Rumen Sampling Methods Bias Bacterial Communities Observed

Jill V. Hagey[1],

Maia Laabs[1],

Elizabeth A. Maga[1]

Edward J. DePeters[1*]

[1] Department of Animal Science, University of California Davis, California, USA

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*Correspondence:

Edward J. DePeters

ejdepeters@ucdavis.edu

Abstract

The rumen is a complex ecosystem that plays a critical role in our efforts to improve feed efficiency of cattle and reduce their environmental impacts. Sequencing of the 16S rRNA gene provides a powerful tool to survey shifts in the microbial community in response to feed additives and dietary changes. Oral stomach tubing a cow for a rumen sample is a rapid, cost-effective alternative to rumen cannulation for acquiring rumen samples. In this study, we determined how sampling method, as well as type of sample collected (liquid vs solid), bias the microbial populations observed. The abundance of major archaeal populations was not different at the family level in samples acquired via rumen cannula or stomach tube. Liquid samples were enriched for the order WCHB1-41 (phylum Kiritimatiellaeota) as well as the family *Prevotellaceae* and had significantly lower abundance of *Lachnospiraceae* compared with grab samples from the rumen cannula. Solid samples most closely resembled the grab samples; therefore, inclusion of particulate matter is important for an accurate representation of the rumen microbes. Stomach tube samples were the most variable and were most representative of the liquid phase. In comparison with a grab sample, stomach tube samples had significantly lower abundance of *Lachnospiraceae*, *Fibrobacter* and *Treponema.* Fecal samples did not reflect the community composition of the rumen, as fecal samples had significantly higher relative abundance of *Ruminococcaceae* and significantly lower relative abundance of *Lachnospiraceae* compared with samples from the rumen.

Introduction

The ruminant stomach consists of four chambers the reticulum, rumen, omasum, and abomasum. The rumen, which is the largest of the four compartments, is a complex pregastric

anaerobic fermentation chamber that harbors a diverse microbial community of bacteria, archaea, protozoa, and fungi [1]. These microbes exist symbiotically inside the ruminant host and are responsible for fermentation of dietary compounds. During the anaerobic fermentation of chemical constituents in the diet, volatile fatty acids (VFA), B-vitamins, and microbial cell proteins are produced, which serve as sources of nutrients and energy for the host that have a direct effect on physiological and production parameters [2]. As the rumen compartment does not secrete enzymes, ruminants are dependent on the enzymes produced by the various rumen microbes for digestion of feed. These microbial enzymes allow the ruminant to convert a wide variety of both plant- and animal-based feedstuffs into products that will contribute to the synthesis of meat and milk for human consumption. The bacterial population of the rumen comprises nearly 95% of the total microbial community and is diverse. There are many genera of bacteria that have been linked to feed efficiency, milk yield, and milk composition in dairy cattle [3,4].

Factors such as age [5], breed [6–8], health status, season [9] and diet of the animal all contribute to variation in the microbiota of the rumen. Dietary composition was reported to be the primary factor affecting the taxa present in the rumen microbiota as well as the richness of those taxa, with the ratio of forage-to-concentrate in the diet of utmost importance [10–12]. The rumen is home to a stable yet dynamic microbial ecosystem that has adapted to survive in an anaerobic environment with osmotic pressure, high buffering capacity and internal competition for substrate [13]. When dietary changes occur slowly, rumen conditions change causing microbial populations to shift in response to the new feed ingredients by favoring the growth of certain taxa over others, which subsequently affects the organic acid profiles produced [14]. However, when a dietary change occurs rapidly, for example changing from high forage diet

(high cellulose and hemicelullose substrate) to a high concentrate diet (high starch and sugar), the shift in microbial community often causes simple indigestion in the cow, which can lead to ketosis. This occurs in dairy production when cows transition from a high forage diet fed prepartum to a lower forage, higher concentrate lactation diet in a matter of hours, which can contribute to indigestion. Management strategies at the farm level have evolved to minimize perturbations to the rumen microbial environment that reduce health and production performance when ingredients in the diet change due to cost or availability of a feed ingredient. Methods to quickly sample and diagnose microbial perturbations due to dietary transitions could improve the management strategies of these high-risk animals.

The composition of the rumen microbiota was first described by Hungate in 1966, and has been studied more extensively in recent years, in part due to the reduced costs associated with next generation sequencing techniques such as the pyrosequencing and Illumina platforms [15]. Much of the recent interest in the rumen microbiota has been generated by research related to climate change and the potential to reduce methane emissions from ruminant livestock as a greenhouse gas mitigation strategy. Next generation sequencing has thus far been a successful tool for characterizing the diversity of the microbial community within the rumen in greater detail through 16S rRNA gene amplicon profiling [16,17]. This technology is advantageous in that it allows the identification of a broader array of rumen microbial taxa, given that only a small fraction of the total species have been successfully cultured. However, the most appropriate method of obtaining a representative rumen sample is still widely debated [18]. It is well known that the bacterial populations between the solid and liquid portions of the rumen digesta differ in microbial composition, suggesting that the sampling method used will affect the

97 characterization of the microbial community [19–24]. Thus, identifying sampling methods that
98 accurately represent both the liquid and solid fractions of the rumen digesta are necessary.
99 Much of the existing research describing the rumen microbiome was performed on
100 animals surgically fitted with rumen cannula, which offer the accuracy and convenience of
101 sampling both liquid and solid rumen digesta directly from the rumen chamber. However, the
102 surgical fistulation procedure is invasive, and the costs associated with the procedure as well as
103 the ongoing animal care limit the number of animals that can feasibly be used in an experiment.
104 Importantly, if microbial biomarkers of health or disease are identified for on-farm testing,
105 retrieving rumen fluid through a cannula is not a practical approach on commercial dairy and
106 livestock farms. Alternatively, many studies have used an oral stomach tube to collect rumen
107 fluid without the need for a rumen fistula [8,18,25]. Oral stomach tubes are a cheaper, less
108 invasive approach to rumen sampling that can be performed on as many cows as necessary, thus
109 economically increasing the experimental sample size. In terms of bacterial community
110 composition and diversity, rumen fluid extracted via the fistula was comparable to fluid extracted
111 via the oral stomach tube [8,26]. Some of the disadvantages of the oral stomach tube include
112 possible contamination by saliva (which affects the pH of the sample), inconsistent sampling
113 region within the rumen, stress to the animal, skilled labor associated with use, and limited
114 representation of particulate matter in samples, though the importance of these concerns to the
115 microbial composition of the sample are widely debated among researchers [18,27].
116 The collection of fecal material from cattle is another non-invasive, simple, and
117 inexpensive technique that is not as commonly regarded as a viable tool for collecting samples
118 representative of the rumen microbiota. Although fecal sampling requires minimal equipment, is
119 cost-effective, and can be performed easily on any animal, bacterial populations of the feces

were found to not reflect the rumen digesta [28,29]. However, in these studies, the fecal microbiome was not compared with the liquid and solid fractions of the rumen digesta individually. If the feces reflect the microbial populations in the solid fraction, fecal samples might be useful in evaluating microbial taxa involved in fiber digestion. Conversely, if fecal samples represent the liquid fraction, lactate-producing microbes that contribute to ruminal acidosis could be diagnosed in a less invasive manner.

The aim of this study was to identify and compare the bacterial populations present in samples collected using three methods – an oral stomach tube, fecal samples, and grab sample through a rumen fistula. To the authors' knowledge, no studies have considered this variety of sampling methods on a comparative basis using next generation sequencing. Our results will be useful in helping investigators design experiments that capture their microbial populations of interest.

Materials and methods

Animals

The experimental protocol and all procedures used in this study were approved by the UC Davis Institutional Animal Care and Use Committee. Four non-lactating Holstein (3) and Jersey (1) cows, each ruminally fistulated prior to the study, were used for the collection of samples. For the two-week duration of the study, cattle were housed individually with ad libitum access to water and offered the same maintenance total mixed ration (TMR) twice daily at approximately 08:00 and 16:00. Dietary composition of TMR was analyzed for protein, fiber, mineral, and energy content (Cumberland Valley Analytical Services, Hagerstown, MD; Table 1).

TABLE 1 | Dietary Composition of Total Mixed Ration

Item[a]	Dietary Composition[b]

Moisture	11.1%
Dry matter	88.9%
Crude protein	10.7% DM
Adjusted protein	91.4% CP, 9.8% DM
Soluble protein	28.6% CP, 3.1% DM
ADF	78.5% NDF, 36.7% DM
aNDF	46.8% DM
Ash	9.62% DM
Calcium	0.33% DM
Phosphorus	0.22% DM
Magnesium	0.25% DM
Potassium	1.91% DM
Sodium	0.08% DM
Iron	449 ppm
Zinc	54 ppm
Copper	8 ppm
TDN	57.5% DM
Net energy lactation	0.59 Mcal/lb
Net energy maintenance	0.56 Mcal/lb
Net energy gain	0.30 Mcal/lb
Non fiber carbohydrates	32.9% DM

Table 1: Chemical composition of the total mixed-ration (TMR) fed to the rumen-fistualted dry cows. Dietary analysis conducted by Cumberland Valley Analytical Services (Hagerstown, MD) completed 12/01/2016. Ingredient composition of TMR on an as is a basis was 50% wheat hay, 25% alfalfa hay, 21.4% almond hulls and 3.6% mineral supplement. [a]Acid detergent fiber

(ADF); Ash free Neutral Detergent Fiber (aNDF); Total Digestible Nutrients (TDN). [b]Dry Matter (DM); Crude Protein (CP); Neutral Detergent Fiber (NDF).

Sampling

Cows were given a one-week period for environmental adaption prior to sampling. This adaptation period was necessary to allow them to acclimate to an individual (rather than group) feeding approach and to reduce sorting of the feed. All cows were fed the maintenance TMR diet (Table 1) prior to and throughout the study. Sampling of fecal and ruminal contents occurred on days 7, 9, and 11 of the experiment, and took place approximately 4 hours after morning feeding. Fecal samples were collected from the rectum with sterile polyethylene gloves and stored in plastic bags. Grab samples (containing both liquid and particulate matter) from the fistula were collected from the medioventral region of the rumen and stored in plastic bags. Rumen liquid was collected from the fistula using a PVC pipe, Tygon® tubing, and a large syringe, and stored in 240 ml sterile plastic vials. The Tygon® tubing was thoroughly rinsed and bleached between cows to avoid cross-contamination of samples. For liquid strained samples, about 250 ml of liquid sample was squeezed through 4 layers of cheesecloth to remove large particles, as is common done [19,30,31]. For solid samples, similar squeezing through a cheese cloth was applied to remove liquid from the solid digesta content, before being stored in plastic bags. On days 9 and 11, the first aliquot of rumen liquid, containing both liquid and solid particulates, was additionally collected as a liquid unstrained sample in 240 ml sterile plastic vials. Lastly, enough rumen liquid to fill a 240 mL sterile plastic vials, which was collected via an oral stomach tube using an oral speculum, Tygon® tubing (1.5cm O.D. and 0.9cm I.D.) and a vacuum pump. A fresh tube was used for each cow to avoid cross-contamination of samples. The pH of each of the liquid-containing samples was measured with a portable pH meter (Milwaukee Instruments,

Rocky Mount, NC). All samples were held on ice during transport and stored in triplicate 60 ml vials at -20°C for DNA extraction and dry matter analysis.

DNA extraction and PCR amplification

DNA extraction was performed using a ZR Fecal DNA MiniPrep™ kit (Zymo Research Corp., Irvine, CA), with slight modifications to the manufacturer's instructions. Samples were thawed at room temperature, and 200 mg of each sample were used for DNA extraction, which included a bead bashing step to facilitate the mechanical lysis of microbial cell walls. As the last step in the procedure, DNA was eluted from the column with elution buffer, and the resulting DNA was evaluated for concentration and purity on a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and stored at -20°C. The V4 region of the bacterial 16S rRNA gene was amplified from each sample using forward primer F515 containing a unique 8 bp barcode (N) and linker region (**GT**) (5'-NNNNNNNN**GT**GTGCCAGCMGCCGCGGTAA-3') and the reverse primer R806 (5'-GGACTACHVGGGTWTCTAAT-3'). The amplification was carried out in triplicate using GoTaq® Green Master Mix (Promega, Madison, WI) as previously described [32]. In brief, PCR conditions were set at initial denaturation for 94°C for 3 min; followed by 35 cycles of 94°C for 45 seconds, 50°C for 1 min, 72°C for 90 seconds with final extension step at 72°C for 10 min. Triplicates were combined in equal concentrations and amplicons were evaluated for off target bands by gel electrophoresis, pooled and then purified using a QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany). A 50µl aliquot of the final pooled PCR product was sequenced at the UC Davis Genome Center DNA Technologies Core via the Illumina MiSeq PE250 platform (Illumina, CA).

