Digestive Adaptations of Both the Fore- and Hind-gut in a Temperate Colobine Monkey

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Research

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Abstract

Background: In mammal herbivores, the digestion of fiber usually occurs predominantly in either the foregut or in the hindgut. However, how both gut regions function synergistically in the digestion of fiber and other nutrients has rarely been reported in wild mammals. This requires an integrative study of host anatomy, physiology and gut microbiome. Colobine monkeys (Colobinae) are folivorous, with fiber fermentation primarily occurring in the foregut, with residual fermentation in the hindgut. For the few colobine species that live in temperate regions obtaining energy from fiber during winter is critical but the mechanisms enabling this remain unclear.

Results: We studied microbial and morphological digestive adaptations of golden snub-nosed monkeys (GSMs), *Rhinopithecus roxellana*, a temperate forest colobine from central China. We tested for synergistic foregut and hindgut fiber digestion in a species that experiences high thermal energy demands while restricted to a fibrous, low-energy winter diet. We found that the GSM's colon has a significantly greater volume than that of other foregut fermenting colobines, and both gut regions of GSMs are dominated by microbial taxa producing enzymes to enable active digestion of complex carbohydrates. The microbiomes of the fore- and hindgut differed significantly in composition and abundance. Although the expression of microbial gene functions for fiber digestion were higher in the foregut than in the hindgut, our microbiome analysis in conjunction with that for morphology, enzyme activity and fiber-protein digestion, suggests complementary fiber and protein metabolism in both gut regions.

Conclusions: Our results support that both the GSM fore- and hindgut facilitate fiber digestion, with an enlarged colon consistent as an adaptation to accommodate high throughput of fiber-rich food during winter.

Background

Mammals rely on microbes in the gastrointestinal (GI) tract to metabolize dietary structural carbohydrates such as cellulose and hemicellulose and produce host-available energy in the form of short-chain fatty acids (SCFAs)[1]. GI microbes also neutralize dietary toxins and digestive inhibitors [2, 3], further improving host digestive efficiency. These functions are particularly important for host species that consume food containing relatively high proportions of fiber and toxins such as grass and leaves. As a result, a wide variety of host adaptations have evolved to optimize the digestive efficiency of symbiotic gut microbiota, including sacculated stomachs, enlarged caeca and colons, and rumination[4, 5] in ungulates and coprophage mammals [6].

Among primates, lineages have evolved either a large, multi-chambered foregut (stomach [7]) or a voluminous hindgut (caecum-colon)[8], in which high volumes of fiber are fermented by symbiotic microorganisms. Colobine primates have a sacculated foregut and a relatively small hindgut compared with hindgut fermenting primates[7, 8]. The foregut has thus been suggested as the most important GI

chamber for microbial fermentation and subsequent contributions to host nutrition in colobines [9–11]. Nevertheless, colobines are likely to be less efficient at foregut microbial fermentation than ruminants [7, 11]. Although often termed “ruminant-like” [7], with the exception of the proboscis monkey (*Nasalis larvatus*)[5], colobines do not ruminate (regurgitate and re-masticate a bola of previously-consumed food). Moreover, although colobines typically exhibit three or four stomach fermentation chambers, these chambers do not exhibit the same strong functional division as do ruminants [7, 8], and increased mixing of food particles occurs[12]. As a result, while only the smallest, most completely digested particles are allowed to pass from the rumen into the rest of the ruminant digestive tract, particles of any size and stage of digestion can pass from the colobine foregut to the rest of the digestive tract. Given this reduced efficiency of the colobine foregut, it is still not fully understood how colobines extract sufficient energy and nutrients from their high-fiber, high-toxin diets[13].

For any animal consuming a diet high in fiber and toxins, it has been posited that the most efficient digestive anatomy should include both a foregut and hindgut of similar size[14]. It is known that many ruminants benefit nutritionally from microbial fermentation in both the rumen and the hindgut [1, 2, 4]. In these species, the hindgut continues to ferment structural carbohydrates that pass undigested from the foregut and to also digest proteins that have been released from compounds such as tannins by microbial metabolism [2, 15]. The hindgut also digests microbial material originating in the foregut [9]. However, in most ruminants, the hindgut exhibits a reduced volume compared to the rumen, likely as a result of reduced food volume in the distal GI tract as well as the incomplete mixing of food particles in the rumen that allows only the most completely digested food particles to pass on to the rest of the GI tract. In short, the increased digestive efficiency that would be conferred by an enlarged hindgut is unnecessary.

