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Research Article

Gut microbiome biogeography in reindeer supersedes millennia of ecological and evolutionary separation

Stefaniya Kamenova [1,2,3], Eric J. de Muinck[1,4], Vebjørn Veiberg[5], Tove Aagnes Utsi[6], Sam M.J.G. Steyaert[7], Steve D. Albon[8], Leif Egil Loe[2], Pål Trosvik[1,*]

[1]Centre for Ecological and Evolutionary Synthesis, Department of Biosciences, University of Oslo, 0371 Oslo, Norway
[2]Departments of Ecology and Natural Resource Management, Norwegian University of Life Sciences, 1433 Ås, Norway
[3]National Museum of Natural History, Bulgarian Academy of Sciences, 1000 Sofia, Bulgaria
[4]Department of Pharmacy, University of Oslo, 0371 Oslo, Norway
[5]Norwegian Institute for Nature Research, 7034 Trondheim, Norway
[6]Department of Arctic and Marine Biology, Faculty of Biosciences, Fisheries, and Economics, UiT The Arctic University of Norway, 9510 Alta, Norway
[7]Faculty of Biosciences and Aquaculture, Nord University, 7713 Steinkjer, Norway
[8]The James Hutton Institute, Craigiebuckler, Aberdeen AB15 8QH , United Kingdom
*Corresponding author. Centre for Ecological and Evolutionary Synthesis, Department of Biosciences, University of Oslo, 0371 Oslo, Norway. E-mail: pal.trosvik@ibv.uio.no
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Abstract

Ruminants are dependent on their gut microbiomes for nutrient extraction from plant diets. However, knowledge about the composition, diversity, function, and spatial structure of gut microbiomes, especially in wild ruminants, is limited, largely because analysis has been restricted to faeces or the rumen. In two geographically separated reindeer subspecies, 16S rRNA gene amplicon sequencing revealed strong spatial structuring, and pronounced differences in microbial diversity of at least 33 phyla across the stomach, small intestine, and large intestine (including faeces). The main structural feature was the Bacteroidota to Firmicutes ratio, which declined from the stomach to the large intestine, likely reflecting functional adaptation. Metagenome shotgun sequencing also revealed highly significant structuring in the relative occurrence of carbohydrate-active enzymes (CAZymes). CAZymes were enriched in the rumen relative to the small and large intestines. Interestingly, taxonomic diversity was highest in the large intestine, suggesting an important and understudied role for this organ. Despite the two study populations being separated by an ocean and six millennia of evolutionary history, gut microbiome structuring was remarkably consistent. Our study suggests a strong selection for gut microbiome biogeography along the gastrointestinal tract in reindeer subspecies.

Keywords: 16S rRNA gene amplicon sequencing; digestion; herbivory; High Arctic; ruminants; shotgun metagenome sequencing

Introduction

Ruminants possess remarkably complex digestive systems, whose functioning is reliant on a diverse community of gut microbial symbionts for the efficient conversion of energy from plants, largely contributing to their evolutionary success and ecological diversification (Ley et al. 2008, Chen et al. 2019). Although domesticated ruminants have been extensively studied, we currently lack basic knowledge about the gut microbiomes of most non-domestic herbivores. For wild herbivores, the composition and diversity of microbial communities remain largely undescribed, and the general understanding of the factors driving microbiome dynamics and functionality in natural conditions is limited.

The ruminant gastrointestinal tract (GIT) is highly compartmentalised, comprising four stomach chambers (i.e. rumen, reticulum, omasum, and abomasum), small intestine, cecum, and large intestine (including the colon and the rectum sections) (Staaland et al. 1979, Hofmann 1989, van Soest 1994). The stomachs in ruminants, especially livestock, are relatively well understood, where anaerobic bacteria play a prominent role in food digestion, host metabolism and health (Dearing and Kohl 2017; Valdes et al. 2018, Zeineldin et al. 2018). However, comparatively little is known about the microbiomes of the other GIT compartments. Differences in pH levels and physiology (Kararli 1995, Kohl et al. 2013) together with documented structuring in the composition, diversity and function of microbial communities suggest a unique role in food digestion for each compartment (Xie et al. 2021, Hu et al. 2022). Although several studies have indicated that structured microbial communities across the GIT may be a conserved trait across ruminant species (Li et al. 2014, Malmuthuge et al. 2014, Mao et al. 2015, Kim et al. 2019, Wang et al. 2019, Xie et al. 2021), no studies have compared multiple sections of the GIT between subspecies subjected to differential selection pressures of diet, behaviour, and energy budgets.

Here, we characterise and compare the microbiome structuring through the GIT compartments, as well as the faeces, of two geographically separated subspecies of reindeer (*Rangifer tarandus*). Reindeer are the most abundant large ruminant within the Arctic tundra, very well adapted to cope with the extreme seasonality in food availability, weather and day length (Blix 2016, Lin et al. 2019). Also, reindeer are the only cervid that has been

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domesticated throughout parts of Eurasia (Røed et al. 2008) and plays a central role in supporting the livelihoods and the cultural identity of Arctic communities. The Svalbard reindeer (*Rangifer tarandus platyrhynchus*) is endemic to the Svalbard archipelago and has been isolated from its ancestral population (*Rangifer tarandus tarandus*) on the Norwegian mainland for 6 000 years (Kvie et al. 2016). Major differences between the two subspecies are that Svalbard reindeer lead a sedentary lifestyle without long-distance migrations (Tyler and Øritsland 1989), typical of semi-domesticated reindeer (Eira et al. 2022). The two subspecies also have markedly different diets due to major differences in resource availability. Lichen is available on the mainland and is consumed by semi-domesticated reindeer, especially in late winter (Mathiesen et al. 2000). On Svalbard, lichen is relatively scarce and of marginal importance with diets dominated by moss, graminoids, and the polar willow (*Salix polaris*) (Bjørkvoll et al. 2009).

Research on the reindeer microbiome so far has only focused on the rumen or faeces (Sundset et al. 2007, Sundset et al. 2009, Pope et al. 2012, Zielińska et al. 2016, Glendinning et al. 2021), and the structuring and functioning of the gut microbiome across the entire GIT are currently unknown.

Nonetheless, based on the available studies of wild ruminants, we predicted a significant structuring of microbiome composition and diversity across the GIT in reindeer. We expected the differences to be mainly driven by variations in the relative proportions of the two major bacterial phyla—Bacteroidota and Firmicutes. Considering the significant ecological differences, we also expected that microbiome structuring across the GIT might differ between the two reindeer subspecies. Finally, to test if microbiome structuring is partly linked to the specialist digestive function of the different compartments, we also carried out shotgun metagenome sequencing of the rumen, small intestine, and colon on a subset of Svalbard reindeer samples. We expected significant differences among sections of the GIT in the abundance of microbial enzymatic pathways for the carbohydrate breakdown.

