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A study comparing the healthy and diseased equine glandular gastric microbiota sampled with sheathed transendoscopic cytology brushes

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Highlights

- The gastric microbiota was altered in association with equine gastric glandular disease lesions
- Sarcina was associated with lesions but not normal glandular mucosa
- Sheathed cytology brushes are an effective sampling method for 16S rRNA sequencing studies

Abstract

Background: The role of the equine gastrointestinal microbiota in the pathogenesis of equine glandular gastric disease (EGGD) is poorly understood.

Objectives: To investigate whether the glandular gastric microbiota is altered in horses with EGGD.

Study design: Prospective longitudinal study

Methods: Five Thoroughbred racehorses from one training centre underwent gastroscopy as part of poor performance investigation. Samples were taken from EGGD lesions and adjacent normal mucosa using sheathed 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. The same horses were sampled six months later.

Results: Normal glandular mucosal samples were characterised by a higher proportion of *Proteobacteria* (46.3 %) than EGGD lesions (18.9 %). Relative abundance of *Firmicutes* was lower in samples from normal mucosa (20.0 %) than EGGD lesions (41.2 %). Linear discriminant analysis effect size (LEfSe) confirmed a greater proportion of *Firmicutes* species was characteristic of samples collected from EGGD lesions due to a very high relative abundance of *Sarcina* (up to 92.4 %) in two horses with EGGD. We were unable to comment on the stability of the glandular gastric microbiota over time.

Main limitations: Small sample population. None of the horses examined had grossly normal gastric mucosa.

Conclusions: The gastric microbiota appears altered in EGGD, although we are unable to demonstrate a causative effect. *Sarcina* was particularly increased in abundance in EGGD and may be a useful biomarker of disease. Sheathed cytology brushes were an effective method for sampling the gastric mucosa.

Keywords: *horse, 16S rRNA sequencing, gastric ulceration, Sarcina*

1. Introduction

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 colic compared to horses presenting for routine elective procedures [3]. Equine gastric ulceration syndrome (EGUS) is commonly diagnosed in horses, and is further defined as equine squamous gastric disease (ESGD) or equine glandular gastric disease (EGGD) depending on the anatomical location of the lesions [4]. ESGD and EGGD are distinctly different diseases; the pathogenesis, risk factors, and treatment of ESGD are well established, but the pathogenesis of EGGD, and the role of the gastric microbiota, is poorly understood and there is a higher rate of treatment failure.

High throughput sequencing techniques have become increasingly utilised to investigate the microbiota, and the use of cost-effective culture independent techniques has expanded our knowledge significantly [5]. Previously, much of the information published on the equine gastric glandular microbiota was derived from *post mortem* studies [6, 7], and microbial composition may differ significantly from that in living horses [7]. The role of bacterial pathogens in the aetiopathogenesis of EGGD has not been definitively proven. Recently, there has been some preliminary evidence to suggest a dysbiosis associated with EGUS without differentiating between ESGD and EGGD [8], and another study identifying a lesion-associated and global gastric dysbiosis associated with EGGD [9]. Both studies used transendoscopic biopsy forceps for sample acquisition, which sample a very limited surface

area and are likely to underrepresent rare taxa [10]. Sample collection using transendoscopic cytology brushes is likely to mitigate for this, as well as minimising tissue damage and contamination with host DNA, and has proved superior in human studies [10].

The primary aim of this study was to further investigate the glandular gastric microbiota of horses in race training and identify whether a particular bacterial profile was associated with EGGD. We aimed to explore the use of sheathed transendoscopic cytology brushes for acquisition of glandular gastric mucosal samples suitable for use in 16S small subunit ribosomal RNA sequencing studies in horses. A secondary objective was to assess whether the bacterial population of the glandular mucosa was stable in these horses over time.