Because colobines do not ruminate and exhibit particle mixing in the foregut, more undigested structural carbohydrates and proteins are likely to reach the hindgut compared with other foregut-fermenting mammals such as ruminants. Therefore, in colobines, microbial fermentation in the hindgut may play a more important role in complementing the function of the foregut. Although hindgut microbial community composition is distinct in colobines compared with hindgut fermenting primates, the colobine hindgut still harbors microbial taxa and genes typically associated with fermentation in other primate hindguts[16] [17]. Additionally, high SCFA concentrations in both the foregut and hindgut of king colobus monkeys *(Colobus polykonos*) indicate substantial microbial fermentation of fiber in both compartments[7]. However, like ruminants, most colobines possess a hindgut with a smaller volume than the foregut, suggesting that the hindgut plays a secondary role to the foregut in the digestion of high fiber and high toxin foods. Importantly, though, most previous studies have not directly compared microbial activity in both the foregut and hindgut[18]. Without integrated data describing the gut morphology, microbiome, and enzymatic activity in the same individuals, it is difficult to determine the extent to which microbial functions in the colobine hindgut repeat and/or complement microbial functions in the foregut.

The relationship between the foregut and hindgut may also vary across colobine species in response to [illegible] all colobines consume hard-to-digest diets

high in fiber and toxins, some inhabit more seasonal environments in which shifts in food availability and climate can make meeting energy demands more difficult. For these species, a hindgut of increased volume with substantial microbial activity to complement the digestive function of the foregut could be advantageous to survival.

To explore this possibility, we collected data describing the digestive function of the foregut and hindgut of wild golden snub-nosed monkeys (GSM; *Rhinopithecus roxellana*). The GSM is an ideal model species to examine the digestive relationship between the colobine fore- and hind-gut. The GSM is endemic to the temperate forests of China where it endures the longest winters and lowest temperatures of any non-human primate [19]. Whilst the GSM diet is dominated by leaves during much of the year, leaves are unavailable during winter. GSMs are therefore forced to switch to a fibrous diet of lichens, buds, and bark [20, 21]. Thermal demands also result in GSMs requiring twice as much energy during winter compared to the spring. Winter thus presents these monkeys a considerable challenge to extract the required amount of energy from an enforced ultra-high-fiber diet [22, 23]. Satisfying energetic demands during the winter is partially achieved by increasing daily food consumption [18], but GSMs are also likely to depend heavily on microbial functions to extract sufficient nutritional resources from their diets during the winter. This harsh nutritional environment increases the likelihood that GSMs are reliant on the complementary functions of the foregut and hindgut microbiomes compared with other (tropical / sub-tropical) colobines who do not experience such pronounced seasonal challenges to their nutritional demands.

We collected data on the morphology, microbiome composition and function, and enzymatic activity of the foreguts and hindguts of recently deceased wild GSMs from a single population in the Qinling Mountains, Shaanxi, China to test the hypothesis that the hindgut plays an important digestive role for GSMs despite the presence of a sacculated foregut. Specifically, we predicted that the hindgut of GSMs would be relatively large compared to other colobines to allow for increased microbial fermentative activity and subsequent nutrient absorption. Because the foregut is exposed to the highest concentrations of dietary structural carbohydrates and toxins, we expected that it would be enriched in microbial genes and taxa associated with structural carbohydrate degradation and xenobiotic metabolism, compared to the hindgut. However, given that the morphology of the colobine foregut reduces the efficiency of digestion of these compounds, we expected to detect similar microbial genes and taxa in the hindgut, albeit at lower relative abundances. Furthermore, we expected the hindgut to be enriched in microbial genes and taxa associated with protein metabolism to aid in the digestion of microbially liberated dietary proteins as well as microbial proteins from the foregut. Finally, we predicted that enzymatic activity targeting structural carbohydrates would be present in both the foregut and hindgut, although at a reduced capacity in the hindgut.

Methods

Sample Collection. Over the last 15 years, the GSMs of the Zhouzhi National Nature Reserve (ZNNR) in the Qinling Mountains of Shaanxi province have been studied [23]. During the winters of 2012 and 2013, GSM individuals that had each recently died of

injuries incurred from fighting and/or falling out of a tree (for details see Supplementary Material). Immediately upon discovery, we carefully slit open each dead body to take samples of the contents from different regions of the digestive tract. We took a total of 39 samples from five individuals in the foregut (Sacus gastricus I, Sacus gastricus II, proximal gastric, distal gastric, pylorus sinus, and gastric tube) and the hindgut (cecum, colon and rectum) (Table S1). Each sample was placed into a 2 ml centrifuge tube and then taken to the laboratory and stored in liquid nitrogen prior to DNA extraction.

Comparative morphology of the major components of the GSM digestive system.

We measured the length and mass of each body in the field. Each body was then taken to the laboratory, and the digestive tract was removed. We then measured the volumes of the stomach, caecum and colon using previously described methods [24]. These methods allow us to compare the estimated volumes of organs using their surface areas [11]. The small intestine, caecum and colon were treated as cylinders (with $\mathrm{V} = \pi(b/2\pi)^2 \times 0.1$, and the stomach as a sphere, $\mathrm{V} = 4/3\pi\left(\sqrt{A/4\pi}\right)^3$ ($\mathrm{A} = b \times 1$)). To estimate surface areas, we cut the inner surface of each organ into fragments that could be flattened. The flattened fragments were arranged adjacent to each other and organized into either a triangle or rectangle. We first quantitatively described the gut morphology of the GSM and then compared our findings to a database of 47 species from all major primate subfamilies [11] (Detail see Supplementary Methods; Table S2).