Materials and methods

Sample collection

Wild, free-ranging Svalbard reindeer were sampled from the population inhabiting a 150 km^2 area in the valleys of Reindalen, Semmeldalen and Colesdalen in Nordenskiöld land, Svalbard (77°50′–78°20′N, 15°00′–17°30′E). Mean daily temperatures in October oscillate between −6.1°C and −1.5°C (average −3.8°C, weather station Svalbard Airport, https://www.sios-svalbard.org). The area supports a relatively high density of reindeer compared to other parts of Svalbard (Van der Wal and Brooker 2004), with an estimated population of about 2 500 in 2020 (Loe et al. 2021). Twenty individuals were culled in October 2021 as part of a scientific campaign. All but two were females. Among the eighteen females, sixteen were adults and two were yearlings. Each animal was weighed, and its entire GIT was carefully removed, and placed on a new, clean plastic sheet. In total eight sections of the GIT were sampled: the four stomach chambers, the small intestine, the cecum, and the large intestine (i.e. the colon and the faeces). Each section was cut open using a disposable, sterile scalpel, and approximately 50 g of digested GIT content was scooped from each compartment. Faeces were collected directly from the rectum. Digesta were placed in clean plastic containers filled with 96% laboratory-grade ethanol and stored at room temperature.

Semi-domesticated reindeer GIT samples were collected from a herd in the Fiettar herding district, in West Finnmark (70°37′N–24°30E′), the North Norwegian part of Sápmi. The closest settlement is Alta, where September mean daily temperatures vary between 10.8°C and 4.9°C (average 7.9°C, Norwegian Meteorological Institute, https://www.met.no/en). Four one and a half years-old males were sampled in late September 2017 during the traditional autumn culling. Each GIT section was cut open using a knife, which was cleaned before sampling each section. Care was taken to avoid touching the GIT content during opening. The contentPermutational multivariate analysis of variance was sampled directly using disposable nitrile gloves, along nine sections: the four stomach chambers (i.e. rumen, reticulum, omasum, and abomasum), different locations across the upper and lower small intestine as well as the caecum and the large intestine compartments (i.e. the ascending and descending colon, the latter corresponding to faeces, collected directly from the rectum). Gloves were changed before sampling each segment. Digesta were placed into clean plastic bags and stored at −18°C within a few hours after collection. The animals did not receive any pelleted food and only had access to natural pastures with plants, mushrooms, and lichen until just before they were slaughtered.

DNA extraction and 16S rRNA gene amplicon sequencing

Approximately 250 mg of wet weight of digesta was subsampled from each GIT segment using disposable plastic spatulas (Chemglass, UK) in a laminar flow cabinet. DNA from the semi-domesticated reindeer samples was extracted using the DNeasy PowerSoil kit (Qiagen, Germany) according to the manufacturer's instructions. DNA from the Svalbard reindeer samples was extracted using the MagAttract version of the DNeasy PowerSoil DNA kit (Qiagen, Germany) according to the manufacturer's instructions. Library preparation for DNA sequencing was carried out as previously described (de Muinck et al. 2017), targeting the V4 region of the 16S rRNA gene with the 515f (GTGCCAGCMGCCGCGGTAA)-806r (GGACTACHVGGGTWTCTAAT) primer pair. A 2 × 300 pair-end sequencing was performed using the MiSeq platform at the Norwegian Sequencing Centre, Oslo. Sequence read demultiplexing was carried out using a custom routine developed at the Norwegian Sequencing Centre (https://github.com/nsc-norway/triple_index-demultiplexing). Further sequence data processing was performed using the Divisive Amplicon Denoising Algorithm as implemented in the dada2 v1.16 R-package (Callahan et al. 2016). This involved a filtering and a trimming step, denoising, forward and reverse reads merging and chimera detection. Filtering and trimming were carried out using default parameters, with truncation of read 1 (Forward) to a length of 240 bp and read 2 (Reverse) to a length of 160 bp. Chimera removal was carried out with the dada2 function 'removeBimeraDenovo' and the consensus method. Taxonomic classification of amplicon sequence variants (ASVs) was done using the SILVA v138.1 16S rRNA gene reference database (Quast et al. 2013) and the 'assignTaxonomy' function in dada2, with a minimum of 50% bootstrap support for an ASVs to be assigned to a taxon. All ASVs classified as either chloroplasts or mitochondria were removed from the dataset.

Shotgun metagenome sequencing

The rumen, the small intestine, and the colon sections from three of the Svalbard reindeer adults (one male and two females) were randomly selected and subjected to shotgun metagenomic profiling. Library preparation for shotgun sequencing was carried out using a tagmentation-based protocol (Picelli et al. 2014), and li-

braries were sequenced on a NovaSeq6000 in 2 × 150 PE mode. Raw sequences were trimmed and filtered using Trimmomatic v0.39 (Bolger et al. 2014) with default settings and clipping of Nextera PE adapter sequences. All nine libraries were co-assembled using MEGAHIT v1.2.9 (Li et al. 2015). Reads were mapped back to contigs using BWA v0.7.17 (Li and Durbin 2009) and the resulting SAM files were sorted by coordinate and converted to binary format (BAM) using SAMtools v1.11 (Danecek et al. 2021). Contigs were binned using MetaBAT2 v2.14 (Kang et al. 2019) and CONCOCT v1.1.0 (Alneberg et al. 2014), and the resulting bin sets were integrated and optimised with the dereplication, aggregation and scoring strategy DAS Tool (Sieber et al. 2018), in order to produce the final set of metagenome-assembled genomes (MAGs). Bins were inspected for completeness and contamination using CheckM v1.1.3 (Parks et al. 2015). Taxonomic classification of MAGs was done with GTDB-Tk v1.7 (Chaumeil et al. 2019). The blastx function in BLAST+ v2.11.0 (Camacho et al. 2009) was used to identify carbohydrate-active enzymes (CAZymes) in MAGs by aligning translated DNA sequences to the dbCAN-PUL database of genes identified as parts of polysaccharide utilisation loci (PUL) (Ausland et al. 2021). As a search criterion, for each identified protein sequence, the top hit with an e-value no higher than 0.01 was considered a positive hit. Since MAG binning was relatively inefficient, we also annotated the full complement of assembled contigs, both binned and unbinned with dbCAN-PUL, in order to get a more realistic view of differences in CAZyme carriage among the gut regions. This was done using the BLAST+ approach described above. Contig coverage was computed using the 'coverage' function in SAMtools, and the mean coverage on each contig was normalized by dividing by the total coverage to get the mean relative coverage of contigs.

