thankful for the opportunities my family have worked so hard to provide for me.

Sources of funding

This study has received support from the School of Veterinary Medicine Vet Fund Small Grants Scheme, project number 145974-01, University of Glasgow; and the Petplan Charitable Trust, project number 2017-579-617. Without their financial support this study would not have been possible, thank you.

Author's declaration

I declare that, except were explicit reference is made to the contribution of others, that this dissertation is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Name: Sarah J. Voss

Signature:

Ethical approval

This study was approved by the Glasgow University Animal Ethics and Welfare Committee, reference 42a/17.

Written informed consent was given by owners/trainers for the acquisition of samples and use of the data derived from those samples.

Data accessibility

These sequence data have been submitted to the EMBL databases under accession number PREJEB38790.

List of abbreviations/definitions

- ACTH – adrenocorticotropic hormone
- ECEIM – European College of Equine Internal Medicine
- EGGD – equine glandular gastric disease
- EGUS – equine gastric ulceration syndrome
- ESGD – equine squamous gastric disease
- FDR – False discovery rate
- LEfSe – linear discriminant effect size
- NSAID – Non-steroidal anti-inflammatory drug
- OTU – operational taxonomic units
- PCoA – Principle coordinates analysis
- SAA – Serum amyloid A
- SD – standard deviation
- TMPS – trimethoprim sulfadiazine

1 Introduction

Equine gastric ulceration syndrome (EGUS) is a common condition, affecting sports and leisure horses (B W Sykes et al., 2015), as well as working equids (Al-Mokaddem et al., 2015), worldwide. The equine stomach is lined by two different epithelial linings; the dorsal squamous (non-acid producing) portion, and the glandular (acid producing) portion ventrally, with the *margo plicatus* forming the junction between the two epithelial linings. EGUS is now recognised as two different syndromes defined by which portion of the stomach is affected; equine squamous gastric disease (ESGD) and equine glandular gastric disease (EGGD) (B W Sykes et al., 2015). There has been a move away from describing lesions as glandular ulceration, and EGGD is now known to be characterised by erosive and inflammatory histopathological change (H Martineau et al., 2009; H. Martineau et al., 2009; Crumpton et al., 2015).

Treatment of ESGD and EGGD is currently similar, with reduction of gastric acidity using oral omeprazole (a proton pump inhibitor) forming the mainstay of treatment for both syndromes. EGGD lesions are often less responsive to treatment with omeprazole with a higher treatment failure rate than ESGD (B. W. Sykes et al., 2014b; B. W. Sykes, Sykes, et al., 2015). Without a more thorough understanding of the aetiopathogenesis of EGGD it is challenging to improve and optimise treatment protocols. We have an incomplete understanding of the aetiopathogenesis of EGGD, which has a high prevalence across multiple equine disciplines, and a high treatment failure rate, making it a disease of interest.

Proposed mechanisms for the pathogenesis of EGGD include breakdown of mucosal defences, altered perfusion, and the role of pathogenic bacteria has also been questioned (B W Sykes et al., 2015; Rendle et al., 2018; Banse and Andrews, 2019). *Helicobacter pylori* is a well-established human gastric pathogen, and this is known to affect other veterinary species. *H. pylori* has been identified inconsistently in horses (Dong et al., 2016), but the association with gastric lesions has so far remained unconvincing. Bacterial involvement in gastric disease in other species has made the potential of a bacterial EGGD

pathogenesis of significant interest, however, we currently have incomplete and inconsistent evidence to support or refute a bacterial aetiology.

Alterations in the gastrointestinal microbiome are increasingly associated with a number of gastrointestinal and extra-gastrointestinal disease processes (Costa and Weese, 2018). However, to date there remains very limited data describing the normal gastric mucosal microbial community profile *in vivo*, despite evidence to suggest that significant *post mortem* change in microbial community is likely to occur, limiting the value of *post mortem* sampling.

The studies included in this thesis expand on the current knowledge of the equine gastric glandular microbial population at normal sites of normal glandular mucosa, and at sites affected by EGGD lesions.

2 Literature review

2.1 Overview of gastric anatomy

The equine stomach is a u-shaped segment of the gastrointestinal tract, which may be divided into four histological regions (Merritt, 1999). The most oral portion of the stomach is termed the squamous (previously the 'non-glandular') mucosa, is comparable to the mucosa lining the oesophagus, and is generally not in contact with gastric content and ingesta (Merritt, 1999). The remaining three mucosae, the cardia, fundic, and pyloric mucosa, form the glandular mucosa aborally (Merritt, 1999). The demarcation between the squamous and glandular portions is termed the *margo plicatus*, and is a common site of ulcerative lesions in the adult horse (REF (Murray 1998; Hammond et al. 1986; Murray et a/. 1989).

There is a narrow portion of glandular mucosa immediately aboral to the *margo plicatus*, which is termed the cardiac glandular region, which becomes the fundic region further distally (Merritt, 1999). The glandular portion is often discussed as one type mucosa, however it comprises four distinct histological regions. The fundic region is the largest portion of the glandular mucosa, and extends from the cardiac region distally along the greater curvature, before becoming the pyloric region immediately oral to the duodenum (Merritt, 1999). The fundic region contains chief cells, which release pepsinogen, and parietal

cells which are stimulated to release hydrochloric acid (HCl) following release of histamine from enterochromaffin-like (ECL) cells (Merritt, 1999). The pyloric mucosa, where EGGD lesions are most commonly identified (Begg and O'Sullivan, 2003; Luthersson, Nielsen, et al., 2009) is characterised by G cells, which release gastrin (a prosecretory hormone stimulating parietal cells to release HCl), and D cells which monitor gastric acidity and release somatostatin, which inhibits HCl secretion (Kitamura et al., 1984). There is a higher abundance of cells expressing immunoreactivity to somatostatin, glicentin, and glucagon in the cardiac and fundic regions than in the more aboral pyloric region (Kitamura et al., 1984). The pyloric region is the most aboral portion, and expresses some somatostatin immunoreactivity, but secretes more gastrin than the other gastric mucosae (Kitamura et al., 1984).

2.2 Clinical Signs of EGUS

Clinical signs associated with EGUS are often subjective or multifactorial, and in order to conclude that EGUS is the cause of clinical signs in a given case it is important to assess improvement of clinical signs in association with repeat gastroscopy and documented resolution of lesions (B W Sykes et al., 2015). Researchers began correlating the endoscopic identification of EGUS lesions with clinical signs in adult horses in the late eighties and it was soon established that EGUS was common in asymptomatic animals, and well as in association with abdominal discomfort (Murray et al., 1989).

One endoscopic survey of symptomatic and asymptomatic horses revealed a high proportion of EGUS (predominantly ESGD) lesions in asymptomatic yearlings and adult horses, with a prevalence of 52% (compared to a 92% prevalence in symptomatic horses) (Murray et al., 1989). Lesions in this study were described but predated the currently established grading system therefore it is hard to draw direct comparisons to more recent studies. The use of a 2-metre endoscope limited examination of the glandular mucosa, as with many earlier studies. The study concluded that there was a causal relationship between the presence of EGUS and clinical signs of inappetence, colic, and poor body condition, with a higher incidence of clinical signs in animals with lesions identified endoscopically (Murray et al., 1989). The same study also reported resolution of clinical signs in a proportion of these horses in which endoscopic resolution of EGUS lesions was

evidence after treatment of ranitidine, supporting a direct link between the clinical signs described and EGUS. Another large case series reported EGUS in 91 of 111 horses presenting for investigation of abdominal discomfort, and 31 of those horses EGUS was determined to be the primary cause of colic signs based on the absence of any other identifiable cause, and an improvement in clinical signs following endoscopic resolution of EGUS lesions after treatment (Murray, 1992).

Clinical signs associated with ESGD are well reported, however, there has been little published describing clinical signs specific to horses with EGGD and signs are often vague and non-specific (Rendle et al., 2018). There is little evidence providing a definitive link between EGGD and clinical signs reported by owners. One study examining 63 horses reported a positive relationship between EGGD lesion resolution ($p= 0.002$) and lesion improvement (0.006) and owner reported resolution of clinical signs (Varley et al., 2019). Clinical signs included poor performance (65%) and apparent girthing pain (31.7%), and less commonly weight loss, altered appetite, changes in hair coat, and signs of colic/bruxism. This study reported that 68% of horses presented with two or more of the listed clinical signs; the most common combination reported was behavioural change and poor performance in 19% of the cases examined. Many of these clinical signs are well established to be associated with ESGD; this study does not make it clear how many of the horses with these reported clinical signs also had ESGD lesions, and concurrent presence of ESGD was listed in the exclusion criteria for the study. Therefore it is possible that the association of these signs with EGGD is overstated. While externally visible characteristics have been reported of horses affected by ESGD, this has not been shown to be the case for EGGD. One study reported that all horses affected by EGGD were in good body condition and did not look poor (Malmkvist et al., 2012). The same authors reported that there was not a difference in behaviour, or increased baseline cortisol faecal concentrations in horses with more severe glandular ulceration (Malmkvist et al., 2012).

There is a concern regarding presence of EGUS and impact on performance. One study reported an association between EGUS and poor performance in one racing population, however performance in this case was defined by trainer

expectation and is therefore subjective (Vatistas et al., 1999). The same study did not find a correlation between lesion severity and poor performance, and concluded the effect on performance was likely to be an "all or nothing" phenomenon (Vatistas et al., 1999).

2.3 Diagnosis of EGUS

The principle method of diagnosing gastric ulceration in horses is gastroscopy (Andrews et al., 1999); and the ECEIM consensus statement recommends that a diagnosis of EGUS can only be made by gastroscopic examination (B W Sykes et al., 2015). Gastroscopy allows the clinician to establish whether ESGD or EGGD is present, and therefore formulate an appropriate treatment plan, and assess response to treatment.

Although there are an increasing number of first opinion equine practices with access to gastroscopy, it is still not universally available, and performing gastroscopy and interpreting clinical findings requires additional expertise (Hewetson et al., 2017). Gastroscopy is also associated with expense to the client, and the with client interest in EGUS increasing this has lead to some animals being treated empirically without diagnostic investigation (Hewetson et al., 2017), which cannot be justified (B W Sykes et al., 2015). It is important to note that EGUS is often asymptomatic, and a high rate of EGUS has been identified in working equids with no reported signs of EGUS (Sgorbini et al., 2018), therefore role in limiting performance is likely to often be part of a number of factors.

For these reasons, there has been some interest in establishing use of screening tests (Hewetson et al., 2017). Urine sucrose concentration was initially validated for diagnosis of ESGD, and was shown to have a sensitivity and specificity of 83% and 90% respectively in a model using horses with EGUS induced by the feed deprivation model (O'Conner et al., 2004). It is technically demanding for use as a screening test, and requires bladder catheterisation in order to collect urine at specific time point (O'Conner et al., 2004), and has not been used in general practice. This study used the feed deprivation model to induce gastric ulceration in the study population, and so may not accurately reflect naturally occurring disease. Unfortunately, the authors only endoscopically evaluated the squamous

mucosa, and therefore the usefulness of this test in application to EGGD lesions, or the effect that undiagnosed EGGD lesions may have had on the results, cannot be determined.

Following O'Conner et al.'s study, blood sucrose concentration was evaluated as a more convenient means of assessing increased sucrose absorption associated with gastric ulceration (Hewetson et al., 2006). In contrast to O'Conner et al's study, Hewetson et al. used horses with naturally acquired lesions, however only a small number of horses (ten) were examined in this study, none of which were affected by EGGD lesions diagnosed endoscopically. Therefore the utility of this test in the context of horses affected by EGGD cannot be determined from this study. Building on these initial findings, blood sucrose concentration has been evaluated in a larger number (101) of horses, and compared to gastroscopy as a gold standard diagnostic test, and found that diagnostic sensitivity and specificity was poor for ESGD and EGGD (Hewetson et al., 2017). EGGD lesions were reported in 70% of the study population, and the authors postulated that the lack of reliability of the diagnostic test in this population was due to a difference in permeability to sucrose of the glandular compared to the squamous mucosa (Hewetson, 2017).

A commercially available faecal blood and albumin assay has been marketed for detection of hindgut and foregut disease, including EGUS. However, there has been no correlation found between identification of faecal blood or albumin using the Succeed® faecal blood and albumin kit and the presence of EGUS as diagnosed by gastroscopy (B. Sykes et al., 2014).

It has been postulated that Serum Amyloid A (SAA) (an acute phase protein) concentration is elevated in association with severe EGUS. Initial work supported a variable association between increased SAA concentration and the presence of EGUS in a small population of racehorses (Pollock, 2017). In particular, this study suggested the elevations in SAA concentration may be associated with pyloric EGGD lesions. However, there was found to be no association between elevated SAA concentration and either presence or severity of either EGGD or ESGD lesions (Spanton et al., 2020). SAA is also elevated by a large number of conditions associated with inflammation, and as such lacks specificity. Therefore use of SAA as a screening test cannot be recommended.

More recently, the serum proteome has been investigated with respect to experimentally induced EGGD, and identified 14 biomarkers that may be associated with EGGD (Tesena et al., 2019). However, this study investigated lesions experimentally induced with doses of phenylbutazone at significantly higher doses than would be considered safe, and therefore it is challenging to extrapolate this data to clinical scenarios.

Transendoscopic biopsies are not routinely performed in the diagnosis of EGGD, with EGGD being diagnosed based on gross gastroscopic appearance. Biopsies are however advised for any lesions which are non-responsive to treatment for three or more months, or if severe pathology is suspected (Rendle et al., 2018). A protocol has been suggested by Rendle *et al.*, briefly, samples should initially be acquired from the surface of a lesion and used to create an impression smear, before deeper 'bites' are taken from freshly exposed mucosa for histopathological and bacteriological analysis. There is limited information regarding the usefulness of mucosal biopsies, however, preliminary work indicates that there is a poor correlation between gross appearance and underlying histopathological change (Crumpton et al., 2015).

With specific reference to EGGD, further exploration of the underling aetiopathogenesis may lead to development of additional screening tests for EGGD, however, currently, gastroscopic examination remains the only effective method of assessing presence and extent of EGGD lesions, in addition to monitoring treatment efficacy.

2.3.1 Grading systems

It is well recognised that endoscopic appearance does not correlate well with histological description of EGUS lesions (Pietra et al., 2010; Crumpton et al., 2015). It has also been demonstrated that although clinicians are inclined to focus on gross pathology visible at the pylorus, diffuse gastritis throughout the glandular mucosa is often associated with grossly normal mucosa (Crumpton et al., 2015).

There have been a number of proposed grading systems for EGUS. Andrews at al. proposed a 0-3 grading systems for EGUS, where the system used was based on

the most severe lesion identified on gastroscopy, this included EGSD and EGGD lesions (Andrews, Sifferman, et al., 2010). Murray et al. first applied a grading system that took into account severity and number of lesions, also taking into account their anatomical location (Murray et al., 1989). This approach was subsequently criticised due to the likelihood that it may over or underestimate overall severity when the scores assigned are tallied (Macallister et al., 1997). Subsequently, Murray et al. formed a 0-10 grading system, which gave more importance to individual lesion severity over total number of lesions identified (Murray et al., 1996). The grading systems developed in these papers were primarily to facilitate data analysis and reporting in these studies and were not initially designed for use by practitioners.

An initial attempt to formally develop a grading score for EGUS assigned scores 0-4 for lesion number, followed by a score 0-5 for lesion severity, with the intention that ESGD and EGGD lesions should be graded separately, with each gastroscopic examination generating four scores (Macallister et al., 1997). In this study five assessors who were experienced in equine gastroscopy reviewed 16 horses, before inter-user agreeability was assessed. They found good agreement between observers for scoring number of EGGD lesions, and severity of ESGD and EGGD lesions, but there was less good agreement when assessing the number of ESGD lesions present.

Following this, the EGUS Council reported a 0-4 grading system designed to be used by both researchers and practitioners, and designed to be applicable to EGGD and ESGD lesions (The Equine Gastric Ulcer Council, 1999). This grading system was compared to the number and severity system, and was found to be more repeatable, and clinicians found implementation of the EGUC system simpler to use (Bell et al., 2007). A comparison between endoscopic grading scores, and *post mortem* examination and a histopathologic grading system found that endoscopic assessment tends to underestimate severity in terms of lesion number and depth for ESGD lesions, and number of EGGD lesions (Andrews, Reinemeyer, et al., 2010).

Currently, the ECEIM consensus statement advises use of lesion descriptors for EGGD rather than a linear grading system, as there is poor correlation between gross appearance and underlying pathology (H. Martineau et al., 2009; B W Sykes

et al., 2015; Crumpton et al., 2015). Current advice is to describe anatomic location of EGGD lesions (cardia, fundus, antrum, or pylorus) and then to give a gross lesion description according to number (focal/multi-focal/diffuse), perceived severity (mild/moderate/severe), and a gross description (flat and haemorrhagic/flat and fibrinosuppurative/raised and haemorrhagic/depressed ± blood clot/depressed and fibrinosuppurative) (B W Sykes et al., 2015). These descriptors are relatively complex in comparison to a linear grading system and are likely to be more challenging for horse owners to understand. It is also important to acknowledge that no work exists to validate intra or inter-user agreeability or repeatability of these descriptors. It is more challenging to apply these terms to EGGD lesions in the context of studies evaluating EGGD, and as such, many studies are still using linear grading systems to record and assess EGGD lesions.

2.4 Prevalence of EGGD

There are multiple studies reporting EGUS prevalence in horses competing in various disciplines, as well as in leisure horses and donkeys. EGUS is a well described and prevalent syndrome in leisure and sports horses (B W Sykes et al., 2015), less is known about prevalence in other equids. Studies reporting prevalence of gastric lesions in horses were previously often biased towards description of ESGD, and EGGD was previously frequently overlooked (Sykes and Jokisalo, 2015). Different grading systems have been used historically, making drawing conclusions and comparisons between prevalence and severity data difficult.

Multiple different equestrian disciplines have been evaluated for prevalence of EGUS, and data are variable. Two percent of horses used in showing were found to have EGGD (58% of horses examined were affected by EGGD (McClure et al., 1999). One study reporting 87 Thoroughbred racehorses reported only eight animals affected by EGGD; in contrast, 80 were affected by ESGD (Murray et al., 1989). In contrast, a later study examining 63 racing Thoroughbreds reported 51% of the population were affected by EGGD (Murray et al., 1996). In a larger population of racehorses (345 Thoroughbreds) there was an overall incidence of EGUS in 86% of animals, however, not all of these horses underwent gastroscopic examination of the pylorus (Begg and O'Sullivan, 2003). In the 175

animals that did, 47% had EGGD lesions diagnosed. The authors noted that the pylorus was the most common site for EGGD lesions in this population (Begg and O'Sullivan, 2003), highlighting the importance of thorough gastroscopic evaluation.

The previously discussed Thoroughbred studies were performed in Australia, however, EGGD has been found to be prevalent in other global populations. A population of 113 horses from three UK and five Australian training yards found no significant difference between prevalence of ESGD, but found that Australian animals had a significantly increased prevalence of EGGD (Habershon-Butcher et al., 2012). One paper reported 29 Thoroughbreds affected by EGGD out of a total of 201 examined in a hospital in Denmark (Luthersson, Nielsen, et al., 2009). When the ulcer score developed by Macallister et al. (1997) was applied, the same authors identified more severe glandular lesions present at the pylorus compared to the visible fundic or cardiac regions. Again, this highlights the care required when interpreting older literature which frequently does not include the pylorus in gastroscopic examination.

In a small population of endurance horses only eight horses out of a cohort of 30 were found to be affected by EGGD (Nieto et al., 2004). This was in similar to a study of the same size reporting up to 33.3% of endurance horses during the competition season (Tamzali et al., 2011).

In a study surveying 60 domesticated and 29 feral horses at a UK abattoir 70.6% of domesticated and 29.6% of feral horses were affected by EGGD (Ward et al., 2015). This exceeded the proportion of both of these populations affected by ESGD (60.8% and 22.2% of domesticated and feral horses respectively). Interestingly, EGUS is often thought of as a disease of domestication, but Ward et al. provide evidence to suggest that studies investigating both ESGD and EGGD should not neglect less commonly considered equine populations. Another frequently neglected population with respect to studies reporting prevalence are working equids. A study reporting frequency of gastric lesions in a small cohort of working male donkeys in Egypt reported hyperkeratosis and acanthosis of the squamous mucosa in 34% of animals, histological evidence of inflammation in 49% of animals, erosions in 6%, and squamous ulceration in 14% of donkeys (Al-Mokaddem et al., 2015). None of the animals reported by Al-Mokaddem et al.

were affected by gross EGGD lesions. In a population of donkeys in Italy EGUS was present in approximately 51% of animals, but only one donkey examined had EGGD. A large retroscpective study describing *post mortem* findings of 1444 donkeys in the UK reported EGUS to be a common pathology, with 608 (42.1%) animals affected, but did not describe lesion distribution. Similar prevalence has been reported in other *post mortem studies*, with lesion distribution describing 10% of donkeys to be affected by EGGD (Burden et al., 2009). This indicates that it is important to remember other equids when discussing prevalence, diagnosis, and treatment of EGGD.

2.5 Risk factors for EGGD

Risk factors for EGGD in the racing Thoroughbred population are not as well described as for ESGD. Many risk factors described for ESGD have not been found to be associated with an increased risk of EGGD (Rendle et al., 2018). It can be challenging to compare risk factors directly, as studies reporting at a population level have many other variables that may predispose horses to gastric disease, however, common themes have emerged from multiple studies.

2.5.1 Horse factors

A large UK study of sport and leisure horses did not identify any significant effect of signalment on the presence of EGGD (Hepburn, 2014), however, this is in contrast to other publications. In a population of horses presenting to a hospital in Finland, the Warmblood breed has been identified as at higher risk, although this likely reflects a difference in management practices rather than a genuine breed-specific reason (Mönki et al., 2016). One study found that Warmbloods and sports ponies (9.6% and 20.9% respectively) were at increased risk of having EGGD in the absence of ESGD (Luthersson, Nielsen, et al., 2009). Statistically significant effect of sex has been identified in UK and Australian racehorses, with mares and geldings noted to be at increased risk of being affected by EGGD compared to colts (Habershon-Butcher et al., 2012), although the authors did not elaborate on a possible effect of age. Increasing age has previously been noted to increase the likelihood of ESGD and EGGD being present concurrently (Luthersson, Nielsen, et al., 2009).

It has been suggested that horses that are more easily stressed are more prone to developing EGUS. However, more recently it has been found that, in a small population, hair cortisol concentration is negatively associated with ESGD; although this just achieved statistical significance (Prinsloo et al., 2019). Unfortunately, this study did not investigate association with EGGD. Stress and associated cortisol concentrations have been proposed as the reason that amount of experience appears to be protective against EGGD in a population of polo ponies (Banse et al., 2018). Similarly, lower level of competition has been found to be a risk factor in Canadian Warmbloods, which may be a consequence of lack of experience that animals competing to a higher level may be expected to have (Pedersen et al., 2015) Having an increased number of handlers was associated with having EGGD in a population of horses in Finland (Mönki et al., 2016), and it is possible this is an effect of stress, whether as a consequence of having contact with an increased number of people, or by virtue of the types of premises and disciplines that mean horses tend to be handled by more people.

2.5.2 Discipline and exercise

Interestingly, undertaking fast exercise less frequently and swimming have been identified as risk factors in UK and Australian Thoroughbreds (Habershon-Butcher et al., 2012) In the same study, Habershon-Butcher et al. reported an effect of trainer on likelihood of EGGD lesions being present. Again this is likely to be a consequence of a large number of variables. In contrast to the findings of Habershon-Butcher et al., exercising more than six days per week was found to increase EGGD risk in show jumping Warmbloods (Pedersen et al., 2015). In a small population of endurance horses the prevalence of EGGD was found to increase nearly two fold, from 16% to 33%, when horses progressed from the interseason to competition season periods (Tamzali et al., 2011). More work is needed to further elaborate on the effect of exercise on EGGD.

2.5.3Association with the presence of ESGD

There is a lack of consensus regarding a potential association between the presence of ESGD being associated with a concurrent diagnosis of EGGD. Some studies initially reported no association between the presence both syndromes (Murray et al., 2001; Begg and O'Sullivan, 2003). Where both syndromes are

present in the same animal, there does not appear to be a correlation between severity of ESGD and the severity of EGGD lesions (Begg and O'Sullivan, 2003), however, as discussed elsewhere, defining the severity of EGGD present in an individual animal based on gross appearance alone is now thought to be inappropriate. More recently, Sykes et al. have since stated there is an association between EGGD and ESGD and postulated that this may be effected by pyloric lesions causing delayed gastric emptying and therefore cause the squamous mucosa to acidic gastric contents (Sykes et al., 2019). Other work has identified an association between increasing age and likelihood of having ESGD and EGGD concurrently (Luthersson, Nielsen, et al., 2009), indicating that this is likely to be more complex and multifactorial.

2.5.4 Diet

Suboptimal diet has been established as a risk factor for ESGD, and changing diet has been shown to be a useful adjunct to treatment with omeprazole in horses with ESGD (Luthersson et al., 2019), however the interplay between diet and EGGD is less well described. Feeding a diet high in starch only increased the incidence of ESGD under standardised conditions, but not EGGD (Malmkvist et al., 2012). A significant effect of not receiving any grass turnout, not being fed haylage, being fed unprocessed grains, and not being fed a complete ration frequently have all been identified in a population of 113 Thoroughbred racehorses in the UK and Australia (Habershon-Butcher et al., 2012).

The availability of water has been shown to have an effect of the likelihood of EGUS, with one paper finding horses were nearly three times as likely to have EGUS lesions if water was unavailable (Luthersson, NIELSEN, et al., 2009). This study includes EGGD lesions in the analysis, however based on the results presented it is not possible to infer EGGD specific risk factors in the population studied. Horses that had radiographic evidence of sand in the colon had a decreased risk of having EGGD concurrently, although this did not achieve statistical significance (Mönki et al., 2016). This is likely to represent a difference in management and may reflect either time at pasture or type of pasture, rather than a genuine protective effect of sand.