DNA extraction and sequencing. To identify the mechanisms by which carbohydrates are digested in the fore- and the hindgut, we examined the microbial composition and functional potential of both the fore- and the hindguts from the five individuals. Genomic DNA was extracted from each sample using a QIAamp DNA Stool Mini Kit (Qiagen, Germany). To describe the microbial composition of each sample, we sequenced the V3-V4 region of the 16S rRNA gene (Supplemental Material). The resulting amplicons were sequenced on an Illumina MiSeq using 2x300 paired-end reads. To describe the microbial functional potential in each digestive compartment, we used shotgun metagenomic sequencing. We pooled the DNA from all of the samples collected from the same digestive compartment for each individual, resulting in five foregut sample pools and five hindgut sample pools. Metagenome library preparations for each pool were constructed following the manufacturer's protocol (NEBNext® Ultra™ II DNA Library Prep Kit for Illumina®; Supplmental Material). DNA sequencing was performed on Illumina HiSeq using 2x150 paired-end reads.

Data analysis of 16S rRNA. We sequenced 47 samples and produced a total of 9, 298, 270 reads. 3, 732, 400 reads (79, 413 reads per sample) were retained after quality filtering with an average length of 448bp. DNA sequences were demultiplexed and quality filtered using MiSeq Control Software. We used the *search* function for chimerism checks to remove low-quality sequences, the *flash* function for splicing, and the *trimmomatic* function for quality control [25]. Sequences were clustered into amplicon sequence variants (ASV) using the DADA2 wrapper in QIIME2 [26] (https://benjjneb.github.io/dada2/tutorial.html). Taxonomy was assigned using a pre-trained Bayesian classifier in QIIME2 and the Silva 138 database

Sequences were rarefied to 46, 256 reads prior to the calculation of alpha and beta diversity statistics. Alpha diversity indices were calculated in QIIME2 using the Shannon index for diversity, and the Chao1 index for richness. Principal coordinate analysis (PCoA) and an Unweighted Pair Group Method with Arithmetic mean (UPGMA) tree were used to visualize the data based on both weighted and unweighted UniFrac distances [27]. PERMANOVAs on weighted and unweighted UniFrac distances were used to test overall differences in composition. We tested for differences in microbial diversity between the hindgut and foregut using a linear mixed effects model (nlme, R v. 3.5.4) with the fixed effect defined as gut compartment and the random effect defined as individual GSM. We also tested for differences in the relative abundances of specific microbial ASVs present in at least ten samples between fore- and hind-guts using a linear mixed effects model (nlme, R v. 3.5.4) with the fixed effect defined as gut compartment and the random effect defined as the individual GSM. We tested every ASV using a loop and corrected the resulting p values for multiple tests (fdrtool, R v. 3.5.4). Because only 22 ASVs were present in at least ten samples, we repeated this process at both genus and family levels.

Data Analysis of metagenomic data. We obtained a total of 928, 965, 472 raw reads (150 bp) across ten samples from the foreguts and hindguts of the five individuals. Raw shotgun sequencing reads were trimmed using cutadapt (v1.9.1). Specifically, low-quality reads, N-rich reads and adapter-polluted reads were removed as well as host contamination reads. After filtering, we had a total of 417, 333, 904 reads with an average length of 146 bp for the ten samples (Table S3). Sequences from each sample were assembled de novo separately. Whole genome de novo assemblies were performed using SOAPdenovo (v2) with different k-mers. The best assembly result of Scaffold, which has the largest N50, was selected for the subsequent analysis. CD-HIT was used to cluster scaftigs derived from assembly with a default identity of 0.95.

To analyse the relative abundance of scaftigs in each sample, paired-end clean reads were mapped to assembled scaftigs using the Burrows-Wheeler Aligner (BWA version 0.7.12) to generate read coverage information for assembled scaftigs. Paired forward and reverse read alignments were generated in the SAM format using the BWA-SAMPE algorithm with default parameters. The mapped reads counts were extracted using SAMtools 0.1.17. The corresponding scaftigs were mapped to the mass of bacterial data extracted from the NT database of NCBI. LCA algorithm (Lowest Common Ancestor, applied in MEGAN software system) was used to ensure the annotation significance by picking out the lowest common classified ancestor for final display.