Statistical analyses

Generalised additive models were fitted to investigate the potential non-linear relationship between the sampling depth (predictor variable fitted as a spline function) and the number of ASVs, using the 'gam' function in the mgcv v1.8.31 R-package. Permutational multivariate analysis of variance (PERMANOVA) and computation of Bray–Curtis distances were carried out using the 'adonis2' and the 'vegdist' functions, respectively, in the vegan v2.5.6 R-package. Non-metric multidimensional scaling (NMDS) was carried out using the MASS v7.3.51.6 R-package. Random forest analysis was carried out using the 'randomForest' and 'rf.significance' functions in the randomForest v4.6.14 and rfUtilities v2.1.5 R-packages, respectively. The number of trees used for the model was 501. For computing the *P*-values of the random forest models, we used the 'rf.significance' function with default settings and 1001 permutations. For computing UniFrac (phylogenetic) distances ASV sequences were aligned using MUSCLE v5.1.0 with the super5 algorithm (Edgar 2004) and phylogenies were estimated using FastTree v.2.11.1 (Price et al. 2009). Weighted and unweighted UniFrac distances were computed with the R-package GUniFrac v1.4. Standard testing of group differences was carried out using unpaired t-tests, Kruskal–Wallis and Wilcoxon rank sum tests with the t.test, wilcox.test and kruskall.test functions in R. In the case of the shotgun metagenome data analysis, when testing for differences in CAZyme abundances among the gut regions, we used Wilcoxon tests, while in testing for differences in CAZyme carriage among MAGs we used t-tests. In the former case, we were dealing with poorly defined distributions and thus opted for the non-parametric approach. In the latter case, we found that assuming genomic CAZyme carriage distribution to approximate normality was reasonable. In order to control for the false discovery rate in scenarios of multiple testing, *P*-value correction was done with the R function p.adjust with the method argument set to 'BH' (Benjamini–Hochberg). For visualization purposes only, in the graphs for which individual data points were heavily overlapping, random normally distributed noise was added with a standard deviation of 0.03 using the 'rnorm' function.

Table 1. Sample size for each of the gastrointestinal tract sections for the Svalbard and the Finnmark semi-domesticated reindeer.[a]

Svalbard	*N* = 91	Finnmark	*N* = 32
Rum	15	Rum	4
Ret	12	Ret	4
Oma	14	Oma	4
Abo	6	Abo	4
Sin	9	Sin_U	4
		Sin_L	3
Cec	13	Cec	4
Lin	11	Lin_AC	3
		Lin_DC	2
Fec	[a]15	Fec	-

[a]Rum, rumen; Ret, reticulum; Oma, omasum; Abo, abomasum; Sin, small intestine; Sin_U, small intestine upper part; Sin_L, small intestine lower part; Cec, cecum; Lin, large intestine; Lin_AC, large intestine ascending colon; Lin_DC, large intestine descending colon; and Fec, faeces.

Results

Sequencing of Svalbard reindeer samples

16S rRNA gene amplicon sequencing of the 91 samples (Table 1) resulted in a total of 25 956 238 merged reads after quality filtering and chimera removal (mean 285 233 ± 63 075 s.d.), representing a total of 15 349 ASVs. Even at this level of sampling depth, there was still a highly significant relationship between the sampling depth and the number of ASVs identified in a sample ($P < .001$, generalised additive model; Fig. S1), indicating that the system is still insufficiently sampled for describing the full diversity at a sampling depth of up to 400 000 sequences. To account for these differences in sampling depth we used common scaling (McMurdie and Holmes 2014) to the lowest sample read number (126 180 reads). After the removal of singleton ASVs a total 15 011 ASVs, distributed across 33 known phyla, remained. Out of these ASVs, 99.3% were classified successfully at the phylum level, while only 37.2% were reliably classified at the genus level (Fig. S2).

Microbiota structuring across the GIT in the Svalbard reindeer

We observed very pronounced structuring of the GIT along the three main regions; 1. The four stomach compartments. 2. The small intestine. 3. The large intestine (grouping the cecum, the colon, and the faeces) (Fig. 1). Using all seven gastrointestinal sections plus faeces as factor levels in a PERMANOVA analysis with Bray–Curtis distances produced an R^2 of 0.55 ($P < .001$). Using only the three main regions as factor levels R^2 went down just 0.04, to 0.51 ($P < .001$) (Table 2), despite having reduced the number of factors on which to partition variance from eight to three. The spatial structure was also evident when using phylogenetic distances ($P < .001$ for all PERMANOVA tests with weighted and unweighted UniFrac distances; Table 2). This strongly supports the notion that the three main regions are the dominant structuring factor (Fig. 2). This observation was confirmed by using random forest classification models for predicting the GIT provenance of