2. Materials and methods

2.1. Selection of cases

The study cohort consisted of five Thoroughbred racehorses (four geldings, one mare) from a single training yard in Scotland, aged between two and five years (mean age three years). Horses were presented by the trainer for gastroscopic evaluation due to recent poor performance and the absence of any localising clinical signs. All horses were fed a similar diet, stabled at the same yard, and received no grass turn out. None of the horses had received any medications, including antimicrobials, for at least four weeks prior to presentation.

Dietary intake was the same for all horses, consisting 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.

2.2. Sample collection

(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®[a] and butorphanol, Dolorex®[b], dose adjusted according to horse temperament), all horses were examined on the same day.

The gastroscope was passed via a naso-oesophageal tube into the stomach, which was insufflated to allow visualisation of the *margo plicatus* and 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 recommendations [4].

Sheathed 3 metre cytology brushes (Eickemeyer Endoscopy Cytology Brush[c]) were plugged with sterile Amies medium to prevent contamination, and then 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 8-10 sweeps over the target area, the brush was withdrawn into the sheath prior to pulling the sheathed 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 then moved to a -80 °C freezer and stored until DNA extraction.

If more than one EGGD lesion was identified, samples were taken individually from each lesion type. Control samples were taken from grossly normal adjacent mucosa, such that they would have been exposed to the same local environmental conditions as the sampled lesions. The total number of samples collected from each horse was dependent on horse behaviour and speed of sample collection; total procedure length was limited to 30 minutes to minimise impact on horse welfare. Following sample collection, the squamous mucosa was inspected and ESGD lesions were graded according to published recommendation [11]. Gastroscopy findings were reported to the trainer, who was advised of the optimum treatment and management approach for each animal. The trainer elected to not treat any of the horses for ESGD or EGGD for financial reasons.

The horses were examined for a second time (S2) approximately six months later once they had returned to training. No medications were administered between S1 and S2. Gastroscopy was repeated and samples collected as described above. One additional horse on the same training and dietary regimen, a seven-year-old gelding, was presented for examination at S2 and data from this case is also included in the present study.

2.3. DNA Extraction

After removal from -80 °C storage, samples were thawed for 15 minutes at room temperature prior to DNA extraction. The brushes were folded into the bottom of the cryotubes using sterile forceps so that they would be immersed in the initial solution. DNA was extracted using a commercially available kit (Qiagen® Blood and Tissue Kit[d]) using a modified protocol. Proteinase K was added to the samples prior to vortexing 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. Brushes remained in the sample solutions until the samples were transferred into spin columns, at which time they were discarded. Samples were finally eluted in 40 µL of buffer AE. The DNA concentration in the final solution was assessed using a Qubit™ 4[e] benchtop fluorometer. A modified ethanol precipitation technique using a polyacramide carrier, GenElute LPA[f], was performed on all samples with a DNA yield less than 10 ng/µL 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 confirmed an increase in DNA concentration in the majority of samples.

2.4. Sequencing 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. 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[h]), which were then quantified using Qubit (ThermoFisher Scientific[e]), and library size was determined using an Agilent 2100 Bioanalyser (Agilent[i]). 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 were excluded before the table was rarefied to 5,000 sequences per sample. Alpha and beta diversity analyses were performed on the rarefied OTU tables. Weighted and

unweighted UniFrac distances were established and principal co-ordinate analysis (PCA) plots created to compare similarity between individual samples. Group comparisons were corrected using the Benjamin-Hochberg false discovery rate (FDR) procedure for multiple comparisons. Weighted analysis was undertaken to give a quantitative assessment of sample composition similarity, and unweighted was performed as a more qualitative measure to ensure low abundance phyla were not obscured. 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 (p-value) was set at < 0.05. 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.

3. Results

3.1. Sample collection

Horse signalment and gastroscopic findings are detailed in Table 1. Twenty four normal mucosa samples (eight at S1, 16 at S2) and ten EGGD lesion samples (seven at S1, two 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 there were no horses in training, at S2 all horses were in training except TB04.

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.

3.3. Alpha and beta 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. 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. 2.