We conducted a detailed metagenomic study of carbohydrate digestion from the functional genes in the foregut and the hindgut (Figure S3A), and used an online database to identify carbohydrate-active enzymes (CAZymase; Fig. 2C). Genes were predicted using Meta Gene Mark, and BLASTP was used to search the protein sequences of the predicted genes with the NR database, CAZy database, eggNOG database and KEGG (Kyoto Encyclopedia of Genes and Genomes) database with E < 1e-5. For glycosyl hydrolase (GH) families, the representative sequences selected from the CAZy Web site were used in BLAST searches of the metagenomic data to identify GH families using an E-value cutoff of 1e-5 (Table

ences in the functional potential (overall gene

families as well as CAZymes) of the microbiome between the foregut and the hindgut. *P*-values were corrected for multiple tests [28]. We further applied correspondence analysis, a form of multidimensional scaling to determine whether the variation in the abundance of GH families distinguished the gut regions[29].

Enzyme activity of fiber digestion in the gut. Dietary fiber contains cellulose, hemicellulose and lignin. Microbial pathways for the degradation of cellulose and hemicellulose are relatively common, while pathways for lignin degradation are rare. To test whether the fore- and hindgut microbiomes are simultaneously involved in fiber digestion we measured the activity of enzymes that digest cellulose (β-glucosidase) and hemicellulose (xylanase), in the foreguts and hindguts of three individuals. We took samples from three sections of the foregut (Presaccus, Saccus, Tubus gastricus) and four sections of the hindgut (Caecum, Ascending colon, Trasverse colon, Descending colon) in each of the three individuals, respectively.

Endo-cellulase (endo-β-1,4-glucanase) and hemi-cellulase (endo-β-1,4-xylanase) activities were assayed by measuring the amount of reducing sugar released from 2 % CMC sodium salt (Sigma-Aldrich, USA) and 2% xylan (Sigma-Aldrich, USA), respectively, using the DNS method [30]. β-glucosidase activity on p-nitrophenyl-d-glucopyranoside (pNPG; Sigma) was assayed [31]. The contents of the fore- and the hindgut were each diluted with NaAc buffer (50 mM, pH 6.0) at a concentration of 0.5 g/mL and the enzyme activities were assayed immediately after dilution. A unit of specific enzyme activity (U) was defined as the number of micromoles of reducing sugar (or p-nitrophenol) released per minute. All data shown are the means of triplicate experiments. Wilcoxon rank-sum tests were used to compare enzyme activity between the foregut and hindgut (Table S9).

Digestion ratio. Using sterile spoons, we took fresh samples (> 5g) of gut contents from the three sections of the foregut (Presaccus, Saccus, Tubus gastricus) and the four sections of the hindgut (Caecum, Ascending colon, Trasverse colon, Descending colon) from the same three monkeys as used for the enzyme activity measurements (Table S10). We directly measured crude protein (CP), lipids (CL), ash, acid detergent fiber (ADF), neutral detergent fiber (NDF) and acid detergent lignin (ADL) from 0.5g dry mass of each sample [21, 32](for details see Supplementary Materials). All analyses were repeated three times. Cellulose was determined as the difference between ADF and ADL; hemicellulose was determined as the difference between NDF and ADF. For wild monkeys, it is impossible to use artificial markers to indicate a dry matter diet. We therefore used faecal ADL as an internal marker to estimate the digestibility of hemicellulose and cellulose [15, 33]. We assumed that the monkeys had stable daily nutrient intakes because they mainly fed on tree bark during the sampling period [23]. To estimate the digestion of hemicellulose and cellulose in the foregut, we measured the reduction of hemicellulose and cellulose from the first stomach chamber - the presaccus - to the small intestine. To estimate the digestion of hemicellulose and cellulose in the hindgut, we measured the reduction of hemicellulose and cellulose from the small intestine to the end of descending colon [15, 33]. Either Wilcoxon rank-sum or paired t-tests were used to quantify differences in mean reduction of hemicellulose and cellulose between the [illegible] small intestine to colon).

Results

Gut morphology. GSMs have a typically enlarged colobine stomach (Figure S1A, GSM, n = 3, Table S2), with a caecum volume that would be expected for a primate of the same size (Figure S1B). However, the GSM also possesses a particularly voluminous colon for its size, averaging 68.8% of the volume of the foregut. Considering the relationship between colon volume (log ml) and body size (log body length cm), the residual for the GSM (0.558) was significantly higher than the mean residual value of the ten other colobine species included in the analysis (-0.143 (s.e. = 0.04); range − 0.360 to 0.047; one-sample t-test: $t_8 = 16.44$, $P < 0.001$). Thus, the GSM has a considerably larger colon than the other colobines included in the analysis (Fig. 1).