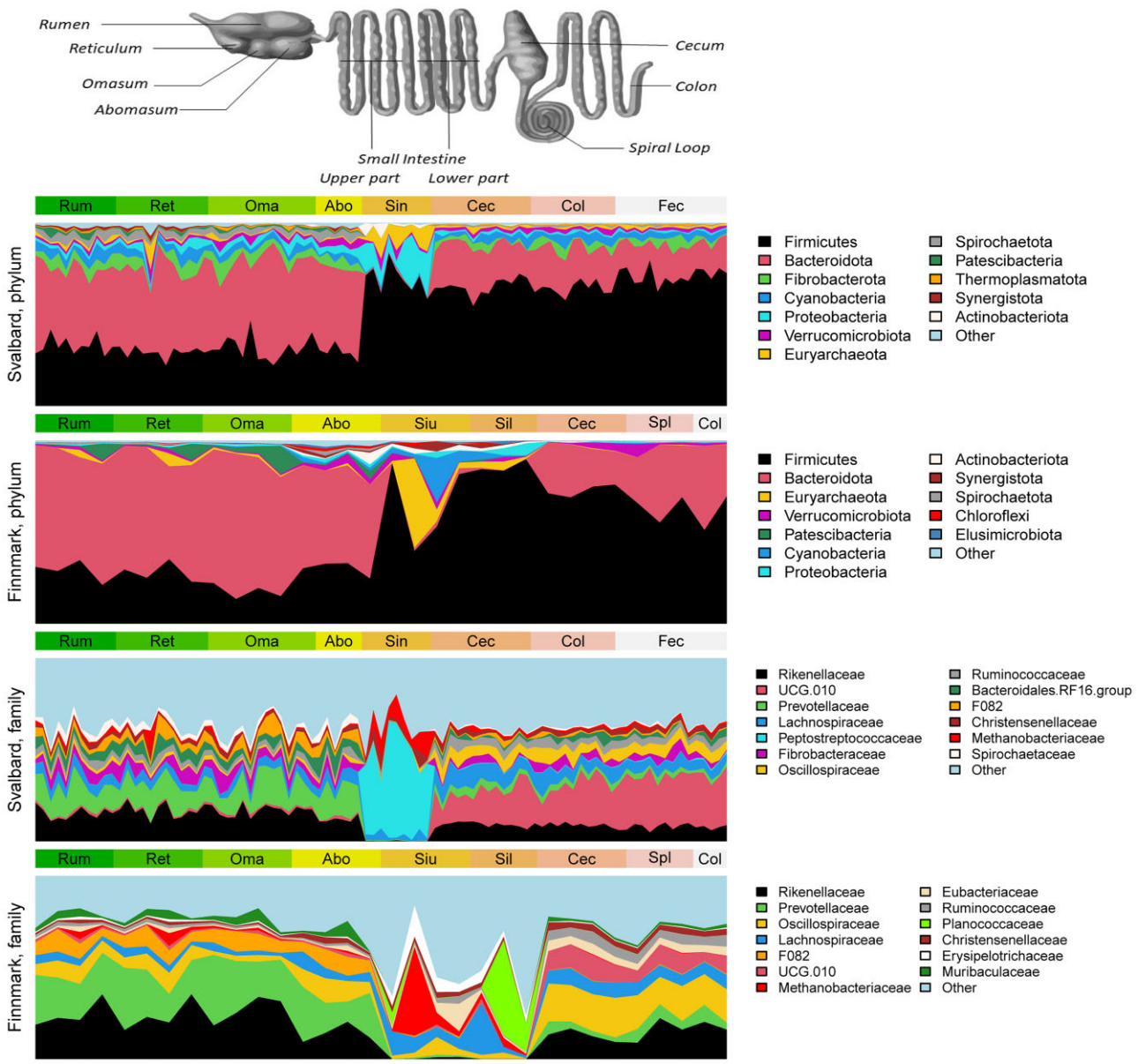

Figure 1. Phylum and Family level composition in the sampled GIT sections and faeces of Svalbard and Finnmark semi-domesticated reindeer. The category 'Other' includes ASVs that could not be reliably assigned to a taxon at the relevant level. The sampled sections are indicated in the colored boxes above each filled curve plot. Rum, rumen; Ret, reticulum; Oma, omasum; Abo, abomasum; Sin, small intestine; Siu, small intestine upper part; Sil, small intestine lower part; Cec, cecum; Col, colon; Spl, spiral loop; and Fec, faeces.

individual samples. This method uses decision trees to produce majority vote predictions with a bootstrap approach, and a so-called 'out-of-bag error rate' can be estimated from the number of erroneous predictions. Prediction accuracy was 100% (out-of-bag error estimate of 0%, $P < .001$) for classification to the three main regions, meaning that 91 out of 91 samples were classified to the correct region. When classifying samples to the seven GI sections plus faeces, classification accuracy was 51.6% (out-of-bag error estimate of 48.4%, $P = .004$).

When examining the phylum level composition of the microbiota in the seven GI sections and the faecal samples, the differences among the three main regions were noticeable (Fig. 1). Out of 33 identified phyla, 25 occurred at a significantly different relative abundance in at least one region ($P < 0.05$, Kruskal–Wallis rank sum tests with Benjamini–Hochberg corrected P-values). The main contrasts were differences in the relative abundances of Firmicutes and Bacteroidota (Fig. 1). There was no significant difference in Firmicutes abundance between the small intestine and large intestine, but both had significantly higher abundances than the four stomach compartments ($P < .001$ for all comparisons, Wilcoxon rank sum test with Benjamini–Hochberg corrected p-values). The stomach had significantly more Bacteroidota than the large intestine, which in turn had higher levels than the small intestine ($P < .001$ for all comparisons), where this phylum was relatively rare. Proteobacteria levels were increased in the small intestine relative to the two other regions ($P < .002$ for both comparisons). Fibrobacterota was rare in the small intestine relative to both other regions ($P < .001$ for both comparisons), while this phylum was more common in the stomach than in the large intestine ($P < .001$). Euryarchaeota was significantly enriched in the small intestine ($P < .001$ for both comparisons). Spirochaetota were rare in the small intestine relative to both other regions ($P < 0.001$ for

Table 2. PERMANOVA results for the reindeer population from Svalbard and Finnmark.[a]

	Bray–Curtis		Weighted UniFrac		Unweighted UniFrac	
Svalbard	R^2	*P*-value	R^2	*P*-value	R^2	*P*-value
All sections	0.55	<.001	0.67	<.001	0.58	<.001
Main regions	0.51	<.001	0.63	<.001	0.55	<.001
Stomach	0.1	.002	0.14	<.001	0.11	<.001
Large intestine	0.07	.04	0.11	<.001	0.06	.19
Stomach individual	0.79	<.001	0.66	<.001	0.64	<.001
Large intestine individual	0.86	<.001	0.74	<.001	0.72	<.001
Finnmark						
All sections	0.49	<.001	0.64	<.001	0.52	<.001
Main regions	0.42	<.001	0.55	<.001	0.6	<.001
Stomach	0.12	.94	0.26	.091	0.22	.284
Large intestine	0.13	.93	0.17	.87	0.17	.98
Stomach individual	0.78	<.001	0.56	<.001	0.56	<.001
Large intestine individual	0.86	.002	0.73	<.001	0.69	.001
Both populations						
Between populations	0.09	<.001	0.09	<.001	0.12	<.001
Main regions	0.37	<.001	0.47	<.001	0.41	<.001

[a]The listed statistics are based on Bray–Curtis distances, as well as weighted and unweighted UniFrac distances. The tests are for differences among the gut segments, the three main gut regions, among individuals within the stomach and large intestine, and between the two populations.