When phylum-level composition of the samples from each group was assessed, irrespective of date sampled or EGGD lesion description, normal mucosa was associated with a higher proportion of *Proteobacteria* (46.3 %) compared to lesions (18.9 %) (Fig. 3). In contrast, the relative abundance of *Firmicutes* was lower in samples from normal mucosa (20.0 %) than in EGGD lesions (41.2 %), these differences in community composition were found to be significant (Table 2). When abundance was examined at class, order, and 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, Fig. 4). In contrast, *Sarcina* accounted for 0.2% of the total counts in all the normal mucosa samples combined.

Principle coordinates analysis plots were produced to represent the similarity between samples in a three-dimensional space (Fig. 5). There was 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 lesions 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. 6).

3.4. Longitudinal study results

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. 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, however, we were unable to obtain enough EGGD lesion and normal mucosa samples across both dates to establish this.

4. Discussion

This study adds to current knowledge of the equine gastric microbiota and provides evidence of bacterial dysbiosis associated with EGGD. In addition, this study explores the use of transendoscopic cytology brushes for collection of equine gastrointestinal tract mucosal samples for 16S SSU rRNA sequencing, a technique found to be useful in human studies. Cytology brushes are beneficial for sampling wider areas of mucosa than with transendoscopic biopsy forceps and are less invasive [10]. Additionally, sheathed devices are preferred in order to minimise cross-contamination and increase sampling precision [12]. Mucosal and luminal microbiota are not comparable [6], increasing the importance of targeted sampling techniques when investigating mucosal disease.

Paul et al. also identified a significant difference in community structure associated with EGGD lesions in a study using transendoscopic biopsy forceps, but did not identify a candidate pathogen [9]. It is possible that using methods that sample a very small area of mucosa may not give a representative cross-section of the local microbiota at lesion sites, and may underrepresent rare taxa.

The relative abundance of the genus *Sarcina* (a gram-positive bacterium belonging to the *Firmicutes* phylum [13]) was higher in samples taken from glandular mucosal lesions in this study. Costa *et al.* identified *Sarcina* in stomachs of horses euthanased for non-gastrointestinal disease [6]. 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. *Sarcina* was also identified in normal mucosal samples in this study, but accounting for just 0.2% of the counts in total, suggesting it may be part of the normal gastric microbiome, with excessive proliferation under certain conditions. *Sarcina* has been postulated to be of increasing importance in humans with delayed gastric emptying and has been associated with glandular ulceration and erythema [14] , emphysematous gastritis [15], perforated gastric ulceration post-bariatric surgery [16], gastric rupture and peritonitis [17, 18] and found co-existent with *Helicobacter pylori* in two cases of gastritis/duodenitis [19]. *Sarcina* has been recognised with increasing frequency in histopathological samples from humans with gastric disease and although considered unlikely to be causative, appears to be a possible marker of functionally or structurally delayed gastric emptying [20]. Increased cortisol stress response has been recognised in some horses with EGGD [21], and increased cortisol production is thought to prolong gastric emptying time in this species [22]. 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 disease in other veterinary species [23, 24, 25]. Vatn *et al.* identified *Sarcina*-like organisms in a horse with acute gastric dilatation, as well as in lower numbers in five control animals [26]. Other studies have identified *Sarcina* in both normal and diseased equine stomachs, but not made an association between this organism and the presence of EGUS lesions [5, 9, 27].

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 [28, 29], has been identified in equine stomachs [30], but has not been associated consistently with gastric pathology [31, 32]. We did not identify *Helicobacter* in this study. No *Escherichia* organisms were identified, as such we were unable to support the findings of Husted *et al*. [27], who identified a potential association between *Escherichia* and glandular lesions. *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 EGGD lesions and presence of *Streptococcus* or *Enterococcus* species. These findings therefore do not support the postulated involvement of *Enterococcus faecium* or *Streptococcus bovis* as per Rendle *et al*. [33]. 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 [34].

One limitation of this study was the low overall sample number, and a low number of horses with EGGD in particular. 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. As it is probable that the gastric microbiota is widely affected in diseased stomachs, future studies should seek to include horses without EGUS [9]. 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 affect the gastrointestinal microflora.