Microbial compositions and functional genes. The compositions of the bacterial communities in the GSM fore- and hindgut differed significantly (PERMANOVA, F = 40.53, R2 = 0.522, P < 0.001; Fig. 2A). At both the ASV and genus levels, the hindgut microbiome community was more diverse than that of the foregut (Shannon index; $\chi^2 = 91.1$ (ASV), 94.4 (genus), df = 1, P < 0.001; Figure S2A). The relative abundance of every prevalent microbial ASV (19 ASVs present in at least ten samples) differed significantly between the foregut and hindgut, as did 129 of the 132 genera that collapsed from all ASVs. When we considered only the genera with at least a ten-fold change in relative abundance, we found that 39 were more abundant in the hindgut whilst only seven were more abundant in the foregut (Table S4). Taxa including the known carbohydrate degraders *Clostridium, Roseburia, Faecalibacterium, Blautia, Dorea, Bacteroides*, and *Ruminococcus* were more abundant in the hindgut while *Atopobium, Acidaminococcus, Syntrophococcus, Shuttleworthia*, two unknown *Selenomonaceae*, and an unknown *Lachnospiraceae* were more abundant in the foregut (Fig. 2B). The same pattern was observed at the family level.

We identified 356 KEGG pathways present in the foregut and/or the hindgut. LDA showed that there is no significantly different pathway when using a threshold of two. We then performed a Wilcoxon rank-sum test and found 137 pathways differed significantly in relative abundance between the foregut and the hindgut (Table S5; P < 0.05). Although the hindgut exhibited richer and more abundant functional genes (Figure S5), the metabolic pathways represented in each gut section appeared to be complementary. For example, genes for carbohydrate digestion and absorption as well as glycan, pentose and glucuronate metabolism were enriched in the foregut, whereas genes for the metabolism of starch and sucrose, peptidoglycan, pyruvate, fructose and mannose, amino sugar and nucleotide sugar, carbon and glycosaminoglycan were all more abundant in the hindgut. With respect to xenobiotic metabolism, genes for monoterpenoid and phenylpropanoid biosynthesis, geraniol degradation, and xenobiotic metabolism by cytochrome were enriched in the foregut. In contrast, genes for the biosynthesis of ansamycins, zeatin, flavone and flavonol as well as genes for the metabolism of other compounds such as caffeine were enriched in the hindgut; styrene, nitrotoluene and polycyclic aromatic hydrocarbon degradation genes were also enriched in the hindgut. Finally, although some pathways associated with protein and amino acid metabolism were more abundant in the hindgut (ribosome ko03010, nonribosomal peptide structures ko01054, aminoacyl-tRNA biosynthesis ko00970; ko03020, ko00270), many more pathways absorption ko04974, protein processing in

endoplasmic reticulum ko04141; ko00480, ko00290). Genes for the metabolism of vitamins and lipids also showed the same pattern. (Table S5A Wilcoxon rank-sum test, $P < 0.05$). Because of our focus on cellulose and hemicellulose degradation, we also specifically tested for differences in the relative abundance of genes involved in the metabolism of cellulose and hemicellulose between gut sections. Of the 18 relevant KEGG Orthology that we detected in both gut sections, the relative abundances of all but one were significantly higher in the foregut than in the hindgut (Table S6).

Using a CAZyme analysis, overall, we identified gene modules from 107 GH families. The bulk of these enzymes are involved in the digestion of oligosaccharides (25%), starch (16%), 8% cellulose and hemicellulose, 5% debranching enzymes, and 4% pectin digesting (GH 28) (Table S7). Although the relative abundances of those GH in both the foregut and the hindgut show similar patterns (Figure S4), the composition of these CAZyme arrays differed between the two gut regions (PERMANOVA, $F = 40.54$, $R2 = 0.83$, $P < 0.01$; Fig. 2C). Specifically, the relative abundances of 40 out of 107 identified GH gene families that digest starch, cellulose, and oligosaccharides, differed significantly between the foregut and the hindgut (Welch's t test, $P < 0.01$; Figure S3B; Table S7). For example, β-mannosidase, β-glucuronidase (GH2) had higher relative abundance in the foregut while amylomaltase (GH77) had higher relative abundance in the hindgut.

Activity of fiber degrading enzymes. The activity of both β-glucosidase and xylanase were significantly higher in the foregut than in the hindgut (Table S9). The endo-β-1,4-glucanase that digests lignin was not detected in either the foregut or the hindgut.

Cellulose and hemicellulose digestibility. Cellulose and hemicellulose were digested in both the fore- and the hindgut. Although not statistically significant, both cellulose and hemicellulose exhibited a trend of being digested more completely in the foregut than in the hindgut (Table S10A, B).

Discussion

To evaluate the digestive roles of the foregut and hindgut in the GSM, we gathered data describing the anatomy, the microbiome compositions and function, and the enzyme presence and activity in the foreguts and hinguts of five wild individuals. Recent work on four *Rhinopithecus* species, including the GSM, shows that these colobines possess derived genetic adaptations associated with an efficient ability to metabolize fatty acids and xenobiotics and to enable the digestion of high levels of RNA derived from their stomach microbiome[34]. Our investigations demonstrate that a specialized gut morphology and microbiome accompany these genetic adaptations, enabling snub-nosed monkeys to subsist on a diet very high in cellulose, hemi-cellulose, and lignin. As hypothesized, the hindgut appears to play an important digestive role. The relative size of the GSM hindgut is larger than that of other colobines. Additionally, while the microbial communities of the GSM foregut and hindgut differ, that of the hindgut appears to perform key digestive functions. Specifically, the foregut microbiota appears to initiate the degradation of dietary fiber and toxins with a broad array of microbial genes, whereas the hindgut is

enriched with a specific subset of microbiota that may target dietary compounds that have been incompletely digested in the foregut.