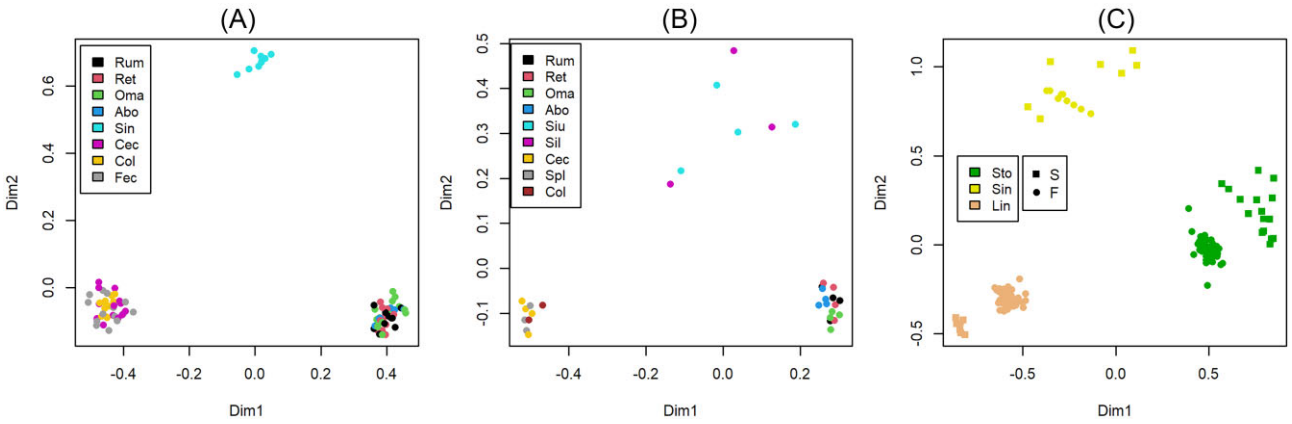

Figure 2. Non-metric multidimensional scaling of the Bray–Curtis distance matrix of the (A) Svalbard population, (B) Finnmark population, and (C) both populations together. In (A), a small amount of normally distributed noise was added to all data points in order for samples within the same main region to be distinguishable. Without added noise, the data points basically form three dots with all of the samples from a main region on top of each other. In each plot, the structuring is highly significant (see Table 2 for details). Rum, rumen; Ret, reticulum; Oma, omasum; Abo, abomasum; Sin, small intestine; Siu, small intestine upper part; Sil, small intestine lower part; Cec, cecum; Col, colon; Spl, spiral loop; Fec, faeces; Sto, stomach; Sin, small intestine; Lin, large intestine; S, Svalbard reindeer; and F, Finnmark reindeer.

both comparisons) and more abundant in the stomach than in the large intestine ($P < .001$).

At the Family level, the taxonomic assignment was relatively poor (62.4% assigned ASVs). Nevertheless, looking at differences among GI regions at this level can be informative (Fig. 1), with 140 of 221 classified families occurring at significantly different relative abundance in one main GI region relative to at least one other ($P < .05$, Kruskal–Wallis rank sum tests with Benjamini–Hochberg corrected *P*-values). The most abundant family overall was Rikenellaceae with a higher relative abundance in the stomach region relative to the colon, and almost no occurrence in the small intestine. Prevotellaceae and Spirochaetes were also very predominant in the stomach relative to the colon and the small intestine. While the small and large intestines had similar levels of Firmicutes, the former was dominated by Peptostreptococcaceae with a significant contribution of Methanobacteriaceae, while the latter was dominated by UCG-010 (order Oscillospirales), Lachnospiraceae and Oscillospiraceae. The Proteobacteria in the small intestine were mostly Enterobacteriaceae while this group was rare in the stomach and large intestine.

Although taxonomic classification was inefficient on the genus level (37.2% assigned ASVs), we did some analysis of spatial structure (Fig. S3). Out of 486 identified genera 284 were found at significantly different relative abundance in one main GI region relative to at least one other ($P < .05$, Kruskal–Wallis rank sum tests with Benjamini–Hochberg corrected *P*-values). The genus with the highest overall abundance was the Rikenellaceae RC9 gut group, which was particularly prevalent in the stomach. *Prevotella* was also abundant in the stomach, as well as the related Prevotellaceae UCG-1 and Prevotellaceae UCG-3. The colon, on the other hand, had relatively high abundances of Prevotellaceae UCG-4, Bacteroides and the putative genus UCG-5. The small intestine was dominated by a few genera, including *Romboutsia*, *Escherichia-Shigella*, *Paeniclostridium*, *Methanobrevibacter* and *Clostridioides*. It is also noteworthy that the combined relative abundance of ASVs without a genus-level taxonomic assignment differed markedly

among the three main gut regions (Fig. S3), with the lowest mean proportion in the small intestine (10.0%), the highest in the colon (59.7%), and 43.1% in the stomach.

We observed significant structuring, in terms of microbiota composition, among the four stomach compartments ($R^2 = 0.10$, $P = .002$; PERMANOVA) and the cecum, large intestine and faeces ($R^2 = 0.07$, $P = .01$). This structure was weak compared to what we observed among the main GI regions. Within the stomach compartments by far the strongest structuring factor was the individual from which samples were collected ($R^2 = 0.79$, $P < .001$). This was also the case for the cecum, colon, and faecal samples ($R^2 = 0.86$, $P < .001$). The age and sex of the animals did not have any impact on the observed spatial structure (Fig. S5).

Microbiota diversity, as measured by Shannon entropy (Fig. 3A, Fig. S7) and the number of observed ASVs (richness; Fig. 3B, Fig. S7), was much lower in the small intestine than in the large intestine and stomach ($p<0.001$ for all comparisons; two-sided t-test), while diversity was significantly higher in the large intestine than the stomach ($P < .001$ for Shannon entropy and richness). We did not observe significant differences in diversity among the four stomach or large intestine compartments (i.e. cecum, colon, and faecal samples).

Sequencing and spatial structure of the semi-domesticated reindeer GIT microbiota

For the four semi-domesticated reindeer, 16S rRNA gene sequencing produced a total of 2 039 724 sequences distributed among the 32 samples that were successfully sequenced (mean 63 742±20 699 s.d.) (Table 1). The scaling was carried out to the lowest sample read number (34 155 reads), and a single sample with less than 30 000 reads was discarded. After removal of singleton ASVs a total 4 812 ASVs, distributed across 24 known phyla, remained. Out of these ASVs, 99.3% were classified successfully at the phylum level, while only 46.8% were reliably classified at the genus level (Fig. S4). As with the Svalbard samples, there was strong structuring according to the three main regions of the GI tract (stomach, small intestine, and large intestine) ($R^2 = 0.42$, $P < .001$, PERMANOVA with Bray–Curtis distances; Fig. 2B, Table 2, Fig. S6). Similar results were obtained when using phylogenetic distances ($P < .001$ for weighted and unweighted UniFrac). This structure was confirmed with a random forest model that produced a 100% classification accuracy to the three main regions ($P < .001$). Differences in phylum level composition between the main regions (Fig. 1) generally reflected those observed for Svalbard reindeer. Firmicutes were more common in the large intestine ($P < .001$, Wilcoxon rank sum test with Benjamini–Hochberg corrected P-values) and small intestine ($P = .002$) than the stomach. Bacteroidota was the dominant phylum in the stomach ($P < .001$ for comparison with both other regions) and this group was almost absent in the small intestine ($P = .001$ for comparison with the large intestine). Euryarchaeota was more prevalent in the small intestine ($P = .02$ vs the stomach, $P = .002$ vs the colon). Also, it is noteworthy that in the semi-domesticated reindeer, we observed a relatively high abundance of sequences classified as Cyanobacteria in the small intestine (Fig. 1). Family level differences were also similar to those observed in Svalbard reindeer, e.g. with respect to Rikenellaceae, Prevotellaceae, Oscillospiraceae, Lachnospiraceae, Methanobacteriaceae and UCG-010 (Fig. 1). On the genus level, 11 of the 15 most abundant genera were shared between animals from Finnmark and Svalbard (Fig. S3), and the most abundant genus in both populations was Rikenellaceae RC-9 gut group. Interestingly, the combined abundance of ASVs without a genus-level classification did not follow the pattern seen in the Svalbard population, with the highest proportion seen in the small intestine (58.4%), and similar proportions in the stomach and colon (34.9% and 36.4%, respectively). Diversity patterns were like those seen in the Svalbard animals (Fig. 3). The highest Shannon entropy was observed in the large intestine ($P < .001$ for both comparisons, two-sided t-test) and substantially higher values in the large intestine than the small intestine ($P < .001$) (Fig. 3C, Fig. S8). However, ASV richness was marginally higher in the stomach than the large intestine (Fig. 3D, $P = .1$), but significantly higher in both the stomach and large intestine relative to the small intestine ($P < .001$ for both comparisons) (Fig. 3D). Consistent with the results for the Svalbard reindeer, we did not observe strong structuring within any of the three main GI regions, and the main structuring factor was the individual (Fig. 2b, Fig. S6). Interestingly, when analysing the Svalbard and semi-domesticated reindeer samples together, samples from the same main GI region clustered together, regardless of the population from which the samples were collected (Fig. 2C). PERMANOVA models using main GI region as the explanatory variable explained a much higher proportion of variance than models using population (Table 2). This result was corroborated by a random forest model, trained on data from both populations, with 100% classification accuracy for assigning samples to the three main GI regions, regardless of which population the sample came.