5. Conclusion

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. Further investigation of the inter-relationship between these factors and the gastric microbiome may improve understanding of the aetiopathogenesis of EGGD.

6. Manufacturers' addresses

a Dechra Veterinary Products, Shrewsbury, UK, b MSD Animal Health, Milton Keynes, UK, c Eickemeyer, Surrey, UK, d Qiagen, Hilden, Germany, e Thermo Fisher Scientific, Loughborough, UK, f Sigma-Aldrich Company Ltd, Dorset, UK, g Illumina, San Diego, USA, h Beckman Coulter, High Wycombe, UK, i Agilent Technologies LDA UK Limited, Cheshire, UK

Declaration of Competing Interest

none

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Ethical approval

Study approved by the Glasgow University Animal Ethics and Welfare Committee, reference 42a/17. Written owner informed consent was obtained for all animals included in this study.

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Table 1: signalments of horses and gastroscopic findings on both sampling dates.

ID	Age at S1	Sex	Sample ID	Sampling date	Sample	ESGD grade

	(years)					
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	

†are associated with a significantly increased abundance of *Sarcina*. Samples marked

‡were not successfully sequenced.

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

Table 2: Comparison of the median abundance of the most common phyla between normal mucosa and EGGD lesions.

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	0.638
Verrucomicrobia	2.8	1.7	0.712
Other non-specified phyla	12.4	5.6	N/A

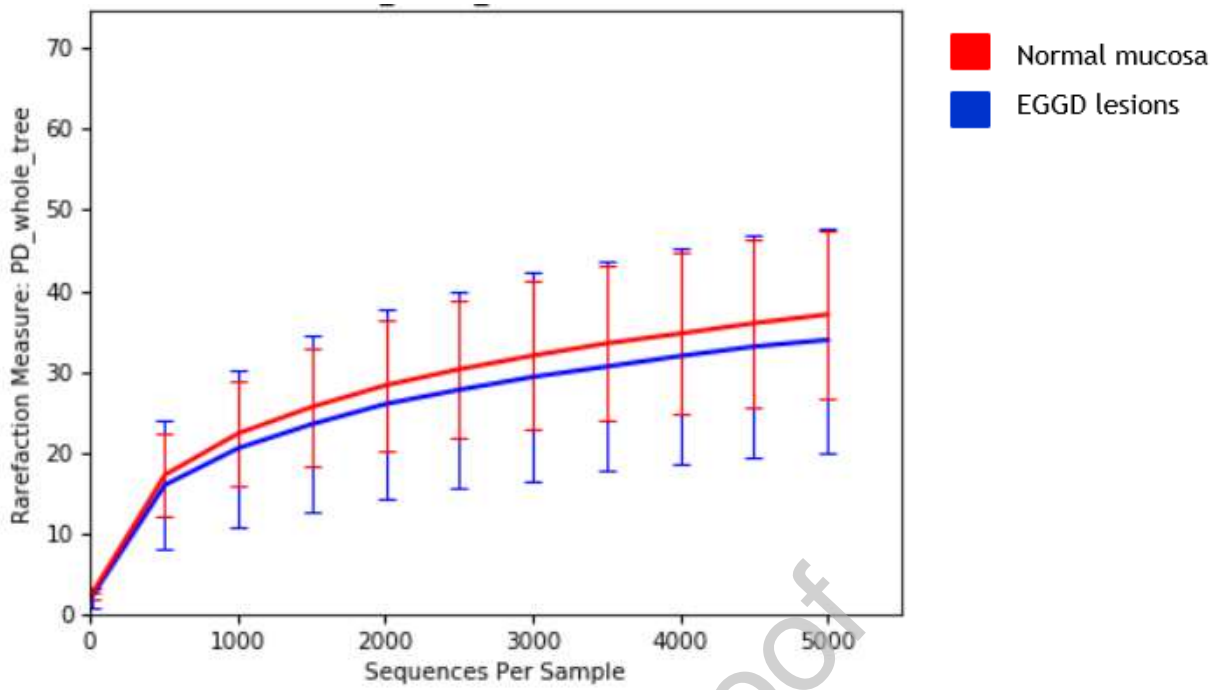


Fig 1. PD Whole Tree metric rarefaction curve comparing sample richness of EGGD lesion samples and normal mucosa, which are comparable. Rarefaction curve indicates samples were sequenced in adequate depth.

