Herbivorous rodents (*Neotoma spp.*) harbour abundant and active foregut microbiota

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Summary

Symbiotic gut microbes have facilitated the success of herbivorous mammals, which are generally grouped into foregut- and hindgut-fermenters. However, rodents are primarily herbivorous and exhibit a variety of gastrointestinal anatomies. Most rodents house microbes in hindgut chambers, such as the caecum and colon. Some rodents also exhibit stomach segmentation with a foregut chamber proximal to the stomach. For over a century, scientists have hypothesized that this foregut chamber houses a microbial community, yet this has never been explicitly examined. We investigated the capacity of each of the gut regions to house microbes by measuring size, pH, bacterial cell density, concentrations of microbial metabolites and digesta transit time in woodrats (*Neotoma spp.*). We also compared microbial communities across gut chambers, as well as faeces, using 16S rRNA sequencing. This allowed us to test the appropriateness of using faeces as a proxy for microbial communities of other gut chambers. We found that woodrats house foregut microbial communities with similar density and volatile fatty acid concentrations to rumen ecosystems. Resident microbial communities varied between gut chambers, and faecal bacterial communities were significantly different from caecal and colonic communities. The foregut microbiota may provide a number of physiological services to the host.

Introduction

Mammalian herbivores have repeatedly evolved symbiotic relationships with gut microbes that contribute significantly to the digestion of fibre (Stevens and Hume, 2004). These gut microbes may reside in stomach chambers, such as in ruminants, macropod marsupials and some primates, or in distal gut chambers, such as in equids, elephants and rabbits (Stevens and Hume, 2004). The location of these gut chambers can have a profound influence on gut microbial communities such that foregut- and hindgut-fermenting mammals harbour unique communities (Ley *et al.*, 2008). Most gut microbial ecology studies focus on ruminants and other large-bodied herbivores (Pope *et al.*, 2010; Hess *et al.*, 2011; Zhu *et al.*, 2011). Rodents are generally herbivorous and form the most diverse and abundant mammalian order (Stevens and Hume, 2004). However, their gut microbial ecology remains understudied compared with other groups.

Rodents are an especially interesting group to study gut microbial communities, as they exhibit a wide variation in gut anatomy. Most rodents house microbes in a caecum in the hindgut; however, some also exhibit segmentation of the stomach and have a foregut chamber. It has been proposed for over a century that the rodent foregut houses a microbial community, presumably for fibre digestion (Toepfer, 1891; Carleton, 1973). Woodrats (*Neotoma spp.*) exhibit foregut segmentation (Kohl *et al.*, 2011) and represent an ideal system for studying adaptations to herbivory (Dearing *et al.*, 2000). The idea of a foregut microbial community is supported by a slightly elevated pH of ~4.5 (Kohl *et al.*, 2013) and documentation of a diverse microbial community in the woodrat foregut (Kohl and Dearing, 2012). Additionally, the microbial communities of woodrat faeces more closely resemble those of foregut- rather than hindgut-fermenting mammals (Kohl *et al.*, 2011). However, the functional nature of the rodent foregut has not been determined.

This unique gut chamber is likely to harbour a novel microbial community compared with other gut regions. Previous studies have documented that microbial communities change along the length of the gut. Mice colonized with eight bacterial species show different relative and absolute abundances of microbes between gut regions (Sarma-Rupavtarm *et al.*, 2004). Likewise, the

Table 1. Mean ± 1 SEM pH and relative masses (percent of body mass) of luminal contents of various regions of the gut of *N. albigula*.