Amplicon library processing

Raw paired end reads were screened to remove phiX, human and host contamination using Kneaddata v0.6.1 by aligning reads to the phiX174 (NCBI ACC: NC_001422.1), bovine (ARS-UCD1.2) and human (GRCh38) reference genomes [33]. Reads were demultiplexed followed by trimming of primers and barcodes with Cutadapt v1.18 [34]. Ends of reads were trimmed for quality, any read smaller than 150bp was discarded and a max expected error of 2 was used as a quality filter using the filterAndTrim function from DADA2 v1.8.0 [35]. Sequences were merged, denoised, chimeras were removed and exact amplicon sequence variants (ASVs) were identified using DADA2. Taxonomy was assigned using the RDP native Bayesian classifier algorithm in the DADA2 assignTaxonomy function with Silva reference database v.132 training set. A phylogenetic tree of unique ASVs was made using FastTree with default options in QIIME v.1.9.1 [36]. The ASV table, sequences and tree produced by DADA2 were imported into the R package Phyloseq v.1.24.2 for further analysis [37].

Microbial community analyses and statistics

First, unsupervised exploratory analysis was conducted with double principal coordinates analysis (DPCoA), which was calculated and graphed with the phyloseq R package [37,38]. Both modeling and hypothesis testing of differentially abundant ASVs between sample types was determined using the Corncob R package [39]. All genera-level and ASV-level relative abundances were modeled using a beta-binomial regression with a logit-link for mean and dispersion as described by Martin et al [39]. Differential abundance was modeled as a linear function of sample type, cow and day. Significant differentially abundant ASVs were determined with the parametric Wald test with bootstrapping (n=1000) as described by Martin et al [39]. Within the Corncob algorithm the Benjamini-Hochberg (BH) adjustment for multiple comparisons was used to calculate adjusted p values. An adjusted p value ≤ 0.05 was considered

significant. This model has the benefit of accommodating the absence of a taxon in samples without zero-inflation or pseudocounts, accounts for differences in library sizes, give valid inference even with small samples [39]. Richness of sample types was estimated with the R package breakaway and evenness was calculated using the R package DivNet, which accounts for the structure of microbial communities [40,41]. Hypothesis testing of alpha diversity (richness and evenness) metrics was done using the betta() function using sample type, cow and day as fixed effects in the breakaway R package [42]. Beta diversity was calculated by using unweighted UniFrac distances and graphed by PCA clustering in the Phyloseq R package [37]. The number of clusters in the data was determined with the gap statistic using the gapstat_ord() function in Phyloseq [43].

Data availability

Scripts for sequence processing and analysis, interactive graphs, R objects as well as an Rmarkdown file to reproduce figures in this paper can be found at https://doi.org/10.5281/zenodo.4026849. Raw sequencing files are available through the Sequence Read Archive under the study accession number PRJNA692782.

Results

Sequence processing of rumen and fecal samples

After filtering with Kneaddata and demultiplexing the single run of MiSeq yielded 747,961 250bp raw paired-end reads that entered the DADA2 pipeline. After the quality trimming, initial filtering, and chimera removal, the library size ranged from 2,189 to 24,624 reads, with a median library size of 7,197 and an average size of 8,110 reads. The median read length of quality filtered merged reads was 257bp. A total of 5,607 AVSs were identified, of

which 94 weren't assigned to a phylum and thus were removed for analysis along with 12 ASVs assigned to chloroplasts and mitochondria. The 94 unassigned taxa were found in all sample types with solid samples having the most reads of unknown taxa. This suggests there are is still a diverse group of microbes attached to solid particles that have yet to be identified. The final feature table had 5,485 ASVs across 68 samples.

Community composition of all sample types

The 5,485 ASVs were assigned to 21 phyla, 78 orders, 117 families, and 293 genera. Here we define major phyla as those with a mean relative abundance in at least one sample type of greater than 3%. Major phyla were Firmicutes, Bacteroidetes, Kiritimatiellaeota, Proteobacteria, Euryarchaeota and Spirochaetes (Fig 1A). Of these major phyla, Firmicutes was significantly lower in relative abundance ($P \leq 0.0001$; Fig 1C) while Bacteroidetes and Proteobacteria had significantly higher relative abundance in feces and liquid samples as compared with grab samples ($P \leq 0.002$; Fig 1C). In addition, Kiritimatiellaeota was significantly higher in relative abundance in stomach tube and liquid samples compared to grab samples ($P \leq 0.001$; Fig 1C). Spirochaetes was significantly lower in relative abundance in feces, stomach tube and solid samples compare with grab samples ($P \leq 0.003$; Fig 1C). While Euryarchaeota had significantly lower relative abundance in feces, it had significantly higher relative abundance in stomach tube samples compared with grab samples ($P = 3.24\text{x}10^{-8}$). Minor phyla were those with less than 3% relative abundance in all samples (Fig 1B). An interactive version of Figure 1B with mean and standard deviations for each phyla is available at https://doi.org/10.5281/zenodo.4026849 as interactive Fig 1 – minor phyla. The phylum Gemmatimonadetes was only found in stomach tube samples and Deferribacteres was only found in fecal samples (Figure 1B). For the minor phyla in feces Tenericutes, Patescibacteria,

Actinobacteria, Fibrobacteres, Chloroflexi and Synergistetes were significantly lower in relative abundance and Verrucomicrobia, Epsilonbacteraeota, Cyanobacteria, Planctomycetes and Lentisphaerae were significantly higher in relative abundance compared with grab samples ($P \leq 0.001$; Figure 1C). Samples acquired with the oral stomach tube had significantly lower relative abundance of Patescibacteria and Fibrobacteres and significantly higher relative abundance of Verrucomicrobia, Epsilonbacteraeota, and Fusobacteria compared with grab samples. Only 1.68% of ASVs were assigned a species, but 67.3% were able to be assigned to a genus.

Fig 1. **Relative abundance of (A) major and (B) minor phyla and (C) their differential abundances.** (A) Relative abundance of major phyla defined as those phyla found at greater than 3% relative abundance and graphed as relative abundance ± SE. (B) Minor phyla defined as those found below 3% relative abundance present in sample types. (C) Phyla that are significantly differentially abundant compared with grab samples. Graphed as coefficients with a 95% confidence interval from the corncob model. Families with negative coefficients for a sample type are expected to have a lower relative abundance when compared to the grab samples while positive coefficients suggest a higher relative abundance in that sample type compared to grab samples.

Liquid unstrained and fecal samples were the least variable samples as they shared 510 and 441 ASVs, respectively, with samples of their own type. On the other hand, stomach tube and liquid strained samples were the most variable as these sample types only shared 225 and 307 ASVs, respectively, with samples of their own type. Moderately variable sample types were grab and solid samples, which shared 319 and 405 ASVs, respectively, with samples of their own type.

Diversity

304 The evenness of fecal samples was significantly lower than all rumen sample types ($P \leq$
305 0.001; Fig 2A). Fecal, stomach tube, and liquid strained samples had significantly lower
306 evenness than grab samples ($P \leq 0.001$; Fig 2A). Solid and liquid unstrained samples did not
307 have significantly different evenness compared with grab samples ($P \geq 0.05$; Fig 2A). Both the
308 individual cow sampled and day of sampling significantly affected the evenness of a sample ($P \leq$
309 0.05; Fig 2A).

310 **Fig 2. Differences in estimated alpha diversity among sample types.** (A) DivNet estimate of
311 Shannon diversity plotted as mean with a 95% confidence intervals and (B) mean breakaway
312 estimate of species richness with 95% confidence intervals. Both the richness and evenness of
313 fecal samples were significantly lower than all other rumen sample types ($P \leq 0.001$). Stomach
314 tube and Liquid strained samples had significantly lower evenness than grab samples ($P \leq$
315 0.001). Solid and stomach tube samples were estimated to have significantly fewer species than
316 grab samples ($P = 0.02$ and $P \leq 0.001$, respectively).

317 The richness of samples from the rumen were estimated to be significantly higher than
318 that of fecal samples ($P \leq 0.001$; Fig 2B). Fecal samples were estimated to have a mean of 2,021
319 species, which was significantly lower than the grab samples estimated mean of 4,119 species (P
320 ≤ 0.001; Fig 2B). Liquid strained and unstrained samples did not have a significantly different
321 mean number of estimated species compared with grab samples ($P \geq 0.05$; Fig 2B). However,
322 solid and stomach tube samples were estimated to contain a significantly lower number of
323 species compared with grab samples, 286 and 506, respectively ($P = 0.02$, $P \leq 0.001$; Fig 2B).
324 Neither the day sampled nor individual cow had a significant effect on the number of species in a
325 sample ($P \geq 0.05$; Fig 2B).

Weighted UniFrac distances were calculated to determine beta diversity. Calculations of eigenvalues showed that 86.8% of the variance between samples was contained in the first two principle components, thus a two-dimensional visualization was deemed appropriate (Fig 3). Two distinct groups were present with fecal samples clustering away from all rumen sample types (Fig 3). Grab and solid samples exhibited low variability and overlapped each other, forming one group. Liquid samples were further down the second axis, which might indicate that there were distinct phylogenetic differences between these samples and grab samples. Stomach tube samples were the most variable with some of these samples found within the grab and solid sample cluster, while other stomach tube samples were more closely associated with liquid samples. The gap statistic of the weighted UniFrac strongly suggested there were at least 3-5 clusters in the data. As there are six sample types in the dataset, this suggests that grab and solid samples are likely one cluster as these samples overlap the most (Fig 3). The unweighted UniFrac showed a similar pattern, with less variation explained in axis one and two, 45.8% and 6.9%, respectively (data not shown).

Fig 3. Beta diversity as weighted UniFrac distances between samples. To faithfully reflect the variance in the coordinates, the height-to-width ratio was based on the ratio between the corresponding eigenvalues.

Overall differences between sample types

As an exploratory first step, DPCoA was performed (Fig 4A). An interactive version of this graph with taxon identification is available at https://doi.org/10.5281/zenodo.4026849 as interactive figure 2 – DPCoA. Additionally, since Firmicutes and Bacteroidetes dominated a majority of the graph, a version without these phyla was created at and is available at https://doi.org/10.5281/zenodo.4026849 as interactive Fig 3 – DPCoA_NoFrimBact with the aim

to allow a better visualization of minor phyla. This phylogenetic ordination method provides a biplot representation of both samples and taxonomic categories. The DPCoA was used to identify the underlying structure of these data and identify taxa that could be contributing to differences between sample types that will be specifically examined with differential abundance testing.

Fig 4. Double principal coordiant analysis (DPCoA) of the Bray-Curtis distances among samples. DPCoA is a phylogenetic ordination method and that provides a biplot representation of both (A) samples and (B) taxonomic categories. Note that while the biplots are a square shape to fit the page the CS1 explains roughly twice the variation of CS2 similar to what is seen in Fig 1. The 1st axis discrimates fecal from rumen samples while the 2nd axis separtes liquid strained samples from other rumen sample types. Samples that have larger scores on CS1 have a subset of taxa from Bacteroidetes and Firmicutes that is different than rumen samples. Liquid strained samples have lower values on CS2 suggesting they are distinguished from other rumen sample types by taxa in the phylum Kiritimatiellaeota and *Prevotellaceae*. Fecal samples are predicted to have lower abundance of *Lachnospiraceae* and greater abundance of *Ruminococcaceae*.