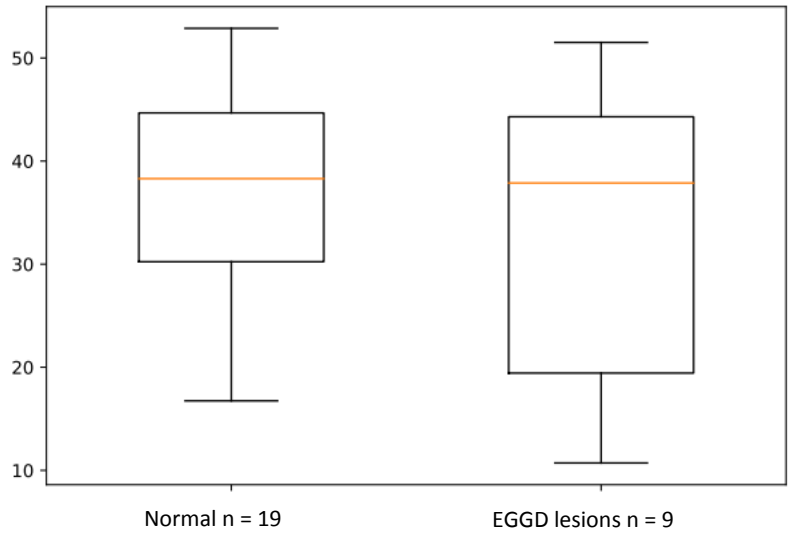


Fig 2. PD Whole Tree alpha diversity metric boxplots comparing normal mucosa and EGGD lesion samples. There was no significant difference between the two groups.