Functional differentiation

To compare differences in the abundance of microbial CAZymes along the Svalbard reindeer tract, samples from the rumen, the small intestine, and the colon from each of the three animals were subjected to shotgun metagenomic sequencing. Shotgun sequencing resulted in a total of 261 820 473 reads (mean 29 091 164 ± 17 050 042 s.d.) after quality filtering. Alignment to the dbCAN-PUL database identified 168 105 candidate carbohydrate-active enzyme genes. The mean relative coverage of contigs containing CAZymes was significantly higher in the rumen samples relative to the colon and small intestine ($P < .001$ for both comparisons, Wilcoxon paired rank sum test). The small intestine had significantly higher coverage of these contigs relative to the colon ($P < .001$, Wilcoxon paired rank sum test). The same pattern was observed, at a high level of significance, for all the five main CAZyme classes: glycoside hydrolases, glycosyl transferases, polysaccharide lyases, carbohydrate esterases, and carbohydrate-binding modules (Fig. S9). The main substrate groups in the rumen samples included relatively complex carbohydrates, for example, xylan, beta-glucan, fructan, glucomannan, and cellulose (Fig. 4), while the small intestine and colon were dominated by enzymes for processing simpler carbohydrates like raffinose, melibiose, sucrose, and galactose (Figs. S10 and S11).

Bin selection with the DAS tool resulted in 76 MAGs of acceptable quality, with mean completeness of 81.4% (median 88.4%) and mean contamination of 8.6% (median 2.9%). Among the 76 MAGs, Bacteroidota was the most common phylum (33 MAGs), followed by Firmicutes (17), the candidate phylum Patescibacteria (7), Actinobacteria (5), and Proteobacteria and Verrucomicrobia (3 for each). All but 5 of the MAGs were classified to the genus level but only 18 were classified at the level of the species (Table S1). Annotating MAGs with the dbCAS-PUL database did not result in significantly different mean numbers of CAZymes in Bacteroidota and Firmicutes (299 vs 317, $P = .76$ unpaired t-test). Proteobacteria and Patescibacteria stood out by having a mean of only 65 and 61 CAZyme genes, respectively, which was significantly lower than Bacteroidota and Firmicutes ($P < .001$ for both comparisons,

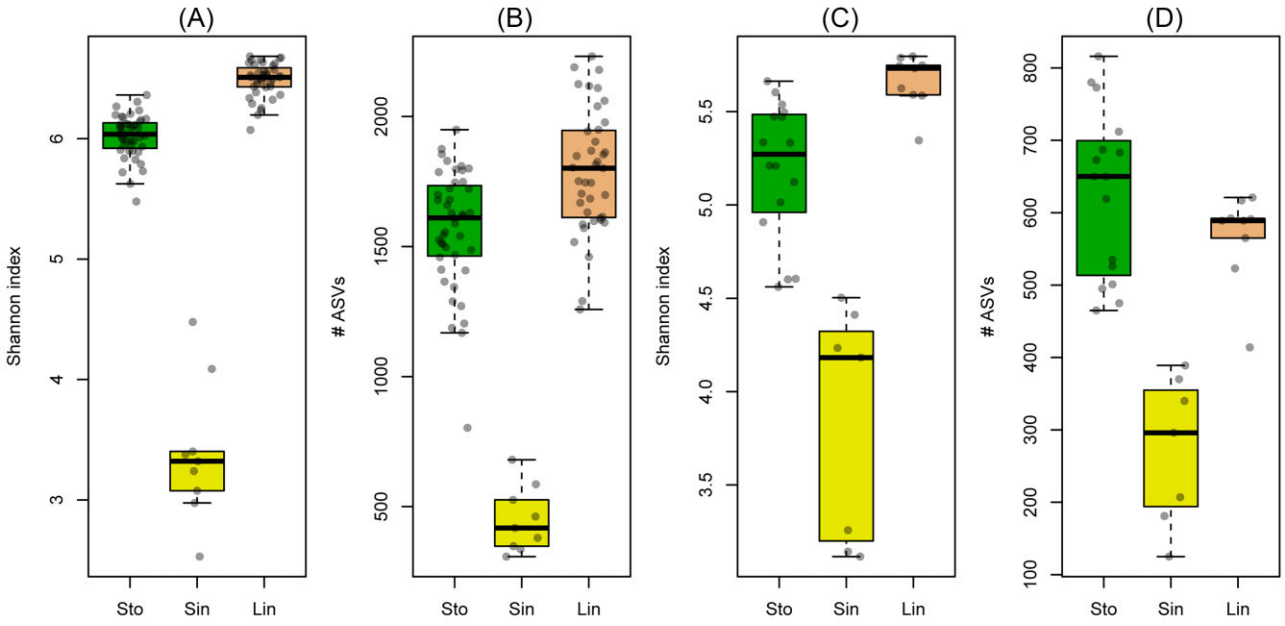

Figure 3. Shannon entropy and ASV richness of microbiome communities in the three main gut regions (Sto, stomach; Sin, small, and Lin, large intestine) in (A, B) Svalbard reindeer and (C, D) Finnmark semi-domesticated reindeer. Diversity in each individual section can be seen in supplementary Figs. S7 and S8.