Fecal samples clustered away from samples that were collected from the rumen, which was primarily driven by differences in the relative abundance of a subset of Firmicutes in the families *Ruminococcaceae*, *Lachnospiraceae* and *Christensenellaceae* and a subset of families in the phylum Bacteroidetes, mainly *Rikenellaceae* and *Prevotellaceae* on the 1st axis (Fig 4B). Additionally, fecal samples separate from samples from the rumen based on having more taxa from the family *Akkermansiaceae* and phylum Tenericutes and fewer from the families *Fibrobacteraceae,* and *Spirochaetaceae* (Fig 4B and S1 Fig). Liquid samples were found lower on the 2nd axis of the DPCoA, indicating these samples had more taxa from the phylum

372 Kiritimatiellaeota and a subset of Bacteroidetes most of them in the family *Prevotellaceae* (Fig
373 4B). Also, the separation of liquid strained samples away from other rumen samples was due to
374 fewer taxa from the phylum Euryarchaeota and the family *Eggerthellaceae* that is within the
375 phylum Actinobacteria (S1 Fig).

376 To test the significance of these differences, differential abundance testing was performed
377 with Corncob. All sample types were compared to grab samples as a base line, because it is
378 considered the gold standard for surveying microbial communities in the rumen. The relative
379 abundance of *Prevotellaceae* was significantly lower in feces and was significantly higher in
380 liquid samples compared with grab samples ($P \leq 0.0004$; Fig 5A and 6). Stomach tube ($P = 0.06$)
381 and solid ($P = 0.77$) samples were not significantly different in the relative abundance of
382 *Prevotellaceae* compared with grab samples. The relative abundance of *Prevotellaceae* was
383 highest in liquid strained samples compared with other sample types (Fig 5A). In comparison to
384 grab samples, the relative abundance of *Ruminococcaceae* was significantly higher in feces ($P \leq$
385 0.001; Fig 5B) and solid ($P = 0.003$; Fig 5B) samples while liquid strained samples had
386 significantly lower relative abundance ($P \leq 0.001$; Fig 5B). Neither stomach tube nor liquid
387 unstrained samples had significantly different relative abundance of *Ruminococcaceae* compared
388 with grab samples. Fecal samples were lower in relative abundance of *Lachnospiraceae*
389 compared with all other samples ($P \leq 6.96 \times 10^{-15}$), while relative abundance was higher for grab
390 samples compared with all other sample types ($P \leq 0.03$; Fig 5C). Neither day of sampling nor
391 individual animal significantly affected the relative abundance of *Prevotellaceae* ($P \geq 0.05$). In
392 contrast, the relative abundance of *Ruminococcaceae* and *Lachnospiraceae* was significantly
393 affected by individual animal ($P \leq 0.03$), but not day of sampling.

Fig 5. Significant differences in the relative abundance of specific bacterial families. Relative abundance of (A) *Prevotellaceae* (B) *Ruminococcaceae* and (C) *Lachnospiraceae* as modeled by corncob. Points are the estimated relative abundance and bars are a 95% prediction interval for each cow on different days of sampling.

Specific community differences between grab and fecal samples

To further distinguish what taxa were contributing to the separation of fecal samples from rumen samples on the DPCoA, we identified taxa that were found in one sample type and not the other. Within the phyla Firmicutes and Bacteroidetes, families *Barnesiellaceae*, *Chitinophagaceae*, *p-2534-18B5_gut_group*, *GZKB124,* and *Hymenobacteraceae* were found in fecal samples, but were not found in grab samples. Conversely, *Leuconostocaceae*, *Carnobacteriaceae*, *Aerococcaceae*, *Syntrophomoadaceae*, *Bacteroidetes_DB2-2*, *PeH15*, *M2PB4-65_termite_group*, *COB_P4-1_termite_group*, *Spirosomaceae,* and *Porphyromonadaceae* were found in grab samples, but were not found in fecal samples.

Next, we identified ASVs, genera and families that were differentially abundant between sample types. There were 657 significant differentially abundant ASVs in fecal samples compared with grab samples, as well as 114 differentially abundant genera ($P \leq 0.05$; S2 Fig). At the genera level, 131 ASVs were unable to be fit to the Corncob model for differential abundance testing. Primarily, this was due to either limited or lack of reads in one of the sample types. Of these genera that didn't fit the model, *Acetatifactor*, *Shuttleworthia*, *Succinivibrio, Veillonellaceae UCG-001,* and *Lachnospiraceae UCG-006* were found in all grab samples with greater than or equal to 50 reads across all samples, but absent in fecal samples. Similarly, there were 11 genera found in all fecal samples with 50 or more reads, but these were not found in any grab samples including *Coprococcus 3*, *Cellulosilytium*, *Clostridioides*, *Paeniclostridium*,

417 *Parasutterella*, *Aeriscardovia*, *Odoribacter*, *Harryflintia*, *Negativibacillus, Pygmaiobacter,* and
418 *Ruminococcaceae UCG-011*.

419 The most common families with differentially abundant ASVs were *Lachnospiraceae*,
420 *Ruminococcaceae*, *Christensenellaceae*, *Family XIII, Rikenellaceae,* and *Prevotellaceae*. These
421 families are in the phyla Firmicutes and Bacteroidetes, which had the most significant
422 differentially abundant ASVs. However, as a percent of total ASVs these phyla only had 4.9%
423 and 16.3% significant differentially abundant ASVs, respectively. In contrast, 25.6% of the
424 ASVs assigned to Chloroflexi and 29.5% of ASVs assigned to Euryarchaeota were significantly
425 different between grab and fecal samples. The significant ASVs in Chloroflexi were all assigned
426 to the genus *Flexilinea*. In addition to the significantly lower abundance of some Chloroflexi
427 ASVs in fecal samples compared with grab samples, another 51.3% of the ASVs in the phyla
428 were not found in any fecal samples (S2 Fig). In the phylum Euryarchaeota, feces had
429 significantly lower abundance of *Methanobrevibacter*, *Methanosphaera,* and were almost devoid
430 of *Methanomethylophilaceae*.

431 There were 30 families that had significantly lower relative abundance while 18 families
432 had higher relative abundance between fecal and grab samples (Fig 6). Families that had the
433 strongest positive relationship with fecal samples were *Peptostreptococcaceae* ($P = 1.76\text{x}10^{-7}$;
434 Fig 7A), *Akkermansiaceae* ($P = 6.95\text{x}10^{-5}$; Fig 7B), and *Bacteroidaceae* ($P = 7.87\text{x}10^{-12}$; Fig
435 7C), which were significantly higher in relative abundance compared with grab samples.
436 Conversely, the families with largest negative relationship between fecal and grab samples that
437 had significantly lower relative abundance were *Veillonellaceae* ($P = 1.66\text{x}10^{-11}$; Fig 7D) and
438 Bacteroidales_BS11_gut_group ($P = 6.20\text{x}10^{-11}$; Fig 7E). Additionally, fecal samples separated
439 from rumen samples on the DPCoA (S1 Fig) due in part to differences in the families

440 *Spirochaetaceae* and *Fibrobacteraceae* both of which had significantly lower relative abundance
441 than grab samples ($P = 7.88x10^{-9}$; $P = 4.73x10^{-8}$, respectively Fig 7F).

442 **Fig 6. Families that were significantly differentially abundant across sample type compared**
443 **with grab samples.** Graphed as coefficients with a 95% confidence interval calculated from the
444 corncob model. Families with negative coefficients for a sample type are expected to have a
445 lower relative abundance when compared to the grab samples while positive coefficients suggest
446 a higher relative abundance in that sample type compared to grab samples.

447

448 **Fig 7**. **Significant differences in the relative abundance of specific bacterial families between**
449 **fecal and grab samples.** Fecal samples had significantly higher relative abundance of (A)
450 *Peptostreptococcaceae*, (B) *Akkermansiaceae*, (C) *Bacteroidaceae*, compared to grab samples.
451 Also, there was significantly lower relative abundance of (D) *Veillonellaceae*, (E)
452 Bacteroidales_BS11_gut_group and (F) *Spirochaetaceae* compared to grab samples. Points are
453 the estimated relative abundance and bars are a 95% prediction interval for each cow on different
454 days of sampling.

455 Based on the DPCoA findings, the phyla Spirochaetes and Actinobacteria also played an
456 important role in distinguishing feces from grab samples (Fig 1A and 4B). In the phylum
457 Spirochaetes, there were 10 ASVs, all of which were from the genera Treponema_2, that had
458 significantly lower relative abundance in fecal samples compared with grab samples. Within the
459 phylum Actinobacteria, there were 4 ASVs in the genera *Olsenella*, 5 ASVs in *Atopobium*, 7
460 ASVs in the genera *DNF00809,* and 1 ASV assigned to *Raoultibacter*, which were all
461 significantly lower in relative abundance compared with grab samples.

Specific community differences between grab and stomach tube samples

Oral stomach tube samples were composed of 20 phyla, 65 orders, 98 families, and 236 genera. There were 255 ASVs found in grab samples that were not found in the stomach tube samples. Likewise, 404 ASVs in stomach tube samples were not present in the grab samples. There were 3,615 ASVs that were in common between stomach tube and grab samples. Three families *Rhodobacteraceae*, *Bacteriovoracaeae,* and *Spirosomaceae* were found in grab samples, but were not present in stomach tube samples. The 5 families found in stomach tube samples, but not in grab samples were *Cellvibrionaceae*, *Neisseriaceae*, *Bifidobacteriaceae*, *Micrococcaceae*, and *Solirubrobaceraceae*.

In addition to the taxa not found in a particular sample type, there were 13 families, 43 genera, and 199 ASVs significant differentially abundant between stomach tube and grab samples. *Lachnospiraceae*, *Ruminococcaceae*, *Prevotellaceae,* and *Erysipelotrichaceae* were the most common families to have significant differentially abundant ASVs in stomach tube versus grab samples. The relative abundance of 39 ASVs in the family *Lachnospiraceae* were significantly lower while 15 were significantly higher in comparison to grab samples. At the genus level, 15 genera in the family *Lachnospiraceae* were significantly lower in abundance, while *Blautia*, *Acetitomaculum* and *Howardella* were the only genera that had higher relative abundance (S2 Fig). While *Ruminococcaceae* in stomach tube samples was not significantly different from grab samples at the family level (Fig 5B), eight genera in this family were significantly higher in relative abundance between the two sample types. *Prevotellaceae* in stomach tube samples was not significantly different from grab samples at the family level ($P = 0.055$; Fig 5A*),* but at the genus level, two were significantly lower and one significantly higher.

Three genera in the family *Erysipelotrichaceae, Catenisphaera*, *Erysipelotrichaceae* _UCG-009, and *Erysipelotrichaceae* _UCG-004 were all significantly higher in stomach tube compared with grab samples. The only assigned genera in the family *Fibrobacteraceae*, *Fibrobacter,* was significantly lower in abundance in stomach tubes compared to grab samples (S1 Fig). The genus *Streptococcus* had significantly higher relative abundance compared with grab samples (Fig 6).

The only genus in the phylum Euryarchaeota that had significant differences in abundance in samples from the stomach tube as compared with those collected from the rumen was *Methanobrevibacter*. This genus was significantly higher in stomach tube samples. At a finer resolution, there were only four ASVs assigned to *Methanobrevibacter* and one ASV assigned to *Methnomethylophilaceae* that were significantly higher in abundance in stomach tube samples compared with grab samples. However, at the family level three methanogenic families, *Methanomethylophilaceae*, *Methanobacteriaceae* and *Methanocorpusculaceae*, were not significantly different between the two sample types.

Comparing sub-fractions of the grab sample

Grab samples of rumen contents were placed in cheesecloth and squeezed to create the liquid strained sample and the solid particulate sample. There were 283 ASVs found in the grab sample that were not identified in the liquid strained samples. Conversely, there were 3,587 ASVs found in common between grab samples and liquid strained samples.

Based on the DPCoA, separation of liquid samples from other rumen sample types was driven in part by taxa from the phylum Kiritimatiellaeota (Fig 4). ASVs in this phylum were only assigned down to the order level with all ASVs assigned to WCHB1-41. Seventeen ASVs from Kiritimatiellaeota were significantly higher in liquid samples compared with grab samples while these ASVs were not significantly different in solid samples versus grab samples.