2.5.5 Non-steroidal anti-inflammatory drug administration

Non-steroidal anti-inflammatory drugs (NSAIDs) act on the arachidonic acid cascade to inhibit cyclo-oxygenase (COX) -1 and -2 enzymes, and therefore lead to reduced mucosal concentration of prostaglandins, mediated by COX inhibition. Prostaglandins are essential for regulation of mucosal blood flow and mucous secretion, as well as regulation of pH through the release of bicarbonate and inhibition of acid secretion (Wallace, 2008). It therefore would follow that administration of NSAIDs leading to reduced mucosal prostaglandin concentration would result in EGGD. As such, NSAID administration is frequently discussed as a potential risk factor for the development of EGGD, and it was postulated historically that glandular ulceration was less common in adult horses than in foals, and was associated with NSAID administration in younger animals (Andrews and Nadeau, 1999). Other studies have failed to detect an association between NSAID administration and EGUS, attributing this finding to shorter endoscopes not allowing full examination of the glandular mucosa in clinical cases (Murray et al., 1996; Vatistas et al., 1999). However, reduced prostaglandin E_2 concentrations were not identified in a phenylbutazone induced model of EGGD in two separate studies (Meschter et al., 1990; Pedersen et al., 2018). In a study of elderly donkeys in the UK NSAID administration was not found to be associated with increased risk of either ESGD or EGGD (Burden et al., 2009). As well as phenylbutazone, EGGD lesions have also been experimentally induced in ponies with off-label doses of flunixin (1.8 mg/kg intramuscularly q8 hours) (MacAllister et al., 1992).

2.5.6 Stereotypies

There is evidence to suggest that crib biting horses have increased gastrin secretion compared to non-crib biting horses following a meal, which may logically be expected to increase the risk of EGUS (Wickens et al., 2013). However, this study did not find a difference in ESGD lesion scores or baseline gastric pH. Unfortunately, this study did not describe EGGD lesions in the study population.

One study identified an increasing severity of ESGD lesions in foals that displayed crib biting behaviour, however, they did not use the 1999 EGUS council grading

system, and did not report on lesions affecting the glandular mucosa (Nicol et al., 2002), limiting the conclusions that can be drawn from this paper. In order to further dissect the link between crib biting and EGUS, one study examined the mucosal and ingesta pH difference between crib-biting and non-crib-biting horses *post mortem* (Daniels et al., 2019). The authors did not identify a significant difference between the gastric mucosae histopathologically of the two groups. They concluded that this supported EGUS and crib-biting being prevalent in the same population, but did not establish a causative link between the two entities, and that it is likely that EGUS and stereotypies are often present concurrently due to sharing an association with stress (Daniels et al., 2019).

2.5.7 Gastrointestinal parasitism

G. intestinalis attaches to the gastric mucosa, resulting in necrosis and erosion at the point of parasitic attachment to the stomach, and all donkeys examined *post mortem* in one study had histological evidence of chronic gastritis (Abuowarda, 2020). In contrast, a *post mortem* study reporting gastric pathology in a population of working donkeys in Egypt noted only one animal with squamous ulceration at a site of parasite attachment, describing hyperkeratosis and eosinophilic infiltration to be characteristic of *Gasterophilus* infestation (Al-Mokaddem et al., 2015). The same study noted that *Draschia megastoma* was associated with chronic glandular necrosis, and that *Habronema* was associated with increased glandular mucous production (Al-Mokaddem et al., 2015). A smaller *post mortem* study reporting gastric pathology in Spain reported EGUS in 9/10 animals, with 3/10 of those donkeys affected by *Gasterophilus* spp., however no clarification of EGUS severity was made, or whether lesions were considered to be a consequence of parasitic attachment to the gastric mucosa (Morales Briceño et al., 2015). No association between EGUS and gastrointestinal parasitism was reported in a large scale *post mortem* study of the UK donkey population (Morrow et al., 2011)

2.6 Aetiopathogenesis of EGGD

A single aetiopathogenic cause of EGGD is unlikely (Rendle et al., 2018), and a number of potential contributing factors have been investigated, largely based

on known aetiopathologic factors extrapolated from other species. The mucosal epithelial barrier is key to defence against ulceration (Dyck, 1979). As the glandular mucosa is exposed to acidic conditions under normal circumstances, a breakdown of mucosal defences has been proposed as an inciting cause, as well as possible colonisation with bacteria, inflammation and stress (B W Sykes et al., 2015; Banse and Andrews, 2019). The poor response of EGGD lesions to acid suppression as a lone therapeutic approach also supports the hypothesis that acidic conditions are not as important in the development of EGGD as for ESGD (Banse and Andrews, 2019). EGGD lesions have been examined histopathologically, and has demonstrated the presence of erosions and both focal and diffuse gastritis, eosinophilia, and glandular metaplasia (H Martineau et al., 2009; H. Martineau et al., 2009).

2.6.1 Breakdown of normal mucosal defences

Mucosal barrier function is essential for stopping toxins and bacteria within the lumen reaching the subepithelium and circulation (Reed et al., 2018), and breakdown of normal mucosal defence systems has been proposed as a cause of EGGD. Full discussion of gastric mucosal barrier function is beyond the scope of this review. In summary, the gastric mucosae are hydrophobic; a property imparted by surface adsorbed surface-active phospholipid (Ethell et al., 2000). At the glandular mucosa these hydrophobic properties are proposed to stop back-flow of hydrochloric acid, and therefore act as a protective mechanism against acid-induced injury (Hills et al., 1983; Goddard et al., 1990). The stomach frequently sustains mucosal injury, but the speed of healing and multiple layers of primary and secondary defences mean that these insults do not frequently lead to more significant mucosal damage (Wallace and Granger, 1996; Wallace, 2008). Prostaglandins are another important factor maintaining a healthy gastric mucosa, and are known to be integral to effective mucosal barrier function, being important for mucous production, regulation of blood flow, and bicarbonate secretion (Wallace, 2008).

2.6.2 Effect of pH

Although acid-suppressive therapy, primarily omeprazole, remains the mainstay of treatment for both ESGD and EGGD, there is no evidence that decreased pH is

causative of EGGD. The glandular mucosa is exposed to acidic conditions constantly, and this, as well as the fact that EGGD responds poorly to acid suppressive therapy (B. W. Sykes et al., 2014b; B. W. Sykes, Sykes, et al., 2015), implies that it is unlikely that increased exposure to acidic conditions is causative. Some work has been undertaken to map the pH of the equine stomach (Murray and Schusser, 1993; B. W. Sykes, McGowan, et al., 2015), however the majority of glandular lesions occur at the pylorus, and there are no published data describing normal pH at this site. It is logical to assume mucosal pH will be more alkaline at the pylorus compared to the ventral fundus due to the proximity to the duodenum and sporadic reflux of bile into the stomach. However, there is some data to suggest that concentration of bile acid does not significantly affect pH (Baker and Gerring, 1993). More work is required to determine whether pH fluctuation has a significant effect on the aetiopathogenesis or healing of EGGD lesions.

2.6.3 Exercise-related factors

In humans, splanchnic blood flow decreases during exertion (Clausen, 1977), and gastric ischaemia arises as a consequence during strenuous exercise (Otte et al., 2001). This may be a contributory factor in equine athletes, as there is some evidence to suggest that increasing exercise is a risk factor for EGGD (Chapter 2.3.2).

2.6.4 Proposed bacterial pathogenesis

Despite the well-established link between pathogenic bacteria and gastric ulceration in people, there is currently no strong evidence to suggest that bacteria have a causative role in EGUS. Initially, there were unsuccessful attempts to identify an infectious cause of gastroduodenal ulceration in foals when it was considered that the clusters of cases seen on some premises likely indicated an infectious aetiology (Acland et al., 1983; Gross and Mayhew, 1983). However, these studies implemented culture-based methods, which are likely to underestimate the bacterial population present. Now, most studies investigating potential bacterial causes will use more advanced techniques, such as 16S sequencing.

It has been previously proposed that horses with EGUS refractory to first line treatment may benefit from treatment with antimicrobials (Andrews et al., 2005), and although this was at one time a relatively popular clinical practice, there is no evidence to support this being necessary or effective. More recently, one study investigated the use of orally administered trimethoprim-sulphadimidine (TMPS,15g total dose per horse) combined with omeprazole (2g total dose per horse) compared to omeprazole monotherapy, and there was no statistically significant difference in healing of EGGD lesions (Ben W. Sykes et al., 2014). This study only provides weak evidence that addition of antimicrobials to a treatment regime does not improve healing. TMPS was dosed once daily; this is the licensed dosing regime, however it is well accepted that the short half-life of trimethoprim necessitates 12 hourly dosing (DUIJKEREN et al., 1994). Doxycycline has been frequently added to treatment protocols of horses with EGGD refractory to treatment in practice and its use has largely been extrapolated from the supposition that *Spirochaetes* may play a role in EGGD pathogenesis. However, there have been no studies assessing the use of doxycycline to treat EGGD.

There have been many attempts to identify pathogenic bacteria in association with EGUS. *H. pylori* has been a particular target due to its well-established link with gastric disease in other species. When discussing *H. pylori* it is important to consider whether bacteria identified are urease producing strains, as production of urease during colonisation improves bacterial survival and contributes to pathogenesis of gastric disease (Follmer, 2010). *Helicobacter* species have previously been found to be prevalent in the stomachs of horses, however initial work did not find an association between clinical signs of EGGD and lesions noted were described as mild (R Hepburn, 2004).

In one equine study using PCR to identify *H. equorum*, and the *cagA*, *ure*, and *vacA H. pylori* genes the *ure* gene was identified in three horses affected by EGGD, however a relatively small proportion of the study population affected by EGGD tested positive (Bezdekova et al., 2007). A small study undertaken on Thoroughbred horses in Venezuela identified *H.pylori* in a total of 11 samples from 20 horses using PCR, but failed to identify the *cagA* or *glmM* gene (Contreras et al., 2007). The positive PCR samples correlated well with

identification of spiral-shaped organisms that were positive for Warthin Starry stain, as would be expected of *H. pylori*. Samples from seven horses that were positive for *H. pylori* on PCR were further interrogated with 16S rRNA sequencing, and shared 99% similarity with *H. pylori*. The detection of *Helicobacter* in this study was correlated with presence of EGUS in two out of seven horses, and gastritis in five out of six horses. One flaw which makes it challenging to extrapolate the results to clinical practice is the lack of accurate documentation of whether these samples were from squamous or glandular mucosa. Building on this work, 136 racing Thoroughbreds in Venezuela were evaluated for the presence of *Helicobacter* using histopathologic techniques combined with urease testing (Morales et al., 2010). One hundred and thirty four horses had gastric lesions, and urease activity was demonstrated in 52 horses. However, again the study lacks detail and does not specifically describe ESGD or EGGD as separate sites. A later study was unable to replicate these results using fluorescence *in situ* hybridisation (FISH) (Husted et al., 2010). In the same study, Husted *et al*. identified *Lactobacilus salivarius* and *Sarcina ventriculi* using FISH in *post mortem* glandular mucosa samples, however, these organisms were identified in both EGGD lesion samples and normal mucosa, and no statistical analysis was performed in order to establish whether these organisms were significantly associated with EGGD in particular.

Other proposed pathogens have included *Streptococcus bovis*, and *Enterococcus faecium* (Rendle et al., 2018), however, evidence is lacking to support their involvement in the pathogenesis of EGGD.

2.7 Treatment of EGGD

As the pathogenesis of EGGD is incompletely understood, it is perhaps unsurprising that treatment protocols are variable and are often associated with poor rates of success. Treatment of EGGD has revolved around acid suppressive therapy, which has a largely been extrapolated from the treatment of ESGD, however there is evidence in humans to support that degree of acid suppression can be used to predict glandular mucosa lesion healing in patients with gastro-oesophageal reflux disease (Bell et al., 1992). Treatment of EGGD therefore has largely relied on the use of oral omeprazole, more recently commonly with the

addition of oral sucralfate. Misoprostol has also been reported as a successful monotherapy.

2.7.1 Proton Pump Inhibitors

Treatment with oral omeprazole alone carries a poorer success rate for EGGD lesions than for ESGD lesions. One study reported a 21% EGGD lesion healing rate, where healing is defined as an improvement in grade of lesions rather than resolution, of EGGD lesions, compared to 80% of ESGD lesions in the same study (B. W. Sykes et al., 2014a). The same study also reported worsening in EGGD grade of 13% of the horses, which is a novel finding, however this represents a very small number of horses and has not been borne out in other studies. Despite recording the weight of all animals enrolled, all received 2.0g omeprazole, which is approximately 4 mg/kgf, . This study employed the use of *post hoc* power calculations, showing that although power was appropriate for investigating treatment efficacy for ESGD, the EGGD arm of the study was underpowered.

Commented [VS(oVM1]: Make sure it hasn't

Sykes et al. compared two doses (4.0 mg/kg vs. 1.6 mg/kg) of oral omeprazole, and again found 60% of horses with EGGD lesions improved with 4mg/kg dose, compared to 100% of ESGD lesions at the same dose using a buffered omeprazole paste (B. W. Sykes et al., 2014b). The authors reported a more consistent dose-dependent improvement in lesion grade with ESGD lesions than EGGD lesions, and caution must be exercised when extrapolating results from very few horses. The same authors went on to further investigate this dose-dependent relationship, and examined the use of 1.0, 2.0, and 4.0 mg/kg oral omeprazole in a larger number of Thoroughbred racehorses. Similarly to the previous study, the lower studied doses were found to be noninferior to the licensed dose of 4 mg/kg in the treatment of ESGD (B. W. Sykes, Sykes, et al., 2015). It is important to note that the methods of each study are not directly comparable, with the 4.0 vs 1.6 mg/kg study using a buffered oral paste, and the study comparing 1.0, 2.0, and 4.0 mg/kg using a preparation of enteric coated granules suspended in a paste. It is not possible to draw direct comparisons between these two preparations with the data currently available (Sykes et al., 2016). The ECEIM consensus statement highlighted the need for direct measurement of duration and extent of acid suppression achieved in horses with

different dosing regimen and preparations of omeprazole, as previously much of this information has been derived from clinical trials (B W Sykes et al., 2015).

There are multiple factors that may affect the reported success of oral omeprazole in the treatment of EGGD. The preparation of omeprazole used is important, and use of coated or buffered preparations are preferred and have been shown to be more effective (Merritt et al., 2003). Merritt et al. also demonstrated a positive effect of feeding a small grain-based meal approximately 30 minutes after dosing, and not exercising horses until 4-8 hours after omeprazole administration. It is also important to consider the practicalities of orally administered drugs; many owners may struggle to administer oral medications by syringe, and whether report efficacy corresponds to efficacy seen in practice is likely to be affected by all of the above factors. It has also been established that individual horses acid-supress more than others following treatment with oral omeprazole, and this may be vary up to ten fold (Sykes et al., 2016).

A more recent round-table article has recommended that horses should receive no feed for eight hours prior to oral omeprazole administration, and that they should not receive feed for a minimum of 30 minutes afterwards (Rendle et al., 2018). Typically, periods of feed deprivation cause concern amongst owners of horses with EGUS. However, it has been demonstrated that night-time fasting has minimal effect on intragastric pH compared to daytime fasting , and as such periods of feed deprivation prior to morning administration of omeprazole are not likely to be deleterious (L. et al., 2009).

3.1.1.1 Long-acting intramuscular omeprazole

Injectable omeprazole is available in the UK as an unlicensed special preparation, and as such is used according to the prescribing cascade. Initial work showed that intramuscular omeprazole had superior bioavailability to orally administered omeprazole (SANDIN et al., 2010)

A recent retrospective clinical study has subsequently reported favourable healing of ESGD lesions with long-acting injectable omeprazole (97% healed)

when compared to oral omeprazole at 28 days (67% healed), however the authors are yet to report their data regarding EGGD lesions (Gough et al., 2020).

Injectable omeprazole may be associated with transient injection site swelling (Sykes et al., 2017; Rendle et al., 2018). Although this has been reported to be non-painful, this may make the intramuscular route less desirable to owners if muscle pain and swelling results in days lost from training.

It is anticipated that the rate of lesion improvement and resolution will be superior with long-acting injectable omeprazole, however, as there is no data available to support this currently, this preparation should be used according to the cascade, and in the author's opinion should be reserved for cases that show an unsatisfactory response to oral omeprazole. Injectable omeprazole may also be useful to consider for horses that are non-compliant with oral dosing.

3.1.1.2 Omeprazole and sucralfate combination therapy

Omeprazole and sucralfate combination therapy has been recommended as a first line treatment for EGGD (Rendle et al., 2018). Sucralfate is a basic aluminium salt complexed with sucrose (Jewell, 2007) which has been shown to increase gastric mucosal PGE_2 release (Hollander et al., 1984), and binds to sites of ulceration forming a protective barrier (Steiner et al., 1982), and upregulates bicarbonate and mucus secretion (Shorrock and Rees, 1989; Szabo and Hollander, 1989). Sucralfate has also been shown to have beneficial effects in humans affected by *H. pylori* gastritis by reducing the mucosal expression of TNF-α, reduction in mucosal cell apoptosis, and overall reduction in mucosal injury (Slomiany et al., 1998) and increase in endothelin-1 (Slomiany et al., 2000). Sucralfate has also been demonstrated to restore gastric barrier function in a canine *ex vivo* model (Hill et al., 2018) Sucralfate has been shown to result in a brief increase in intragastric pH in horses (Clark et al., 1996).

There is relatively little evidence to support the use of sucralfate for treatment of EGGD in the horse, one small clinical study found sucralfate was non-superior to corn syrup for the treatment of subclinical gastroduodenal ulceration in a 12 foals (Borne and MacAllister, 1993). There may be some benefit to sucralfate monotherapy in cases of phenylbutazone toxicity in foals, and one study showed

the mucosa-sparing benefits of sucralfate to be superior to ranitidine (Geor et al., 1989). A study comparing treatment of EGGD with misoprostol versus sucralfate and omeprazole combination therapy reported an 80% failure in healing and 35% failure in EGGD lesion improvement in 20 horses (Varley et al., 2019). The combination of omeprazole and sucralfate was inferior to misoprostol in terms of lesions healing. A larger study reported an overall EGGD 80% improvement rate (with a lower proportion of pyloric lesions improving 67.5%) in a population of 204 horses treated with omeprazole and sucralfate (Hepburn and Proudman, 2014). These two studies are contrasting in the apparent success of this therapeutic combination, however, it is challenging to draw direct comparisons between the two as they use different definitions of improvement and healing. However, it can be reasonably concluded that this therapeutic combination has moderate efficacy for improvement of EGGD lesions.

Dosing regime must be considered if administering omeprazole and sucralfate concurrently. As sucralfate is minimally absorbed and coats the mucosa of the gastrointestinal tract it might be expected to inhibit the absorption of omeprazole (Sulochana et al., 2016); as such, sucralfate should be administered at least 30 minutes after a proton pump inhibitor (Rendle et al., 2018). It has also been stated that sucralfate requires an acidic environment to be effective, however there is some evidence in rats that it may still exert beneficial effects at near neutral pH (Danesh et al., 1988).

Sucralfate is not a licensed veterinary medication and therefore must be used with owner informed consent according to the cascade.

2.7.2 Synthetic prostaglandin analogues

Misoprostol is a synthetic prostaglandin E_1 analogue (Varley et al., 2019) which is used as a treatment for NSAID-induced gastrointestinal disease in the horse (Martin et al., 2019). Misoprostol has been demonstrated to significantly reduce gastric acidity in the horse (Sangiah et al., 1989). It has also been demonstrated that misoprostol exerts cytokine-specific anti-inflammatory effects on equine leukocytes and inhibits neutrophil effector functions *in vitro* (Martin, Messenger, et al., 2017; Martin, Till, et al., 2017).

There is recent increasing interest in the use of misoprostol in the treatment of EGGD (Rendle et al., 2018), however, there is little evidence to support the efficacy of this unlicensed medication. One relatively small prospective clinical study demonstrated that misoprostol was superior to a combination of omeprazole and sucralfate for both EGGD lesion healing ($p<0.0001$) and improvement ($p<0.001$) (Varley et al., 2019). Although misoprostol appears promising based on the results of this study, the authors still reported that 28% of the 43 horses enrolled on the misoprostol arm of the study failed to respond to treatment. This study used client owned clinical cases, and as such there was no placebo group in the study, and a larger multicentre study ideally is required to fully elucidate the efficacy of misoprostol for treatment of EGGD. Preliminary work indicates that a dose of 5 µg/kg orally or per rectum is safe, however, ideal dosing regime and effects of long term administration have not been investigated in the horse (Lopp et al., 2019). The same study indicated that when dosed orally in fed horses there was an adverse effect on drug absorption, therefore until further information is available it is sensible to administer misoprostol before feed in clinical cases. This dose (5 µg/kg orally) has not been shown to reduced *ex vivo* TNF-α mRNA synthesis by equine leukocytes, however, this was based on a single dose in a small group of horses. Clinical and anecdotal evidence indicates that misoprostol should be considered as a monotherapy and has been advocated as a first line treatment option for EGGD (Rendle et al., 2018).

2.7.3 Histamine type 2 receptor antagonists

One early study reported significant improvement in both ESGD and EGGD lesions following treatment with cimetidine and ranitidine, and the authors reported 20% of glandular lesions had healed within two weeks, and described 100% resolution of all glandular lesions following four weeks of treatment (FURR et al., 1989). Therefore histamine type 2 receptor antagonists showed some initial promise as a treatment for EGGD. However, only five horses in this study were affected by EGGD lesions affecting the glandular fundus, and there was no description of lesions at the pylorus, likely because a two metre endoscope was used. It is also not clear from the report which horses received ranitidine or cimetidine; it is therefore challenging to use the results to inform treatment.

In a recent roundtable article discussing EGGD, it was concluded by an expert panel that there was no evidence to support the use of ranitidine, and there was no further discussion regarding other histamine receptor antagonists in the context of EGGD (Rendle et al., 2018).

Furthermore, in the UK prescribing decisions should be guided by the cascade; as there are no histamine receptor antagonists licensed for use in the horse, and no evidence to support their efficacy, it is not appropriate to include them in treatment protocols for EGGD.

Commented [VS(oVM2]: How do I reference this... find out

2.7.4 Diet

Feeding corn oil may decrease gastric acid production and result in increased PGE_2 concentrations, therefore enhancing gastric mucosal defence mechanisms (Cargile et al., 2004). However, the addition of corn oil to the diet was not sufficient to counteract the negative effect of feeding a high starch diet in one study (Luthersson, NIELSEN, et al., 2009). Unfortunately, although both glandular and squamous portions of the stomach were examined in this study, lesion grading combined both ESGD and EGGD lesions; therefore it is not possible to establish the effect on EGGD lesions directly. Additionally, there is evidence to suggest that feeding large meals induces increased gastrin release compared to feeding smaller meals (Sandin et al., 1998), which would be expected to reduce gastric pH to a greater extent. Therefore when treating horses with EGGD it is intuitive to provide smaller more frequent meals, and to avoid single large meals. There is also data describing an increased gastrin secretion response when horses are fed after treadmill exercise as opposed to when they are fed before exercise (Furr et al., 1994). This emphasises the potential importance of timing of feed as well as meal composition and size, and highlights the multifactorial nature of optimising management when treating horses with EGUS.

With regards to meal composition, there is little evidence to guide feeding protocols specifically for horses with EGGD (Rendle et al., 2018). However, as discussed previously, the aim of acid-suppression is still a cornerstone of treatment for EGGD. As such, advising owners to feed alfalfa forage may be appropriate, as this has been shown to increase gastric pH and ESGD scores were

significantly lower in horses on an alfalfa diet in one study (Nadeau et al., 2000). Unfortunately, this study enrolled only six horses and only one was affected by EGGD, meaning it is not possible to draw conclusions regarding feeding alfalfa and EGGD lesion healing.

It is intuitive to provide access to grazing for horses with EGGD, however there is no peer reviewed evidence to inform the clinician with respect to dietary optimisation in the treatment and management of horses with EGGD (Rendle et al., 2018). However, horses with EGGD are often concurrently affected by ESGD, and dietary management is well established for management of squamous disease. As such, owners of horses with EGGD often receive similar dietary advice.

2.8 The gastrointestinal microbiome

The microbiota of the gastrointestinal tract comprises an enormous number of organisms, including parasites, fungi, bacteria, archaea, and viruses (Costa and Weese, 2018). The genetic material derived from these species makes up the microbiome. The advent of accessible and affordable sequencing techniques has significantly expanded our knowledge of the microbiome, with previously used culture-based methods not accurately informing on either richness or relative abundance of the species comprising the microbiota.

The gastrointestinal microbiome is of increasing interest in both human and veterinary medical fields. There is growing evidence that changes in the gastrointestinal microbiota occur in many disease processes in the horse. Rapid alteration in the faecal microbiota has been demonstrated in association with post-partum colic,[1] non-surgical colic,[2] and in horses presenting with surgical or non-surgical colic compared to horses presenting for routine elective procedures.[3] Subsequently, there has been interest in using changes in the microbiome associated with conditions such as post-partum colic to pre-empt the onset of disease (Weese et al., 2015).

The equine gastrointestinal microbiome varies markedly according to gut compartment (Costa et al., 2015). The faecal microbiome is of particular interest due to the ease and non-invasive nature of sample collection, and

potential ease of clinical applications. The faecal microbiome has also been shown to vary within individuals over time. It has been established that the microbial population present in the lumen of the gastrointestinal tract compartments is not the same as the mucosal population at the same level. Ericsson et al. assessed different gut compartments in order to build a 'microbiological map' of the equine gastrointestinal tract. In this study luminal and mucosal samples were taken from all gastrointestinal tract compartments other than the mouth, oesophagus, and duodenum and submitted for 16s rRNA sequencing (Ericsson et al., 2016). A small number of horses (nine) were examined in order to establish the alpha and beta diversity of the samples. The most striking finding was the sudden change in the microbial population between the small and large intestine. They also highlighted that although the richness of species was greater in the large intestine, the species present in the large intestine were more closely related to one another.

2.8.1 Equine gastric microbiome

Less work has been undertaken to characterise the gastric microbiome in horses than other gut compartments. At first, the harsh environment of the stomach would intuitively mean that the stomach would harbour a less diverse microbiota than other gastrointestinal tract compartments, such as the mouth and large intestine. The gastric juice is able to significantly reduce the bacterial population of the stomach to bacteria capable of surviving in the harsh acidic environment (Wallace, 2008). Initially, it was believed that the large intestine had a richer and more diverse microbiome then the stomach, however, more recent work has demonstrated that this is not statistically significant (Ericsson et al., 2016). Ericsson et al. found that there was a greater number of operational taxonomic units in luminal and mucosal gastric and small intestinal samples than in the large intestinal compartments examined. However, this finding did not prove to be statistically significant.

Yuki et al. describe four further animals that were examined in the same study, however, no results were formally published, and only the squamous mucosa was investigated. The equine stomach has subsequently been found to have a far more diverse bacterial population than was initially thought.