Region	Percent of body mass	pH
Foregut	1.88 ± 0.28	4.40 ± 0.14
Stomach	2.07 ± 0.18	1.37 ± 0.09
Small intestine	2.42 ± 0.48	6.98 ± 0.15
Caecum	6.13 ± 0.32	6.33 ± 0.07
Large intestine	2.05 ± 0.25	6.42 ± 0.11
Faeces	–	6.37 ± 0.16

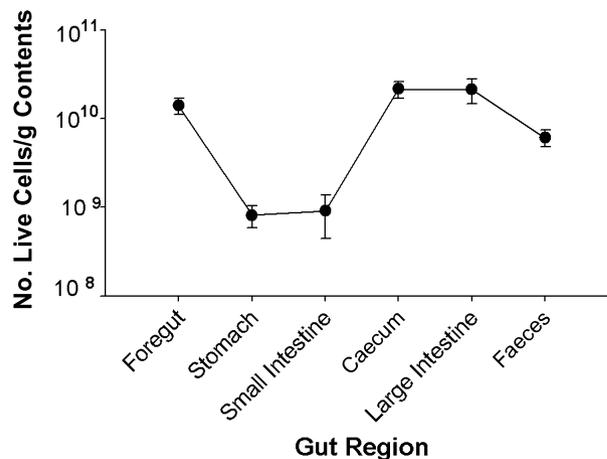**Fig. 1.** The density of live microbial cells in each gut region.

human large intestine exhibits greater microbial diversity than the small intestine (Wang *et al.*, 2005). These differences are the result of several factors, such as changes in nutrient concentration, pH and flow rate (Harrison *et al.*, 1975; Palframan *et al.*, 2002). However, studies comparing gut microbial communities across gut regions, as well as validating the use of faeces as a representation of various gut regions, have not been conducted.

We conducted a thorough investigation into the capacity of various gut regions of woodrats to house microbial communities by measuring the size, pH, bacterial cell density, concentrations of microbial metabolites and digesta transit time. Additionally, we compared bacterial diversity among gut regions by conducting bacterial inventories with 16S rRNA sequencing. This study also allowed us to investigate the appropriateness of using faeces as a proxy for the communities residing in other gut chambers.

Results

Gut regions varied significantly in their capacity to house microbial communities. The relative mass of luminal contents varied across regions, with the caecum being the largest chamber (Table 1). The pH of various gut regions also differed, with the gastric stomach having an extremely low pH (1.4), the foregut having a moderately low pH (4.4) and the rest of the gut being near neutral (Table 1). The majority of microbial cells in the gastrointestinal tract were dead or injured, as shown by flow cytometry (Supporting Information Fig. S1). However, the gut still harboured a dense community of live microbial cells (Fig. 1). The woodrat foregut harboured a microbial community with a density of live cells on par with that of other, well-known microbial chambers, such as the caecum and large intestine (Fig. 1).

Measurements of microbial metabolites revealed that the foregut microbial community is quite active. Concentrations of acetate, valerate, total volatile fatty acids (VFAs) and NH₃-N were all higher in the foregut chamber, whereas concentrations of butyrate were higher in the caecum (Table 2).

Digesta passed through the woodrat gut relatively quickly. The last meal (food eaten < 1.5 h before dissection) generally filled the foregut and reached the stomach and small intestine in this time (Fig. 2). Only $13 \pm 4\%$ of foregut contents were from food consumed more than 1.5 h before dissection, and no food eaten more than 3 h before dissection was found in this chamber. Meals progressed through the anterior gut relatively quickly and were then retained in the hindgut for several hours, such that food eaten between 3 h and 7.5 h before dissection all tended to be found in the large intestine (Fig. 2).

Sequencing effort resulted in 619 625 high-quality sequences (average of $20\,654 \pm 715$ sequences per sample. These sequences were assigned to 15 799 operational taxonomic units (OTUs) at 97% sequence similarity. We were able to accurately assign 83.4% of OTUs to bacterial phyla and only 7.7% of OTUs to microbial genera. Relative abundances of 5 of the 12 most dominant phyla showed significant differences between gut regions (Fig. 3A, Table 3). Firmicutes was the dominant phylum in the stomach, caecum and large intestine, while Bacteroidetes comprised the majority of the community in the foregut, small intestine and faeces. Diversity

Table 2. Metabolite concentrations of volatile fatty acids and ammonia nitrogen (mM) in the foregut and caecum of *N. albigula*.