In addition to Kiritimatiellaeota, the DPCoA suggested that the families *Lachnospiraceae* and *Prevotellaceae* were also a major cause of differences between liquid and grab samples (Fig 4). Differential abundance testing found that indeed *Lachnospiraceae*, *Ruminococcaceae*, and *Prevotellaceae* were the most common families to have significant differentially abundant genera in liquid strained versus grab samples. *Lachnospiraceae* was significantly lower in liquid samples compared with grab samples ($P < 2.0\text{x}10^{-16}$; Fig 5C). Liquid samples had the most significant differently abundant genera with 22 that had lower relative abundance compared with grab samples and three with higher. One of these genera with significantly higher relative abundance was *Howardella*, which was also higher in relative abundance in the stomach tube samples. Liquid unstrained and liquid strained samples had significantly higher relative abundance in *Prevotellaceae* than grab samples (Fig 5A). Within that family there was higher relative abundance of the genera *Prevotella_1*, *Prevotellaceae_UCG-003, Prevotellaceae_UCG-001* and lower relative abundance of *Prevotellaceae_NK3B31_group* ($P \leq 0.01$; S2 Fig). In the family *Ruminococcaceae*, there were 7 genera with significantly lower relative abundance.

Liquid samples were also differentiated from grab samples by a significantly lower abundance of Actinobacteria, specifically the family *Eggerthellaceae*, and significantly higher abundance of Lentisphaerae and Cyanobacteria (Fig 1C). ASVs in the phylum Cyanobacteria were all within the order *Gastranerophilales* and were not classified any lower. Likewise, ASVs in the phylum Lentisphaerae were only assigned to the family *Victivallaceae* which were significantly lower in abundance in liquid samples compared with grab samples (Fig 6).

In liquid strained samples there were roughly an equal number of ASVs assigned to the genera *Methanobrevibacter* that were significantly higher and lower in relative abundance compared with grab samples (Fig 6). Therefore at the genus level there was not a significant

531 difference observed in the abundance of the genera *Methanobrevibacter*. Also, in the same
532 phylum Euryarchaeota, there was significantly lower relative abundance of *Methanosphaera* in
533 liquid strained samples when compared with grab samples.

534 Discussion

535 While other studies looked at differences in the rumen microbiome due to rumen
536 sampling method, they usually involved different diets and did not include all the sampling
537 methods presented in the current study. As diet is an important factor that affects the rumen
538 microbiome, we choose to keep the diet consistent during the study to fully investigate the
539 differences between sampling methods. To the authors' knowledge, this is the first study to
540 compare rumen sampling methods utilizing ASVs rather than OTUs. Therefore, this study has
541 the advantage of identifying AVS that are comparable across studies, which will improve the
542 reproducibility of sequencing studies of the rumen [44].

543 Kim et al. detected 19 bacterial phyla in the rumen with Firmicutes (57.8%),
544 Bacteroidetes (26.7%) and Proteobacteria (6.9%) in greatest abundance with the remainder of the
545 16 phyla less than 3% of the total sequences [45]. In the present study, 21 phyla were identified
546 in the grab sample and only three phyla were over 3% abundance: Firmicutes (64.3%),
547 Bacteroidetes (20%) and Spirochaetes (4.1%). This differed from fecal samples where the top
548 four phyla were Firmicutes (61.2%), Bacteroidetes (32.1%), Verrucomicrobia (1.3%) and
549 Proteobacteria (1.1%) with the remainder of the phyla observed at less than 1% mean relative
550 abundance (Fig 1A and 1B). The relative abundance of Firmicutes and Bacteroides in the fecal
551 samples were similar to what Wong et al. found in fresh manure, but they found Actinobacteria
552 among the top four phyla rather than Verrucomicrobia [46].

553 The day a sample was collected did not affect the number of species sampled and did not
554 impact the abundance of *Prevotellaceae, Ruminococcaceae* and *Lachnospiraceae.* These
555 observations agree with previous work that found there was little day-to-day variation in both the
556 solid and liquid fraction of rumen samples from the same animal [47]. Previous work found
557 differences between breeds [6,7], while others found minimal to no influence of breed [8,48]. As
558 we only had one Jersey as part of this study we are unable to determine the impact of breed on
559 the community composition.

560 Diversity

561 In the present study, fecal samples had lower richness when compared to grab samples.
562 This is in agreement with a study that used Faith's Phylogenetic Diversity to compare samples
563 from esophogeal tubing or feces of beef calves [49]. The same result was found using the
564 number of ASVs present in fecal compared to rumen condense after slaughter [50]. Similar to
565 fecal samples, we found that samples collected via the esophageal tube had lower richness than
566 grab samples. Such a finding was expected as microbes adhered to particles would be in low
567 proportion or excluded in the stomach tube sample, even though the tube used did not have a
568 screen. Using a stomach tube without a screen allowed the collection of small size particulates
569 only, whereas the grab samples included small to large particulate sizes. Our finding contradicts
570 Paz et al. who reported no difference in richness between a rumen sample collected from a
571 rumen cannula compared with a sample collected via esophageal tube [8]. However, in Paz et al.,
572 solid particles that adhered to the metal strainer of the esophageal tube were recovered and added
573 to the esophageal sample to create a sample that was "more adequately representative of the
574 rumen content", which suggests the authors acknowledge that a sample collected by an
575 esophageal tube that did not contain particles would not represent rumen contents. However, the

576 research did not address this suggestion by analyzing the rumen contents collected with a
577 stomach tube without the added solids.

578 Our work also differed from that of Ji et al. who reported the diversity of the bacterial
579 population was not affected by sample type [51]. Samples in their study included rumen digesta
580 collected from a cannula that was squeezed through cheese cloth to create a liquid and a solid
581 fraction for comparison with rumen digesta from a cannula. However, we determined that both
582 liquid sample types did not have significant differences in the number of taxa observed compared
583 to grab samples, while solid samples had significantly lower estimated species than grab
584 samples. The work of Weimer et al. (2017) used a sample cup to collect 100 ml of digesta from
585 the medio-ventral region of the rumen followed by squeezing through cheese cloth to create a
586 liquid and a solid sample [31]. While this study found that community diversity and community
587 richness were greater in solids than liquid, our data showed the opposite. Greater richness in
588 liquid samples could potentially be explained by the greater relative abundance of
589 *Prevotellaceae*, the most abundant species in the rumen, compared with the estimated number of
590 species in solid samples. Jewel et al. found liquid samples to have higher richness than solid
591 samples in agreement with our data [52].

592 Some of these discrepancies are in part due to differences in the metric used to estimate
593 richness. All these previous studies reported Chao1 as a measure of richness, but the current
594 study used breakaway to estimate richness. Many alpha diversity estimates that are ubiquitous in
595 the literature are highly biased and require statistical adjustments to address this bias, which
596 Chao1 does not [40,41]. Further, the strong negative bias of Chao1 is even further increased by
597 the use of rarefying as a means of normalization in the previous studies [53]. It is true that Dr.
598 Anne Chao proposed that Chao1 could be a useful metric for datasets that skewed toward low-

599 abundance classes as microbiome data does; however, these low abundance counts aren't reliable
600 due to sequencing platform and PCR errors. Breakaway addresses some short comings of Chao1
601 by providing an estimate of variance of richness estimates for hypothesis testing, estimating the
602 number of missing taxa, and adjusting the richness estimate accordingly (bias correction) to
603 provide a more accurate estimate of richness [41]. While this approach produces large error bars,
604 the breakaway estimate provides a more accurate reflection of the uncertainly associated with
605 estimating a true value that can never be known (Fig 2B).

606 # Bacterial populations

607 ## Rumen samples

608 Based on the exploratory analysis with the DPCoA, differences between rumen liquid
609 strained samples and other rumen samples types were driven mainly by *Lachnospiraceae*,
610 *Prevotellaceae* and Kiritimatiellaeota. *Lachnospiraceae* was significantly lower in liquid
611 samples and *Prevotellaceae* had significantly higher relative abundance compared with grab
612 samples (Fig 5A and 5C). Other studies that examine differences between the microbial
613 communities in liquid and solid phases have reported both *Lachnospiraceae* and
614 *Ruminococcaceae* in higher and in lower abundance in the liquid samples compared with the
615 solid [19,54]. These conflicting results could be due to the different diets used in these studies.
616 Animals on all forage diets had higher abundances of both families in liquid phase, while cattle
617 on a diet with a forage to concentrate ratio of 70:30 had lower abundances of these families in
618 the solid phase [19,54]. Lower resolution of the taxa might lend clues as to the cause of these
619 differing results.

620 In agreement with our study, others have found that *Prevotellaceae* were most abundant
621 in liquid phase compared with solid phase and the dominant family in the liquid fraction

622 [19,21,22]. *Prevotella sp.* are capable of degrading a wide variety of substrates including pectin,
623 hemicellulose, protein, fatty acids, and starch [55]. Readily fermentable carbohydrates including
624 sugars and soluble fiber in the liquid fraction likely support the presence of *Prevotella*. Thus, the
625 lower abundance of *Prevotella* in samples with increased solid fraction, including grab samples
626 and solid strained was logical.

627 Our data show that ASVs from Kiritimatiellaeota had significantly higher abundances in
628 liquid strained samples, but these ASVs were not significantly differentially abundant in solid
629 versus grab samples (Fig 1A and 1C). These data are in agreement with a study that found
630 Kiritimatiellaeota in higher proportion in the liquid compared with the solid phase of a yak
631 rumen [56]. Additionally, an order in this phyla, WCHB1-41, was identified to be part of the
632 "core microbiome" in liquid samples from the rumen [57]. Kiritimatiellaeota was found in rumen
633 samples and was in higher abundance from samples of higher methane producers making it a
634 potentially important microbe to understand in order to possibly reduce methane emissions [58].
635 Bioinformatic analysis has hypothesized that this phyla uses sodium for a coupling ion to
636 generate the electrochemical gradient to produce ATP, rather than the typical H^+ [59]. Therefore,
637 in circumstances when concentrations of H^+ are relatively lower, as when methane emission are
638 high, this phylum could have a competitive advantage of using sodium as a coupling ion. The
639 role of this rumen microbe has yet to be understood and our data demonstrates that for
640 investigators interested in elucidating the role of this microbe in the rumen ecosystem, samples
641 can be enriched with Kiritimatiellaeota by filtering rumen samples through cheese cloth.

642 ## Stomach tube samples

643 In a previous study, when sampling was done by either rumen cannula or esophageal tube
644 *Prevotellaceae*, *Lachnospiraceae* and *Ruminococcaceae* were the predominate families

645 regardless of the sampling method [8]. Importantly, these authors made a point to include
646 particles attached to the strainer to capture a representative sample in the rumen. Similarly, in the
647 present study *Prevotellaceae* and *Ruminococcaceae* (Fig 5A and 5B) were not significantly
648 different at the family level, while *Lachnospiraceae* was significantly lower in stomach tube
649 samples (Fig 5C). The lower relative abundance of *Lachnospiraceae,* specifically the genera
650 *Butyrivibrio* and *Coprococcus*, in samples collected by esophageal tube rather than through a
651 rumen fistula was also determined in another study (S2 Fig) [60]. However, at a finer resolution
652 our data showed that these three families had the most significant differentially abundant ASVs
653 when comparing the stomach tube and grab samples.

654 In agreement with De Menezes et al. who found *Fibrobacter* and *Spirochaetes* in the
655 solid fraction, the only assigned genera in the family *Fibrobacteraceae*, *Fibrobacter,* was
656 significantly lower in abundance in stomach tube samples compared with grab samples (Fig 1C,
657 6 and S2 Fig) as was the family *Spirochaetaceae* (Fig 1C and 6) due to a lower abundance of the
658 genus *Treponema* (S2 Fig) [61]. Initially, we hypothesized the lower abundance of *Fibrobacter*
659 species in stomach tube samples would largely be driven by the exclusion of fibrous particles in
660 the sample as *Fibrobacter* facilitates cellulose degradation in the rumen [62–64]. However,
661 significantly lower abundances of the family *Fibrobacteraceae* and *Fibrobacter* at the genus
662 level were seen in solid and liquid unstrained samples compared to grab samples (Fig 6 and S2
663 Fig). Alternatively, the differences could be attributed to location of rumen sampling.