Perkins et al. used fluorescence *in situ* hybridisation (FISH), using probes directed against 16s rRNA for all gastric regions from all horses. This study aimed to characterise the bacterial populations within specific mucosa-associated environments; glandular mucus, glandular epithelium, superficial squamous (?) epithelium, adherent mucus, and free mucus. This was only performed in a small number of horses; six sampled gastroscopically using a transendoscopic grab biopsy instrument, and three sampled *post mortem* using punch biopsy instruments. Findings common to all of the horses included a significantly higher proportion of *Streptococcus* spp. present in the glandular portion of the stomach compared to the squamous portion. Interestingly, this study also found that bacteria were only deeply invasive within the mucosa at sites of gastric ulceration, although only sites of squamous ulceration were identified (four of the nine horses had grade 2-3/4 squamous ulceration adjacent to the *margo plicatus*). Interestingly, this study did not find *Helicobacter* spp. to be abundant in healthy or ulcerated equine stomachs. The huge drawback to this study is that there were significant differences between the two different groups of horses, however, as factors such as husbandry were not controlled for, it is impossible to determine whether those differences are due to inter-horse variability, or sampling method.

A more recent study examining the entire gastrointestinal tract and its compartments, and examined samples taken *post mortem* from 11 horses using 16s rRNA sequencing of the V4 hypervariable region (Ericsson et al., 2016). The gastric mucosal samples in these horses were collected by scratching a collection tube against the glandular mucosa immediately ventral to the *margo plicatus*, with the large disadvantage that this method is not informative regarding different bacterial populations at different sites within the stomach. The most common phylum identified at this site was *Firmicutes*, with *Lactobacillus* and *Sarcina* spp. predominating. Interestingly, *Streptococcus* spp. became more abundant in the duodenum. Considering this information in conjunction with the findings of Perkins et al. it seems probable that if a more ventral portion of the glandular mucosa was sampled *Streptococcus* may have been identified in greater abundance; it is intuitive to believe that the different environments present at the dorsal and ventral aspects of the glandular mucosa would support different bacterial populations. However, both Perkins et al. and Costa et al.

agree that *Firmicutes* is the predominant mucosally associated phylum present in the equine stomach.

More recently, there has been increasing interest in applying culture-independent techniques to specifically investigate the gastric microbiome of horses affected by gastric ulceration. One study used 16S rRNA sequencing, targeting the V1-V3 hypervariable regions in 52 horses with the specific aim of identifying the presence of *Helicobacter*, as well as to describe the normal gastric microflora. One limitation of this study was using primers targeting the V1-V3 hypervariable regions, with V4-V6 described as the most reliable for showing full-length 16S rRNA sequences when performing phylogenetic studies, although frequently, techniques are not directly comparable between sequencing studies. Unfortunately, this paper also does not make it clear whether the horses are affected by squamous or glandular ulceration specifically, and horses were allocated a combined score based on grades (1999 EGUS council) assigned by three clinicians, which was translated to a descriptive healthy, mild, moderate, or severe score. All horses had the pyloric antrum sampled only using transendoscopic biopsy forceps, and no lesion-orientated sampling was undertaken. However, data are provided describing the glandular gastric microbiota, with *Firmicutes* (mean abundance 50%), *Proteobacteria* (18.7%), *Bacteroidetes* (14.4%), and *Actinobacteria* (9.7%) identified as the most common phyla.

Commented [VS(oVM3]: Out of sequence, reorder

Commented [SV4]: This reference needs chasing up

2.9 Variables affecting the gastrointestinal microbial community

Factors affecting the gastrointestinal microbiome have largely been investigated via faecal sampling, intended as a proxy for the hindgut microbiome; this does not accurately reflect the communities residing in the upper gastrointestinal tract. There is limited information to indicate that diet, including frequency of concentrate feed and roughage, amount of water drunk, and bedding substrate used affect the glandular gastric microbiota community (Dong et al., 2016). Interestingly, the same study did not identify an effect of antimicrobial administration.

Dietary change has been shown to induce rapid change in the faecal microbiome (Fernandes et al., 2014), and a change in the hindgut microflora was similarly identified after an abrupt change in diet in a small group of fistulated geldings (Muhonen et al., 2009). There has been little investigation in the 'natural' temporal stability of the microbiome according to normal annual fluctuations in diet. Other studies has identified that seasonal variation in pasture (Kobayashi et al., 2006) and environmental conditions (Salem et al., 2018) are significantly associated with changes in the faecal microbial community profile, suggesting that there is likely to be some temporal change of the individual microbiome corresponding to changes in management. There is data to support forage based diets promoting increased microbiome stability over time, however, this study only examined a small number of horses (six) over a relatively short time period (29 days) on each diet (Willing et al., 2009). Intuitively, these factors may be expected to affect the upper gastrointestinal tract, as well as the large intestinal and faecal microbiome, although supporting data is lacking. Principally studies have explored the relationship between the microbiome and grain versus forage based diets, with rapid changes induced secondary to feeds such as barley leading to increases in lactobacilli and streptococci in particular (De Fombelle et al., 2001; Van den Berg et al., 2013).

An effect of age has also been identified on the faecal bacterial community composition in one study investigating three different diets using 454-pyrosequencing (Dougal et al., 2014). Although there was a reduction in diversity with age, the authors identified the relative stability of a core microbial community, irrespective of the other factors investigated. Again, comparable information is not available for the proximal gastrointestinal tract.

2.10 Methods of sampling the gastrointestinal microbiota

There are no studies comparing methods of sampling the mucosally associated microbiota in horses, and to date most studies have only investigated the microbiota *post mortem*. There is no established gold standard sampling technique in the horse, and samples have been collected either by transendoscopic biopsy (Perkins et al., 2012; Dong et al., 2016), or by excisional biopsy and by scraping the mucosa *post mortem* (Costa et al., 2015; Ericsson et al., 2016).

There have been a larger number of studies investigating the mucosally associated microbiota *in vivo* in human patients, and methods of sample collection have been compared. A study comparing sampling of the ileal pouch in patients with a history of ulcerative colitis following proctocolectomy identified demonstrated that there were no significant taxonomic differences between biopsy and brush collected samples (Huse et al., 2014). The same study identified that significantly less host DNA was incorporated in brush collected samples, and as such there was a far higher proportion of bacterial DNA in brush samples, which were gathered over a wide surface area. The authors proposed that as well as causing less mucosal injury, brushes would be less likely to introduce sampling bias and less likely to underrepresent rare taxa by sampling a wider area (Huse et al., 2014). Similarly, a more recent study comparing three different methods in order to identify a technique that minimised contamination from luminal contents when sampling the duodenum transendoscopically found that samples collected with brushes collected the largest proportion of bacterial DNA compared to sheathed and unsheathed biopsy forceps (Shanahan et al., 2016).

2.10 Specific aims and objectives

In summary, the aims of this thesis are to:

- Investigate the equine gastric glandular microbiota *in vivo* in a population of Thoroughbred racehorses subject to the same management conditions
- Evaluate the microbial population at sites of EGGD lesions compared to normal mucosa, and investigate whether a dysbiosis or specific candidate pathogen may be implicated in the pathogenesis of EGGD
- Compare the equine gastric glandular microbiota of two different racehorse populations in order to explore similarities and differences between two populations that would be expected to have similar microbial community profiles
- Explore the use of transendoscopic cytology brushes for acquisition of mucosal samples appropriate for 16S sequencing
- Gather preliminary information on the temporal stability of the equine gastric microbiota

3 Yard One: pilot study

3.1 Introduction

High throughput sequencing techniques have become increasingly utilised to investigate the microbiome, and the use of cost-effective culture independent techniques has expanded our knowledge significantly (Costa and Weese, 2012). Most of the information published to date on the equine glandular gastric microbiota is derived from *post mortem* studies (Perkins et al., 2012; Costa et al., 2015), and community composition may differ significantly from that in living horses (Perkins et al., 2012).

Previous studies undertaken in horses investigating the mucosal microbial population have used samples taken by transendoscopic or *post mortem* biopsy (Richard J Hepburn, 2004; Perkins et al., 2012; Ericsson et al., 2016), or by scraping the gastric mucosa with collection pots *post mortem* (Costa et al., 2015). Previous suggestion that gastric bacterial microbiota is altered *post mortem* (Perkins et al., 2012) highlights the need to establish a mucosal sampling technique that can be used in live horses to gain the most accurate description of the gastric microbiota possible. Studies in other species have used cytology brushes to good effect for sampling the mucosally associated microbiota, and this has been shown to be superior to transendoscopic biopsy samples for this purpose (Huse et al., 2014).

The primary aim of this pilot study was to investigate whether cytology brushes could be used as an effective method for sampling the equine glandular gastric mucosally associated microbiota transendoscopically in horses presenting for gastroscopy. A secondary aim was to describe the gastric glandular microbiota at normal mucosa and sites affected by EGGD lesions.

3.2 Materials and Methods

3.2.1 Case selection

Eight horses were presented for gastroscopy having been identified by the trainer as performing poorly, or due to a specific concern regarding EGUS. No

horses had recently received antimicrobial treatment and none were receiving concurrent medications.

3.2.2 Gastroscopy and sample collection

Gastroscopy was performed at the Weipers Centre Equine Hospital, University of Glasgow, UK. All horses were transported to the hospital specifically for the purpose of gastroscopy by the trainer.

Horses were sedated (detomidine, Domidine®, Dechra, UK; and butorphanol, Dolorex®, MSD Animal Health, UK; dose adjusted according to temperament). The gastroscope was passed via a naso-oesophageal tube into the stomach, which was insufflated to allow visualisation of the *margo plicatus* and access to the pylorus. The glandular portion of the stomach was inspected first and lesions described according to the European College of Equine Internal Medicine consensus statement (B W Sykes et al., 2015); descriptors were agreed by one ECEIM or ACVIM specialist, and another experienced vet. Lesions were not lavaged to remove ingesta until samples had been collected, and samples were only collected from lesions and mucosa that were not coated with feed material.

Sheathed three metre cytology brushes (Endoscopy Cytology Brush, Eickemeyer, UK) were plugged with sterile Amies medium to prevent contamination, and passed into the stomach via the endoscope biopsy channel. When the sheath was approximately 1 cm clear of the endoscope the cytology brush was advanced through the sheath, dislodging the plug. The brush was then swept over the EGGD lesion to be sampled. After eight to ten sweeps over the target area, the brush was withdrawn into the sheath prior to pulling the guarded brush back out of the endoscope. The brush was cut off into a labelled sterile cryotube using wire cutters and snap frozen in liquid nitrogen. The cryotubes were moved to a -80 °C freezer as soon as possible and stored until DNA extraction.

After samples were obtained, ESGD lesions were graded according to published recommendation (The Equine Gastric Ulcer Council, 1999). Before the gastroscope was withdrawn from the naso-oesophageal tube, excess air remaining in the stomach was removed to minimise the risk of post-procedural colic. Horses were re-fed after the effects of the sedation had waned.

Treatment plans were established for each horse according to hospital policy, and the trainer was informed of the gastroscopic findings and an appropriate treatment plan for each horse.

The cryotubes containing the cytology brushes were removed from liquid nitrogen and stored in a freezer at -80ºC until ready for DNA extraction.

3.2.3 DNA extraction

Samples were removed from -80ºC storage and were allowed to thaw in the cryotubes for 15 minutes at room temperature prior to DNA extraction. The brushes were then folded into the bottom of the cryotubes using sterile forceps in order that they would be immersed in the initial solution. DNA was extracted using the Qiagen Blood and Tissue Kit (Qiagen, Germany).

20μL of proteinase K and 180μL of Buffer ATL was added to the cryotubes which were then vortexed vigorously for 30 seconds. The tubes were incubated at 56ºC for one hour. Samples were then vortexed for 15 seconds and pulse centrifuged, prior to adding 200μL of Buffer AL and vortexing again for 15 seconds. Samples were then pulse centrifuged, prior to adding 200μL of 100% ethanol. Samples were vortexed again for 15 seconds and pulse centrifuged.

The mixtures were then pipetted into spin columns in 2 ml collection tubes, and the brushes discarded. The tubes were then centrifuged at 8 000 rpm for one minute. The flow through and collection tubes were then discarded. The spin columns were placed in new collection tubes and 500 μL of Buffer AW1 was added and the tubes centrifuged at 8000 rpm for one minute. The flow through was again discarded and the spin columns were placed in new 2 ml collection tubes. 500 μL of Buffer AW2 was then added, prior to centrifugation for three minutes at 14 000 rpm. The flow through and collection tube were again discarded, and the spin column added to a 1.5ml Eppendorf. 200 μL of Buffer AE was added to the membrane, and then the columns were incubated at room temperature for five minutes prior to centrifugation at 8 000 rpm for one minute. Spin columns were then discarded.

The DNA concentration in the final solution was assessed using a benchtop fluorometer (Qubit 4 Fluorometer, Thermo Fisher Scientific, UK). Initially a broad range assay was performed, however DNA concentration was too low to be quantified. Subsequently, high sensitivity assays were performed on all samples.

3.2.4 Ethanol precipitation

Due to the low concentration of DNA acquired; a modified ethanol precipitation technique was performed in order to provide a concentration sufficient for sequencing. Ethanol precipitation was performed on all samples with a DNA yield less than 10 ng/μL.

20 μL of sodium acetate and 500 μL of 100% ethanol were added to 200 μL of the DNA sample solution, and 1 μL of linear polyacramide as a carrier (GenElute LPA, Sigma-Aldrich Company Ltd., UK). The sample was then vortexed for 10 seconds and centrifuged for five minutes at 12 000 g. Following this, the DNA pellet was identified, and the supernatant removed carefully with a pipette. The pellet was then washed in 70% ethanol and centrifuged at 12 000 g for five minutes. The supernatant was again decanted, and the DNA pellet was allowed to air dry for 10 minutes. The DNA precipitate was resuspended in 1 x Tris EDTA buffer to a final volume of 30 μL. DNA concentration was reassessed by a bench top fluorometer. This revealed an increase in DNA concentration in the majority of the samples.

3.2.5 DNA extraction and ethanol precipitation: amended method

Due to the low DNA yield and concentration in the final solution, the final elution volume was decreased to 40 μL of buffer AE. The total DNA yield remained too low for sequencing (target 1 ng/μl), and therefore ethanol precipitation was performed as described above, however due to the lower starting sample volume the method was adjusted. 40 μL of sample solution was added to 4 μL of sodium acetate and 100 μL of 100% ethanol. 1 μL of linear polyacramide (GenElute LPA) was then added to this solution. The sample was then centrifuged to form a DNA pellet as described above.

3.2.6 Sequencing, library preparation, and analysis

16S sequencing libraries were prepared from the purified DNA starting with 12.5 ng of DNA per sample. The libraries were prepared using a two-step PCR protocol based on the standard published Illumina protocol (Illumina, USA). The first stage was a PCR performed with locus-specific primers for the V3 and V4 regions of 16S rRNA gene, which also contained the Nextera overhang sequence. The second stage involved the addition of Nextera XT v2 Adapters with unique barcodes to allow multiplexing. Complete libraries were purified using SPRI select beads (Beckman Coulter, UK), which were then quantified using Qubit (ThermoFisher Scientific, UK), and library size was determined using an Agilent 2100 Bioanalyser (Agilent Technologies LDA UK Ltd., UK). Libraries were then combined in an equimolar manner before being sequenced to an average depth of 100,000 reads per sample on an Illumina MiSeq instrument using paired end 2x300 bp reads.

FastQ files were quality filtered before trimming. Reads less than 250 bp in length were discarded. The Qiime (version 1.9.1) pipeline was used to analyse the sequences. Operational taxonomic units (OTUs) were defined on the basis of 97 % similarity and sequences from each OTU referenced against the Greengenes database (v13_5) to assign taxonomy. A Biological Observation Matrix (BIOM) table was generated and samples with fewer than 5,000 sequences excluded before the table was rarefied to 5,000 sequences per sample. Alpha and beta diversity analyses were performed on the rarefied OTU tables. Group comparisons were corrected using the Benjamin-Hochberg false discovery rate (FDR) procedure for multiple comparisons. Weighted and unweighted UniFrac distances were established and principle co-ordinate analysis (PCoA) plots created to compare similarity between individual samples. Weighted analysis was undertaken to give a quantitative assessment of sample composition similarity, and unweighted was performed as a more qualitative measure in order to be less likely to obscure low abundance phyla. Normal mucosa and EGGD lesion populations were compared at the phylum level using Wilcoxon rank tests performed using commercially available statistical software (RStudio 2020) using publicly available scripts. Significance was set at < 0.05.

3.3 Results

Eight Thoroughbred flat racehorses in training were enrolled from Yard One. Horses ranged in age from three to seven years, with a mean age of five years. There were seven geldings and one filly. Horses were presented for gastroscopy in two groups, one month apart. Two of the horses had no glandular lesions, six had EGGD lesions. Three horses had erythematous lesions adjacent to the pylorus, two had haemorrhagic and fibrinosuppurative lesions, and one had a pinprick haemorrhagic lesion. All horses had ESGD lesions documented (12.5% of horses grade 2, 50% grade 3, 37.5% grade 4). Only one horse (SV008) was not in active race training at the time of examination. None of the horses had access to pasture, all were receiving ad lib haylage, and a diet of the same commercial hard feed.

Nineteen samples were submitted for sequencing from Yard One; 17 samples were successfully sequenced and two failed (SV004-1 and SV008-3). Overall, the mean number of reads per sample was 56369.94, the minimum number of counts was 37060.0, and the maximum was 93018.0 (SD 13094.5). A total of 15 bacterial phyla were positively identified. Any phyla that did not achieve a minimum of 1% of total reads were combined into the 'other' category.

ID	**Sample ID**	**Description**	**Age (years)**	**Sex**	**In training?**
SV001	SV001-1	HF	4	M	Y
	SV001-2	N	4	M	Y
	SV001-3	HF	4	M	Y
	SV001-4	E/PH	4	M	Y
SV002	SV002-1	E/PH	6	M	Y
	SV002-2	N	6	M	Y
SV003	SV003-1	E	5	M	Y
	SV003-2	N	5	M	Y
SV004	SV004-1†	E	4	M	Y
	SV004-2	N	4	M	Y
SV005	SV005-1	N	7	M	Y
	SV005-2	N	7	M	Y
SV006	SV006-1	N	4	M	Y
SV007	SV007-1	E/PH	7	M	N
	SV007-2	N	7	M	N
SV008	SV008-1	PM	3	F	Y

SV008-2	H	3	F	Y
SV008-3 †	N	3	F	Y
SV008-4	F	3	F	Y

Table 3.1: Samples acquired from all Yard One horses. Lesion description key: HF, mixed haemorrhagic/fibrinosuppurative; E, erythematous; PH, pinprick haemorrhagic; F, fibrinosuppurative; N, normal mucosa; PM, polypoid mass. Sex: M, male; F, female. Training: Y, in training; N, not currently in full training. † samples that were not successfully sequenced

3.3.1 Individual animal results

3.1.1.3 Alpha diversity analysis

Rarefaction curves were plotted in order to assess species richness across samples from individual animals. Rarefaction curves of observed OTUs demonstrated that samples from all animals were similar in richness when plotted to 5000 sequences per sample. SV006 had a lower number of observed OTUs than the other animals, however, the curve for SV006 represents a single sample (figure 3.1). Rarefaction curves indicated that samples were sequenced in adequate depth.

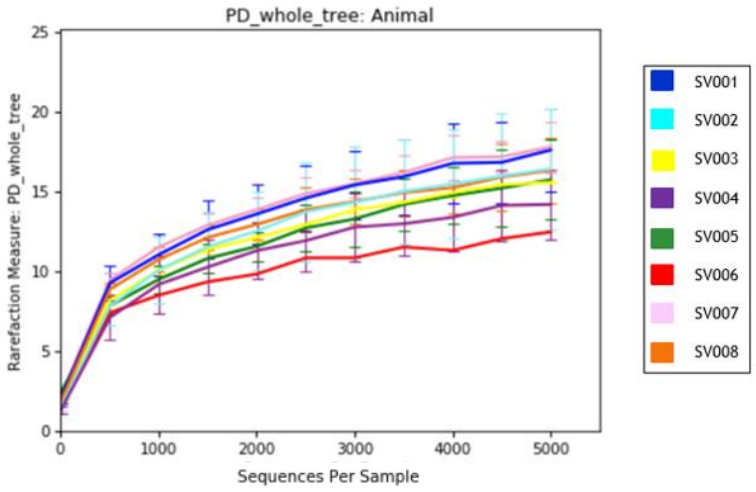


Figure 3.1: Rarefaction curve displaying richness by animal (pooled EGGD and normal samples) to 5000 sequences per sample. Horse SV006 represents a single sample only.

When assessing samples according to animal they were collected from, with EGGD and normal mucosa samples pooled, the predominant phylum present was *Proteobacteria* (mean 64.56%, range 83.2 – 22.0%). *Bacteroidetes* (mean 14.65%, range 4.60 – 19.70%) and *Firmicutes* (mean 11.51%, range 83.2 – 22.0%) were the next most common phyla, followed by *Actinobacteria* and *Fusobacteria* (Fig 3.2). SV005 had a notably different distribution to the other cases, with *Actinobacteria* more prevalent compared to the other horses. No significant differences in management or diet of this horse were noted in the clinical history, on clinical exam, or on gastroscopic examination to account for this difference in bacterial community composition. When the OTU abundances were displayed to a class level (Fig. 3.3), individual animal community differences were apparent. For instance, at a phylum level both SV006 and SV007 initially appear similar with respect to *Proteobacteria prop*, with *Proteobacteria* dominating, however, SV007 samples have a higher proportion of *Alphaproteobacteria* and *Betaproteobacteria*, and inSV006 samples proportion of *Gammaproteobacteria* is greater.

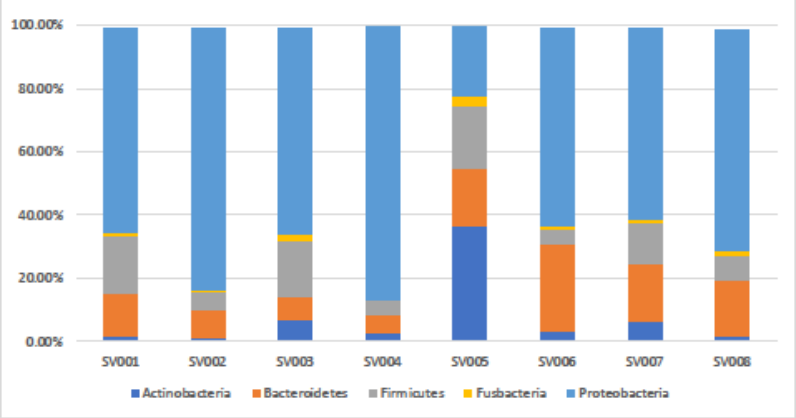


Figure 3.2: 100% stacked bar charts displaying relative abundance to phylum level by individual animal. SV005 has a notably different community composition when compared to other horses.

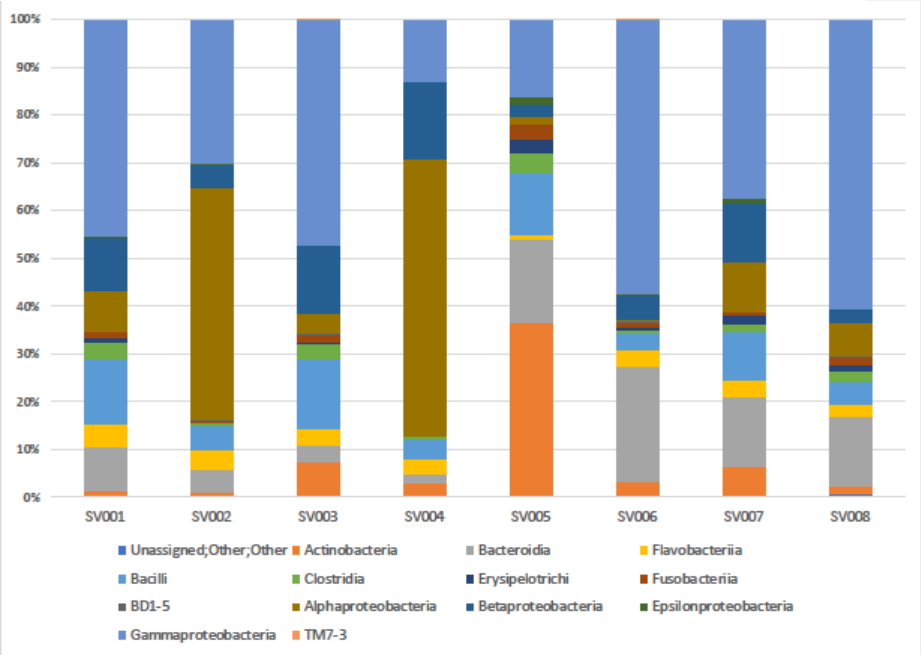
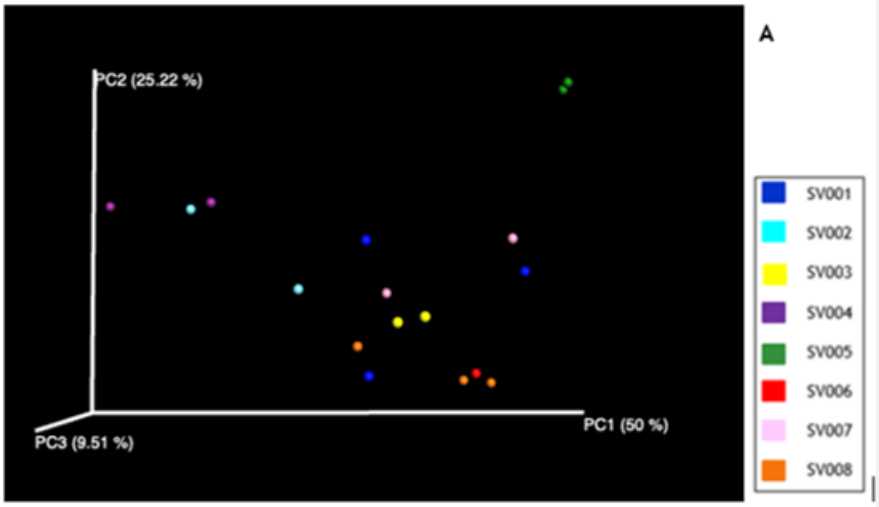


Fig 3.3: 100% stacked bar charts showing differing community composition at a Class level by individual animal (EGGD and normal mucosa samples combined).

3.1.1.4 Beta diversity analysis

Sample similarity was compared using weighted and unweighted UniFrac analyses. PCoA plots were then created (Fig 3.4), plotting sample similarity in a three-dimensional space. Individual animal samples showed some clustering in both the unweighted and weighted PCoA plots, but samples were more tightly clustered in the unweighted plot, indicating that less abundant taxa are likely important determinants in individual community profile and are likely to be obscured when quantitative analysis is undertaken.