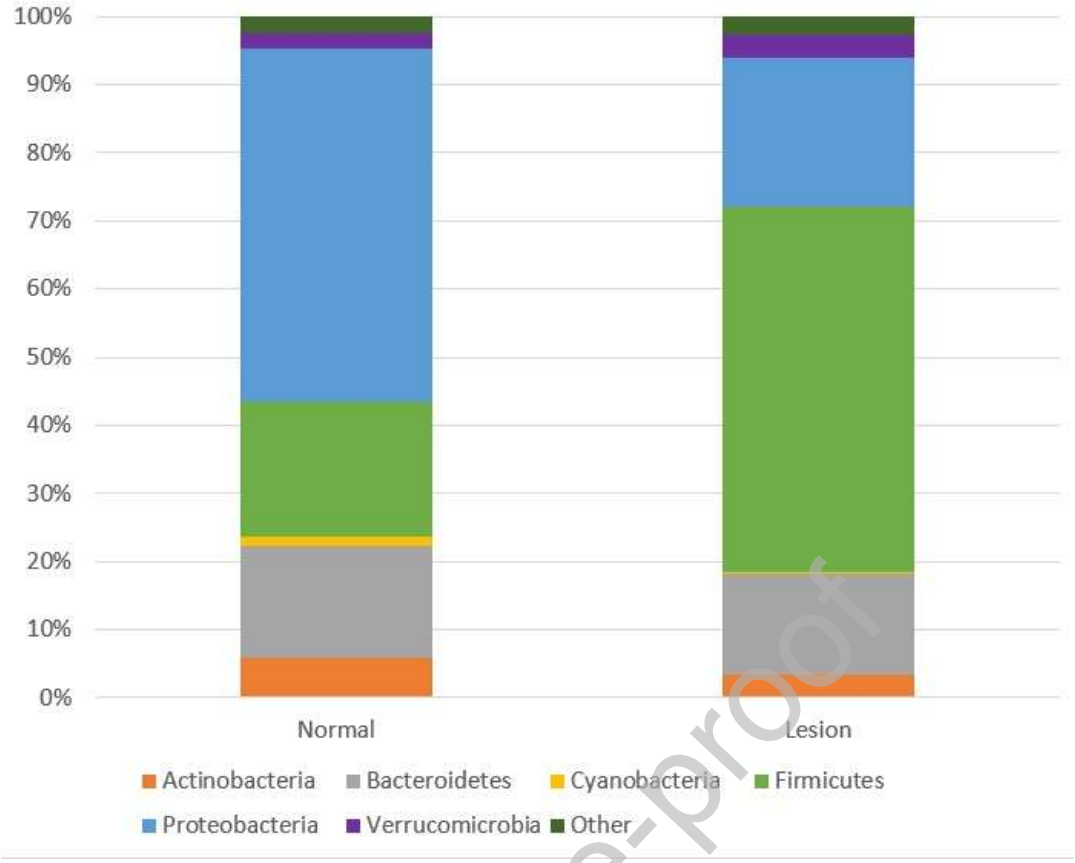


Fig. 3. Stacked bar charts comparing bacterial community composition at phylum level between pooled normal mucosa samples and pooled EGGD lesion samples. *Firmicutes* dominate in EGGD lesion samples, whereas *Proteobacteria* are the predominant phylum in normal glandular mucosa samples.