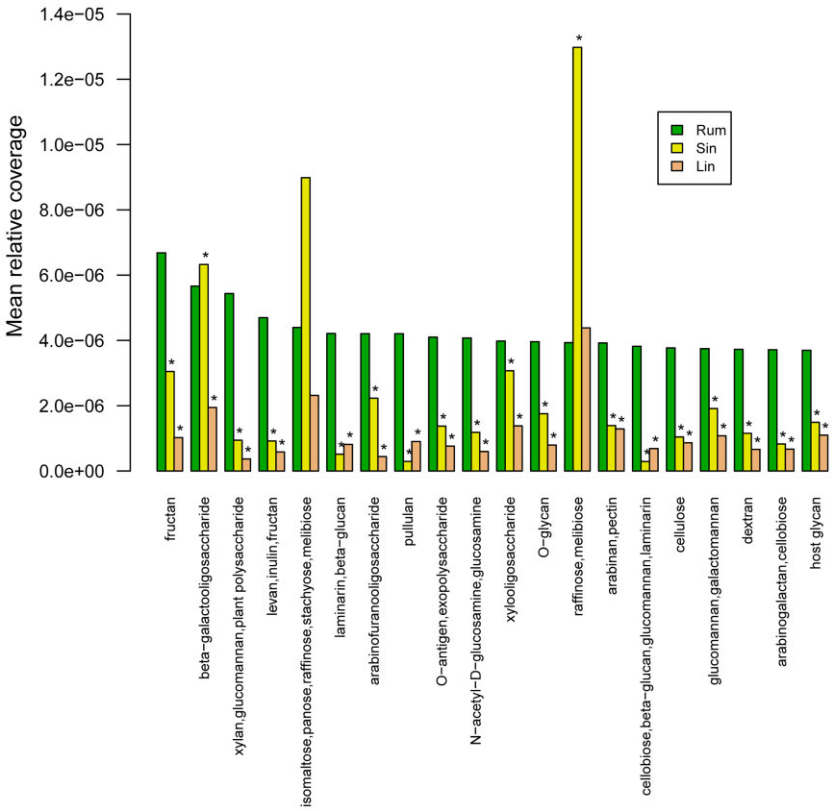

Figure 4. Mean relative coverage of metagenome contigs with annotated CAZymes categorized by the dbCAN-PUL database carbohydrate substrate categories. In the plot, the bars indicating coverage have been sorted to show the 20 substrate categories with the highest CAZyme representation in the rumen samples. Colored bars representing the rumen, small intestine, and colon each represent the means of three Svalbard reindeer samples. Except for beta-galacto-oligosaccharides and the two categories including raffinose and melibiose, all categories show significant relative enrichment in rumen samples relative to the two other GI regions (paired Wilcoxon rank sum test with Benjamini–Hochberg corrected P-values). Asterisks above the yellow- and salmon-colored bars indicate a significant ($P < .05$) difference between the small and large intestines, respectively, relative to the rumen. RUM, rumen; SI, small intestine; and LI, large intestine.

unpaired t-test). The MAG with the highest number of annotated CAZyme-encoding genes (853) was classified as *Fusicatenibacter saccharivorans* (family Lachnospiraceae), while the MAG with the lowest number of such genes (19) was unclassified candidatus *Saccharibacteria UBA2834*, see also Table S1. *Prevotella* was the genus with the highest number of annotated MAGs (6 MAGs), with a mean of 369 CAZyme genes (range 161–552).

Discussion

Ruminants harbour complex and highly diverse gut microbiome communities, but knowledge about the factors driving their composition and dynamics across the gastro-intestinal tract is still limited. Our study identified a very distinct "biogeography" of the microbiome community in the three main regions of the GIT, which contrary to our expectations was also strikingly similar across two reindeer subspecies that have lived >1000 km apart for six thousand years, separated by the Arctic Ocean. Our research adds to the evidence that the GIT microbiome in ruminants is highly structured despite evolutionary isolation and ecological differences.

The four stomachs (rumen, reticulum, omasum, and abomasum) differed markedly from the small intestine, and in turn, from the large intestine (cecum, colon, and faeces). Differences in the microbiome between rumen, reticulum, omasum, and abomasum were relatively small, with all four dominated by Bacteroidota, with Firmicutes the second most common phylum. In contrast, Firmicutes were most common in the small intestine and large intestine compartments. While Bacteroidota were the second most common phylum in the large intestine they were scarce in the small intestine. Thus, GIT microbiome structuring is primarily driven by what seems to be a quasi-ubiquitous pattern of change in the relative abundance of the two dominant phyla—Bacteroidota and Firmicutes. This is in line with earlier studies on other ruminant species [Xie et al. 2021; see also Li et al. 2014 for Chinese roe deer (*Capreolus pygargus*); Kim et al. 2019 for elk (*Cervus canadensis*); Hu et al. 2022 for sika deer (*Cervus nippon*)]. The higher Bacteroidota to Firmicutes ratio in the rumen has also been observed in reindeer across its distributional range (Pope et al. 2012, Glendinning et al. 2021), and the opposite in faeces (Zielińska et al. 2016). However, a higher Bacteroidota to Firmicutes ratio in the rumen of reindeer does not always seem to be the rule. Studies from Sundset et al. (2007) and Salgado-Flores et al. (2016) found Firmicutes to be the dominant phylum in the rumen of both Svalbard and Norwegian semi-domesticated reindeer. Firmicutes were also found to be dominant in the rumen of other species such as Holstein dairy cattle (Mao et al. 2015), free-ranging cattle-yaks (Sha et al. 2020) and muskox (Wu et al. 2022). Seasonality could be one potential factor explaining differences in Bacteroidota to Firmicutes ratios among studies. For instance, reindeer rumen samples in Sundset et al. (2007) were collected during summer, as opposed to autumn in our case. Shifts in the relative abundance of bacterial phyla could be driven by multiple factors. Diet (Lin et al. 2023), often in conjunction with seasonality (Kartzinel et al. 2019), is likely important for determining microbiome composition in free-ranging ruminants, e.g. through changes in dietary fiber content (Ungerfeld et al. 2018, Wu et al. 2022) or food carbohydrates-to-protein ratio intake (Pitta et al. 2014, Hu et al. 2018). The recent study by Lin et al. (2023), for instance, clearly shows that changes in the diet in dairy cattle do lead to a reshuffling of microbiome communities within GIT regions without necessarily disrupting the overall structuring of the GIT. Here, slight differences in seasonality, likely in conjunction with diet (i.e. late autumn in Svalbard *versus* early autumn in Finnmark) might also contribute to explain the differences in microbiome composition, within GIT regions, observed between the two reindeer populations (Fig. 2C). The combined effect of diet and seasonality on the reindeer gut microbiome has not been studied so far. Two earlier cultivation-based studies do suggest the occurrence of major changes in the composition of the gut microbiome, transitioning towards increased capacity for the digestion of fiber during winter, in both the rumen and caecum (Orpin et al. 1985, Mathiesen et al. 1987). Future research should illuminate the extent to which these two factors might affect relative abundances of main bacterial taxa and how this may in turn impact the microbiome structuring across the reindeer GIT.