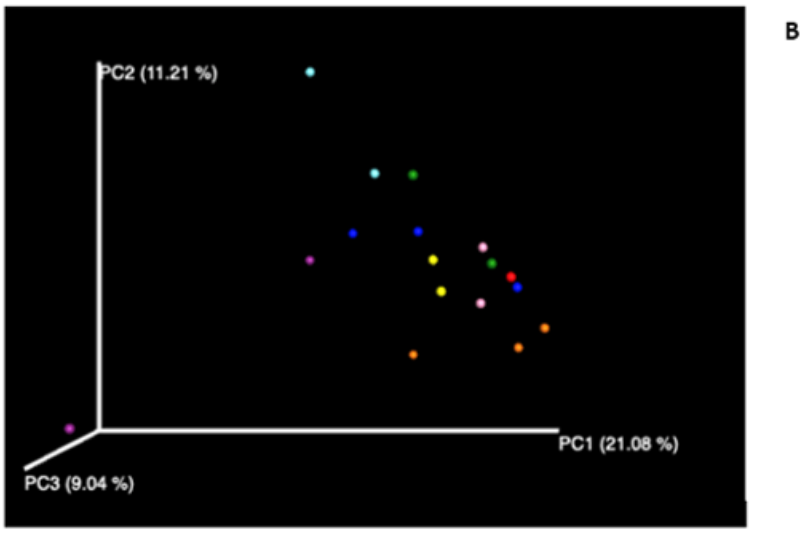


Figure 3.4: (A) weighted and (B) unweighted UniFrac PCoA plots displaying sample similarity in a three dimensional space. Each sphere represents a single sample, and these are colourcoded according to individual animal.

Clustering of samples from individual animals can be noted, principally clustering according to the PC1 axis.

3.3.2 Comparing EGGD lesions to normal mucosa

3.1.1.5 Alpha diversity analysis

The mean number of reads from normal glandular mucosa (55709.9) was similar to the mean number of reads from EGGD lesions (56945.6). Rarefaction plots were created to assess sample richness of normal versus abnormal gastric glandular mucosa. Rarefaction curves were plotted to 5000 sequences per sample, indicating that samples were sequenced in sufficient depth (Fig 3.5). Alpha diversity was calculated using the PD Whole Tree metric, revealing EGGD lesions to have more complex bacterial communities than normal mucosa, this achieved near statistical significance (EGGD lesion mean 17.346, SD 2.126; normal mucosa mean 14.771, SD 2.607; p = 0.052). This is displayed in Fig. 3.6.

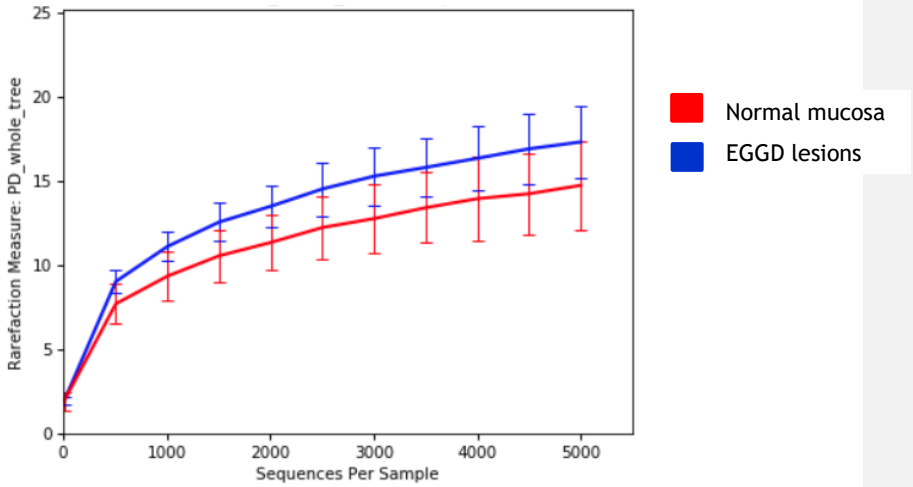


Fig 3.5: PD whole tree metric calculated rarefaction curve, displaying increased richness of pooled EGGD lesion samples compared to normal mucosa.

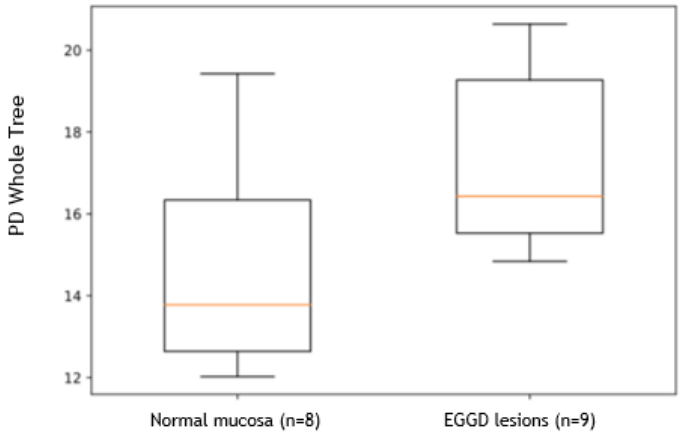


Fig. 3.6 Alpha diversity PD Whole Tree metric boxplot comparing normal mucosa and EGGD lesion diversity.

Bar charts were plotted at a phylum level after removing rare phyla (accounting for less than 1% abundance). This showed *Proteobacteria* to be the most common phylum in EGGD lesion samples and normal mucosa samples (Fig 3.7).

When median abundance was compared for the most abundant phyla between normal mucosa and lesion samples no significant differences were found between the two groups (Table 3.2).

Phylum	Normal (median)	Lesion (median)	p-value
Actinobacteria	2%	5%	0.7722
Firmicutes	8%	13%	0.772
Bacteroidetes	12%	15%	0.847
Fusobacteria	1%	1%	0.815
Proteobacteria	67%	65%	0.743

Table 3.2: Relative phylum abundance at sites of EGGD lesions and normal mucosa. Abundances do not total 100% due to omission of the lower abundance phyla. Comparison of each phylum between normal and EGGD lesion samples did not reveal any significant differences (p = >0.05).

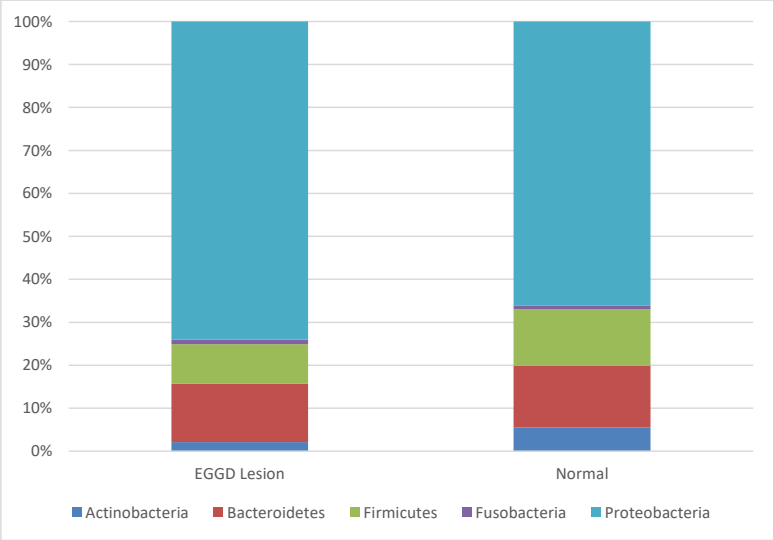


Figure 3.7: stacked 100% bar charts comparing microbial community composition at a phylum level between EGGD lesions and normal glandular mucosa. Community composition appears similar between the two groups.

3.1.1.6 Beta diversity analysis

PCoA plots were generated using the weighted and unweighted UniFrac analysis output (Fig. 3.8). There was no discernible clustering of samples in the weighted analysis, indicating that there was not strong similarity between individual normal mucosa and EGGD mucosa samples. The unweighted PCoA plot indicates stronger clustering of both normal and EGGD samples, indicating again that lower abundance taxa are likely important determinants of the bacterial

community profile. There is a lot of overlap between the normal and EGGD groups even when low abundance taxa are accounted for.

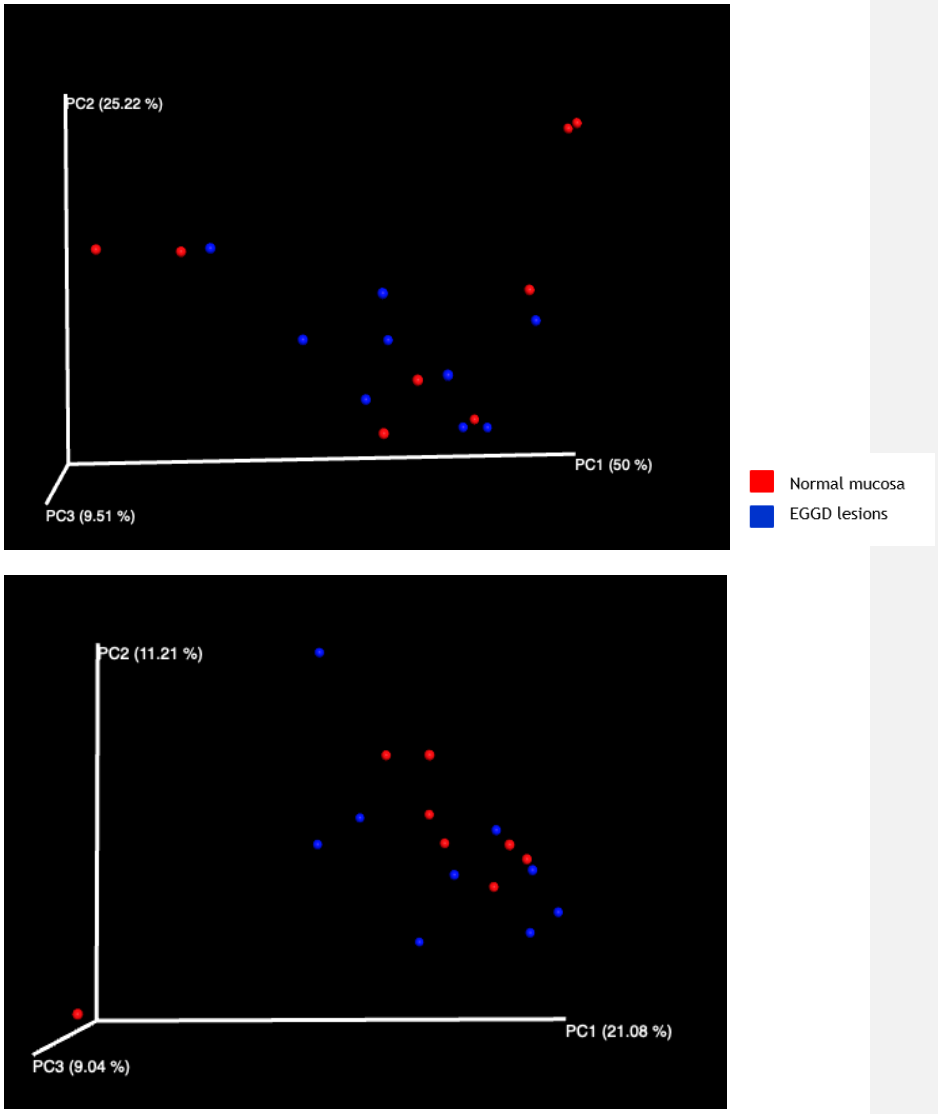


Fig 3.8: weighted (A) and unweighted (B) PCoA plot displaying quantitative sample similarity according when assessed by normal and EGGD lesion samples. There is no discernible clustering of either group.

3.1.1.7 Lesion type

Overall, there were very few samples of each individual lesion type (Table 1), and therefore it was not possible to perform meaningful detailed analysis comparing the microbial population present at different EGGD lesion types. Qiime group significance analysis comparing OTUs present at each different lesion category did not find a significant difference between any of the comparisons ($p = >0.05$). This includes between normal mucosa and any of the individual lesion categories.

3.4 Discussion

The findings of this pilot study are supportive of *Proteobacteria* being the predominant phylum present at the pylorus of equine stomachs, followed by *Bacteroidetes* and *Firmicutes*, and does not support the findings of previous samples collected from live cases, where *Firmicutes* was found to be dominant (Perkins et al., 2012; Dong et al., 2016). There was an indication that samples may be clustering in terms of similarity more by horse than by whether they were collected from normal or abnormal mucosa. Although more data are required in order to explore this further, currently the reported data suggest that irrespective of lesions, individual animals are likely to have a unique glandular mucosal microbial community profile. This echoes findings from other studies investigating other gastrointestinal compartments (Kobayashi et al., 2006; Willing et al., 2009; Blackmore et al., 2013; Salem et al., 2019).

The results of this pilot study do not replicate the findings of the limited *ante mortem* data available describing the live horse glandular gastric microbiota. Dong et al. also reported *Firmicutes* to be the predominant phylum in a small group of racehorses sampled using transendoscopic biopsies (Dong et al., 2016). Other previous work, reporting only two normal glandular samples acquired by transendoscopic biopsy forceps, demonstrated that *Firmicutes* was overwhelmingly more abundant in these two samples, followed by *Proteobacteria* and *Bacteroidetes* (Perkins et al., 2012). In the *post mortem* samples acquired in this study, *Firmicutes* abundance diminishes and the

proportion of *Proteobacteria* and *Bacteroidetes* increases; with the overall most abundant phyla variable between individual animals (Perkins et al., 2012). Inter-individual variation in phylum abundance was also a feature of Dong et al.'s study. There are a number of reasons why the results of our pilot study are different these reports. Firstly, a greater number of samples were acquired from each horse than in both of these studies, and as a greater number of samples were acquired from different sites within the same horse, it is less likely that horse to horse variation would skew our results. It is also possible that our use of cytology brushes has given different results by sampling a wider area and therefore this technique is less likely to be susceptible to sampling bias (Huse et al., 2014). This pilot study is the first documented use of sheathed transendoscopic cytology brushes for this purpose, and there is no data comparing this method to transendoscopic biopsy samples for 16s rRNA sequencing. However, other studies have found cytology brushes to be superior when used for a similar purpose in humans (Huse et al., 2014), and have been found to sample less host DNA and a larger proportion of bacterial DNA (Shanahan et al., 2016).

Additionally, this study provides preliminary evidence of a dysbiosis associated with EGGD lesions, with EGGD lesion samples more diverse than normal glandular mucosa samples. This finding requires more investigation in a larger population of horses to see whether this is a consistent finding, and to date, there is not enough information available in the literature to compare the findings of this pilot study. It is challenging to establish whether changes in the microbiome are causative, or are secondary to another disease process. However, establishing whether the bacterial population is altered in horses with glandular disease provides another piece of the puzzle with respect to the aetiopathogenesis of EGGD in horses. When assessing the sequencing output for the EGGD lesion samples in this cohort of horses it was not possible to identify a candidate pathogen, equally, analysis did not identify a single bacterial phylum to be significantly more or less abundant in EGGD lesion samples compared to normal mucosa. This may be a genuine finding, or this may be an effect of small sample size.

This study has established that cytology brushes provide samples rich enough to use for 16S rRNA sequencing analysis of the equine glandular gastric microbial community. They can be used easily and effectively with minimal training transendoscopically, meaning that this technique can be used in clinical cases presenting for gastroscopy. Previous studies have largely focused on samples taken at necropsy, however, samples collected in this manner are unlikely to best represent the gastric microbiome in life, and as such development of minimally invasive transendoscopic techniques should be prioritised. One study showed that there is a significant difference in gastric mucosal bacterial population of both squamous and glandular mucosa between samples taken from live horses and from samples taken *post mortem* (Perkins et al., 2012). It is likely this methodology can be applied to other sites in the horse, such as the squamous gastric mucosa, proximal duodenum, and respiratory tract. An additional benefit of cytology brushes versus transendoscopic biopsy techniques is that a wider area of mucosa can be sampled effectively which is less likely to introduce sampling bias especially when considering low abundance taxa (Huse et al., 2014). Grab biopsy forceps typically obtain samples a few millimetres in diameter, and of limited depth, limiting the area of mucosa sampled. Additionally, although work in humans has indicated that sheathed biopsy instruments are likely to provide samples useful for mucosal 16S studies, to the author's knowledge, sheathed transendoscopic biopsy instruments are not available for equine gastroscopes. As such, it may be expected that unsheathed samples are prone to contamination from luminal contents and therefore may not accurately reflect the mucosal population. Some mild increase in erythema, and in cases of haemorrhagic lesions, some mild haemorrhage, was observed post-sampling the lesion sites with cytology brushes in our study, however mucosal damage is minimal compared to use of biopsy forceps (Huse et al., 2014).

DNA yield after extraction was low from all of the samples in this study. There may be a number of reasons for this, including low bacterial content in the samples, failure to release biological material from the cytology brushes, or a failure to break down bacterial cell walls during the lysis step. There is a possibility that this reflects an inability of the cytology brushes to collect biological material from the mucosal surface, however, there were large

quantities of mucus visible grossly on the cytology brushes in all cases, and this is considered less likely. In order to improve DNA yield in future studies, adding a bead beating step to the DNA extraction protocol may be beneficial. The duration samples are frozen prior to DNA extraction is unlikely to have had a significant effect on community (Fouhy et al., 2015; Shaw et al., 2016; Anderson et al., 2016). All of the samples from this pilot study were removed from -80°C storage at the same time for DNA extraction, meaning that due to the samples being collected at four week intervals some samples were frozen for four weeks longer. However, this did not have an impact on the DNA yield obtained from these samples as assessed by fluorometer.

The sample population was a small group of Thoroughbred horses from a single race yard in Scotland. For the purposes of the pilot study our intention was to use a single yard in order to minimise the effects of other variables such as environment, management, and diet on the results obtained. Geldings were over-represented in this cohort of horses. There is some evidence to suggest that sex influences individual microbiota composition in other species (Bolnick et al., 2014; Org et al., 2016), however thus far there host genotype does not appear to have a significant effect on microbiota community in horses (Plancade et al., 2019; Mach et al., 2020). Despite this, future studies should seek to include mares, stallions, and geldings in order than any effect of sex on gastric microbiota can be identified.

In this pilot study we attempted to use horses as their own controls, in order to establish whether the bacterial population is altered at sites of diseased mucosa in individual animals. It is not possible to establish whether the samples from grossly normal mucosa represent a truly normal microbial population. Lesions were not taken from mucosa more distant to EGGD lesions due as the local environment is varies according to region of the glandular mucosa therefore it is possible that bacterial population would vary too.

Ideally more samples would be collected from normal and abnormal mucosa, however, taking samples in a manner that does not contaminate the brushes adds time to the gastroscopic examination, and so as not to negatively impact animal welfare, this limited the number of samples that could be collected from each horse. Additionally, the number of individual lesion types identified was

relatively low. In order to get a larger number of each lesion type significantly more horses would need to be recruited to the study. Another disadvantage to this study is that none of the recruited horses had normal stomachs and every animal had ESGD. This was not unexpected based on reported prevalence of ESGD lesions in the UK racehorse population. As it is not possible to screen for the presence or absence of ESGD and EGGD without gastroscopy, it is likely that a very large number of horses would need to be enrolled in future studies to have sufficient representation of each lesion type, as well as animals with and without ESGD in order to establish the effect this may have on results.

4 Yard Two: longitudinal study

4.1 Introduction

Initial investigation at Yard One indicated that a dysbiosis was associated with the presence of EGGD lesions, although a candidate pathogen was not identified, there was increased alpha diversity of EGGD lesion samples which approached statistical significance. The primary aim of investigation at Yard Two was to gather further data describing the glandular gastric microbiota in a comparable population of horses kept under similar management conditions in a different local environment. We sought to collect samples from normal mucosa and EGGD lesions in order to further explore the possibility of a dysbiosis present at EGGD sites. Another aim of the investigation at Yard Two was to gather preliminary information regarding the temporal stability of the glandular gastric bacterial population using time points six months apart. Until recently, there were relatively few longitudinal studies of the equine gastrointestinal microbiome. One study investigating temporal stability of the faecal microbiome of ponies demonstrated a strong stability of individual faecal microbiome profiles over two separate 72 hour periods (Blackmore et al., 2013). Similarly, other studies of the faecal microbiome in periparturient mares suggest relative stability in alpha and beta diversity around the time of foaling (Salem et al., 2019). However, longer term data reporting microbiome stability over the course of a year in animals kept at pasture indicated that annual change in environmental conditions was associated with altered faecal microbial community profiles (Salem et al., 2018).

To the author's knowledge, there are no studies reporting the longitudinal stability of the equine gastric bacterial community.

Following the pilot study, a secondary aim of investigation undertaken at Yard Two was to assess whether use of the cytology brush collection method was repeatable, and to attempt to replicate the method outside of a hospital environment.

We hypothesised that a dysbiosis would be associated with EGGD lesions, as was described at Yard One, and that the dominant phyla identified at Yard One would be identified with similar abundance in another cohort of racehorses kept at a different premises.

4.2 Materials and methods

4.2.1 Selection of cases

The cohort consisted of five Thoroughbred racehorses (four geldings, one mare) from a single training yard in Scotland, aged between two and five years, with a mean age of three years (Table 1). These horses were presented by the trainer for gastroscopic evaluation due to recent poor performance and the absence of clinical signs localising to other body systems. All horses were fed a similar diet, stabled at the same yard, and received no grass turn out. Dietary intake consisted of *ad lib* haylage, with racing cubes and chaff. All horses were first sampled in September 2018 (S1) whilst not in training, and the procedure was repeated in all five horses after a further six months (S2), once they had returned to training. One additional horse was added to the study, which underwent gastroscopy on S2 only.

4.2.2 Sample collection

Horses were first sampled after the end of the Flat season when they were not in training (S1). A clinical examination was performed on each horse by one of the authors (SJV) before gastroscopy was undertaken. Gastroscopy was performed at the home yard under standing sedation (detomidine (Domidine®, Dechra UK) and butorphanol (Dolorex®, MSD Animal Health UK), dose adjusted according to horse temperament) and all horses were examined on the same day. Gastroscopy and

sample collection protocol was the same as for the pilot study protocol at yard one (Chapter 3). Following collection of samples the owner was given a verbal and written report of the gastroscopic findings, and advised of treatment and management changes for each horse as appropriate.

The horses were examined for a second time (S2) approximately six months later once they had returned to training. Gastroscopy was repeated and samples collected as described previously. The trainer had not treated any of the horses with any medications or changed their diet since the S1 sample collection time-point. One additional horse on the same training regimen, a seven-year-old gelding was presented for examination at S2 and data from this case is also included in the present study.

4.2.3 DNA Extraction

DNA extraction was performed on the samples in a similar fashion as described in the Yard One pilot study (Chapter 3), but with an amended method in order to attempt to improve DNA yield.

Following the addition of Proteinase K to the thawed samples the samples were vortexed for for 30 seconds before incubation at 56°C for one hour to improve yield from cytology brushes. DNA extraction was then undertaken as per the manufacturer's protocol. Samples were finally eluted in 40 µL of buffer AE. The DNA concentration in the final solution was assessed using a benchtop fluorometer as per Chapter 3.2.3. A modified ethanol precipitation technique was performed on all samples with a DNA yield less than 10 ng/µL as described in Chapter 3.2.5 to provide a concentration sufficient for sequencing. The DNA precipitate was resuspended in Tris-EDTA buffer to a final volume of 30 µL. DNA concentration was reassessed by fluorometer, which revealed an increase in DNA concentration in the majority of samples.

4.2.4 Sequencing and library preparation

Sequencing, library preparation, and analysis was performed as described in Chapter Chapter 3.2.6. In addition, linear discriminant effect size (LEfSe) analysis was performed in Qiime (ver 1.9.1) using the Koeken tool in order to

look for ‘biomarkers’ associated with EGGD mucosa compared to normal glandular mucosa. A bar chart representing the effect size (LDA) was produced, and a cladogram was generated to provide a visual representation of the branches of the phylogenetic tree associated with EGGD lesion and normal mucosa samples.

4.3 Results

4.3.1 Sample collection

Horse signalment and gastroscopic findings are detailed in Table 1. Three horses had EGGD lesions at S1 examination, one horse had EGGD at S2 examination. Twenty four normal mucosa samples (8 at S1, 16 at S2) and ten EGGD lesion samples (7 at S1, 3 at S2) were acquired (Table 1). All the horses sampled at S1 were sampled again at S2, plus one additional seven-year-old gelding. At S1 none of the horses were in training, at S2 all except one horse (TB04) were in training. All samples were collected at the yard successfully, and the samples were kept frozen in liquid nitrogen until it was possible to place them in storage at -80°C

4.3.2 Sequencing output

A total of 34 samples were submitted for sequencing, five normal mucosa samples (2TB01-1, 2TB03-1, 2TB03-2, 2TB05-3, 2TB06-3) and one EGGD lesion sample (2TB02-2) were not sequenced successfully. The read depth for individual samples was on average 64,227. A total of 19 bacterial phyla were identified in the course of the study and only a small proportion of reads (0.3 %) could not be allocated to a particular phylum. Phyla that did not achieve at least 1% of reads for at least one horse were assigned to the ‘other’ category.

4.3.3 EGGD lesions compared to normal mucosa

4.3.3.1 Alpha diversity analysis

Analysis was performed on all S1 and S2 samples. Three different community diversity indices (PD Whole Tree, Chao 1 and observed OTUs) indicated that there was not a large difference in diversity between normal mucosa and lesion samples, with considerable overlap between the two groups (Fig. 4.1). PD Whole

Tree alpha diversity analysis did not reveal a significant difference in richness between the EGGD and normal mucosa groups (EGGD lesions mean 33.973, SD 13.857; normal mucosa 37.106, SD 10.431; p = 0.525); displayed in Fig. 4.2.

When phylum-level composition of the samples from each group was assessed, irrespective of date sampled or EGGD lesion description, normal mucosa had a higher proportion of *Proteobacteria* (46.3 %) compared to lesions (18.9 %) (Fig. 4.3). In contrast, the relative abundance of *Firmicutes* was lower in samples from normal mucosa (20.0 %) compared to lesions (41.2 %), these differences in community composition were found to be significant (Table 4.2). When abundance was examined at class, order, and at genus level it was apparent that the greater abundance of *Firmicutes* at EGGD lesion sites was due to an over-representation of *Sarcina*, belonging to the order *Clostridiales*, of the *Firmicutes* phylum. In order to explore this further, individual samples were plotted in stacked bar charts at genus level and three samples from EGGD lesions (TB02.1, TB03.4, TB03.5) were found to have a very high relative abundance of *Sarcina* (83.4 %, 92.4% and 89.2 % respectively). In contrast, *Sarcina* accounted for 0.2% of the total counts in all the normal mucosa samples combined.