664 Another fiber adherent bacterium *Ruminococcus flavefaciens* (contained in genus
665 Ruminococcus_1) did follow the expected pattern of significantly lower abundance in stomach
666 tube and liquid samples and significantly higher abundance in solid samples compared with grab
667 samples (S2 Fig). The different distribution of these to cellulolytic species could be reflective of

668 their differential preferences for particular plant tissues, for example structural polysaccharides
669 of the cell wall, as a growth substrate [65]. For studies that are interested in fibrolytic bacteria
670 such as *Fibrobacter*, straining the liquid out of the sample does not enrich for these bacteria, but
671 rather seems to disrupt these communities. Therefore, our data suggests that grab samples are the
672 best option for examining these populations.

673 An important phylum in defining stomach tube samples was Fusobacteria, which was
674 significantly higher in abundance in stomach tube samples compared with grab samples (Fig
675 1C). This difference was driven by the genus *Fusobacterium* (S2 Fig) and to the authors'
676 knowledge this difference between stomach tube and rumen sampling methods has not been
677 previously reported. *Fusobacterium necrophorum* is an important target species for improving
678 rumen efficiency as it degrades lysine, whose dietary deficiency is the most likely to limit milk
679 production [66,67]. In addition, *F. necrophorum* was reported to be an opportunistic pathogen
680 that causes liver abscesses in feedlot cattle [68,69]. Our data have identified a previously
681 unreported difference between rumen and stomach tube samples that would enable monitoring of
682 this important genus with stomach tube sampling and has implications for both dairy and beef
683 cattle.

684 Stomach tube samples more closely reflected liquid samples, but stomach tube samples
685 were highly variable (Fig 3 and S1 Fig). This high variability in microbial community could
686 reflect the fact that the stomach tube did not have a screen, therefore the solid contribution to the
687 stomach tube sample was also highly variable. There were 3,615 ASVs that were in common
688 between stomach tube and grab samples. Two families, *Rhodobacteraceae* and *Spirosomaceae*
689 were found in grab, liquid strained and liquid unstrained samples, but were not present in
690 stomach tube samples. However, *Solirubrobacteraceae* was found only in stomach tube samples.

These differences could reflect differences in the location of the tube placement (cranial ventral) compared with the sampling the rumen from the cannula (central rumen).

Taken together, these data suggest that stomach tube samples could be reflective of rumen samples provided some solid particulate are included and attempts are made to place the tube at a consistent depth. Despite following these precautions, researchers should expect these samples to be more variable than grab samples and increase their sample size accordingly.

Feces vs rumen

In the current study, as anticipated, fecal samples were not representative of the microbial community of the rumen. The differences between fecal and rumen samples were driven by differences in two Firmicute families: *Ruminococcaceae* and *Lachnospiraceae* (Fig 4). Indeed, it was found that there was significantly higher abundance of *Ruminococcaceae* (Fig 5B) and significantly lower abundance of *Lachnospiraceae* in feces (Fig 5C). Similarly, Noel et al. found the abundance of *Ruminococcaceae* to be much higher in feces compared with rumen samples [70]. However, they found no difference in the abundance of *Lachnospiraceae*. A recent preprint found strikingly similar relative abundances of top three most abundant families in feces from dairy cattle: *Ruminococcaceae* (34.9% compared to our 40.7%), and *Rikenellaceae* (11.6% compared to our 15.7%) and *Lachnospiraceae* (6.8% compared to our 7.7%) [71]. These data show that *Ruminococcaceae* is typically found in higher abundance in feces, while fecal *Lachnospiraceae* will have lower abundance than the rumen population.

Both *Lachnospiraceae* and *Ruminococcaceae* are also members of the human gastrointestinal tract and have multiple glycoside hydrolases (GH) and carbohydrate-binding modules (CBM) that allow utilization of complex plant material, and transport degradation products of various sizes and compositions [72]. Their differences in abundance between the

714 rumen and fecal samples was likely a reflection of their specialization in degrading the various
715 types of substrates present in these two niches. As both families contain butyrate producers, the
716 shift in these families could represent a change in the major sources of butyrate in the rumen
717 compared with the lower colon. The reader should note that there are discrepancies in the
718 literature as to the taxonomy of genera in *Lachnospiraceae* [73]. Of note is a prominent butyrate
719 producer *Eubacterium rectale* that is cited as belonging to both *Eubacteriaceae* and
720 *Lachnospiraceae,* despite its placement on a 16S rRNA gene tree near recognized members of
721 *Lachnospiraceae* [74]. These inconsistencies can make appropriate comparisons at the level of
722 family across studies difficult.

723 In addition, to the families that drove the major differences between rumen and feces,
724 other families were also found to be differentially abundant between these two sample types.
725 There was significantly higher abundance of *Akkermansiaceae* in feces compared with grab
726 samples (Fig 6B). Until 2016, *Akkermansiaceae* only contained the species *Akkermansia*
727 *muciniphila,* when a novel strain, *Akkermansia glycaniphila,* was isolated from the feces of a
728 reticulated python [75]. Muciniphila means "mucin-loving" in Latin and as its name suggests *A.*
729 *muciniphila* is a mucin-degrader, which produces acetate and propionate from mucin
730 fermentation [76]. This species is known to be one of the most abundant in the human colon
731 making up 0.5-5% of the total bacteria, which was in agreement with the relative abundance we
732 observed (Fig 7B) [77,78]. Other studies have also noted the higher abundances of *Akkermansia*
733 in feces compared with rumen samples [28,79]. In humans, *A. muciniphilia* had a protective
734 effect against obesity and played a role in both glucose and lipid metabolism [80,81].
735 *Akkermansia* also had anti-inflammatory effects that were in part mediated through a membrane
736 specific protein that interacted with the toll-like receptor-2 and improved gut-barrier function

737 when given orally [82]. Due to the role of *A. muciniphilia* in regulating intestinal inflammation
738 and fat deposition, a better understanding of its function in cattle could identify methods to
739 improve weight gain in cattle.

740 Taken together, fecal samples are not an accurate representation of rumen samples as
741 they have differences in the abundance of predominant families in the phyla Firmicutes and
742 Bacteroidetes. Fecal samples differed from those taken from the rumen as they had significantly
743 lower relative abundance of *Lachnospiraceae, Christensenellaceae, Prevotellaceae, Fibrobacter*
744 and *Treponema* (Fig 5A and C, 6, 7 and S2 Fig). Also, fecal samples had significantly higher
745 relative abundance of *Ruminococcaceae, Rikenellaceae* and *Akkermansia* compared with grab
746 samples (Fig 5B, 6, 7 and S2 Fig). Researchers can access the freely accessible data found at
747 https://doi.org/10.5281/zenodo.4026849 to determine how sampling methods might affect the
748 abundance of their microbe of interest.

749 # Archaeal populations

750 ## Feces vs rumen

751 Methanogens are an important functional group within the rumen as their use of H_2 to
752 reduce CO_2 to methane (CH_4) removes H_2 from the rumen that is generated during fermentation
753 of carbohydrates [83,84]. Methane has a global warming potential 28-34 fold higher than CO_2
754 over 100 years, and therefore its mitigation is important to reducing the environmental impact of
755 animal agriculture. Additionally, methane production is energy inefficient, resulting in a 2-12%
756 loss in gross energy intake in cattle [85]. There is very limited data on differences between the
757 archaeal populations in the rumen compared with the feces, as a majority of studies solely focus
758 on the rumen population.

One study that has examined both the rumen and fecal populations of archaea of Nelore cattle was conducted by Andrade et al. [50]. Like this present study, Andrade et al. also utilized DADA2 to identify ASVs and assigned taxa with the SILVA database v132; however, they used different primers that are specific for archaea and bacteria rather than universal primers and classified archaeal sequences using the Rumen and Intestinal Methanogen database (RIM-DB). Together these choices allowed Andrade et al. to classify archaeal ASVs down to the species level, which contrasted with this present study where methanogenic ASVs were only classified down to the genus level. Other than *Methanobrevibacter* and *Methanosphaera,* the other archaeal genera that this present study and Andrade et al. identified were different. Our data contained *Methanocorpusculum*, *Methanimicrococcus* and Candidatus *Methanomethylophilus* while Andrade et al. observed *Methanomicrobium*. Both studies found that *Methanobrevibacter* and *Methanosphaera* were found in both the rumen and feces; however, there were differences in the relative abundances of the main genera. In contrast to Andrade et al. we found significantly lower relative abundance of *Methanobrevibacter* in fecal samples compared with samples from the rumen. Despite using similar methods there is not clear agreement as to the differences in abundance of genera and which genera are present in the two populations.

As an alternative to 16S rRNA gene sequencing, the mcrA gene can be sequenced to study methanogens [86,87]. The mcrA gene encodes the α-subunit of the methyl coenzyme M reductase, which catalyzes the last step of methanogenesis and is conserved among all methanogens [88]. A study that used mcrA amplicon sequencing found that the most abundant genera in manure was *Methanocorpusculum* while in the rumen it was *Methanobrevibacter* [89]. While we found *Methanocorpusculum* in our fecal samples it was a minor genus and the discrepancy is most likely explained by differences in the gene amplicon sequences. Taken

782 together these data suggest *Methanobrevibacter* is a dominant archaeal genus in both the rumen
783 and fecal populations. The lack of data comparing the rumen and fecal populations suggest that
784 further research is required to understand the archaeal populations.
785

786 ## Rumen samples

787 In the present study relative abundance of archaeal families was similar across rumen
788 samples, both liquid and solid phases, with wide variation in the relative abundance of
789 *Methanocorpusculaceae* (Fig 6). In contrast, Bowen et al. found methanogens to be more
790 abundant in the solid phase [19]. Our data more closely agree with de Mulder et al. who found
791 similar abundance in samples including solid, rumen liquid, and liquid [54]. When we examined
792 the archaeal ASVs in our data at the genus level, *Methanosphera* was significantly lower in
793 relative abundance in liquid samples compared with grab samples. This is in agreement with
794 previous studies that found *Methanosphera* was more abundant in the solid phase, rather than the
795 liquid phase [19,54]. As a whole these data suggest that the collective abundance of methanogens
796 was similar between solid and liquid phases, but that *Methanosphera* are found at higher
797 abundance in the rumen liquid. Studies evaluating feed additives or diet alterations to modulate
798 methanogen populations in the rumen should consider including the liquid fraction of rumen
799 fluid to capture changes in the abundance of *Methanosphera*.

800 At the family level three methanogenic families, *Methanomethylophilaceae*,
801 *Methanobacteriaceae* and *Methanocorpusculaceae*, were not significantly different between the
802 grab sample and samples acquired via a stomach tube (Fig 6). However, there were 4 ASVs
803 assigned to *Methanobrevibacter* that were found to be significantly higher in abundance in
804 stomach tube samples. This is a paradoxical finding as stomach tube samples typically have

805 more liquid than solid particles in them and we previously noted that *Methanosphaera* was in
806 higher abundance in liquid samples. As the coefficient for the difference in relative abundance of
807 *Methanobrevibacter* is low (0.1-0.5), we believe that in practice with higher numbers of animals
808 this difference would be negligible.

809 Many of the differences described thus far have focused on the major genera
810 *Methanobrevibacter* and *Methanosphaera*, which are hydrogenotrophic methanogens. While the
811 hydrogenotrophic pathway for methane production is the most common there are two alternative
812 pathways: methylotrophic and acetoclastic that utilize methylated compounds and acetate,
813 respectively. Thus far, only taxa within the order *Methanosarcinales* have been identified to be
814 capable of acetoclastic methanogenesis [90,91]. An acetoclastic methanogen in our data,
815 *Methanimicrococcus*, was only present in two liquid samples. There was not a strong pattern as
816 to the phase in which this minor genus may be found, and as deep sequencing would be required
817 to determine shifts in its abundance, targeted qRT-PCR would be a better choice to study
818 abundance of this microbe. In addition, there was one ASV assigned to
819 *Methnomethylophilaceae,* a methylotrophic archaeon, that was significantly higher in abundance
820 in stomach tube samples compared with grab samples, although at higher taxonomic levels no
821 differences were found for the family *Methnomethylophilaceae*.