Metabolite	Foregut	Caecum	*P* value
Acetate	164.9 ± 4.6	109 ± 10.1	**0.01**
Propionate	15.5 ± 2.2	12.7 ± 0.5	0.31
Butyrate	12.1 ± 2.2	21.5 ± 4.0	**0.04**
Isobutyrate	2.0 ± 1.4	0.3 ± 0.1	0.31
Valerate	0.3 ± 0.1	0.5 ± 0.1	**0.04**
Isovalerate	0.4 ± 0.1	0.3 ± 0.1	0.25
Total VFA	195.2 ± 4.6	145.1 ± 11.5	**0.02**
NH₃-N	43.5 ± 5.0	20.6 ± 2.6	**0.03**

Concentrations were compared between these chambers with a paired *t* test. Significant differences are in bold.
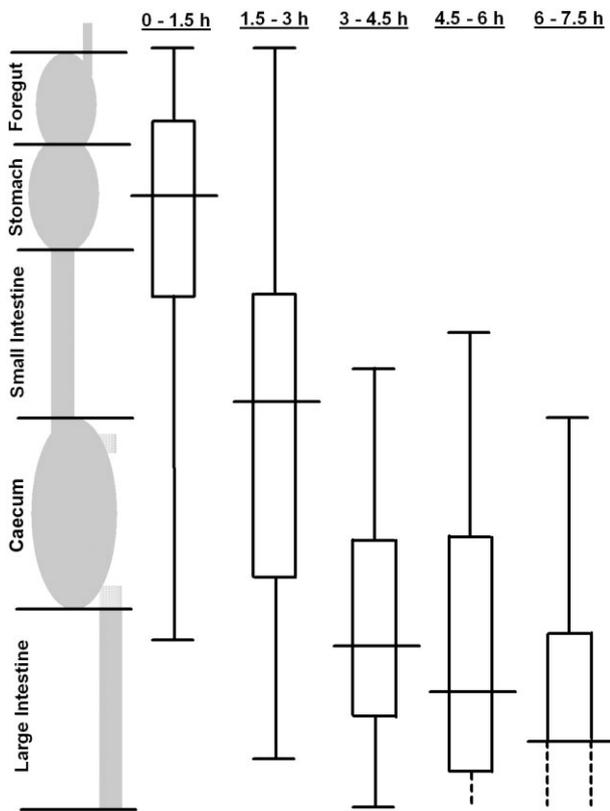
Fig. 2. Digesta movement through the gut of *N. lepida*. Box plots showing the median, quartile, minimum and maximum locations of food eaten at different time points. Dashed lines mean that the measurement includes faecal material. Sizes of gut compartments are not to scale.

also varied at the genus level, with relative abundances of four of the five most dominant identified genera exhibiting significant differences between gut regions ($P \leq 0.01$ for *Oscillospira*, *Lactobacillus*, *Desulfovibrio* and *Ruminococcus*; Fig. 3B). It is also noteworthy that we were only able to accurately identify less than 25% of sequences in any gut region to the genus level. The 'Other' category of genera (Fig. 3B) represents sequences that were identified to the genus level, but are present at very low abundances. This category contains 57 identified genera that collectively make up less than 8% of the community of any region. A large proportion (> 75%) of the community was unable to be identified to the genus level (termed 'Unidentified'), and contained 14 578 OTUs when grouped at 97% sequence identity. Biodiversity metrics (Shannon index, estimated species richness, evenness and phylogenetic diversity) also varied significantly by gut region ($P < 0.001$ for all metrics, Fig. 4), with the small intestine consistently showing the lowest diversity.