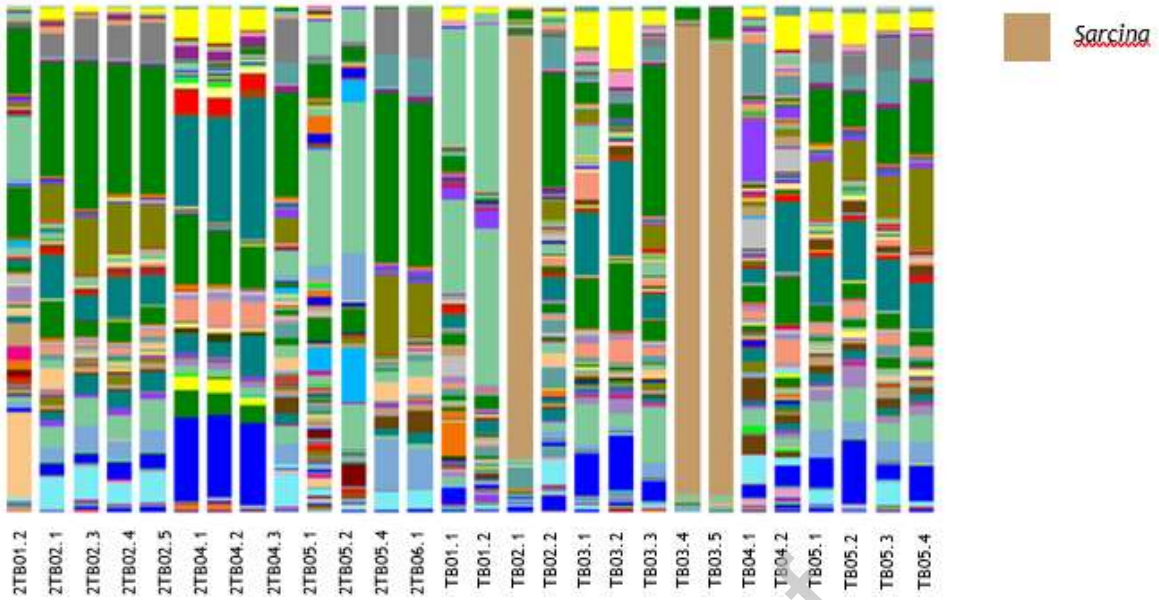
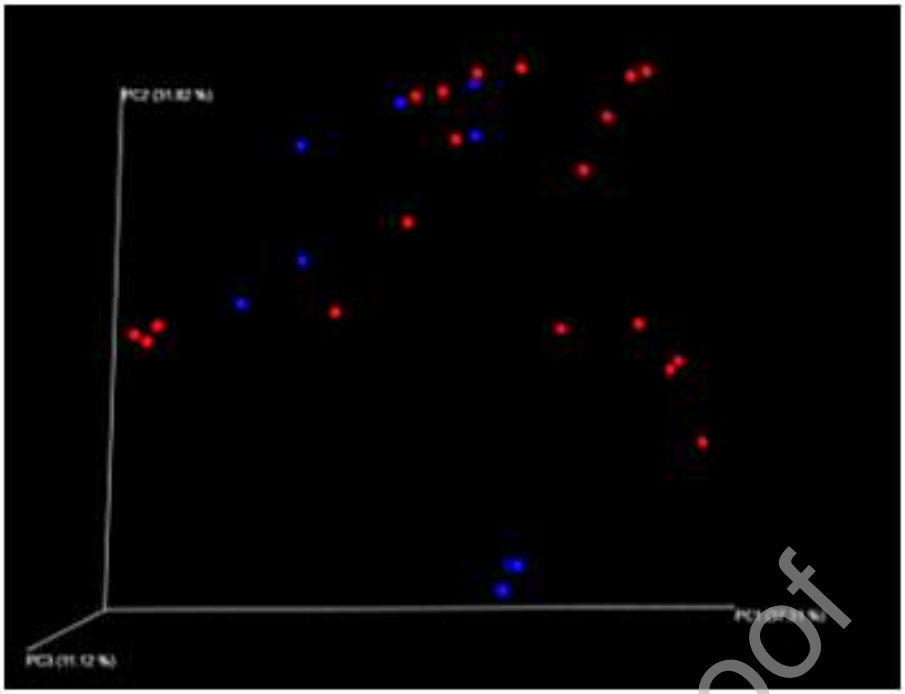
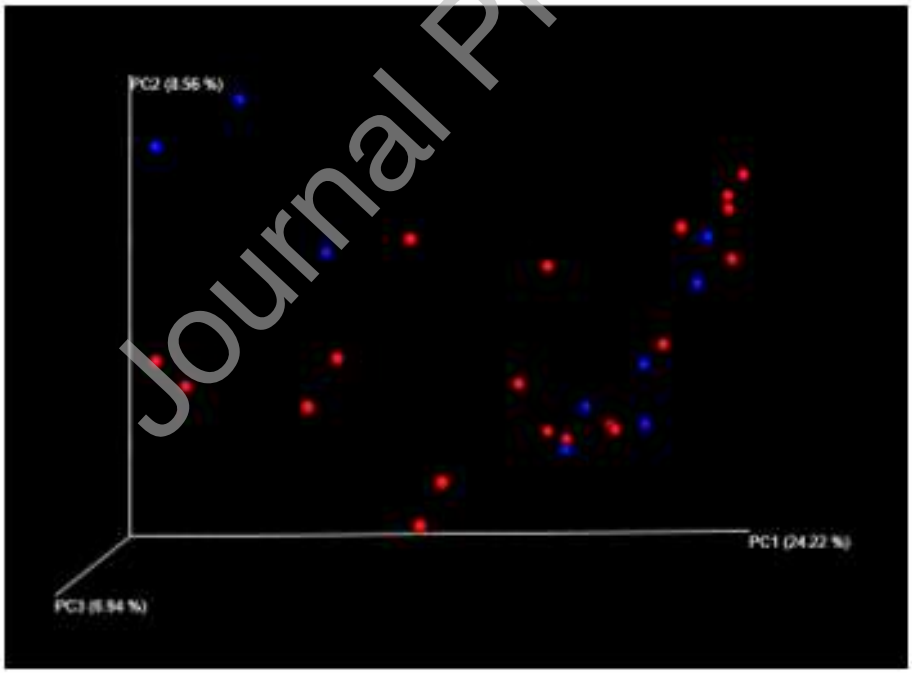


Fig. 4. 100% stacked bar charts displaying individual samples demonstrating the increased abundance of Sarcina in TB02.1, TB03.4, and TB03.5 samples, which represent EGGD lesions.