822 Taken together these results demonstrate that stomach tubing would likely provide a
823 representative community of major populations of methanogens, *Methanosphaera* and
824 *Methanobrevibacter*, compared with grab samples. For minor populations accurate surveys
825 would require more targeted techniques, such as qRT-PCR or mcrA sequencing. While this study
826 added to an understanding of how sampling methods will potentially impact archaea populations
827 observed, it should not be considered a comprehensive evaluation of the microbial communities.

828 Specific archaeal primers and qRT-PCR could be used to clarify discrepancies between this
829 study and past work. However, for those evaluating archaeal communities with 16S rRNA gene
830 sequencing, this study can serve as a guide to help in study design to improve the chances of
831 capturing an accurate picture of the taxa of interest.

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839 Author contribution statement

840 JH analyzed, interpreted data, and wrote manuscript. ML collected samples, prepared libraries,
841 aided in experimental design and edited manuscript. ED designed the experiment, assisted in
842 sample collection helped prepared manuscript. EAM edited manuscript.

843 Conflict of interest statement

844 The authors have not conflicts of interest to declare.

845 Supporting information

846 **S1 Fig. Double principal coordiant analysis of the Bray-Curtis distance after removal of**
847 **the phyla Bacteroidetes and Firmicutes from the dataset.** DPCoA is a phylogenetic
848 ordination method and that provides a biplot representation of both (A) samples and (B)

taxonomic categories. The 1st axis separtes liquid strained samples from other rumen sample types while the 2nd axis discrimates fecal from rumen samples. Samples that have larger scores on the 1st axis have more taxa from the phylum Kiritimatiellaeota and less taxa from the phylum Euryarchaeota. Likewise, samples with higher scores on the 2nd axis have more taxa from the family Akkermansiaceae and less taxa from the families Fibrobacteraceae and Spirochaetaceae. To faithfully reflect the variance in the coordinates, the height-to-width ratio was based on the ratio between the corresponding eigenvalues.

S2 Fig. Significant genera that are differentially abundant across sample type graph as coefficients with a 95% confidence interval calculated from the corncob model. Taxa with negative coefficients for a sample type are expected to have a lower relative abundance when compared to the grab samples while positive coefficients suggest a higher relative abundance in that sample type compared to grab samples. Taxa are presented with phylum, family, genus and species to the lowest assigned level.

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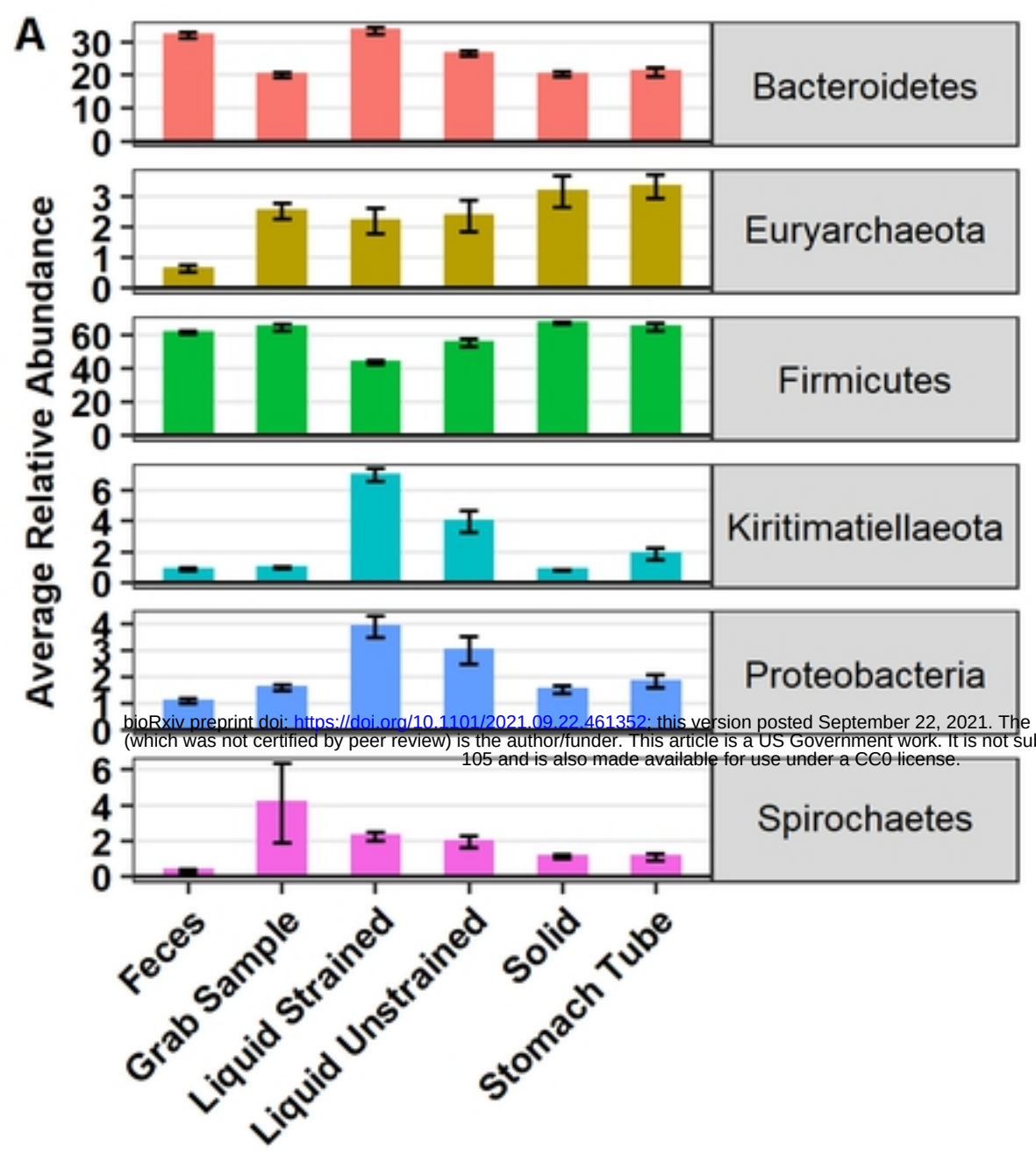