It has been suggested that the most efficient gut anatomy for the digestion of high-fiber, high-toxin diets is one in which the hindgut has a similar volume as the foregut[14]. Although this anatomy has not been observed in any foregut-fermenting animal, including both ruminants and colobines, the hindgut of the GSM is relatively larger than that of any other colobine species that has been studied. This enlarged hindgut is consistent with the GSM's nutritional ecology. The GSM consumes large amounts of high-fiber food during winter when thermal demands are also increased. An enlarged hindgut also enables a longer food retention time and increased microbial activity as well as potentially increased energy and vitamin absorption [35]. These traits likely facilitate survival in such a seasonally variable environment. Data describing the food retention time, microbiome composition and function, and enzymatic activity of both the foregut and hindgut in other colobine species are required for clarification.

Our microbiome data suggest synergistic food processing functions of the foregut and hindgut in the GSM. For example, the GSM foregut is dominated by *Prevotella* and *Selenomonadales*, which are also both abundant in the foreguts of ruminants(Fig. 2B; Figure S2C)[36]. These microbes facilitate hemicellulose and pectin digestion, as well as lactate and succinate transformations that increase energy production from fiber degradation by reducing methane production (Fig. 2B; Figure S2C)[4]. However, KEGG analysis also showed that genes associated with methane metabolism were enriched in the hindgut. This pattern may indicate that microbes in the hindgut help metabolize methane that exits the foregut (Figure S5. e.g. ko00680). A similar relationship is apparent for xenobiotic degradation, increasing digestive efficiency. While some genes for degradation of plant secondary metabolites such as tannins were enriched in the foregut ([37–40]), more were enriched in the hindgut.

Some of the microbial patterns we identified in the GSM hindgut are similar to those described in other colobines. For example, the faeces of *R. bieti*, a congeneric species to the GSM that inhabits higher forests (4,500 m) and feeds mainly on high-fiber lichens during winter[41, 42], contains a microflora with high relative abundances of Fibrobacteres, a lignin-degrader as well as a wide diversity of glycoside hydrolase enzymes for degrading fiber. Similarly, a study of the GSM microbiome found high relative abundances of fiber-degrading *Prevotella* and Ruminococcaceae[43]. The fecal microbiomes of gray (*Semnopithecus priam*) and purple-faced langurs (*S. vetulus*) contain many of the same microbial taxa that we detected in the GSM [44]. Finally, proboscis monkey (*Nasalis larvatus*) foreguts have high abundances of *Prevotella*, which likely function in structural carbohydrate degradation[45]. However, the extent to which these microbial taxa and genes vary between the foregut and hindgut of different colobine species has yet to be systematically determined.

Finally, our enzymatic and digestive efficiency assays concur with the findings of our DNA-based analyses of microbiome composition and function. As predicted, the majority of cellulose and hemicellulose degradation occurs in the foregut (Table S3). However, we did not observe significantly less degradation in the hindgut, suggesting that it also plays an important role in fiber metabolism.

Furthermore, the abundances and distributions of glycosyl hydrolase functional genes within the foregut and the hindgut are similar (Figure S4). Compared with the GH profiles of cows, pandas, termites and wallabies, the GSM GH profile is more similar to the wallaby and cow rumen and contains a higher proportion of cellulases/endohemicellulases than the other two species known to specialize in fiber digestion. Specifically, microbiota associated with pectin digestion at the family level are most abundant in the GSM (Figure S4; Table S8). However, this needs to be confirmed with more precise evaluation of the enzyme activities within the GSM digestive system.

In conclusion, an enlarged foregut enables effective fiber digestion for most leaf eating colobines. An enlarged specialized hindgut, and key complementary microbial functions in both the foregut and hindgut, are likely to contribute to digestive efficiency in any foregut-fermenting mammal. Our observation of these traits in our study GSM population is consistent with the hypothesis that the GSM possesses digestive adaptations to enable individuals to optimize energy and nutrient acquisition from a highly fiber-rich diet during winter. This capacity likely helps enable the GSM to balance energy acquisition and expenditure by microbially extracting energy from cellulose and hemicellulose [18, 20]. Our results support the hypothesis that both the GSM foregut and hindgut play important roles in fiber and toxin digestion. Additional morphological and microbiome data from other colobine and mammal species will clarify the functional importance of the hindgut relative to the foregut in folivores.