Both the individual animal and gut region source of samples influenced bacterial community membership and structure. Community membership, or the presence and absence of bacterial lineages, was primarily driven by gut region (adonis: $R^2 = 0.28$; $P = < 0.001$; Fig. 5) and also by the individual animal ($R^2 = 0.05$; $P = 0.02$). Community structure, which takes relative abundances of taxa into account, was only driven by gut region ($R^2 = 0.55$; $P = < 0.001$) and not by individual animal ($R^2 = 0.04$; $P = 0.27$). These data suggest that gut region determines bacterial community membership and structure, and that individuals have signature bacterial communities memberships across these regions. This is further supported by the principal coordinates analysis (PCoA) results, where samples with similar communities cluster together on the graph. Gut regions parse out based on principal coordinate 1 (15.4% and 54.5% variation explained for membership and structure, respectively), whereas individual animals parse out on principal coordinate 2 (Fig. 5).

Comparisons between gut regions reveal that adjacent chambers rarely share the most similar communities. Similarities between adjacent communities were observed only once, where the bacterial community membership was most similar between the caecum and large intestine (Fig. 6). Rather, disparate regions share similar communities, as shown by the UPGMA trees (Unweighted Pair Group Method with Arithmetic Mean) of both community membership and community structure (Fig. 6). For example, faeces are the most similar to the foregut in terms of community membership and most similar to the small intestine in terms of community structure (Fig. 6).

Discussion

For over a century, scientists have speculated about the microbial dynamics of the rodent foregut. To our knowledge, this study represents the most thorough investigation into the microbial communities and activity of this chamber, as well as elsewhere in the gut. We found that woodrats maintain diverse and dynamic gut communities across the length of their gastrointestinal tracts and that both the gut region as well as individual animal determine these communities.

The woodrat gut varied across regions in its capacity to house microbes. The caecum was the largest chamber by volume, comprising roughly 6% of the animal's body mass. This size is similar to other herbivorous rodents, such as naked mole rats, porcupines and capybara (Stevens and Hume, 2004). The woodrat foregut was smaller than the caecum and made up less than 2% of the animal's body weight. The foregut was comparatively smaller than the foregut chambers in other animals, such as ruminants and kangaroos, which comprise ~10% of the animals' body mass (Stevens and Hume, 2004). Despite its small size, the woodrat foregut houses microbes at a density similar to that of other well-known microbial communities, such as the caecum and large intestine.
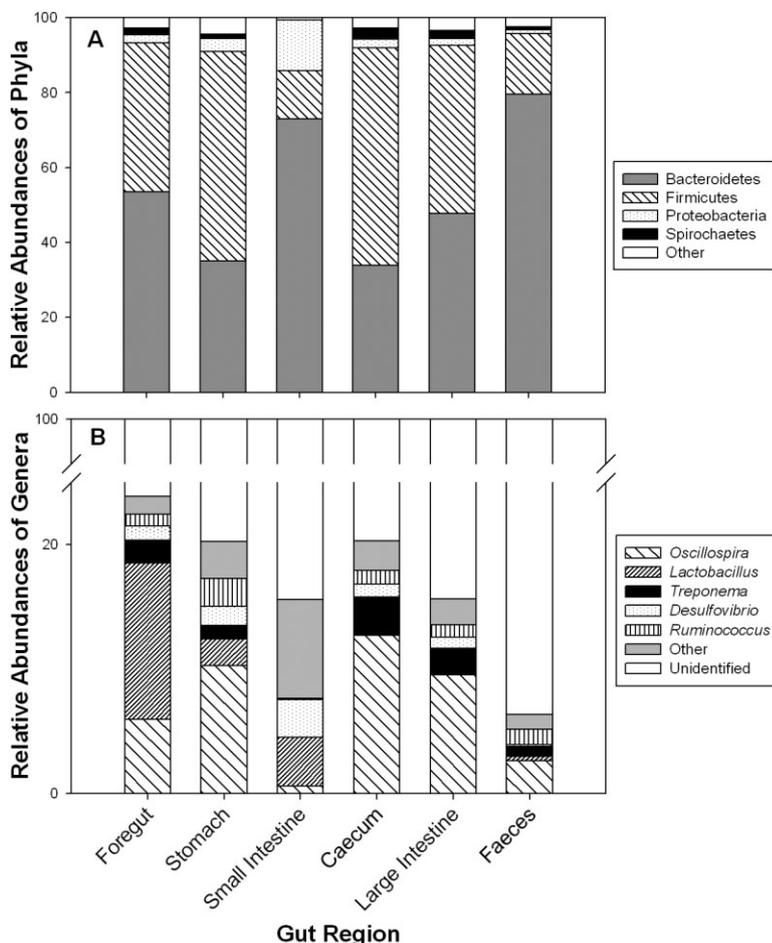
Fig. 3. Relative abundances of dominant bacterial (A) phyla and (B) genera in the gut of *N. albigula*. Rare genera that were assigned with confidence were grouped together.