A



B

Fig. 5. (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.

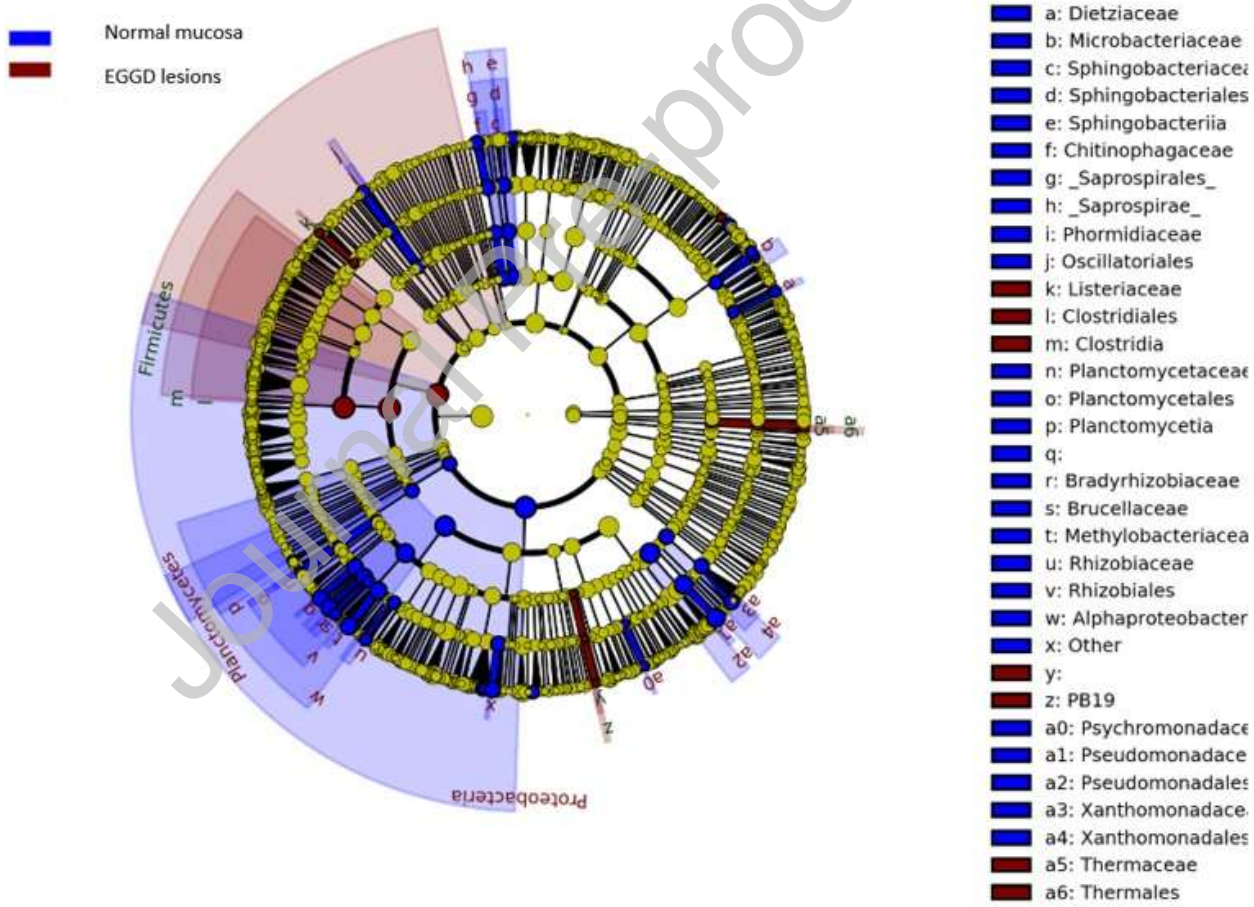


Fig. 6. Cladogram of LefSE analysis results showing taxa significantly associated with normal mucosa and EGGD lesions. Each concentric ring represents a level of taxonomic

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.