Declarations

ETHICS APPROVAL

The present study was approved by Animal Ethics Committee of North Western University of China.

CONSENT FOR PUBLICATION

Not applicable

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AUTHOR CONTRIBUTIONS

S.G. designed the research and wrote the manuscript. S. G., D.W.D, R.L., K.A., A.G., C.A.C., P.A.G, B.L., and [illegible]he revision of the manuscript. S.G., J.Z., R.H, G.F

and G.H. carried out the data collection and research experiments.

COMPETING interests: There are no competing interests.

DATA AND MATERIALS AVAILABILITY: 16s and metagenomic data can be accessed in NCBI PRJNA726190, accession number SUB9554749 and SUB9557011.

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Figures

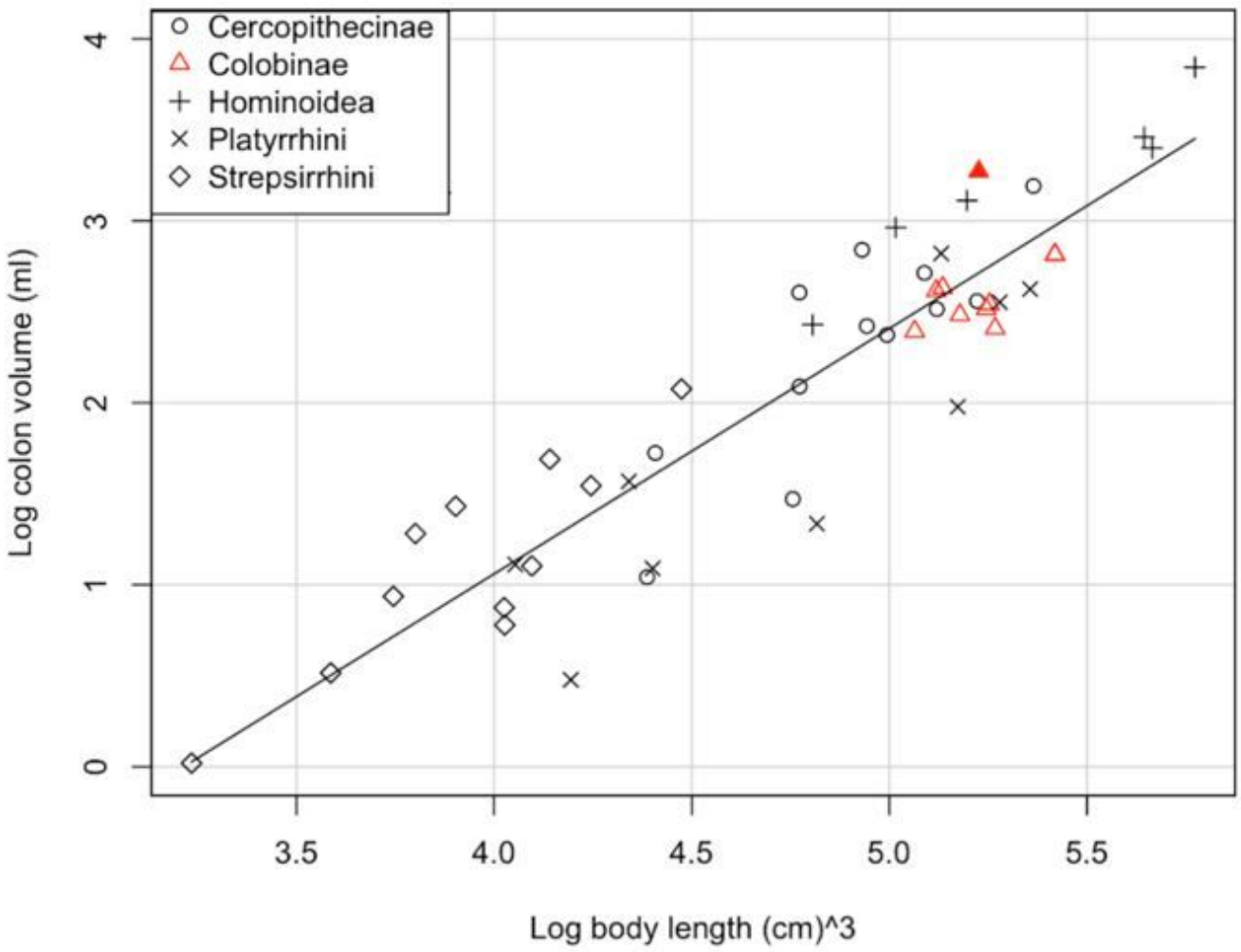

Figure 1

Relationship between the raw values of body size in cm (log of cubed value) and colon volume (log of volume in ml) in 48 primate species. Ten members of the Colobinae are shown as empty red triangles; the datum point for R. roxellana is shown as a filled red triangle. The high positive residual for R. roxellana shows that this species has a relatively large colon for its size compared with other species included in the analysis. The results of a least-squares regression (with the intercept set at zero) using the phylogenetically independent contrasts of these data is: b = 1.44, F1, 44 = 74.32, P < 0.001, R2 = 0.62 (see

methods). The relationship thus remains significant when the effects of phylogeny have been removed. Data used are as presented in Table S1.