Table 3. Statistics from ANOVA testing differences of relative abundances of dominant bacterial phyla between gut chambers.

Phylum	*P*
Bacteriodetes	**< 0.001**
Firmicutes	**< 0.001**
Proteobacteria	NS
Spirochaetes	NS
Tenericutes	**0.007**
Cyanobacteria	**0.015**
Elusimicrobia	NS
Deferribacteres	NS
Actinobacteria	**0.008**
Verrucomicrobia	NS
Fusobacteria	NS
TM7	NS

P values have been corrected using the false discovery rate control. Significant differences are in bold.

Moreover, the microbial density of the foregut was similar to the cow rumen (Stevens and Hume, 2004).

Despite similar cell densities, the concentrations of several isolated VFAs, as well as total VFAs, were higher in the foregut chamber than in the caecum. These metabolites are produced by microbial fermentation of cellulose and other carbohydrates. Foregut VFA concentrations were in the upper range of values measured in a variety of other mammalian herbivores and were greater than those in foregut fermenting herbivores, such as cattle, sheep, kangaroos and sloths (Stevens and Hume, 2004). Further studies should investigate the contributions of foregut fermentation to the energy budget of rodents. Additionally, future studies should investigate the nutritional substrates in the foregut, as well as whether primarily bacteria, or commensal protozoa and fungi accomplish fermentation.

Concentrations of NH_3-N were also higher in the foregut than in the caecum. Ammonia is another index of fermentative digestion and is produced by the microbial degradation of proteins and amino acids. Furthermore, it is indicative of considerable recycling of endogenous urea. Many herbivores recycle urea to conserve nitrogen when

feeding on low-nitrogen plant material (Stevens and Hume, 2004). The extent of urea recycling in the rodent foregut demands further study.

The concentration of microbial metabolites in the foregut is striking given the short residence time of food in this chamber. Many other foregut-fermenting mammals retain food in microbial chambers for extended periods of time to increase the digestion of fibre and liberation of nutrients for absorption in the small intestine (Stevens and Hume, 2004). In contrast, dietary items did not reside in the woodrat foregut for more than 1.5 h, yet we documented indicators of high microbial activity. The rates of microbial processes in the foregut may be rapid and demand further investigation. However, the microbiota may have been fermenting the simple sugars that are present in high abundance in cactus (El Kossori *et al.*, 1998), and not necessarily fibre. Future studies should investigate the substrates of the microbiota.