ID	Age at S1 (years)	Sex	Sample ID	Sampling date	Sample	ESGD grade
TB01	3	Gelding	TB01-1	S1	N	3
			TB01-2		N	
			2TB01-1 ‡	S2	N	2
			2TB01-2		N	
TB02	2	Gelding	TB02-1 †	S1	PH	3
			TB02-2		N	
			2TB02-1	S2	FH	3
			2TB02-2 ‡		FH	
			2TB02-3		N	
			2TB02-4		FH	
			2TB02-5		N	
TB03	3	Gelding	TB03-1	S1	F/PH	3
			TB03-2		F/PH	
			TB03-3		N	
			TB03-4 †		F	
			TB03-5 †		F	
			2TB03-1 ‡	S2	N	3
			2TB03-2 ‡		N	
TB04	2	Gelding	TB04-1	S1	N	0
			TB04-2		N	
			2TB04-1	S2	N	0
			2TB04-2		N	
			2TB04-3		N	
TB05	5	Mare	TB05-1	S1	F	2
			TB05-2		F	
			TB05-3		N	
			TB05-4		N	
			2TB05-1	S2	N	2
			2TB05-2		N	
			2TB05-3 ‡		N	
			2TB05-4		N	
TB06	7	Gelding	2TB06-1	S2	N	4
			2TB06-2		N	
			2TB06-3 ‡		N	

Table 4.1: signalments of horses and gastroscopic findings on both sampling dates. Abbreviations: ESGD, equine squamous gastric disease; S1, first sampling date; S2, second sampling date; N, normal glandular mucosa; PH, pinprick haemorrhagic; FH, flat haemorrhagic; F, fibrinosuppurative. ESGD lesions were graded 0-4, as per 1999 EGUS Council recommendation.11 Samples marked † are associated with a significantly increased abundance of *Sarcina*. Samples marked ‡ were not successfully sequenced.

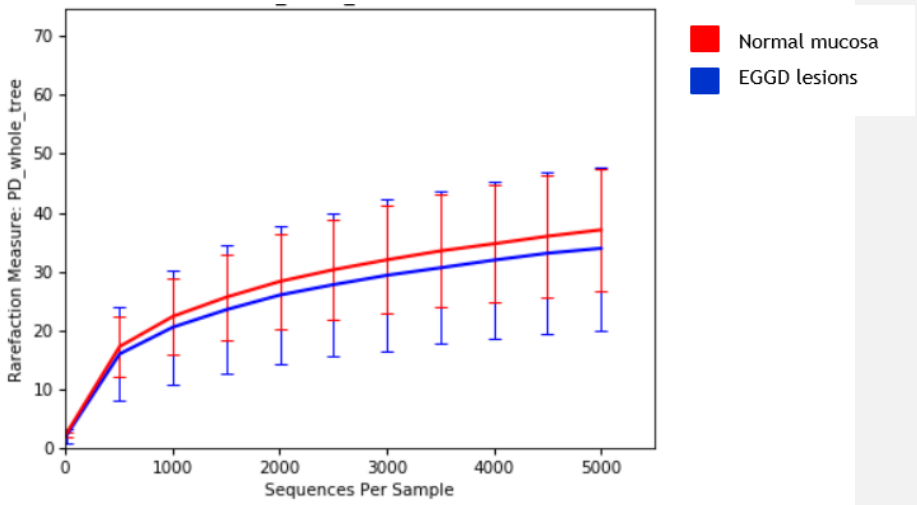


Fig 4.1: PD Whole Tree metric rarefaction curve comparing sample richness of EGGD lesion samples and normal mucosa, which are comparable. Rarefaction curve indicates samples were sequenced in adequate depth.

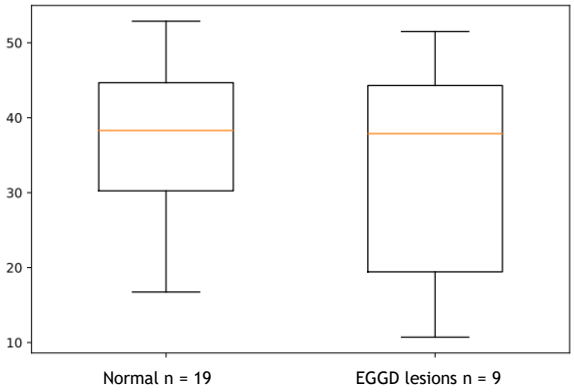


Fig 4.2: PD Whole Tree alpha diversity metric boxplots comparing normal mucosa and EGGD lesion samples. There was no significant difference between the two groups.

Phylum	Lesion median abundance (%)	Normal mucosa median abundance (%)	p-value
Actinobacteria	3.0	4.3	0.325
Bacteroidetes	21.0	18.1	0.623
Firmicutes	41.2	20.0	0.006*
Proteobacteria	18.9	46.3	0.017*
Spirochaetes	0.7	4	0.638
Verrucomicrobia	2.8	1.7	0.712

Table 4.2 Comparison of the median abundance of the most common phyla between normal mucosa and EGGD lesions.

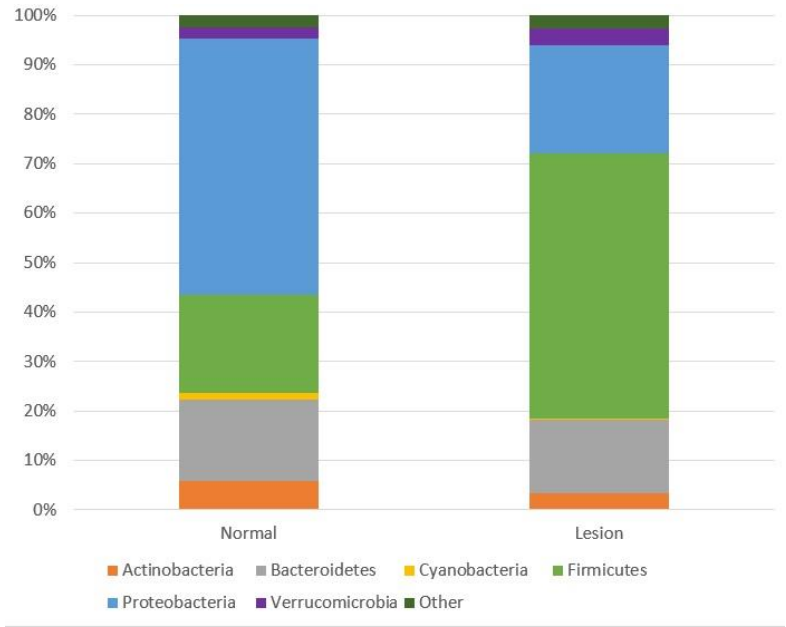


Fig. 4.3: Stacked bar charts comparing bacterial community composition at phylum level between pooled normal mucosa samples and pooled EGGD lesion samples. *Firmicutes* dominate in EGGD lesion samples, whereas *Proteobacteria* are the predominant phylum in normal glandular mucosa samples.

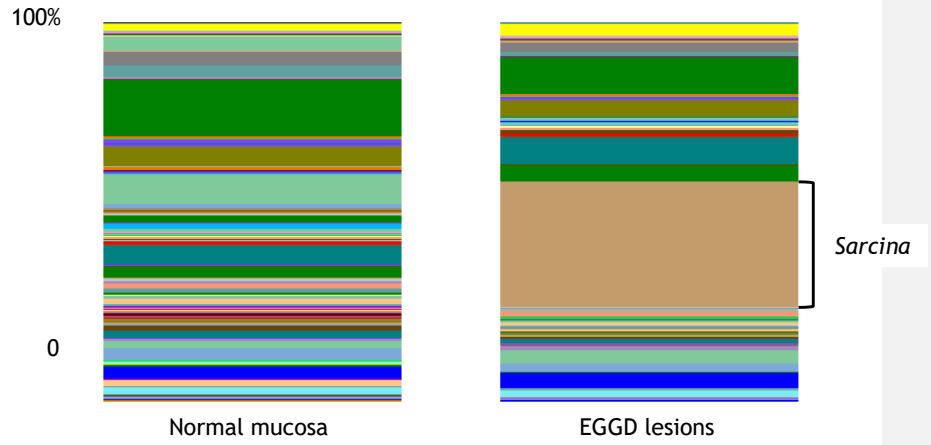


Fig 4.4: Stacked bar charts comparing normal mucosa to EGGD lesions at a genus level. Coloured horizontal bars represent different genera, with each bar representing an individual genus. Due to the extremely large number of different genera identified, a colour coded key is impractical. The tan bar (indicated) denotes the proportion of *Sarcina* in EGGD lesion samples.

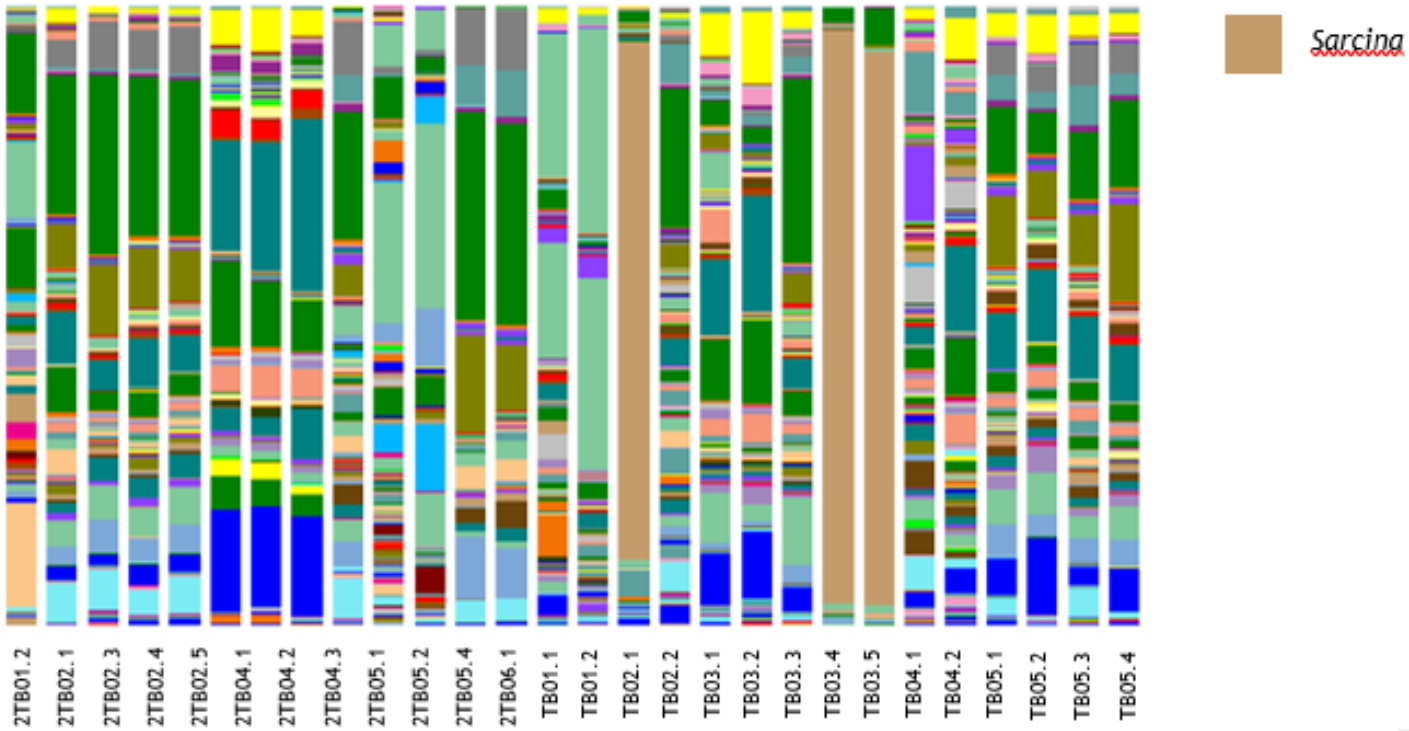


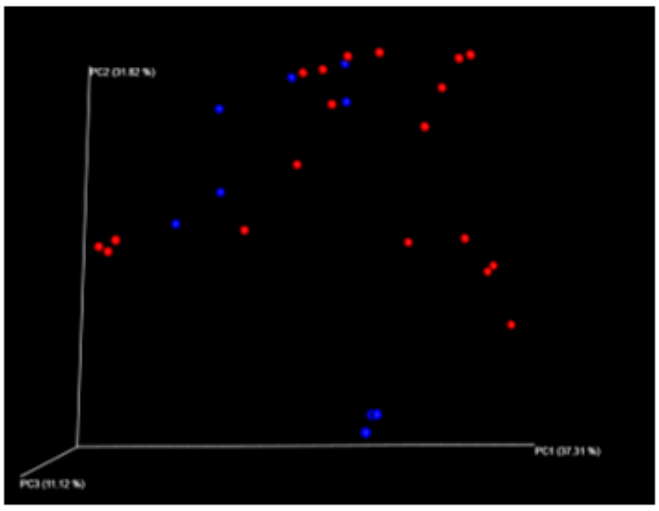
Fig. 4.5: 100% stacked bar charts displaying individual samples demonstrating the increased abundance of *Sarcina* in TB02.1, TB03.4, and TB03.5 samples, which represent EGGD lesions.

3.1.1.8 Beta diversity analysis

Principle coordinates analysis plots were produced to represent the similarity between samples in a three-dimensional space (Fig. 4.6). There is no clustering according to whether the samples were from EGGD lesions or normal mucosa. When all group distances were compared, there were no statistically significant comparisons identified. Group significance tables generated comparing individual OTU abundance compared normal mucosa to EGGD lesion samples indicated that no single OTU was significantly associated with EGGD lesion vs normal mucosal samples when FDR p-values were calculated (P = >0.05).

Linear discriminant analysis effect size (LEfSe) analysis was performed, confirming that a greater proportion of *Firmicutes*, *Clostridiales*, and *Clostridia* species was characteristic of samples collected from glandular lesions (Fig. 4.7).

A



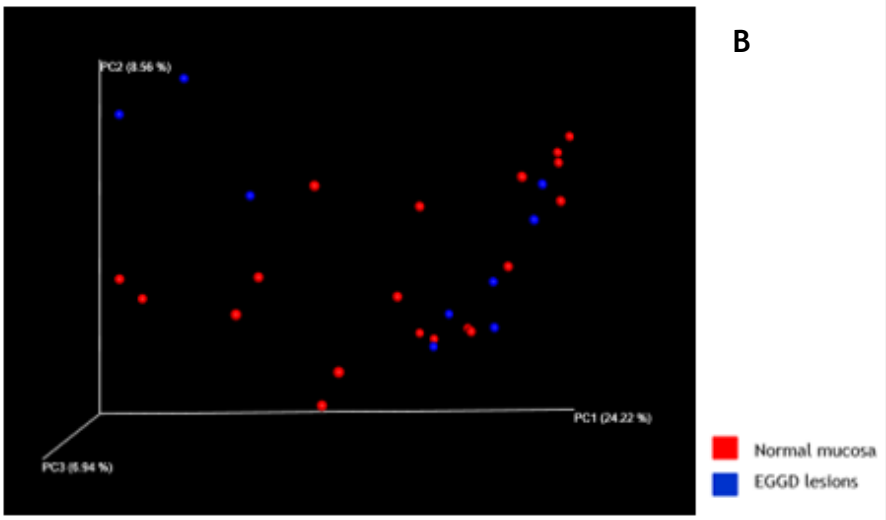


Fig. 4.6: (A) weighted and (B) unweighted PCoA plots indicating sample similarity in three-dimensional space. There is no clustering or separation of samples according to whether they are from normal mucosa or EGGD lesions.

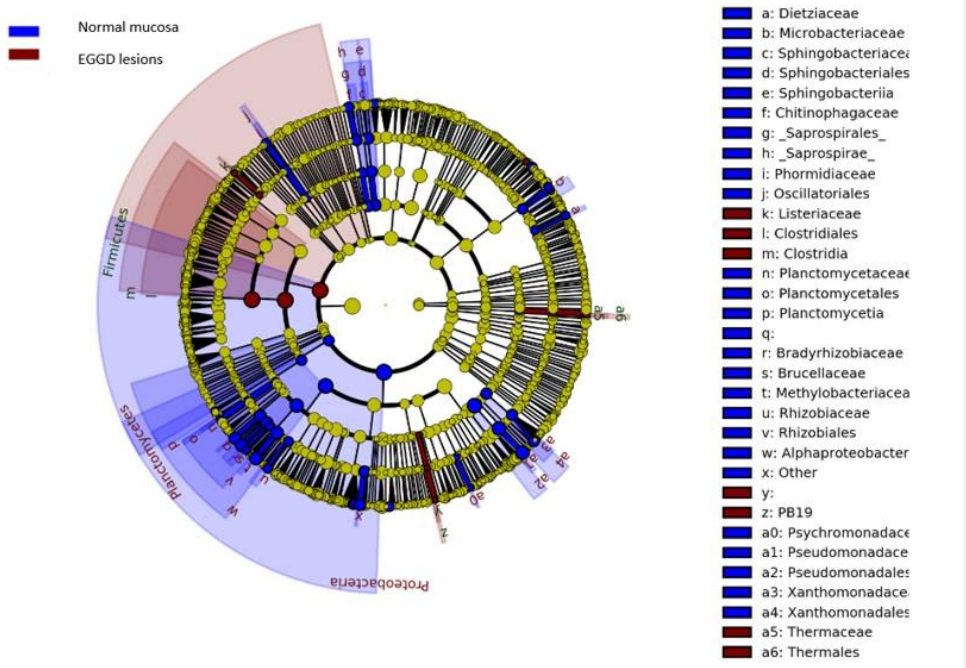


Fig. 4.7: Cladogram of LefSE analysis results showing taxa significantly associated with normal mucosa and EGGD lesions. Each concentric ring represents a level of taxonomic rank, moving from kingdom most centrally, to genus most peripherally. Each small circle represents a single taxon, and circle size is proportional to relative abundance of that taxon.

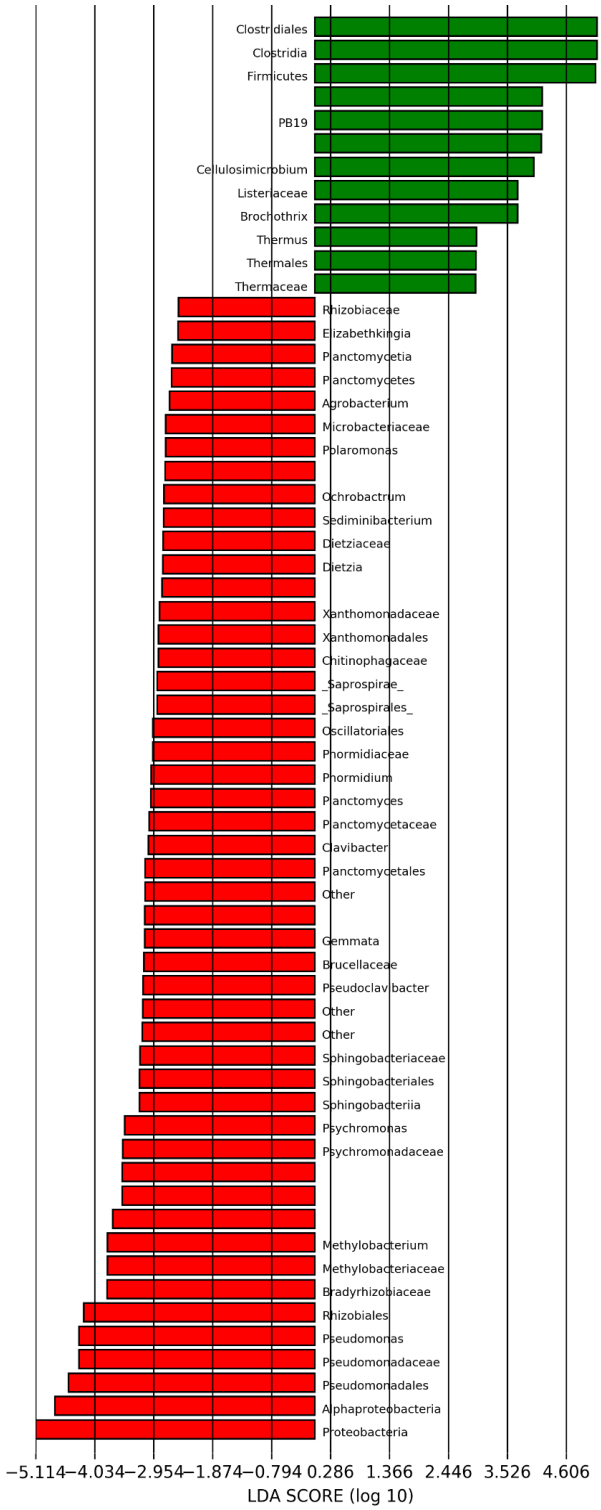

Clostridiales
Clostridia
Firmicutes
PB19
Cellulosimicrobium
Listeriaceae
Brochothrix
Thermus
Thermales
Thermaceae
Rhizobiaceae
Elizabethkingia
Planctomycetia
Planctomycetes
Agrobacterium
Microbacteriaceae
Polaromonas
Ochrobactrum
Sediminibacterium
Dietziaceae
Dietzia
Xanthomonadaceae
Xanthomonadales
Chitinophagaceae
Saprospirae
Saprospirales
Oscillatoriales
Phormidiaceae
Phormidium
Planctomyces
Planctomycetaceae
Clavibacter
Planctomycetales
Other
Gemmata
Brucellaceae
Pseudoclavibacter
Other
Other
Sphingobacteriaceae
Sphingobacteriales
Sphingobacteriia
Psychromonas
Psychromonadaceae
Methylobacterium
Methylobacteriaceae
Bradyrhizobiaceae
Rhizobiales
Pseudomonas
Pseudomonadaceae
Pseudomonadales
Alphaproteobacteria
Proteobacteria
−5.114 −4.034 −2.954 −1.874 −0.794 0.286 1.366 2.446 3.526 4.606
LDA SCORE (log 10)

Fig 4.8 LEfSe analysis bar chart indicating effect size on bacterial community composition for different taxa for normal mucosa and EGGD lesion samples.

4.3.4 Longitudinal bacterial community stability

Samples were grouped according to date collected and rarefaction curves plotted. Diversity was similar across both sample collection dates according to PD whole tree, Chao 1 and observed OTU measures (Fig. 4.9). When samples were examined individually according to sample date, there was a marked level of horse to horse variation. When examining the 100 % stacked bar charts there appears to be a trend for microbiota remaining relatively stable between S1 and S2 sampling dates (Fig. 4.10), however, we were unable to obtain enough EGGD lesion and normal mucosa samples across both dates to establish this.

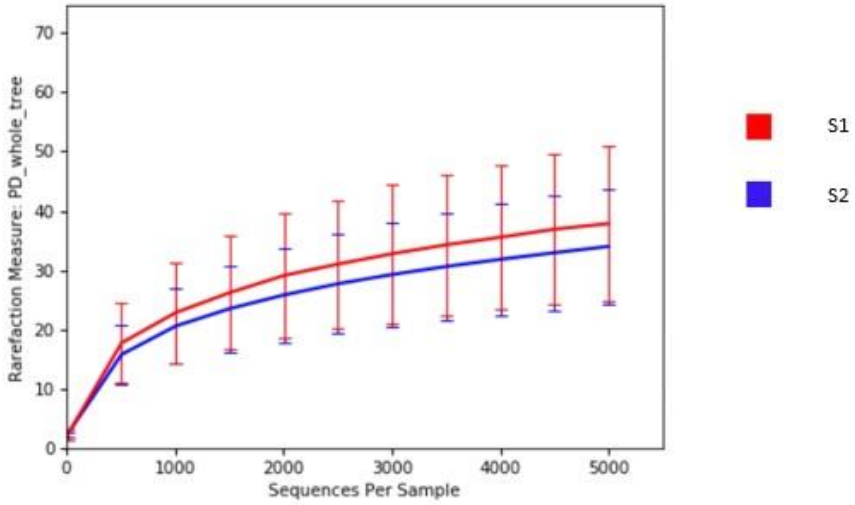


Fig. 4.9: Rarefaction analysis of operational taxonomic units (OTUs) generated using the PD Whole Tree metric from all glandular gastric mucosa samples pooled, comparing the first (S1) and second (S2) sample dates. Overall richness between the two sampling occasions is similar, and samples were sequenced to an adequate depth.

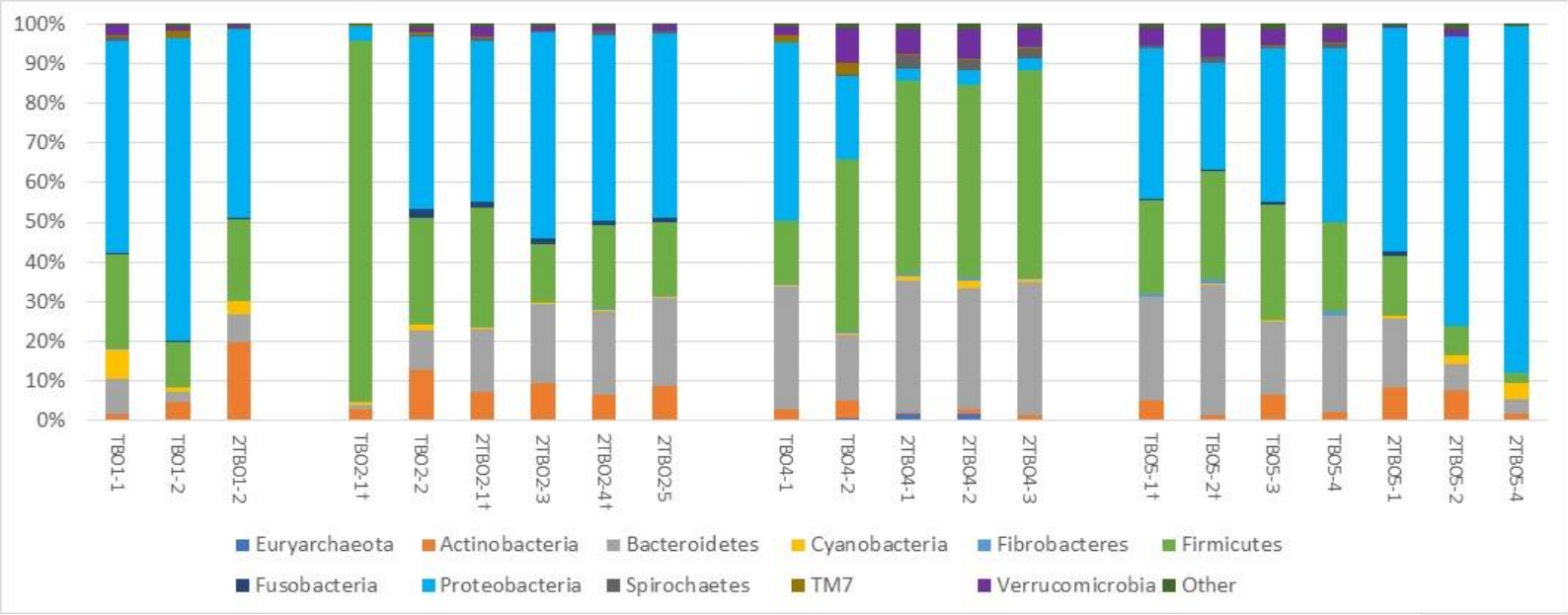


Fig.4.10: 100% stacked bar chart comparing normal mucosa and EGGD lesion samples acquired at S1 and S2 sampling dates for all horses with longitudinal data available. Samples are named according to individual horse ID (e.g. TB01), followed by sequential sample numbers, and correspond to data given in Table 1. Samples are grouped by individual animal. Samples prefixed with a '2' were collected at S2. † denotes an EGGD lesion sample.