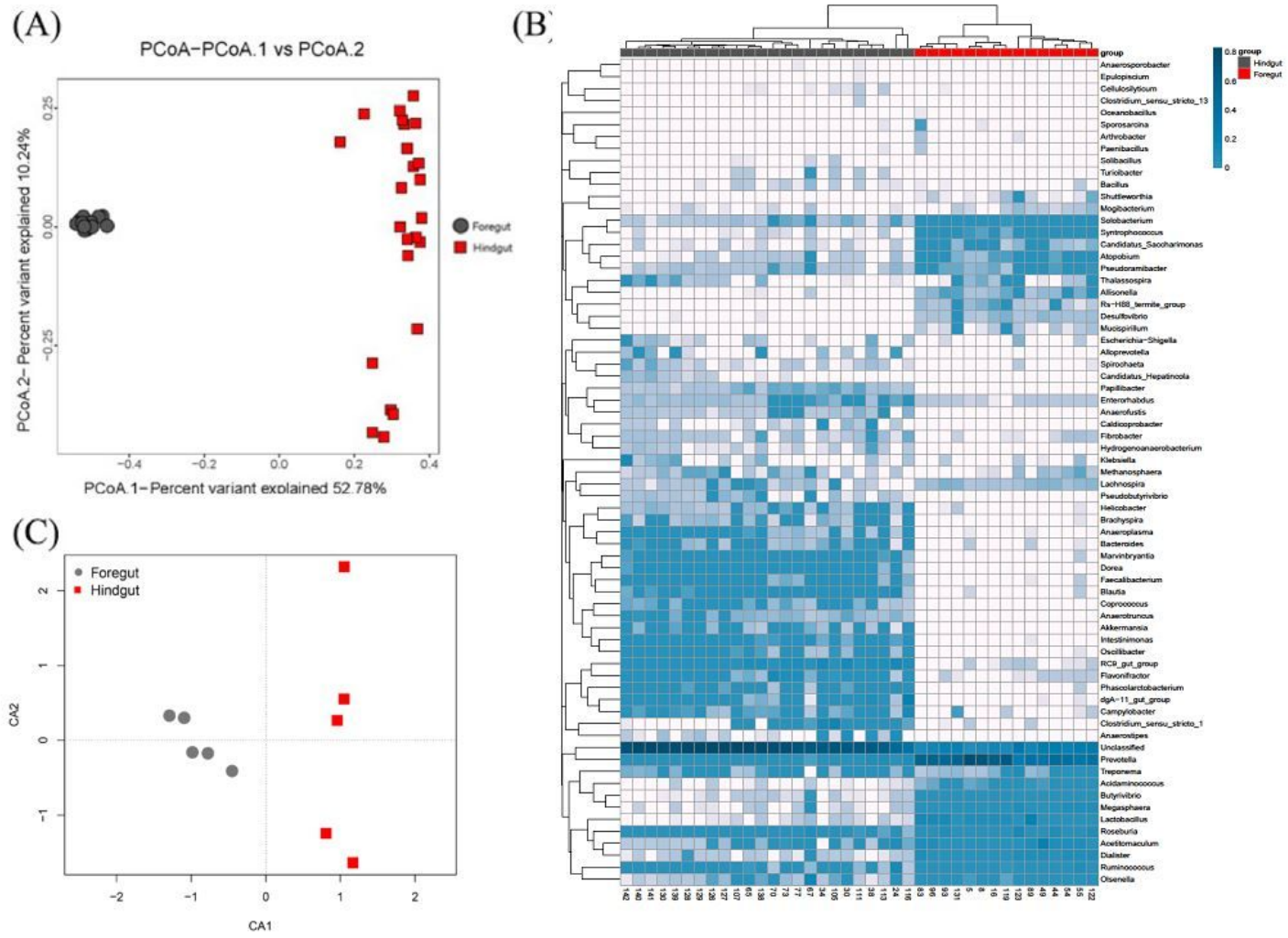

Figure 2

Bacterial community composition and metagenome of the foregut and hindgut of R. roxellana. (A), Principal coordinate analysis showing different bacterial community compositions in each sample of the two gut regions (PERMANOVA, P<0.001; hindgut data from 24 sample of gastrointestinal sites of 5 individuals; foregut data from 15 samples of gastrointestinal sites of five individuals). (B), Bidirectional clustering heatmap showing the relative abundances of indicator genera (Top 70 relative abundances genera) that characterize each gut region. (C), Plot of the results of a correspondence analysis based on the abundances of different GH families in the foregut (gray points) and hindgut (red squares), which shows that bacterial communities in each gut section exhibit different functional arrangements.

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