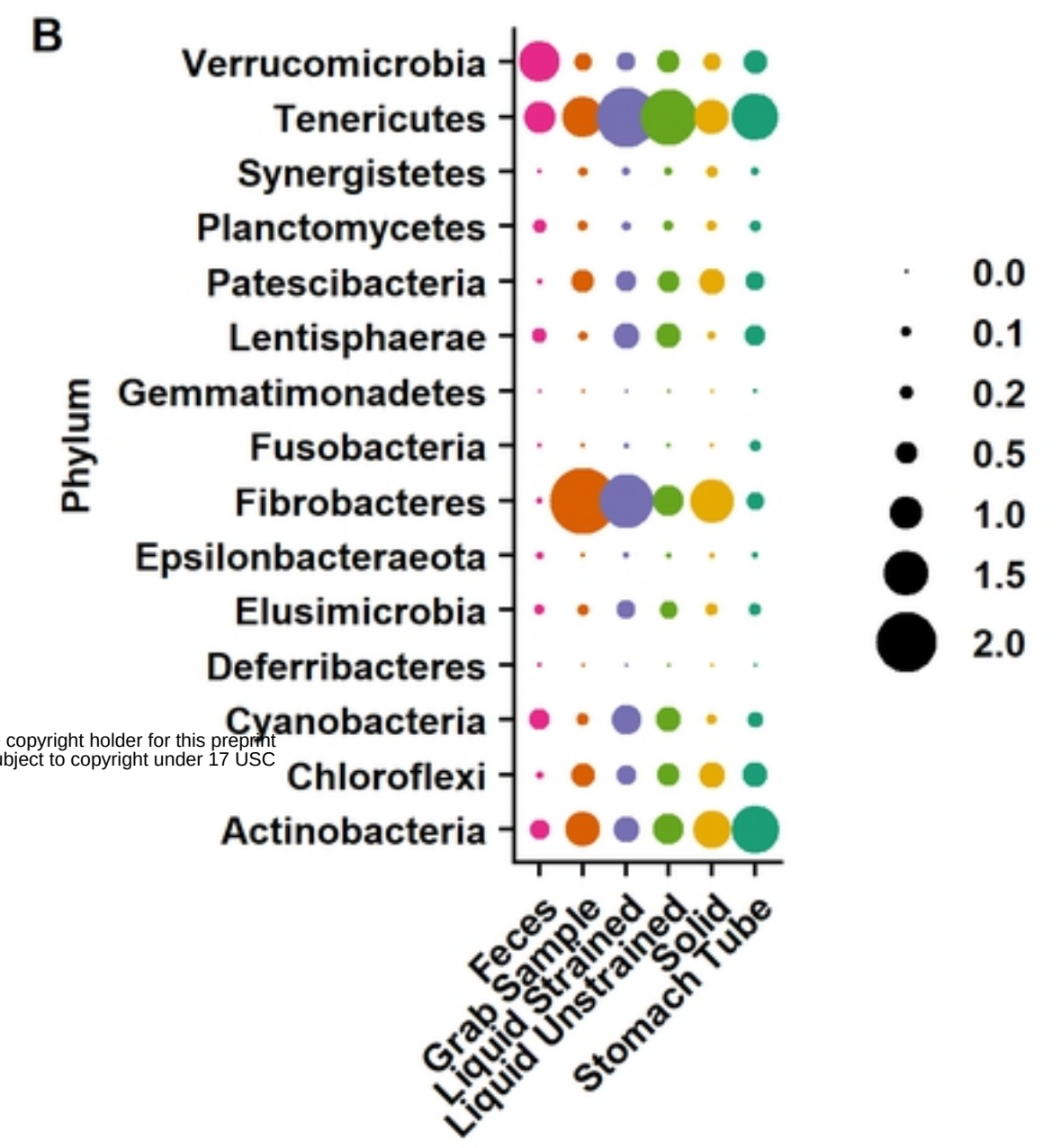

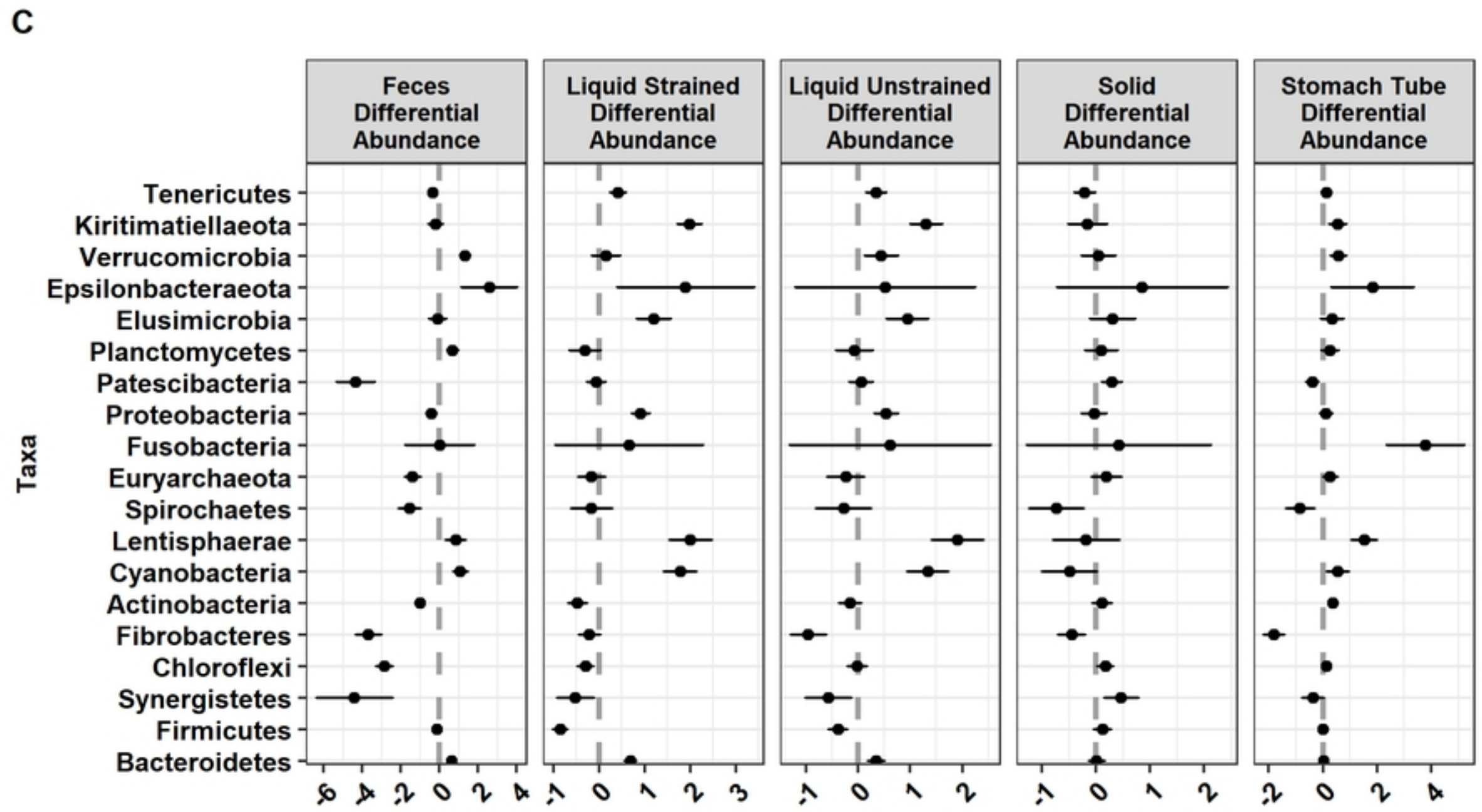

Fig 1

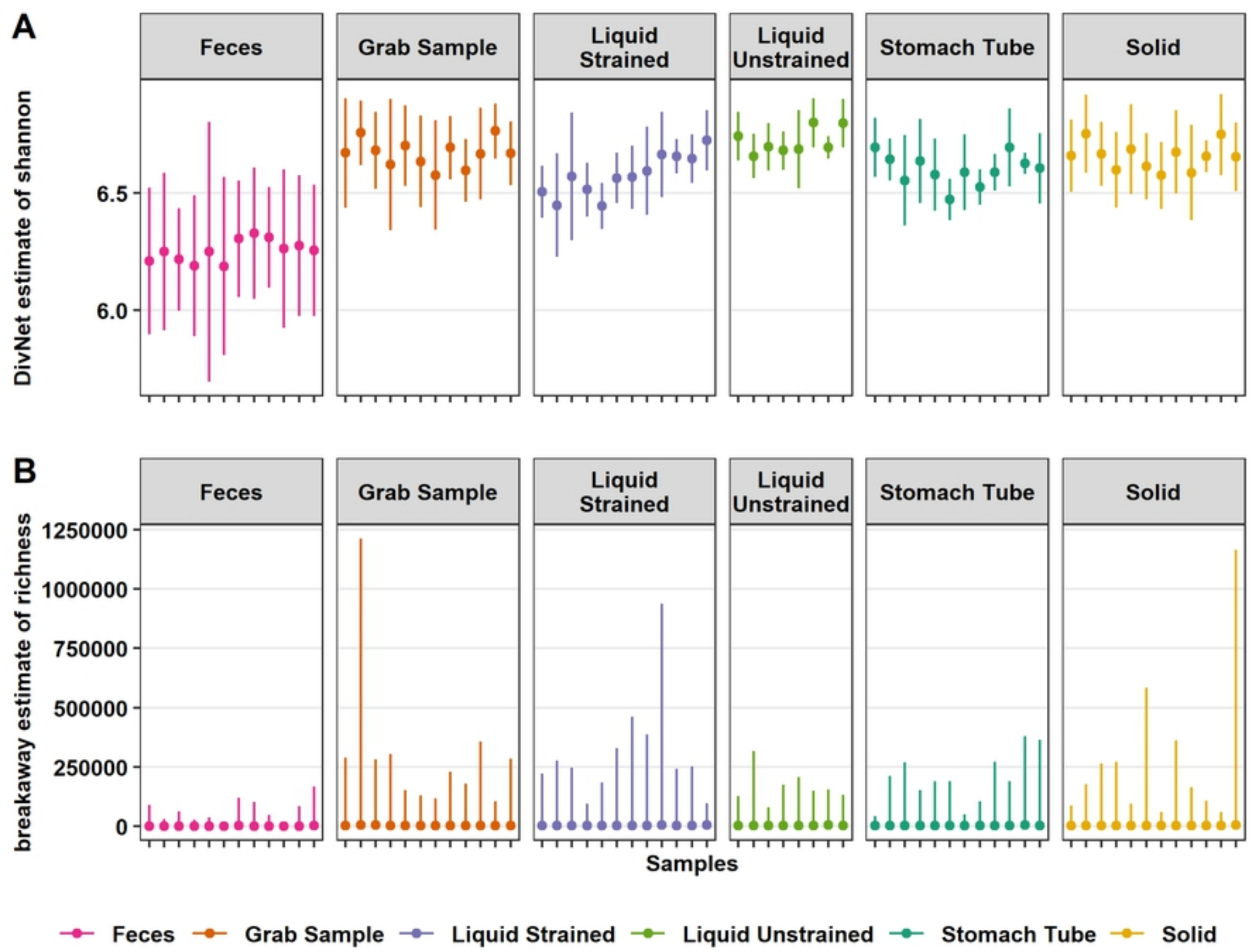
A
Feces
Grab Sample
Liquid Strained
Liquid Unstrained
Stomach Tube
Solid
DivNet estimate of shannon
6.5
6.0
B
breakaway estimate of richness
1250000
1000000
750000
500000
250000
0
Samples
Feces
Grab Sample
Liquid Strained
Liquid Unstrained
Stomach Tube
Solid

Fig 2

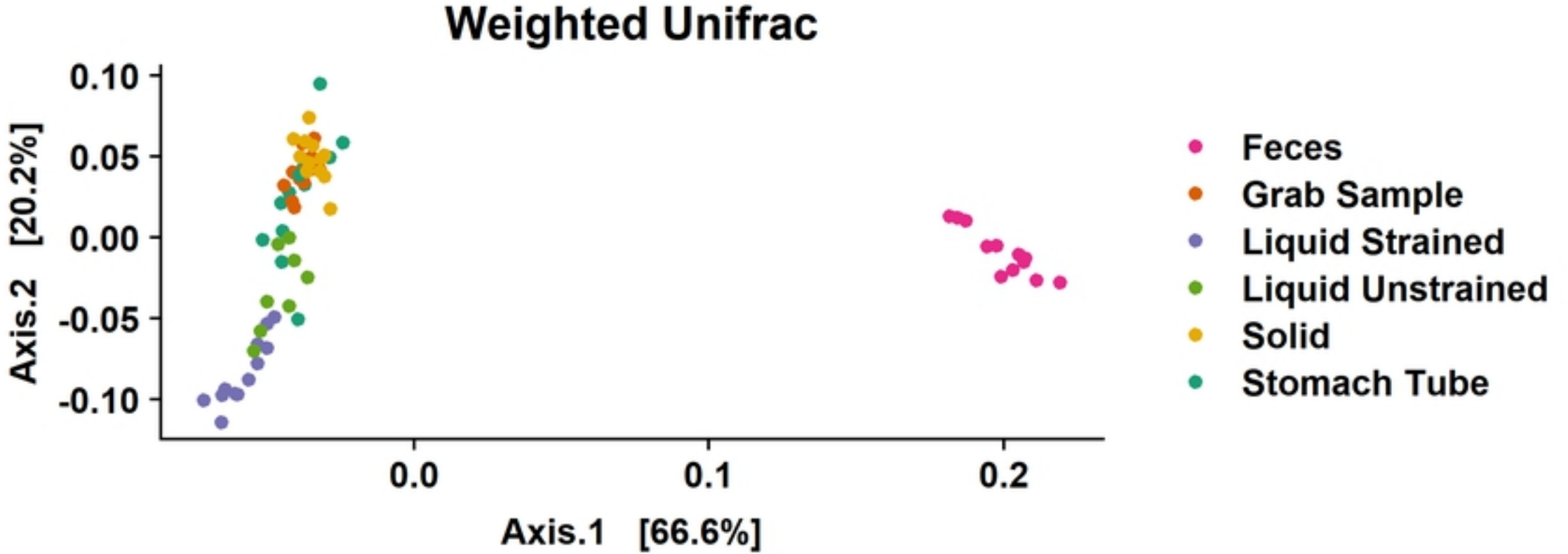
Weighted Unifrac
0.10
0.05
0.00
-0.05
-0.10
Axis.2 [20.2%]
0.0
0.1
0.2
Axis.1 [66.6%]
Feces
Grab Sample
Liquid Strained
Liquid Unstrained
Solid
Stomach Tube