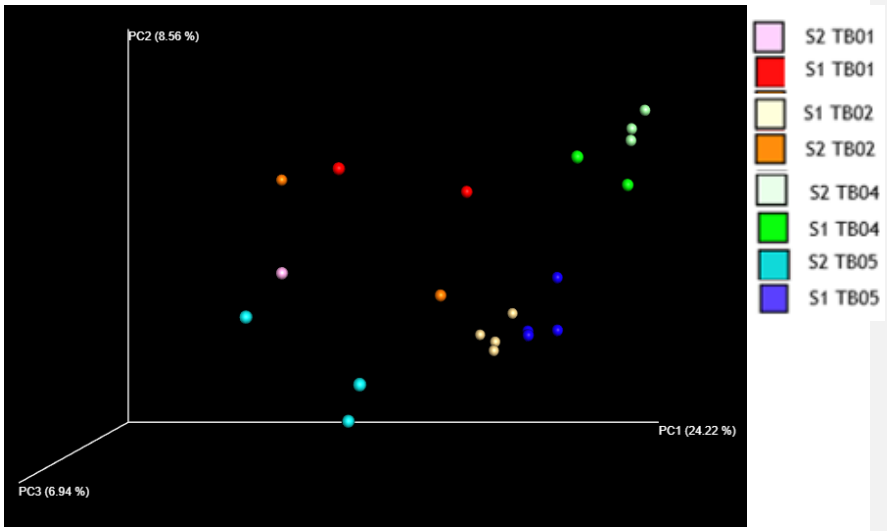


Fig 4.11: Unweighted PCoA plot displaying samples by horses for which longitudinal data were available. There is evidence of clustering according to horse regardless of sampling occasion.

4.4 Discussion

This study adds to current knowledge of the equine gastric microbiota and provides further evidence of bacterial dysbiosis associated with EGGD. Cytology brushes are beneficial for sampling wider areas of mucosa than with transendoscopic biopsies and are less invasive. Mucosal and luminal microbiota are not comparable (Costa et al., 2015), increasing the importance of targeted sampling techniques when investigating mucosal disease. We successfully replicated the cytology brush methodology at a race yard, and established that this technique could be adapted for sampling larger numbers of horses at the home premises.

In contrast to Yard One, EGGD lesions were not richer than normal mucosa, and were very similar in terms of alpha diversity analysis to normal mucosa. However, there were significant differences in the predominant phyla, with the decrease in *Firmicutes* abundance and the increase in *Proteobacteria* abundance achieving statistical significance.

Despite adapting the methodology following the pilot study (Chapter 3), there was a low yield of DNA from all samples, necessitating ethanol precipitation to achieve concentrations adequate for submission for sequencing. In order for the longitudinal study data to be comparable to the pilot study data it was decided that increasing the duration of the lysis step would be likely to yield higher DNA concentrations from the same bacterial phyla but would not alter the species identified significantly. Increasing the lysis step was undertaken to ensure that as few samples as possible failed sequencing.

The relative abundance of the genus *Sarcina* was higher in samples taken from glandular mucosal lesions in this study. *Sarcina* is a gram-positive bacterium (Claus and Wilmanns, 1974), which is ubiquitous in the soil and has been identified in human faeces (Crowther, 1971). *Sarcina* has been postulated to be of increasing importance in humans with delayed gastric emptying and has been associated with glandular ulceration and erythema (Ratuapli et al., 2013), reflux ulcerative oesophagitis (Heidinger et al., 2020), emphysematous gastritis (Laass et al., 2010), perforated gastric ulceration post-bariatric surgery (Sopha et al., 2015), gastric rupture and peritonitis (Tolentino et al., 2003; Al Rasheed and Senseng, 2016), and found co-existent with *Helicobacter pylori* in two cases of gastritis/duodenitis (Sauter et al., 2013). *Sarcina* has been recognised with increasing frequency in histopathological samples from humans with gastric disease and although unlikely to be causative, appears to be a possible marker of functionally or structurally delayed gastric emptying (Lam-Himlin et al., 2011). It has also been identified in association with gastric adenocarcinoma in a woman presenting with gastric outflow tract obstruction (Bhagat et al., 2015). While all the stomachs examined in our study were normal with respect to feed content following the period of starvation, it may be valuable to further investigate the relationship between glandular ulceration and gastric emptying rate in horses.

Sarcina ventriculi has been associated with fatal abomasal bloat and haemorrhagic lesions in calves and lambs (Edwards et al., 2008), goat kids (DeBey et al., 1996), and gastric dilatation in a cat (Im et al., 2017). Vatn et al. identified *Sarcina*-like organisms in a horse with acute gastric dilatation, as well as in lower numbers in five control animals (Vatn et al., 2000). Costa et al. identified *Sarcina* in stomachs of horses euthanased for non-gastrointestinal

disease (Costa et al., 2015). However, these samples were collected up to two hours *post mortem* from the glandular mucosa adjacent to the *margo plicatus*, and so these findings are not necessarily relevant to live clinical cases, where lesions are most commonly identified at the pylorus. Further investigation into the presence of *Sarcina* in horses with EGGD is warranted. To the authors' knowledge there has been no investigation of gastric emptying rate in horses with EGGD and this would be a potential avenue of interest. As *Sarcina* was also identified in normal mucosal samples in this study, accounting for just 0.2% of the counts in total, it may be part of the normal gastric microbiome, with excessive proliferation under certain conditions. *Sarcina* is a challenging bacterium to identify by culture-based methods, and has a characteristic histopathologic appearance. Transendoscopic biopsies should be examined closely for the typical cuboidal stacking appearance of the bacteria (Lam-Himlin et al., 2011), with PCR used to confirm presence when indicated.

There was intra-horse variation in bacterial community profile in samples from both EGGD lesions and normal glandular mucosa. This emphasises the benefit of sampling a wider mucosal area, especially when unable to collect large numbers of samples in clinical cases.

Helicobacter, an important causative agent of human peptic ulcers (McColl, 2010; Chey et al., 2017), has been identified in equine stomachs (R Hepburn, 2004), but has not been associated consistently with gastric pathology (Contreras et al., 2007; Morales et al., 2010). We did not identify *Helicobacter* in this study. No Escherichia organisms were identified, as such we were unable to support the findings of previous studies (Husted et al., 2010). Enterococcus was present in very low abundance in three samples (TB04.2, TB04.1, TB02.2) and was identified in both normal and abnormal mucosa samples. Streptococcus was found in low abundance across all samples. LefSE analysis did not reveal a correlation between glandular pathology and presence of Streptococcus or Enterococcus species. These findings therefore do not support the postulated involvement of *Enterococcus faecium* or *Streptococcus bovis* as per previous suggestions (Rendle et al., 2018). We have identified an increased abundance of *Firmicutes* in association with EGGD lesions. Interestingly, one study has

identified an increased abundance of *Firmicutes* to be associated with non-*Helicobacter* associated gastritis in human patients (Li et al., 2009).

One limitation of this study was the low sample number. We have provided good preliminary evidence for the involvement of *Sarcina* in EGGD, and larger scale studies are required to further understand the potential role of this and other bacteria in the pathogenesis of EGGD. Collecting a greater number of samples from each horse would be beneficial, however this is limited by the welfare implications of prolonging diagnostic procedures such as gastroscopy. Horses enrolled in the study acted as their own controls in order to minimise the effect of inter-individual variation on determining the effects of EGGD on microbial population. Our data suggest that different horses have a different gastric microbiota composition, although the communities appear to be stable over time within each individual. As it is probable that the gastric microbiota is widely affected in diseased stomachs, future studies should seek to include horses without EGUS.

A need for more longitudinal microbiota studies has been identified, and an understanding of the dynamic of the microbial population over time will aid with interpretation of data from cross-sectional studies, which are more commonly performed. This is particularly pertinent when investigating the gastrointestinal microbiota of horses, which commonly experience seasonal dietary changes, which may reasonably be expected to affected the gastrointestinal microflora.

Although our results do not provide evidence for bacterial causation of EGGD lesions, we have identified a novel association between *Sarcina* and EGGD lesions in horses. This is of interest given this bacterium's involvement in gastric pathology in other species. As an association has been made between delayed gastric emptying rate and *Sarcina* abundance in humans, we recommend investigating emptying rate in horses with and without glandular mucosal lesions. Increased cortisol stress response has been recognised in some horses with EGGD (Scheidegger et al., 2017), and increased cortisol production is thought to prolong gastric emptying rate in this species (Padalino et al., 2020). Further investigation of the inter-relationship between these factors and the gastric microbiome may improve understanding of the aetiopathogenesis of EGGD.

5 Combined Analysis: Yard One and Two

5.1 Introduction

Defining what factors influence the microbiota composition in horses is important in order to be able to further understand the effects of disease, and to begin to dissect whether dysbiosis associated with disease may be causative, or an effect of local mucosal environmental change secondary to disease. The aim of this chapter is to analyse the two datasets (Yard One and Yard Two) in combination and assess whether any common themes could be identified. Using combined beta diversity analysis, we aimed to identify whether samples were more similar in terms of which yard they originated from, or whether normal mucosa and lesion samples were more similar in community composition.

Previous evidence has identified that there is significant inter-individual variation in the equine microbiota in other gastrointestinal tract compartments (discussed in Chapter 2.14). This has been found to be the case even when animals were reared under the same conditions from birth (Kobayashi et al., 2006), as well as when animals have been subject to the same diet and conditions as part of prospective studies investigating the faecal microbiome (Willing et al., 2009; Blackmore et al., 2013). Building a picture of the effects of inter-individual variation versus other external factors, such as diet and management system, is important in order to be able to interpret and apply the findings of microbiome studies.

We hypothesised that samples would be more similar if they were collected from the same horse, and that this would have a stronger effect on community profile than whether the samples were collected from EGGD lesions or normal mucosa. A secondary hypothesis of this arm of the analysis was that whether the horse sampled belonged to the Yard One or Yard Two cohort was expected to have an effect on sample similarity, such that horses from the same yard would have less inter-individual variation than when compared to horses from another premises.

5.2 Materials and methods

The samples from Yard One and Yard Two (including S1 and S2 sampling occassions) were combined into one dataset. Gastroscopic images were reviewed from Yard One and Yard Two in order to develop a simplified method of comparing lesion descriptors. Lesions were assigned a score 0 – 3 with similar lesions grouped according to gross appearance. Group 0 – normal glandular mucosa, 1 – erythematous and pinprick haemorrhagic mucosa, 2 – haemorrhagic and fibrinosuppurative lesions. Category 3 was initially applied to the polypoid mass described in horse SV008, however, the polypoid mass sample (SV008-1) was subsequently removed from the analysis as there were no other animals with comparable lesions.

Analysis performed as previously described, but with all samples from Yard One and Yard Two pooled. Repeat attempts were made to sequence samples that had previously failed in order to try to increase the total number of samples included in a relatively small dataset.

5.3 Results: combined analysis

5.3.1 Sequencing output

A total of 47 samples were included in the combined analysis. Previously failed samples (2TB02.2, 2TB05.3, and 2TB06.3; Table 4.1) were included in the analysis. Sample SV008.3 (polypoid mass, Table 3.1) was excluded from the analysis, as no other comparable samples were acquired from the Yard One or Yard Two cohorts.

5.3.2 Alpha diversity analysis

A rarefaction curve was plotted comparing observed OTUs from Yard One and Yard Two samples combined (Fig. 5.1); this revealed that samples from Yard Two were richer than Yard One when sequenced to 5000 sequences per sample. This also indicates that samples were sequenced to an adequate depth, despite addition of the three previously failed samples. Alpha diversity analysis using the PD Whole Tree metric showed the alpha diversity was higher in Yard Two

samples (Yard One mean 17.91, SD 3.16; Yard Two mean 36.45, SD 11.83;, p = 0.001) and boxplots were generated, displaying this.

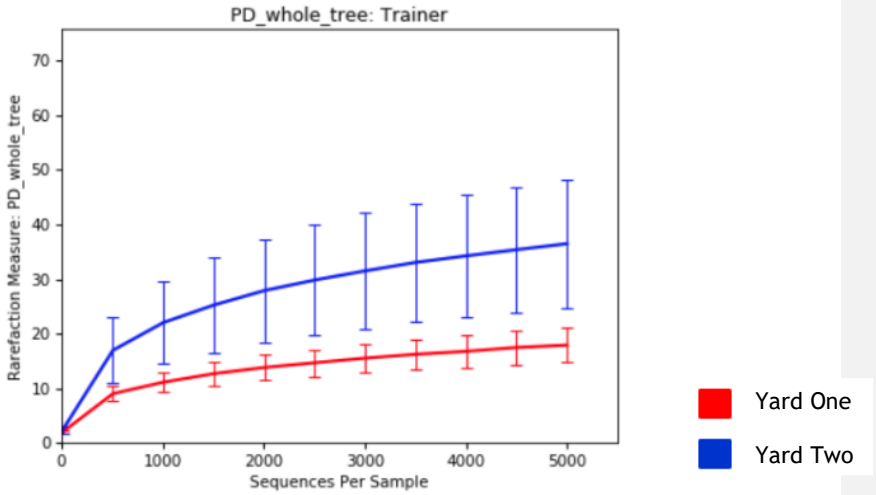


Fig 5.1: rarefaction curve comparing Yard One and Yard Two samples, showing the increased richness of Yard Two compared to Yard One.

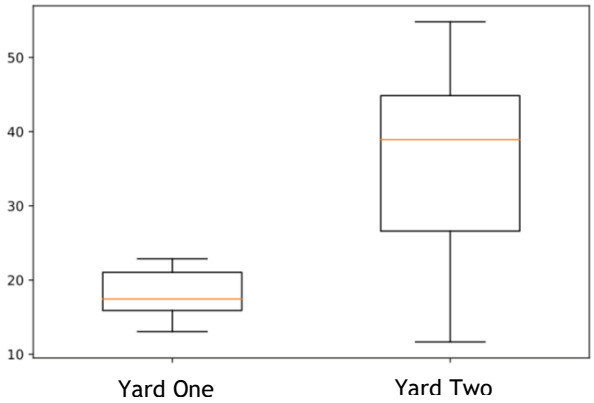


Fig 5.2: PD Whole Tree alpha diversity boxplot comparing yard one and two, showing increased alpha diversity of Yard Two compared to Yard One (p = 0.001)

When alpha diversity of the samples was compared according to lesion score no significant differences were found between groups using the chao 1, PD whole tree, or observed OTU metrics (p = >0.05). Group boxplots displaying sample richness (Fig 5.3) demonstrate that group 0 and group 2 are comparable; group 1 samples appear to be less diverse but this did not achieve statistical significance when compared to the other two groups (p = >0.05).

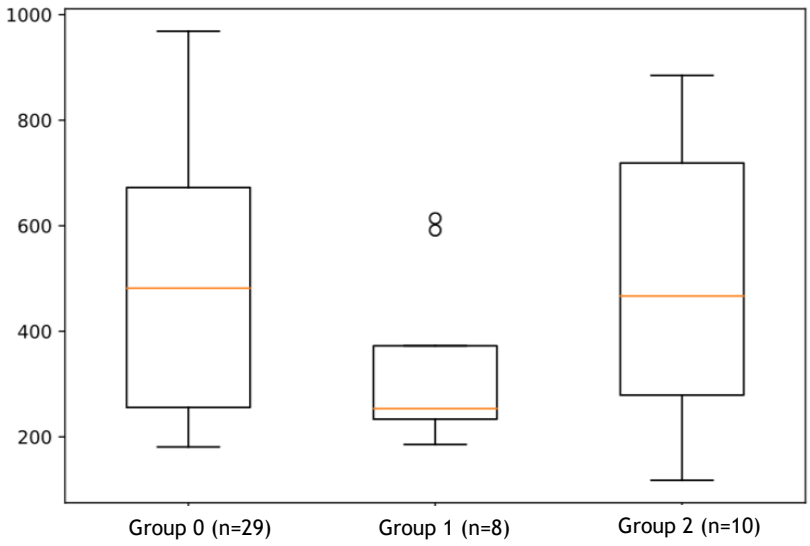
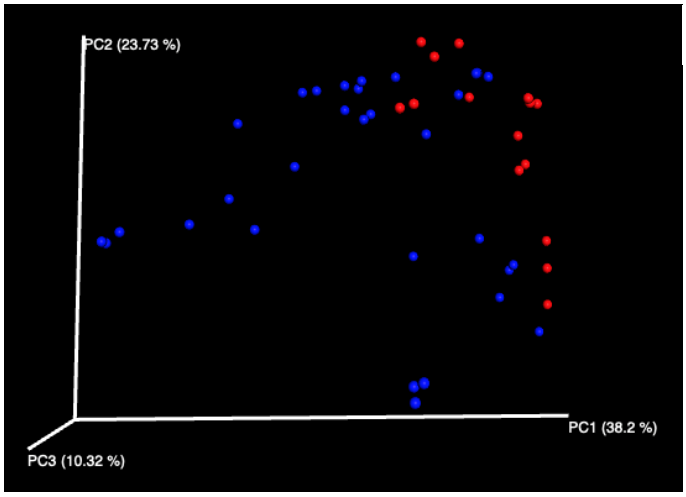


Fig 5.3: alpha diversity boxplot by lesion group. Group 0, normal mucosa; Group 1, erythematous mucosa and pinprick haemorrhagic lesions; Group 2, haemorrhagic and fibrinosuppurative lesions.

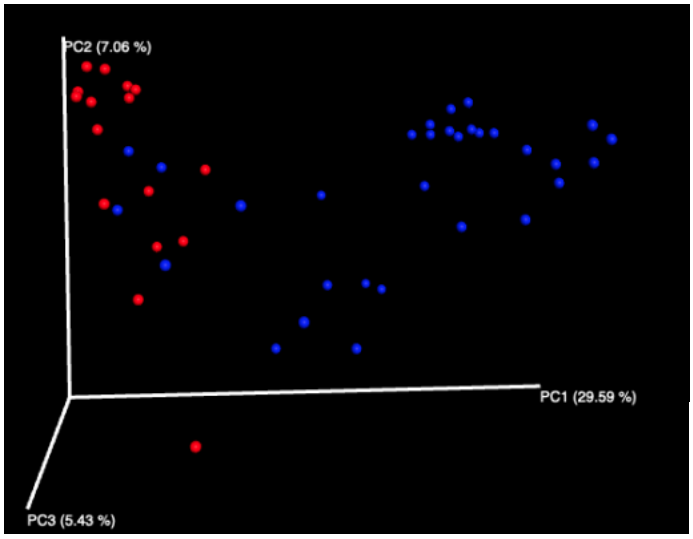
In terms of individual taxa identified, *Sarcina* was not identified in Yard One samples, and increased abundance is a distinct feature of EGGD lesions from TB02 and TB03, as previously described in Chapter 4. One of the samples that was sequenced on second attempt (2TB02.2) contained 0.1% abundance of *Sarcina*; this sample was collected from a horse with high *Sarcina* abundance in other samples, but despite 2TB02.2 representing an EGGD lesion, abundance was low.

5.3.3 Beta diversity analysis

UniFrac analysis indicated samples were similar according to whether they were from Yard One or Yard Two, and as previously described, this effect appears stronger in unweighted (qualitative) than weighted analysis (quantitative) (Fig 5.4)



A



B

Fig 5.4: weighted (A) and unweighted (B) PCoA plots comparing similarity in terms of phylogenetic distance indicated that Yard One samples are more similar to one another than Yard Two samples when considering phylogenetic distance, this was found to be statistically significant (both weighted and unweighted UniFrac distances p = 0.01).

5.3.4 Lesion Group

When PCoA plots were created comparing samples by lesion type group no clustering was observed by group. This remained consistent if samples were considered separately by Yard One or Yard Two, indicating there was no clustering by group at a yard level, or a lesion type level.

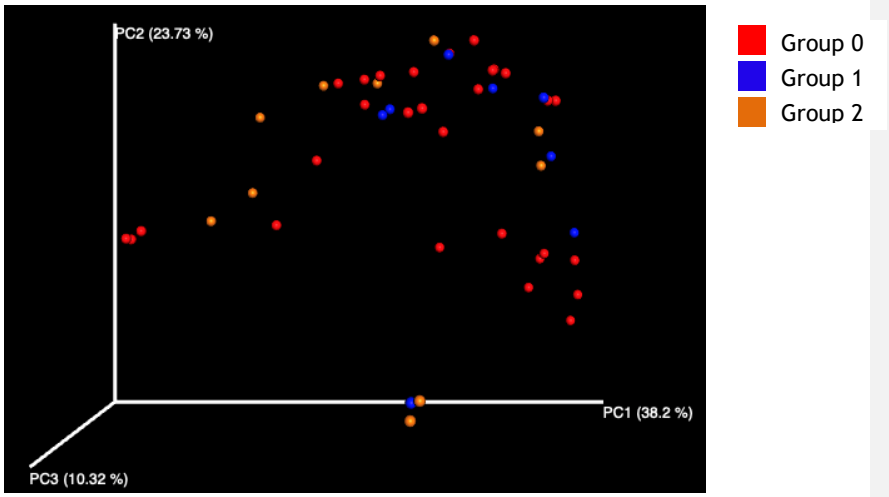


Fig 5.5: Weighted PCoA plot comparing lesion groups - red = 0 (normal), blue = 1 (erythema/mild), orange = 2 (fibrinosuppurative/haemorrhagic). There is no emerging pattern for samples to group according to which sample type they represent.

5.4 Discussion

Comparing Yard One and Yard Two data has highlighted that samples are more similar according to which yard they were collected from, rather than whether

samples were acquired from normal mucosa or EGGD lesions. Additionally, although there were not enough of each individual lesion type to perform meaningful analysis, when lesions were broadly categorised into either erythema and mild/pinprick haemorrhagic (Group 1) and haemorrhagic and fibrinosuppurative (Group 2) groups samples still not display similarity according to these categories. These data indicate that although a dysbiosis may be apparent, through altered alpha diversity and different dominance of phyla where EGGD lesions are present, currently, the overall bacterial community profile appears mostly defined by the specific individual's microbiota community, and at a higher level, the environment and management conditions that that animal is exposed to.

Yard Two samples were significantly richer than Yard One samples, although this may represent a genuine finding between the two populations, there are other variables which may account for these differences. Yard Two samples were also collected at the home yard on both S1 and S2 sampling dates, and it is unknown what effect prolonged time in liquid nitrogen prior to storage may have had on the DNA extracted from each batch. During DNA extraction, initial incubation time of the samples was increased for the Yard Two samples. The aim of this was to increase DNA yield without changing extraction protocol such that different taxa not previously sequenced would be identified. There is currently insufficient information regarding release of bacterial DNA from cytology brush samples in order to assess whether this is likely to have had an influence on sample diversity. If cytology brush sampling were to become commonplace in equine gastrointestinal microbiota studies optimisation of protocols and standardisation of methods should be prioritised in order to ensure future studies are comparable to one another.

There are other factors likely to account for differences in alpha diversity between the two yards, and it would be expected that differing diets, environmental factors, and management practises would affect the gastric microbiota. As discussed in Chapter 2.14, multiple factors have been identified that affect the hindgut and faecal microbiota, but there is no data in live horses to assess the effect of different management practices on the gastric microbial community specifically. In order to explore this further it would be necessary to

expand the current study to more premises in order to elucidate the effects of the multitude of variables that may be reasonably expected to influence the gastric microbiota. Ultimately, performing prospective cross over studies examining the faecal microflora in response to diet change in the same population of horses would be a more rigorous way to examine the effects of different variables, and would reduce the sample size required.

As previously discussed, there were very few horses without ESGD, and none of the animals from Yard One had normal squamous mucosa. The effect that ESGD has on the gastric microbiome is unknown, and it is reasonable to suppose that horses with ESGD lesions affecting a large surface area could have a dysbiosis affecting the entire stomach.

6 General discussion, conclusions, and recommendations

6.1 Clinical applications

Although we have identified a dysbiosis associated with EGGD, we have not identified a specific bacterial community profile associated with EGGD lesions that was consistent across the two populations of horses. We have provided preliminary evidence that the inter-individual differences in microbial community profile persist despite EGGD lesions being present. Use of antimicrobials to treat EGGD would still not be advised on the basis of the data presented in this manuscript. It is possible that if a dysbiosis is associated with EGGD lesions that use of antimicrobials could be detrimental if alteration in commensal bacteria allows proliferation of pathogenic species. Previous work has not identified an effect of antimicrobial administration on the glandular gastric microbiota (Dong et al., 2016); this study was undertaken in a small population of horses, and it seems improbable that the gastric microbiota would remain unaffected by antimicrobial administration, and further investigation is required to investigate whether antimicrobial administration alters the gastric microbiota.

If further work adds support to *Sarcina* being implicated in either the pathogenesis of EGGD, or being a useful biomarker associated with an element

of the pathophysiology of EGGD, it may be useful to attempt to identify *Sarcina* in clinical cases. The methodology used in this study utilised 16S rRNA sequencing to provide in depth description of the microbial community, which is relatively time consuming, requires access to specialist equipment, and is expensive. *Sarcina* has a characteristic histopathological appearance, and in EGGD cases where biopsies are collected, we would encourage clinicians to look for the presence of *Sarcina* in their samples (Lam-Himlin et al., 2011).

6.2 Discussion of methods

Despite adapting the DNA extraction protocol to increase the length of the initial lysis step, there was still a low DNA yield from the Yard Two samples. The DNA sample handling and DNA extraction protocol was not significantly adapted during this study in order to ensure that results were as comparable as possible between yards. The duration of time that samples were stored at -80°C was also not standardised in this study, although as discussed in Chapter 2.10, although some authors initially speculated this may have an effect (Vandeputte et al., 2017) this was not considered to be likely to have affected the community profiles observed (Fouhy et al., 2015; Shaw et al., 2016; Anderson et al., 2016).