It is important to recognise that differences in bacterial proportions between studies could also arise from differences in the laboratory methods used, including DNA extraction protocols (Henderson et al. 2013, Fliegerova et al. 2014). However, in our case, the methodological frameworks used for the analysis of the Svalbard and the semi-domesticated reindeer were highly similar, both using the same DNA extraction procedure and Qiagen PowerSoil kit as well as the same library preparation protocol. In fact, the GIT microbiome structuring observed in the two reindeer subspecies here seems to be highly robust, despite small methodological differences, including sample handling conditions and preservation medium (i.e. frozen versus ethanol-stored samples for the Svalbard reindeer). This, together with findings from other ruminant species, suggests that variations in relative bacterial abundances within gut sections might be common, but with little consequence for the overall microbiome structuring across the GIT.

ASV richness and Shannon diversity were also highly structured across the GIT in both reindeer subspecies, with the highest levels of diversity detected in the colon section, and the lowest in the small intestine. The observed patterns are comparable to other wild ruminants such as the Chinese roe deer (Li et al. 2014) and the Sika deer (Hu et al. 2022). However, only 30 to 40% of ASVs could be assigned to the genus level. Ruminants harbour prodigious levels of microbiome diversity within their guts but our understanding of the identity and the function of this microbial world remains limited, to a large part due to the difficulties of creating appropriate culturing conditions. Our results further emphasize the necessity for a dedicated effort to culture and catalogue the hyperdiverse microbiomes in wild herbivores.

Our metagenome sequencing for Svalbard reindeer does suggest that changes in microbiome composition and diversity across the GIT are linked to digestive functions. We find that the amount of potentially available CAZymes, responsible for the breakdown of carbohydrates, was highest in the rumen compared to the small intestine and the colon. Moreover, rumen-associated CAZymes were mainly enzymes involved in the degradation of highly complex sugars from plant diets such as cellulose, hemi-cellulose or glucans. These results are in accordance with two previous studies on dairy cattle, showing that CAZymes in the rumen are mainly associated with the breakdown of diet-derived carbohydrates as opposed to the large intestine where the main carbohydrate substrate for CAZymes was host-derived (i.e. mucin) (Mao et al. 2015, Lin et al. 2023). Overall, the catabolism of these sugars is known to result in the production of short-chain fatty acids—an essential energy source, providing up to 75% of the total metabolisable energy in ruminants (Bergman 1990), also important for a wider range of metabolic processes (Corrêa-Oliveira et al. 2016; van der Hee and Wells 2021). Our results show the presence of enzymes important for the breakdown of carbohydrates in all re-

gions along the GIT, also suggesting continuous production of various short-chain fatty acids in the small and large intestines as shown in the Sika deer (Hu et al. 2022). Interestingly, the presence of sorbitol, known to be important for liveweight gain and milk production in domestic cattle (Geay et al. 1992, Hussian et al. 2020) was observed in the Svalbard reindeer (Fig. S10). However, the levels could not be compared with the Finnmark reindeer because equivalent CAZymes analyses were not carried out for that population.

Although the shotgun metagenomic sequencing revealed the differential occurrence of CAZymes along the main gut regions, it should be noted that these results are based on a relatively small number of samples. Furthermore, a sampling depth of ~30 000 000 reads per sample should be considered a relatively shallow effort given the extreme complexity of the ecosystems being sampled, in particular in the case of the rumen and large intestine. This limitation is underlined by the relatively low number (76) of good-quality MAGs we were able to produce from the shotgun data, relative to the number of ASVs identified by amplicon sequencing. In future studies, it would be desirable to have sampling depths into the hundreds of millions of sequence reads per sample in order to describe the genome-level composition of the gut microbiome more fully in this particular context.

Interestingly, the distinct and consistent community composition of the large intestine, along with the high taxonomic diversity, suggests equivalence in functional importance with the rumen. However, the role of this "lower-gut microbiome" is still poorly understood (O'Hara et al. 2020). Through a genome-based analysis, Lin et al. (2023) elegantly demonstrate the partitioning of polysaccharides-degrading strategies by the gut microbiome from the stomachs to the small and large intestines, shifting respectively from the mobilization of dietary to endogenous, microbial and host-derived substrates, helping to maintain energy production levels across the GIT. This is in line with our finding of decreasing abundance of microbiome-encoded CAZymes genes in the large intestine, reinforcing the idea that the large intestine seems to select for oligotrophic taxa with distinct fermentation pathways, well adapted to the conditions of nutrient availability within this compartment (Lin et al. 2023). Beyond fermentation, the large intestine might also play an important role in the establishment, and homeostasis, of the immune system in ruminants (Malmuthuge and Guan 2017, Malmuthuge et al. 2019). Heat production could be another important function for the lower-gut microbiome (Rosenberg and Zilber-Rosenberg 2016), which again is an understudied role of the microbiome, but one potentially important for arctic mammals. Further research will help to reveal the extent of these functions within the large intestine of reindeer.

Lastly, an important implication of our study is that it points towards the need to establish efficient, desirably non-invasive, monitoring protocols that explicitly consider GIT microbiome biogeography. Microbial communities associated with the stomachs and the small intestine play important roles for the physiology and the ecology of the host but are not represented in microbiome assessments based on faecal samples. Buccal swabs have been tested as a possible alternative to the invasive sampling of the rumen (Kittelmann et al. 2015, Tapio et al. 2016, Young et al. 2020) but their accuracy in capturing the microbiome composition of the stomachs remains questionable and is yet to be validated for wild ruminants. We found that non-invasive faecal samples were accurately reflecting microbial communities in all parts of the large intestine, but not in GIT compartments further upstream.

In conclusion, we have demonstrated that GIT microbiome structuring seems to supersede evolutionary, ecological, and geographic barriers between reindeer subspecies but also occurs among wild ruminants from other latitudes. This identifies the GIT as an essential selective force of gut microbiome assembly, consistent with the idea of an early evolution of microbial digestion in herbivores (Hume and Warner 1980).

Author's contributions

S.K., E.J.d.M., L.E.L., S.A., and P.T. conceived the ideas and designed the study; S.K., T.A.U., V.V., and S.M.J.G.S. contributed with fieldwork/reindeer expertise and samples acquisition; S.K. and E.J.d.M. carried out molecular analyses; P.T. analysed data with input from E.J.d.M.; S.K. wrote the first version of the manuscript and all authors contributed to improving the manuscript for publication.

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Supplementary data

Supplementary data is available at *FEMSEC Journal* online.

Conflict of interest: The authors declare no conflict of interest.

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Data availability

Raw sequencing datasets are available at the Genbank nucleotide archive under accession numbers PRJNA913801 (amplicon data) and PRJNA913821 (shotgun data). Metadata and scripts can be made available upon request.

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