Fig 3

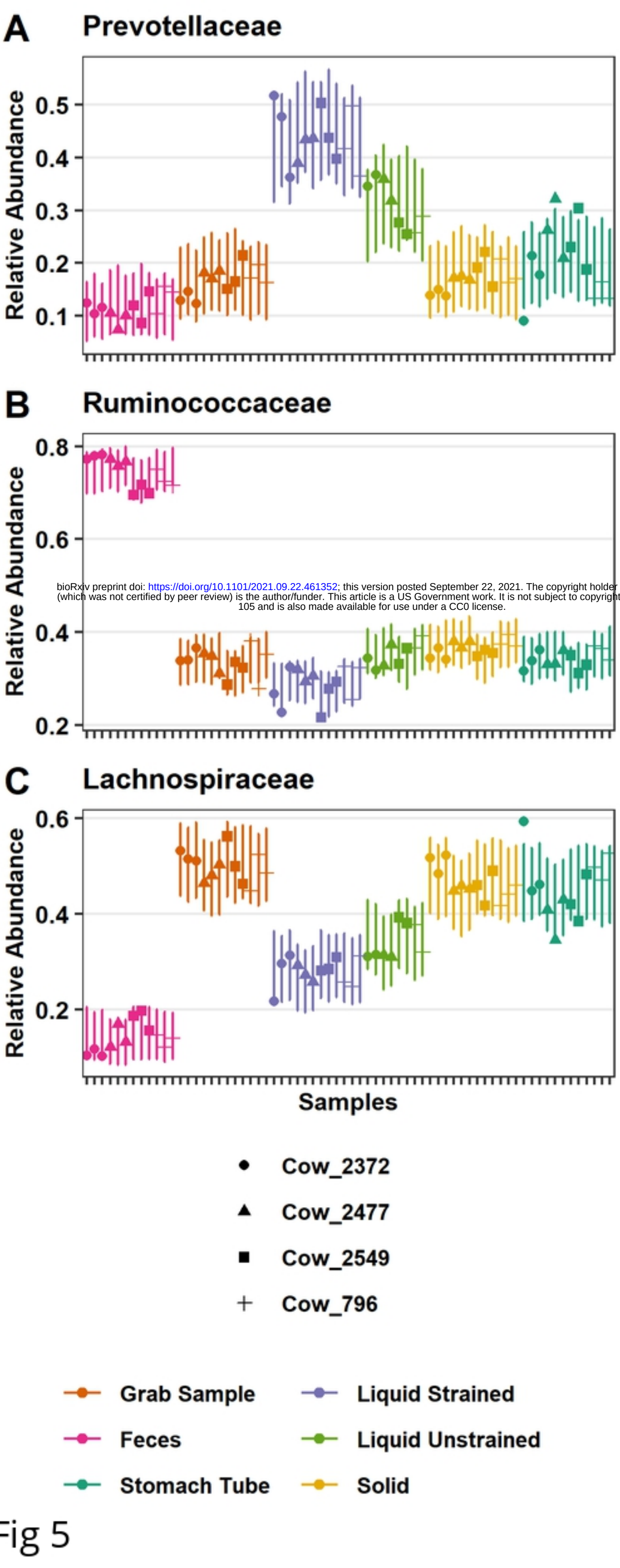

Fig 5

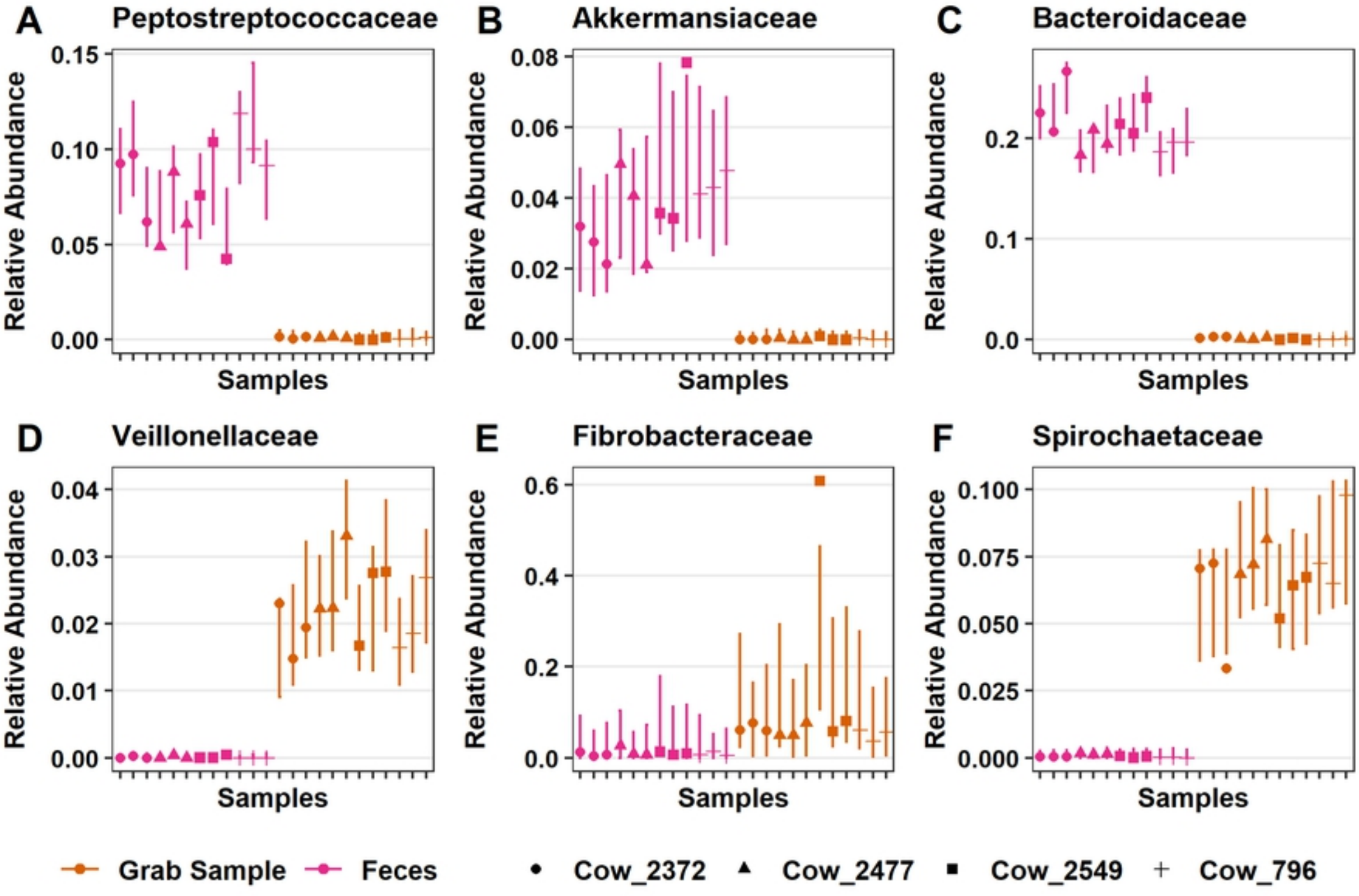
A
Peptostreptococcaceae
B
Akkermansiaceae
C
Bacteroidaceae
D
Veillonellaceae
E
Fibrobacteraceae
F
Spirochaetaceae
Relative Abundance
Samples
0.15
0.10
0.05
0.00
0.08
0.06
0.04
0.02
0.2
0.1
0.0
0.03
0.01
0.6
0.4
0.100
0.075
0.050
0.025
0.000
Grab Sample
Feces
Cow_2372
Cow_2477
Cow_2549
Cow_796

Fig 7

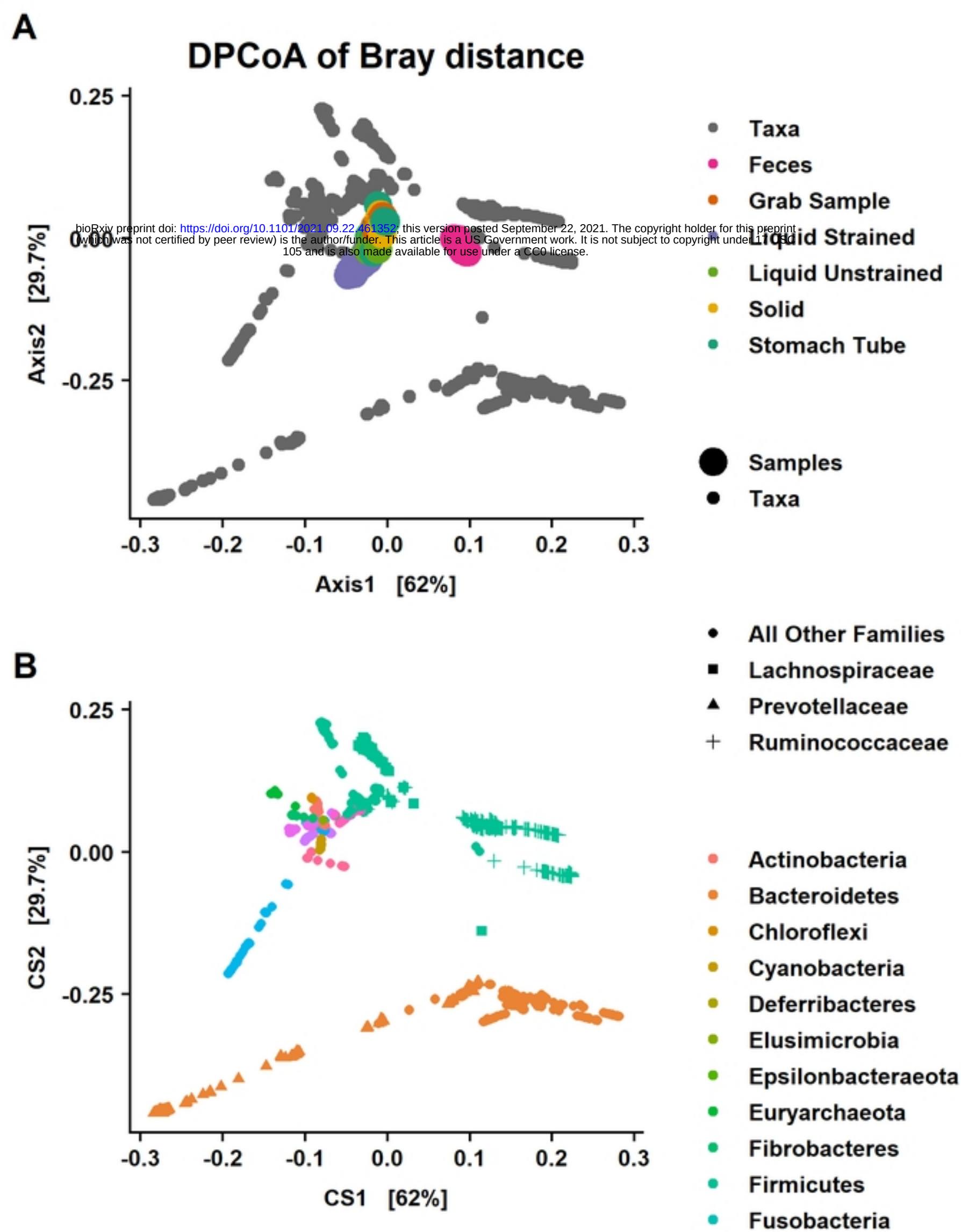

Fig 4

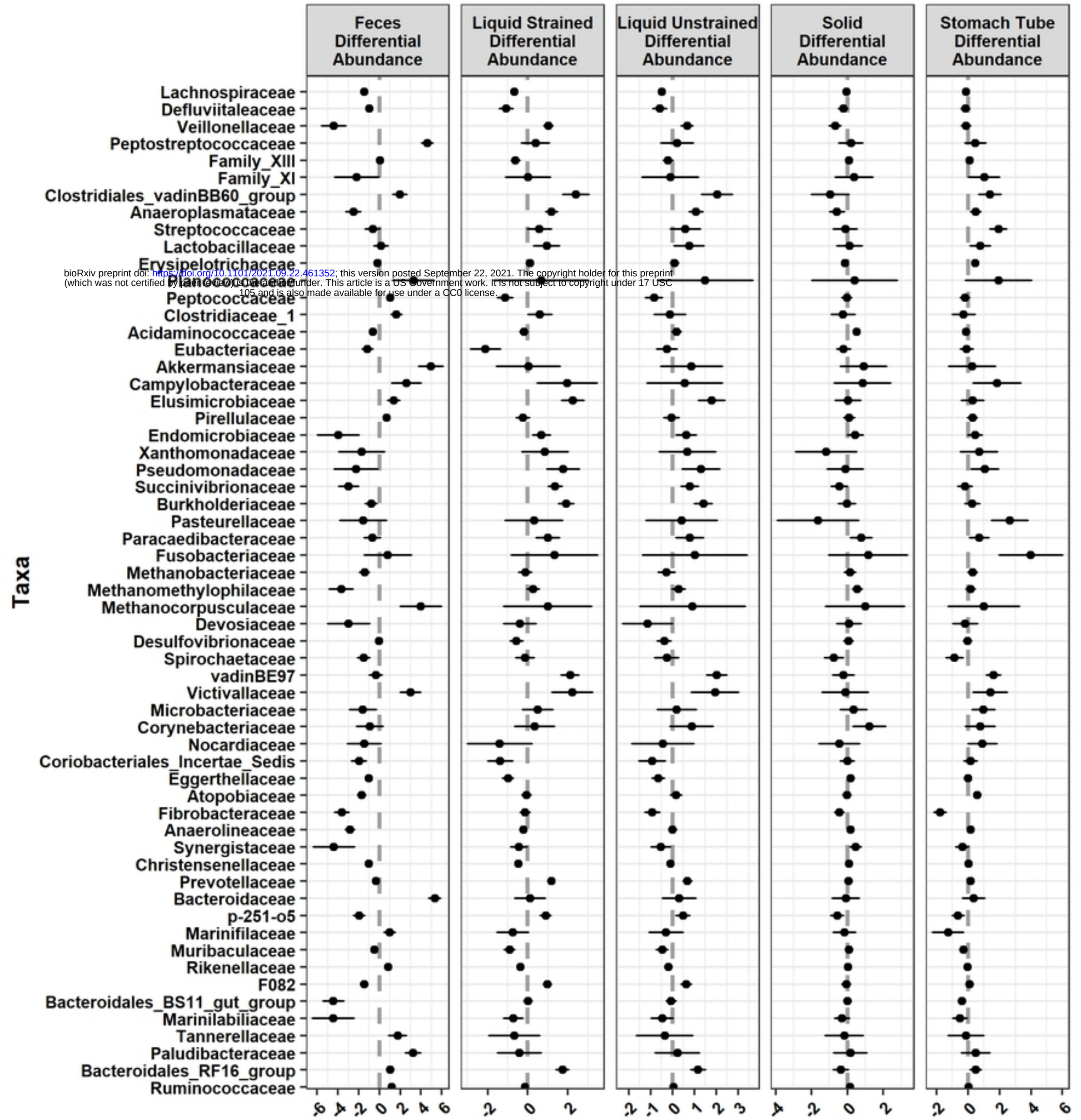

Fig 6