One strength of the studies presented in this thesis is horses being from the same yards, and therefore a large number of variables were controlled for a prospective clinical study of client owned animals. One disadvantage with the sampling of the Yard One horses involving transportation to the hospital is there is no data available to inform whether the act of transporting the horses to the hospital and the short-term change of environment is likely to have altered the gastric microbiome. In order to establish this longitudinal data would need to be acquired before and after transport. Until such time this information is available it is most logical to sample horses at their home yard in order to minimise any effect of transport. In the Yard Two arm of the study we demonstrated this to be possible using the technique described.

6.3 Thesis limitations

The limitations of the Yard One and Yard Two studies have been discussed previously. Overall, the research presented is limited by the number of horses

that we were able to enrol on the study, which in turn had an effect on the number of horses with different EGGD lesion types, and meant that controlling for the presence of ESGD was not practical. This was in part due to the practical limitations of identifying horses from the same premises under the same management conditions. Ultimately, the time required to acquire samples and for those samples to undergo sequencing and subsequent analysis meant that significant expansion was beyond the scope of this thesis. Despite the reduction in price since its inception, sequencing also remains a relatively expensive technique to pursue, and this also creates limitations when considering what is possible in smaller studies. Another large drawback of this thesis is the lack of resolution with respect to the taxa identified. It would be beneficial to aim to identify species down to species level, especially once candidate pathogens have been identified.

6.4 Recommendations for further work

The association between *Sarcina* and delayed gastric emptying in other species indicates that gastric emptying rate should be considered an important avenue of investigation. Delayed gastric emptying has been postulated as a factor linking ESGD and EGGD, with the hypothesis that pyloric EGGD lesions result in delayed gastric emptying and therefore the squamous mucosa is exposed to acidic gastric content (Sykes et al., 2019). However, this has not been robustly investigated.

This study represents preliminary work investigating the microbiota of horses affected with EGGD. Further work should include sampling of a larger number of clinical cases. In the case of these small prospective studies horses were limited to cohorts from the same premises in order to minimise the number of variables likely to affect the microbiota. In order to study horses across a wider population significantly larger numbers of horses must be sampled. A prospective multicentre study investigating the microbial community at sites of EGGD lesions and normal mucosa using the methodology demonstrated in the above study would be required to draw further conclusions. Another potential avenue of interest would be expanding the current study to horses competing in other disciplines, which are likely to have different management regimes, including different diets and are more likely to have access to grazing, which may have an

impact on the gastric microbiome (Dong et al., 2016). Studies investigating horses competing in other disciplines are likely to require enrolment of significantly higher numbers of horses in order to control for management and dietary variables, as large cohorts under similar management conditions are not as readily available as in the racehorse population.

Undertaking a study on a greater number of horses at a larger population level would also potentially allow more conclusions to drawn with respect to whether the presence of concurrent ESGD has an effect on the glandular microbiota. In turn, this would be of interest when attempting to further establish the interrelationship between ESGD and EGGD. This would be of particular interest given the fact that many horses are affected by both syndromes concurrently, and, although reports are conflicting, the presence of ESGD has been identified as a risk factor for EGGD (Sykes et al., 2019). Another benefit of following a larger number of clinical cases would be the ability to investigate changes in the microbial population during treatment and following resolution of EGGD lesions. As the treatment of EGGD still currently largely relies on acid suppression, and it is reasonable to expect that the change in local pH would effect change in the resident mucosally associated gastric bacteria. With the high treatment failure rate associated with omeprazole treatment of EGGD (B. W. Sykes et al., 2014b; B. W. Sykes, Sykes, et al., 2015) this is a particular avenue of interest.

A more robust longitudinal study of the equine gastric microbiome would be beneficial. The profession is aware than longitudinal data are lacking (Costa and Weese, 2012), and it has been shown that seasonal variation occurs in other gastrointestinal tract compartments (Salem et al., 2018). However, there is still a dearth of well-executed longitudinal studies of the healthy equine gastrointestinal microbiome. As such, caution must be exercised when drawing inferences from the available data which represent a single time point. Further work should also aim to include different EGGD lesion types as described by the ECEIM consensus statement (B W Sykes et al., 2015) and attempt to establish whether any changes seen are noted across different categories of lesion. Unfortunately, due to the low numbers of animals and subsequently the low numbers of different EGGD lesion types from Yard One and Yard Two it was not deemed appropriate to try to draw inferences from such low numbers in this

study. It should also be noted that the recommended descriptors have not been validated in terms of inter or intra-user reliability, and to improve the quality of work aiming to compare the microbiota of different lesion types this should be established.

It is clear from the existing literature that the mucosally associated microbial population differs from the luminal population, and that *post mortem* change is likely to affect the results. As such, it should be recommended that investment is directed towards prospective studies collecting samples from live animals, and that *post mortem* studies are only undertaken where this is not possible.

Finally, if *Sarcina* can be consistently identified in association with gastric disease in horses experimental induction of disease should be considered in order to provide further opportunity to study this particular pathogen, and in order to attempt to fulfil Koch's postulates.

6.5 Conclusions

In conclusion, we have provided preliminary evidence of alterations in the glandular gastric microbiota community in association with EGGD, although these changes were not consistent between premises. At one yard a dysbiosis was identified, with increased alpha diversity described in association with EGGD lesions. At the second yard, significant increase in *Firmicutes* abundance, and decrease in *Proteobacteria* abundance were noted in association with EGGD. The large increase in *Firmicutes* was largely due to three samples from two horses, where a large increase in *Sarcina* (belonging to the *Firmicutes* phylum) was associated with EGGD lesions. This is of particular interest as a species associated with gastric disease in other species, and warrants further investigation. We have also provided further evidence for inter-individual variation in the glandular gastric microbiota, such that each animal would be expected to have a unique gastric microflora community profile. Similarity of samples that were collected from horses at the same yard was also identified, and this had a stronger influence of sample similarity than whether a sample represented an EGGD lesion or normal mucosa. These findings are relevant when considering how to interpret the growing body of microbiota studies, and it emphasises the importance of including large numbers of horses when looking

for population level significance until more is understood about the effects of different variables on the gastric microbiota.

7 Appendices

Appendix 1: Abstract presentation at the 2019 World Equine Veterinary Association conference, Verona, Italy.

The role of the gastric microflora in the aetiology of equine glandular gastric ulceration: a pilot investigation using 16s rRNA sequencing

[1]S.J.Voss, [2]W. Weir, [3]D.G.M.Sutton

[1]School of Veterinary Medicine, Weipers Centre Equine Hospital, University of Glasgow, Glasgow, United Kingdom; [2] School of Veterinary Medicine, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, United Kingdom

Background: The pathophysiology of equine glandular gastric ulceration (EGGD), including the potential role of bacteria, has not been elucidated, despite this antimicrobials are frequently used in practice to treat EGGD. **Objectives:** To investigate the microbial community present at sites of glandular disease compared to normal mucosa. **Study design:** Prospective, self-controlled study. **Methods:** Adult horses (n=8) from one yard which were presented for gastroscopy were recruited for this study. EGGD lesions were identified and cytology brushes were swept over the lesions and snap frozen. Similar samples were taken from adjacent normal mucosa. DNA was extracted from these samples and metagenetic analysis of the microbial communities was undertaken using 16S rRNA-based Illumina sequencing approach. **Results:** Six horses had EGGD, two horses had no glandular lesions. Rarefaction curve analysis indicated samples had been sequenced in great depth and illustrated that abnormal mucosa was associated with a higher level of bacterial diversity than normal tissue. Actinobacteria were found to be in greater relative abundance in normal mucosa samples than in lesion samples (11.8% vs 2.3%). Proteobacteria was the predominant phylum across all lesion types, accounting for 65.2% of the population. Weighted and unweighted 'UniFrac' analyses indicated that samples clustered primarily by animal rather than by lesion type. No organisms in the *Helicobacter* family were identified. **Main Limitations:** A low number of each type of EGGD lesion was assessed in this pilot study. **Conclusions:** EGGD samples showed a higher level of species richness than normal mucosal. Although a causal role cannot be deduced, we can establish a link between bacterial community diversity and EGGD. No specific pathogen was identified on the basis of these results. Although animal-to-animal variation accounted for much of the variation in the dataset, this study provides evidence of involvement of the gastric microflora in the aetiology of EGGD.

Ethical Animal Research This study was approved by the University of Glasgow Ethics Committee and horse-owners gave consent for participation in the study. **Competing Interests** None declared. **Sources of Funding** Petplan Charitable Trust pump priming grant, Weipers Centre Equine Hospital Fund

Appendix 2: Consent form signed by owners/trainers of horses enrolled in the studies presented.

University of Glasgow: College of Medical, Veterinary and Life Sciences

Consent Form:
Microbiological and histopathological evaluation of lesions of the equine glandular gastric mucosa

I am the owner / I have authority to act on behalf of the owner (delete as appropriate)

Full name:_____

Address:_____

_____

_____

Telephone: Home: _____ Work:_____

Mobile:_____

Veterinary surgeon's name:_____

_____

Practice name:_____

Animal details:_____

Breed:_____

Name:_____

Colour:_____

_____

Age:_____ Sex:_____

Passport number:_____

Section XI completed? Yes / No

Microchip number:_____

Are you over 18 years of age? Yes/No

I_____(name) agree for tissues, including blood and faeces, taken from my horse to be used for research purposes. Additionally, I give my permission for any further information required to be requested from my primary veterinary surgeon.

Signature: _____

Date: _____

Signature of veterinary surgeon obtaining consent:

_____

Please attach case information sticker here

8 References

Abuowarda, M. 2020. A preliminary study of endoscopic diagnosis of gastric ulcer in egyptian donkeys (Equus asinus) parasitised by gasterophilus intestinalis (Diptera oestridae). *Bulgarian Journal of Veterinary Medicine*. [Online]. [Accessed 11 July 2020]. Available from: https://www.researchgate.net/publication/341685291.

Acland, H.M., Gunson, D.E. and Gillette, D.M. 1983. Ulcerative Duodenitis in Foals. *Vet. Pathol.* **20**, pp.653–66.

Al-Mokaddem, A.K., Ahmed, K.A. and Doghaim, R.E. 2015. Pathology of gastric lesions in donkeys: A preliminary study. *Equine Veterinary Journal*. **47**(6), pp.684–688.

Anderson, E.L., Li, W., Klitgord, N., Highlander, S.K., Dayrit, M., Seguritan, V., Yooseph, S., Biggs, W., Venter, J.C., Nelson, K.E. and Jones, M.B. 2016. A robust ambient temperature collection and stabilization strategy: Enabling worldwide functional studies of the human microbiome. *Scientific Reports*. **6**(1), pp.1–10.

Andrews, F., Bernard, W., Byars, D., Cohen, N., Divers, T., MacAllister, C., McGladdery, A., Merritt, A., Murray, M., Orsini, J. and The Equine Gastric Ulcer Council 1999. Recommendations for the diagnosis and treatment of equine gastric ulcer syndrome (EGUS): The Equine Gastric Ulcer Council. *Equine Vet Educ*. **11**(5), pp.262–272.

Andrews, F.M., Buchanan, B.R., Elliot, S.B., Clariday, N.A. and Edwards, L.H. 2005. Gastric ulcers in horses. *J. Anim. Sci*. **83**, pp.18–21.

Andrews, F.M. and Nadeau, J.A. 1999. Clinical syndrome of gastric ulceration in foals and mature horses. *Equine vet.. I. , Suppl*. **29**, pp.30–33.

Andrews, F.M., Reinemeyer, C.R., McCracken, M.D., Blackford, J.T., Nadeau,

J.A., Saabye, L., Sotell, M. and Saxton, A. 2010. Comparison of endoscopic, necropsy and histology scoring of equine gastric ulcers. *Equine Veterinary Journal*. **34**(5), pp.475–478.

Andrews, F.M., Sifferman, R.L., Bernard, W., Hughes, F.E., Holste, J.E., Daurio, C.P., Alva, R. and Cox, J.L. 2010. Efficacy of omeprazole paste in the treatment and prevention of gastric ulcers in horses. *Equine Veterinary Journal*. **31**(S29), pp.81–86.

Baker, S.J. and Gerring, E.L. 1993. Technique for prolonged, minimally invasive monitoring of intragastric pH in ponies. *American journal of veterinary research*. **54**(10), pp.1725–34.

Banse, H.E. and Andrews, F.M. 2019. Equine glandular gastric disease: prevalence, impact and management strategies.

Banse, H.E., MacLeod, H., Crosby, C. and Windeyer, M.C. 2018. Prevalence of and risk factors for equine glandular and squamous gastric disease in polo horses. *The Canadian veterinary journal = La revue veterinaire canadienne*. **59**(8), pp.880–884.

Begg, L.M. and O'Sullivan, C.B. 2003. The prevalence and distribution of gastric ulceration in 345 racehorses. *Australian Veterinary Journal*. **81**(4), pp.199–201.

Bell, N.J.V., Burget, D., Howden, C.W., Wilkinson, J. and Hunt, R.H. 1992. Appropriate Acid Suppression for the Management of Gastro-Oesophageal Reflux Disease. *Digestion*. **51**(1), pp.59–67.

Bell, R.J.W., Kingston, J.K. and Mogg, T.D.T. 2007. A comparison of two scoring systems for endoscopic grading of gastric ulceration in horses. *New Zealand Veterinary Journal*. **55**(1), pp.19–22.

Van den Berg, M., Hoskin, S.O., Rogers, C.W. and Grinberg, A. 2013. Fecal pH

and Microbial Populations in Thoroughbred Horses During Transition from Pasture to Concentrate Feeding. *Journal of Equine Veterinary Science*. **33**(4), pp.215–222.

Bezdekova, B., Jahn, P. and Vyskocil, M. 2007. Pathomorphological study on gastroduodenal ulceration in horses: Localisation of lesions. *Acta Veterinaria Hungarica*. **55**(2), pp.241–249.

Bhagat, P., Gupta, N., Kumar, M., Radotra, B.D. and Sinha, S.K. 2015. A rare association of Sarcina with gastric adenocarcinoma diagnosed on fine-needle aspiration. *Journal of Cytology*. **32**(1), pp.50–52.

Blackmore, T.M., Dugdale, A., Argo, C.M.G., Curtis, G., Pinloche, E., Harris, P.A., Worgan, H.J., Girdwood, S.E., Dougal, K., Newbold, C.J. and McEwan, N.R. 2013. Strong stability and host specific bacterial community in faeces of ponies. *PloS one*. **8**(9).

Bolnick, D.I., Snowberg, L.K., Hirsch, P.E., Lauber, C.L., Org, E., Parks, B., Lusis, A.J., Knight, R., Caporaso, J.G. and Svanbäck, R. 2014. Individual diet has sex-dependent effects on vertebrate gut microbiota. *Nature Communications*. **5**(1), pp.1–13.

Borne, A.T. and MacAllister, C.G. 1993. Effect of sucralfate on healing of subclinical gastric ulcers in foals. *Journal of the American Veterinary Medical Association*. **202**(9), pp.1465–8.

Burden, F.A., Gallagher, J., Thiemann, A.K. and Trawford, A.F. 2009. Necropsy survey of gastric ulcers in a population of aged donkeys: prevalence, lesion description and risk factors. *Animal*. **3**(2), pp.287–293.

Cargile, J.L., Burrow, J.A., Kim, I., Cohen, N.D. and Merritt, A.M.M. 2004. Effect of Dietary Corn Oil Supplementation on Equine Gastric Fluid Acid, Sodium, and Prostaglandin E_2 Content before and during Pentagastrin Infusion.

Journal of Veterinary Internal Medicine. **18**(4), pp.545–9.

Chey, W.D., Leontiadis, G.I., Howden, C.W. and Moss, S.F. 2017. ACG Clinical Guideline: Treatment of Helicobacter pylori Infection. *American Journal of Gastroenterology*. **112**(2), pp.212–238.

Clark, C.K., Merritt, A.M., Burrow, J.A. and Steible, C.K. 1996. Effect of aluminum hydroxide/magnesium hydroxide antacid and bismuth subsalicylate on gastric pH in horses. *Journal of the American Veterinary Medical Association*. **208**(10), pp.1687–1691.

Claus, D. and Wilmanns, H. 1974. Enrichment and selective isolation ofSarcina maxima lindner. *Archives of Microbiology*. **96**(1), pp.201–204.

Clausen, J.P. 1977. Effect of physical training on cardiovascular adjustments to exercise in man. *Physiological Reviews*. **57**(4), pp.779–815.

Contreras, M., Morales, A., García-Amado, M.A.A., De Vera, M., Bermúdez, V. and Gueneau, P. 2007. Detection of Helicobacter-like DNA in the gastric mucosa of Thoroughbred horses. *Letters in applied microbiology*. **45**(5), pp.553–7.

Costa, M.C. and Weese, J.S. 2012. The equine intestinal microbiome. *Animal health research reviews / Conference of Research Workers in Animal Diseases*. **13**(1), pp.121–128.

Costa, M.C. and Weese, J.S. 2018. Understanding the Intestinal Microbiome in Health and Disease. *Veterinary Clinics of North America: Equine Practice*. **34**(1), pp.1–12.

Costa, M.C.C., Silva, G., Ramos, R.V. V, Staempfli, H.R.R., Arroyo, L.G.G., Kim, P. and Weese, J.S.S. 2015. Characterization and comparison of the bacterial microbiota in different gastrointestinal tract compartments in horses. *Veterinary journal (London, England : 1997)*. **205**(1), pp.74–80.

Crowther, J.S. 1971. Sarcina ventriculi in human faeces. *Journal of medical microbiology*. **4**(3), pp.343–350.

Crumpton, S.M., Baiker, K., Hallowell, G.D., Habershon-Butcher, J.L. and Bowen, I.M. 2015. Diagnostic Value of Gastric Mucosal Biopsies in Horses with Glandular Disease. *Equine Veterinary Journal*. **47**, pp.9–9.

Danesh, B.J.Z., Duncan, A., Russell, R.I. and Mitchell, G. 1988. Effect of intragastric pH on mucosal protective action of sucralfate. *Gut*. **29**(10), pp.1379–1385.

Daniels, S.P., Scott, L., De Lavis, I., Linekar, A. and Hemmings, A.J. 2019. Crib biting and equine gastric ulceration syndrome: Do horses that display oral stereotypies have altered gastric anatomy and physiology? *Journal of Veterinary Behavior*. **30**, pp.110–113.

DeBey, B.M., Blanchard, P.C. and Durfee, P.T. 1996. Abomasal bloat associated with Sarcina-like bacteria in goat kids. *J Am Vet Med Assoc*. **209**.

Dong, H.-J., Ho, H., Hwang, H., Kim, Y., Han, J., Lee, I. and Cho, S. 2016. Diversity of the Gastric Microbiota in Thoroughbred Racehorses Having Gastric Ulcer. . **26**, pp.763–774.

Dougal, K., de la Fuente, G., Harris, P.A., Girdwood, S.E., Pinloche, E., Geor, R.J., Nielsen, B.D., Schott, H.C., Elzinga, S. and Newbold, C.J. 2014. Characterisation of the Faecal Bacterial Community in Adult and Elderly Horses Fed a High Fibre, High Oil or High Starch Diet Using 454 Pyrosequencing A. M. Ibekwe, ed. *PLoS ONE*. **9**(2), p.e87424.

Duijkeren, E.V., Vulto, A.G. and Miert, A.S.J.P.A.M. VAN 1994. Trimethoprim/sulfonamide combinations in the horse: a review. *Journal of Veterinary Pharmacology and Therapeutics*. **17**(1), pp.64–73.

Dyck, W.P. 1979. Pathophysiologic considerations in peptic ulcer disease.

Southern Medical Journal. **72**(3), pp.252–255.

Edwards, G.T., Woodger, N.G.A., Barlow, A.M., Bell, S.J., Harwood, D.G., Otter, A. and Wight, A.R. 2008. Sarcina-like bacteria associated with bloat in young lambs and calves. *The Veterinary record*. **163**(13), pp.391–3.

Ericsson, A.C., Johnson, P.J., Lopes, M.A., Perry, S.C. and Lanter, H.R. 2016. A Microbiological Map of the Healthy Equine Gastrointestinal Tract H. Smidt, ed. *PLoS ONE*. **11**(11), p.e0166523.

Ethell, M.T., Hodgson, D.R. and Hills, B.A. 2000. Evidence for surfactant contributing to the gastric mucosal barrier of the horse. *Equine Veterinary Journal*. **32**(6), pp.470–474.

Fernandes, K.A., Kittelmann, S., Rogers, C.W., Gee, E.K., Bolwell, C.F., Bermingham, E.N. and Thomas, D.G. 2014. Faecal microbiota of forage-fed horses in new zealand and the population dynamics of microbial communities following dietary change. *PLoS ONE*. **9**(11).

Follmer, C. 2010. Ureases as a target for the treatment of gastric and urinary infections. *Journal of Clinical Pathology*. **63**(5), pp.424–430.

De Fombelle, A., Julliand, V., Drogoul, C. and Jacotot, E. 2001. Feeding and microbial disorders in horses: 1-effects of an abrupt incorporation of two levels of barley in a hay diet on microbial profile and activities. *Journal of Equine Veterinary Science*. **21**(9), pp.439–445.

Fouhy, F., Deane, J., Rea, M.C., O'Sullivan, Ó., Ross, R.P., O'Callaghan, G., Plant, B.J. and Stanton, C. 2015. The Effects of Freezing on Faecal Microbiota as Determined Using MiSeq Sequencing and Culture-Based Investigations J. Neu, ed. *PLOS ONE*. **10**(3), p.e0119355.

Furr, M., Taylor, L. and Kronfeld, D. 1994. The effects of exercise training on serum gastrin responses in the horse. *The Cornell veterinarian*. **84**(1),

pp.41–5.

FURR, M.O., Murrray, M.J. and Murray, M.J. 1989. Treatment of gastric ulcers in horses with histamine type 2 receptor antagonists. *Equine Veterinary Journal*. **21**(S7), pp.77–79.

Geor, R.J., Petrie, L., Papich, M.G. and Rousseaux, C. 1989. The protective effects of sucralfate and ranitidine in foals experimentally intoxicated with phenylbutazone. *Canadian journal of veterinary research = Revue canadienne de recherche veterinaire*. **53**(2), pp.231–8.

Goddard, P.J., Kao, Y.C.J. and Lichtenberger, L.M. 1990. Luminal surface hydrophobicity of canine gastric mucosa is dependent on a surface mucous gel. *Gastroenterology*. **98**(2), pp.361–370.

Gough, S., Hallowell, G. and Rendle, D. 2020. A study investigating the treatment of equine squamous gastric disease with long-acting injectable or oral omeprazole. *Veterinary Medicine and Science*. **6**(2), pp.235–241.

Gross, T.L. and Mayhew, I.G. 1983. Gastroesophageal ulceration and candidiasis in foals. *Journal of the American Veterinary Medical Association*. **182**(12), pp.1370–1373.

Habershon-Butcher, J., Hallowell, G., Bowen, I. and Sykes, B. 2012. Prevalence and risk factors for ulceration of the gastric glandular mucosa in Thoroughbred racehorses in training in the UK and Australia. *Journal of Veterinary Internal Medicine*. **26**(3).

Heidinger, M., Gorkiewicz, G., Freisinger, O. and Brcic, I. 2020. Clostridium ventriculi assoziierte ulzerative Reflux-Ösophagitis nach Hiatoplastik: ist die antibiotische Therapie notwendig? Ein Fallbericht. *Zeitschrift fur Gastroenterologie*. **58**(5), pp.456–460.

Hepburn, R. 2014. Endoscopic examination of the squamous and glandular

gastric mucosa in sport and leisure horses: 684 horses (2005–2011). *Proceedings of the 11th International Equine Colic ….*

Hepburn, R. 2004. *Investigation into the Presence of Helicobacter in the Equine Stomach by Urease Testing and Polymerase Chain Reaction and Further Investigation into the Application of the 13C-Urea Blood Test to the Horse*. Virginia Tech.

Hepburn, R.J. 2004. *Investigation into the Presence of Helicobacter in the Equine Stomach by Urease Testing and Polymerase Chain Reaction and Further Investigation into the Application of the 13 C-Urea Blood Test to the Horse INVESTIGATION INTO THE PRESENCE OF HELICOBACTER I*. Virginia Tech.

Hepburn, R.J. and Proudman, C.J. 2014. Treatment of ulceration of the gastric glandular mucosa: Retrospective evaluation of omeprazole and sucralfate combination therapy in 204 sport and leisure horses *In*: *Proc 11th International Equine Colic Research Symposium*.

Hewetson, M. 2017. *Urinary Incontinence in the Horse A Systematic Approach to Diagnosis & Treatment* [Online]. [Accessed 23 January 2019]. Available from: http://www.cpdsolutions.co.za/Publications/article_uploads/Urinary_Incontinence_in_the_Horse_.pdf.

Hewetson, M., Cohen, N.D., Love, S., Buddington, R.K., Holmes, W., Innocent, G.T. and Roussel, A.J. 2006. Sucrose Concentration in Blood: A New Method for Assessment of Gastric Permeability in Horses with Gastric Ulceration. *Journal of Veterinary Internal Medicine*. **20**(2), pp.388–394.

Hewetson, M., Sykes, B.W., Hallowell, G.D. and Tulamo, R.-M. 2017. Diagnostic accuracy of blood sucrose as a screening test for equine gastric ulcer

syndrome (EGUS) in adult horses. *Acta Vet Scand*. **59**, p.15.

Hewetson, M., Venner, M., Volquardsen, J., Sykes, B.W., Hallowell, G.D., Vervuert, I., Fosgate, G.T. and Tulamo, R.-M. 2018. Diagnostic accuracy of blood sucrose as a screening test for equine gastric ulcer syndrome (EGUS) in weanling foals. *Acta Veterinaria Scandinavica*. **60**(1), p.24.

Hill, T.L., Lascelles, B.D.X. and Blikslager, A.T. 2018. Effect of sucralfate on gastric permeability in an ex vivo model of stress-related mucosal disease in dogs. *Journal of Veterinary Internal Medicine*. **32**(2), pp.670–678.

Hills, B.A., Butler, B.D. and Lichtenberger, L.M. 1983. Gastric mucosal barrier: hydrophobic lining to the lumen of the stomach. *American Journal of Physiology-Gastrointestinal and Liver Physiology*. **244**(5), pp.G561–G568.

Hollander, D., Tarnawski, A., Gergely, H. and Zipser, R.D. 1984. Sucralfate protection of the gastric mucosa against ethanol-induced injury: a prostaglandin-mediated process? *Scandinavian journal of gastroenterology. Supplement*. **101**, p.97.

Huse, S.M., Young, V.B., Morrison, H.G., Antonopoulos, D.A., Kwon, J., Dalal, S., Arrieta, R., Hubert, N.A., Shen, L., Vineis, J.H., Koval, J.C., Sogin, M.L., Chang, E.B. and Raffals, L.E. 2014. Comparison of brush and biopsy sampling methods of the ileal pouch for assessment of mucosa-associated microbiota of human subjects. *Microbiome*. **2**(1), p.5.

Husted, L., Jensen, T.K.T., Olsen, S.N., Mølbak, L., Lorenzo-Figueras, M. and Merritt, A. 2010. Examination of equine glandular stomach lesions for bacteria, including Helicobacter spp by fluorescence in situ hybridisation. *BMC microbiology*. **10**(1), p.84.

Im, J.Y., Sokol, S. and Duhamel, G.E. 2017. Gastric Dilatation Associated with Gastric Colonization with *Sarcina* -Like Bacteria in a Cat with Chronic

Enteritis. *Journal of the American Animal Hospital Association*. **53**(6), pp.321–325.

Jewell, R. 2007. Sucralfate *In*: *xPharm: The Comprehensive Pharmacology Reference* [Online]. Elsevier Inc., pp.1–5. [Accessed 1 August 2020]. Available from: http://europepmc.org/books/NBK551527.

Kitamura, N., Yamada, J., Calingasan, N.Y. and Yamashita, T. 1984. Immunocytochemical distribution of endocrine cells in the gastrointestinal tract of the horse. *Equine Veterinary Journal*. **16**(2), pp.103–107.

Kobayashi, Y., Koike, S., Miyaji, M., Hata, H. and Tanaka, K. 2006. Hindgut microbes, fermentation and their seasonal variations in Hokkaido native horses compared to light horses. *Ecological Research*. **21**(2), pp.285–291.

L., H., Sanchez, L.C., Baptiste, K.E. and Olsen, S.N. 2009. Effect of a feed/fast protocol on pH in the proximal equine stomach. *Equine Veterinary Journal*. **41**(7), pp.658–662.

Laass, M.W., Pargac, N., Fischer, R., Bernhardt, H., Knoke, M. and Henker, J. 2010. Emphysematous gastritis caused by Sarcina ventriculi. *Gastrointestinal Endoscopy*. **72**(5), pp.1101–1103.

Lam-Himlin, D., Tsiatis, A.C., Montgomery, E., Pai, R.K., Brown, J.A., Razavi, M., Lamps, L., Eshleman, J.R., Bhagavan, B. and Anders, R.A. 2011. Sarcina organisms in the gastrointestinal tract: a clinicopathologic and molecular study. *The American journal of surgical pathology*. **35**(11), pp.1700–5.

Li, X.X., Wong, G.L.H., To, K.F., Wong, V.W.S., Lai, L.H., Chow, D.K.L., Lau, J.Y.W., Sung, J.J.Y. and Ding, C. 2009. Bacterial microbiota profiling in gastritis without Helicobacter pylori infection or non-steroidal anti-inflammatory drug use. *PLoS ONE*. **4**(11).

Lopp, C.T., Mccoy, A.M., Boothe, D., Schaeffer, D.J. and Lascola, K. 2019.

Single-dose pharmacokinetics of orally and rectally administered misoprostol in adult horses. *American Journal of Veterinary Research*. **80**(11), pp.1026–1033.

Luthersson, N., Bolger, C., Fores, P., Barfoot, C., Nelson, S., Parkin, T. and Harris, P. 2019. Effect of Changing Diet on Gastric Ulceration in Exercising Horses and Ponies After Cessation of Omeprazole Treatment. *Journal of Equine Veterinary Science*.

Luthersson, N., Nielsen, K.H., Harris, P. and PARKIN, T.D.H. 2009. The prevalence and anatomical distribution of equine gastric ulceration syndrome (EGUS) in 201 horses in Denmark. *Equine Veterinary Journal*. **41**(7), pp.619–624.

Luthersson, N., NIELSEN, K.H., HARRIS, P. and Parkin, T.D.H. 2009. Risk factors associated with equine gastric ulceration syndrome (EGUS) in 201 horses in Denmark. *Equine Veterinary Journal*. **41**(7), pp.625–630.

Macallister, C.G., Andrewst, F.M., Deegans, E., §#, W.R., Olovsonfl, S.-G., MacAllister, C.G., Andrews, F.M., DEEGAN, E., RUOFF, W. and Olovson, S.-G.G. 1997. A scoring system for gastric ulcers in the horse. *Equine Veterinary Journal*. **29**(6), pp.430–433.

MacAllister, C.G., Sangiah, S. and Mauromoustakos, A. 1992. Effect of a Histamine H_2 Type Receptor Antagonist (WY 45, 727) on the Healing of Gastric Ulcers in Ponies. *Journal of Veterinary Internal Medicine*. **6**(5), pp.271–275.

Mach, N., Ruet, A., Clark, A., Bars-Cortina, D., Ramayo-Caldas, Y., Crisci, E., Pennarun, S., Dhorne-Pollet, S., Foury, A., Moisan, M.P. and Lansade, L. 2020. Priming for welfare: gut microbiota is associated with equitation conditions and behavior in horse athletes. *Scientific Reports*. **10**(1), pp.1–

19.

Malmkvist, J., Poulsen, J.M., Luthersson, N., Palme, R., Christensen, J.W. and Søndergaard, E. 2012. Behaviour and stress responses in horses with gastric ulceration. *Applied Animal Behaviour Science*. **142**(3–4), pp.160–167.

Martin, E.M., Messenger, K.M., Sheats, M.K. and Jones, S.L. 2017. Misoprostol inhibits lipopolysaccharide-induced pro-inflammatory cytokine production by equine leukocytes. *Frontiers in Veterinary Science*. **4**(SEP), p.28.

Martin, E.M., Schirmer, J.M., Jones, S.L. and Davis, J.L. 2019. Pharmacokinetics and ex vivo anti-inflammatory effects of oral misoprostol in horses. *Equine Veterinary Journal*. **51**(3), pp.415–421.

Martin, E.M., Till, R.L., Sheats, M.K. and Jones, S.L. 2017. Misoprostol inhibits equine neutrophil adhesion, migration, and respiratory burst in an in vitro model of inflammation. *Frontiers in Veterinary Science*. **4**(SEP), p.28.

Martineau, H., Thompson, H. and Taylor, D. 2009. Pathology of gastritis and gastric ulceration in the horse. Part 1: Range of lesions present in 21 mature individuals. *Equine Veterinary Journal*. **41**(7), pp.638–644.

Martineau, H., Thompson, H. and Taylor, D. 2009. Pathology of gastritis and gastric ulceration in the horse. Part 2: a scoring system. *Equine veterinary journal*. **41**(7), pp.646–51.

McClure, S.R., Glickman, L.T. and Glickman, N.W. 1999. Prevalence of gastric ulcers in show horses. *Journal of the American Veterinary Medical Association*. **215**(8), pp.1130–3.

McColl, K.E.L. 2010. Helicobacter pylori infection. *New England Journal of Medicine*. **362**(17), pp.1597–1604.

Merritt, A.M. 1999. Normal equine gastroduodenal secretion and motility. *Equine Veterinary Journal*. **31**(S29), pp.7–13.

Merritt, A.M., Sanchez, L.C., Burrow, J.A., Church, M. and Ludzia, S. 2003. Effect of GastroGard and three compounded oral omeprazole preparations on 24 h intragastric pH in gastrically cannulated mature horses. *Equine Vet J.* **35**(7), pp.691–695.

Meschter, C.L., Gilbert, M., Krook, L., Maylin, G. and Corradino, R. 1990. The Effects of Phenylbutazone on the Morphology and Prostaglandin Concentrations of the Pyloric Mucosa of the Equine Stomach. *Veterinary Pathology*. **27**(4), pp.244–253.

Mönki, J., Hewetson, M. and Virtala, A.-M.K. 2016. Risk Factors for Equine Gastric Glandular Disease: A Case-Control Study in a Finnish Referral Hospital Population. *Journal of veterinary internal medicine*. **30**(4), pp.1270–5.

Morales, A., Garcia, F. and Bermudez, V. 2010. Detection of Helicobacter - like organisms in Thoroughbred horses from Venezuela. *Braz J Vet Pathol Brazilian Journal of Veterinary Pathology*. **3**(1), pp.52–55.

Morales Briceño, A., Lamprea Garrido, A. and Méndez Sánchez, A. 2015. Gastric Ulcers Syndrome in Donkeys . *Revista de Medicina Veterinaria* ., pp.31–35.

Morrow, L.D.D., Smith, K.C.C., Piercy, R.J.J., du Toit, N., Burden, F.A.A., Olmos, G., Gregory, N.G.G. and Verheyen, K.L.P.L.P. 2011. Retrospective Analysis of Post-Mortem Findings in 1,444 Aged Donkeys. *Journal of Comparative Pathology*. **144**(2–3), pp.145–156.

Muhonen, S., Julliand, V., Lindberg, J.E., Bertilsson, J. and Jansson, A. 2009. Effects on the equine colon ecosystem of grass silage and haylage diets after an abrupt change from hay. *Journal of Animal Science*. **87**(7), pp.2291–2298.

Murray, M.J. 1992. Gastric ulceration in horses: 91 cases (1987-1990). *Journal of*

the American Veterinary Medical Association. **201**(1), pp.117–20.

Murray, M.J., GRODINSKY, C., ANDERSON, C.W., RADUE, P.F. and SCHMIDT, G.R. 1989. Gastric ulcers in horses: a comparison of endoscopic findings in horses with and without clinical signs. *Equine Veterinary Journal*. **21**(S7), pp.68–72.

Murray, M.J., Nout, Y.S. and Ward, D.L. 2001. Endoscopic findings of the gastric antrum and pylorus in horses: 162 cases (1996-2000). *Journal of veterinary internal medicine*. **15**(4), pp.401–6.

Murray, M.J. and Schusser, G.F. 1993. Measurement of 24-h gastric pH using an indwelling pH electrode in horses unfed, fed and treated with ranitidine. *Equine Veterinary Journal*. **25**(5), pp.417–421.

Murray, M.J., Schusser, G.R.F.R.F., PIPERS, F.S. and Gross, S.J. 1996. Factors associated with gastric lesions in Thoroughbred racehorses. *Equine veterinary journal*. **28**(5), pp.368–374.

Nadeau, J.A., Andrews, F.M., Mathew, A.G., Argenzio, R.A., Blackford, J.T., Sohtell, M. and Saxton, A.M. 2000. Evaluation of diet as a cause of gastric ulcers in horses. *American Journal of Veterinary Research*. **61**(7), pp.784–790.

Nicol, C.J., Davidson, H.P.D., Harris, P.A., Waters, A.J. and Wilson, A.D. 2002. Study of crib-biting and gastric inflammation and ulceration in young horses. *Veterinary Record*. **151**(22), pp.658–662.

Nieto, J.E., Snyder, J.R., Beldomenico, P., Aleman, M., Kerr, J.W. and Spier, S.J. 2004. Prevalence of gastric ulcers in endurance horses – a preliminary report. *The Veterinary Journal*. **167**(1), pp.33–37.

O'Conner, M.S., Steiner, J.M., Roussel, A.J., Williams, D.A., Meddings, J.B., Pipers, F. and Cohen, N.D. 2004. Evaluation of urine sucrose concentration

for detection of gastric ulcers in horses. *American Journal of Veterinary Research*. **65**(1), pp.31–39.

Org, E., Mehrabian, M., Parks, B.W., Shipkova, P., Liu, X., Drake, T.A. and Lusis, A.J. 2016. Sex differences and hormonal effects on gut microbiota composition in mice. *Gut Microbes*. **7**(4), pp.313–322.

Otte, J.A., Oostveen, E., Geelkerken, R.H., Groeneveld, A.B.J. and Kolkman, J.J. 2001. Exercise induces gastric ischemia in healthy volunteers: a tonometry study. *Journal of Applied Physiology*. **91**(2), pp.866–871.

Padalino, B., Davis, G.L. and Raidal, S.L. 2020. Effects of transportation on gastric pH and gastric ulceration in mares. *Journal of Veterinary Internal Medicine*. **34**(2), pp.922–932.

Pedersen, S., Windeyer, C., Read, E., French, D., Cribb, A. and Macleod, H. 2015. Prevalence of And Risk Factors for Gastric Ulceration In Showjumping Warmbloods. *Journal of Veterinary Internal Medicine*. **29**(4), p.1239.

Pedersen, S.K., Cribb, A.E., Read, E.K., French, D. and Banse, H.E. 2018. Phenylbutazone induces equine glandular gastric disease without decreasing prostaglandin E2 concentrations. *Journal of Veterinary Pharmacology and Therapeutics*. **41**(2), pp.239–245.

Perkins, G.A., den Bakker, H.C., Burton, A.J., Erb, H.N., McDonough, S.P., McDonough, P.L., Parker, J., Rosenthal, R.L., Wiedmann, M., Dowd, S.E. and Simpson, K.W. 2012. Equine stomachs harbor an abundant and diverse mucosal microbiota. *Applied and environmental microbiology*. **78**(8), pp.2522–32.

Pietra, M., Morini, M., Perfetti, G., Spadari, A., Vigo, P. and Peli, A. 2010. Comparison of endoscopy, histology, and cytokine mRNA of the equine gastric mucosa. *Veterinary Research Communications*. **34**(S1), pp.121–124.

Plancade, S., Clark, A., Philippe, C., Helbling, J.C., Moisan, M.P., Esquerré, D., Moyec, L. le, Robert, C., Barrey, E. and Mach, N. 2019. Unraveling the effects of the gut microbiota composition and function on horse endurance physiology. *Scientific Reports*. **9**(1), pp.1–15.

Pollock, P.J. 2017. *Studies exploring the potential use of Serum Amyloid A (SAA) and other equine acute phase proteins for the investigation, monitoring and prognostication of disease in horses*.

Prinsloo, M., Hynd, P., Franklin, S., Weaver, S. and van den Boom, R. 2019. Hair cortisol concentration is inversely related to the severity of equine squamous gastric disease. *The Veterinary Journal*. **249**, pp.58–59.

Al Rasheed, M.R.H. and Senseng, C.G. 2016. Sarcina ventriculi: Review of the Literature. *Archives of Pathology & Laboratory Medicine*. **140**(12), pp.1441–1445.

Ratuapli, S.K., Lam-Himlin, D.M. and Heigh, R.I. 2013. Sarcina ventriculi of the stomach: a case report. *World journal of gastroenterology*. **19**(14), pp.2282–5.

Reed, S.M., Bayly, W.M. and C., S.D. 2018. *Equine Internal Medicine* Fourth. St. Louis: Elsevier.

Rendle, D., Bowen, M., Brazil, T., Conwell, R., Hallowell, G., Hepburn, R., Hewetson, M. and Sykes, B. 2018. Recommendations for the management of equine glandular gastric disease. *UK-Vet Equine*. **2**, pp.2–11.

Salem, S.E., Hough, R., Probert, C., Maddox, T.W., Antczak, P., Ketley, J.M., Williams, N.J., Stoneham, S.J. and Archer, D.C. 2019. A longitudinal study of the faecal microbiome and metabolome of periparturient mares. *PeerJ*. **2019**(4), p.e6687.

Salem, S.E., Maddox, T.W., Berg, A., Antczak, P., Ketley, J.M., Williams, N.J.

and Archer, D.C. 2018. Variation in faecal microbiota in a group of horses managed at pasture over a 12-month period. *Scientific Reports*. **8**(1), pp.1–10.

SANDIN, A., ANDREWS, F.M., NADEAU, J.A., DOHERTY, T.J. and NILSSON, G. 2010. Effects of intramuscular omeprazole on gastric acid secretion in horses over a twenty-four hour period. *Equine Veterinary Journal*. **31**(S29), pp.50–53.

Sandin, A., Girma, K., Sjöholm, B., Lindholm, A. and Nilsson, G. 1998. Effects of Differently Composed Feeds and Physical Stress on Plasma Gastrin Concentration in Horses. *Acta Veterinaria Scandinavica*. **39**(2), pp.265–272.

Sangiah, S., MacAllister, C.C. and Amouzadeh, H.R. 1989. Effects of misoprostol and omeprazole on basal gastric pH and free acid content in horses. *Research in Veterinary Science*. **47**(3), pp.350–354.

Sauter, J.L., Nayar, S.K., Anders, P.D., D'Amico, M., Butnor, K.J. and Wilcox, R.L. 2013. Co-existence of Sarcina Organisms and Helicobacter pylori Gastritis/Duodenitis in Pediatric Siblings. *Journal of clinical & anatomic pathology (JCAP)*. **1**(1).

Scheidegger, M.D., Gerber, V., Bruckmaier, R.M., van der Kolk, J.H., Burger, D. and Ramseyer, A. 2017. Increased adrenocortical response to adrenocorticotropic hormone (ACTH) in sport horses with equine glandular gastric disease (EGGD). *Veterinary Journal*. **228**, pp.7–12.

Sgorbini, M., Veronesi, F., Fratini, M. and Laus, F. 2018. Tick-Borne Diseases and Gastric Ulcer in the Donkey. *Journal of Equine Veterinary Science*. **65**, pp.62–65.

Shanahan, E.R., Zhong, L., Talley, N.J., Morrison, M. and Holtmann, G. 2016. Characterisation of the gastrointestinal mucosa-associated microbiota: a

novel technique to prevent cross-contamination during endoscopic procedures. *Alimentary Pharmacology & Therapeutics*. **43**(11), pp.1186–1196.

Shaw, A.G., Sim, K., Powell, E., Cornwell, E., Cramer, T., McClure, Z.E., Li, M.S. and Kroll, J.S. 2016. Latitude in sample handling and storage for infant faecal microbiota studies: The elephant in the room? *Microbiome*. **4**(1), pp.1–14.

Shorrock, C.J. and Rees, W.D.W. 1989. Effect of sucralfate on human gastric bicarbonate secretion and local prostaglandin E2 metabolism. *The American journal of medicine*. **86**(6), pp.2–4.

Slomiany, B.L., Piotrowski, J. and Slomiany, A. 1998. Effect of sucralfate on gastric mucosal inflammatory responses induced by Helicobacter pylori lipopolysaccharide. *Scandinavian Journal of Gastroenterology*. **33**(9), pp.916–922.

Slomiany, B.L., Piotrowski, J. and Slomiany, A. 2000. Up-regulation of endothelin-1 in gastric mucosal inflammatory responses to Helicobacter pylori Lipopolysaccharide: effect of omeprazole and sucralfate. *Journal of Physiology and Pharmacology*. **51**(2).

Sopha, S.C., Manejwala, A. and Boutros, C.N. 2015. Sarcina, a new threat in the bariatric era. *Human Pathology*. **46**(9), pp.1405–1407.

Spanton, J.A., Smith, L. and Mair, T.S. 2020. Is Serum Amyloid A elevated in horses with equine gastric ulcer syndrome? *Equine Veterinary Education*. **32**(S11).

Steiner, K., Bühring, K.U., Faro, H.P., Garbe, A. and Nowak, H. 1982. Sucralfate: Pharmacokinetics, metabolism and selective binding to experimental gastric and duodenal ulcers in animals. *Arzneimittel-Forschung/Drug Research*.

32(5), pp.512–518.

Sulochana, S.P., Syed, M., Chandrasekar, D. V., Mullangi, R. and Srinivas, N.R. 2016. Clinical Drug–Drug Pharmacokinetic Interaction Potential of Sucralfate with Other Drugs: Review and Perspectives. *European Journal of Drug Metabolism and Pharmacokinetics*. **41**(5), pp.469–503.

Sykes, B., Jokisalo, J. and Hallowell, G.D. 2014. Abstract Programme. The Eleventh International Equine Colic Research Symposium - PDF [Accessed 17 October 2018]. Available from: https://docplayer.net/13449869-Abstract-programme-the-eleventh-international-equine-colic-research-symposium.html.

Sykes, B. and Jokisalo, J.M. 2015. Rethinking equine gastric ulcer syndrome: Part 3 - Equine glandular gastric ulcer syndrome (EGGUS). *Equine Veterinary Education*. **27**(7), pp.372–375.

Sykes, B.W., Bowen, M., Habershon-Butcher, J.L., Green, M. and Hallowell, G.D. 2019. Management factors and clinical implications of glandular and squamous gastric disease in horses. *Journal of Veterinary Internal Medicine*. **33**(1), pp.233–240.

Sykes, B.W., Hewetson, M., Hepburn, R.J., Luthersson, N. and Tamzali, Y. 2015. European College of Equine Internal Medicine Consensus Statement--Equine Gastric Ulcer Syndrome in Adult Horses. *Journal of veterinary internal medicine / American College of Veterinary Internal Medicine*. **29**(5), pp.1288–99.

Sykes, B.W., Kathawala, K., Song, Y., Garg, S., Page, S.W., Underwood, C. and Mills, P.C. 2017. Preliminary investigations into a novel, long-acting, injectable, intramuscular formulation of omeprazole in the horse. *Equine Veterinary Journal*. **49**(6), pp.795–801.

Sykes, B.W., McGowan, C.M. and Mills, P.C. 2015. Placement of an indwelling percutaneous gastrotomy (PEG) tube for the measurement of intra-gastric pH in two horses. *Equine Veterinary Education*. **27**(12), pp.627–632.

Sykes, B.W., Sykes, K.M. and Hallowell, G.D. 2014a. A comparison between pre- and post exercise administration of omeprazole in the treatment of equine gastric ulcer syndrome: A blinded, randomised, clinical trial. *Equine Veterinary Journal*. **46**(4), pp.422–426.

Sykes, B.W., Sykes, K.M. and Hallowell, G.D. 2015. A comparison of three doses of omeprazole in the treatment of equine gastric ulcer syndrome: A blinded, randomised, dose-response clinical trial. *Equine Veterinary Journal*. **47**(3), pp.285–290.

Sykes, B.W., Sykes, K.M. and Hallowell, G.D. 2014b. A comparison of two doses of omeprazole in the treatment of equine gastric ulcer syndrome: a blinded, randomised, clinical trial. *Equine veterinary journal*. **46**(4), pp.416–21.

Sykes, B.W., Sykes, K.M. and Hallowell, G.D. 2014. Administration of trimethoprim-sulphadimidine does not improve healing of glandular gastric ulceration in horses receiving omeprazole: A randomised, blinded, clinical study. *BMC Veterinary Research*. **10**(1).

Sykes, B.W., Underwood, C., Greer, R., Mcgowan, C.M. and Mills, P.C. 2016. Pharmacokinetics and bioequivalence testing of five commercial formulations of omeprazole in the horse. *Journal of Veterinary Pharmacology and Therapeutics*. **39**(1), pp.78–83.

Szabo, S. and Hollander, D. 1989. Pathways of gastrointestinal protection and repair: Mechanisms of action of sucralfate. *The American Journal of Medicine*. **86**(6 SUPPL. 1), pp.23–31.

Tamzali, Y., Marguet, C., Priymenko, N. and Lyazrhi, F. 2011. Prevalence of

gastric ulcer syndrome in high-level endurance horses. *Equine veterinary journal*. **43**(2), pp.141–4.

Tesena, P., Yingchutrakul, Y., Roytrakul, S., Wongtawan, T. and Angkanaporn, K. 2019. Serum protein expression in equine glandular gastric disease (Eggd) induced by phenylbutazone. *Journal of Veterinary Medical Science*. **81**(3), pp.418–424.

The Equine Gastric Ulcer Council 1999. Recommendations for the diagnosis and treatment of equine gastric ulcer syndrome (EGUS): The Equine Gastric Ulcer Council. *Equine Veterinary Education*. **11**(5), pp.262–272.

Tolentino, L.F., Kallichanda, N., Javier, B., Yoshimori, R. and French, S.W. 2003. A Case Report of Gastric Perforation and Peritonitis Associated With Opportunistic Infection by Sarcina ventriculi. *Laboratory Medicine*. **34**(7), pp.535–537.

Vandeputte, D., Tito, R.Y., Vanleeuwen, R., Falony, G. and Raes, J. 2017. Practical considerations for large-scale gut microbiome studies. *FEMS microbiology reviews*. **41**(1), pp.S154–S167.

Varley, G., Bowen, I.M., Habershon-Butcher, J.L., Nicholls, V. and Hallowell, G.D. 2019. Misoprostol is superior to combined omeprazole-sucralfate for the treatment of equine gastric glandular disease. *Equine Veterinary Journal*. **51**(5), pp.575–580.

Vatistas, N.J., Snyder, J.R., Carlson, G., Johnson, B., Arthu, R.M., Thurmond, M., Zhou, H. and Lloyd, K.L.K. 1999. Cross-sectional study of gastric ulcers of the squamous mucosa in thoroughbred racehorses. *Equine Veterinary Journal*. **31**(29), pp.34–39.

Vatn, S., Gunnes, G., Nybo, K. and Juul, H.M. 2000. Possible involvement of Sarcina ventriculi in canine and equine acute gastric dilatation. *Acta Vet*

Scand. **41**.

Wallace, J.L. 2008. Prostaglandins, NSAIDs, and Gastric Mucosal Protection: Why Doesn't the Stomach Digest Itself? *Physiological Reviews*. **88**(4), pp.1547–1565.

Wallace, J.L. and Granger, D.N. 1996. The cellular and molecular basis of gastric mucosal defense. *The FASEB Journal*. **10**(7), pp.731–740.

Ward, S., Sykes, B.W., Brown, H., Bishop, A. and Penaluna, L.A. 2015. A comparison of the prevalence of gastric ulceration in feral and domesticated horses in the UK. *Equine Veterinary Education*. **27**(12), pp.655–657.

Weese, J.S., Holcombe, S.J., Embertson, R.M., Kurtz, K.A., Roessner, H.A., Jalali, M. and Wismer, S.E. 2015. Changes in the faecal microbiota of mares precede the development of post partum colic. *Equine Veterinary Journal*. **47**(6), pp.641–649.

Wickens, C.L., McCall, C.A., Bursian, S., Hanson, R., Heleski, C.R., Liesman, J.S., McElhenney, W.H. and Trottier, N.L. 2013. Assessment of Gastric Ulceration and Gastrin Response in Horses with History of Crib-Biting. *Journal of Equine Veterinary Science*. **33**(9), pp.739–745.

Willing, B., Vörös, A., Roos, S., Jones, C., Jansson, A. and Lindberg, J.E. 2009. Changes in faecal bacteria associated with concentrate and forage-only diets fed to horses in training. *Equine Veterinary Journal*. **41**(9), pp.908–914.