The dominant bacterial taxa in the woodrat gut varied across regions. The phyla Bacteroidetes and Firmicutes comprised the majority of communities across the gut, similar to other mammalian systems (Ley *et al.*, 2008). However, the abundance of Firmicutes was much
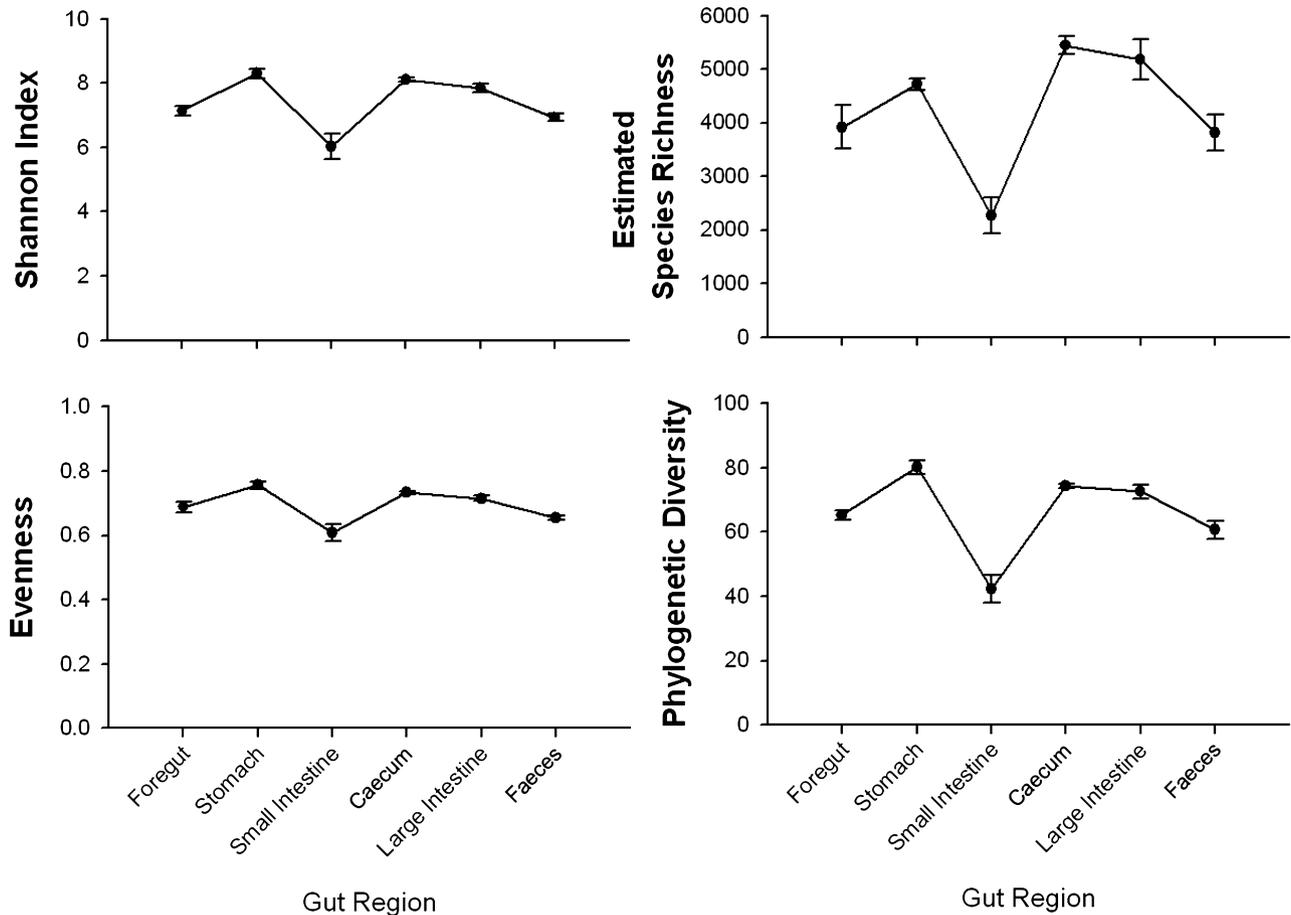
Fig. 4. Various diversity measurements of gut regions of *N. albigula*. The Shannon index is a metric that incorporates both richness and evenness. Estimated species richness was calculated using the Chao1 metric, which estimates the number of OTUs as the asymptote of a species accumulation curve. Evenness measures the variation in relative abundances of OTUs. A community where all OTUs are present in equal relative abundances has an evenness of 1. Phylogenetic diversity measures the cumulative branch lengths from randomly sampling OTUs from each sample.

reduced in the small intestine and faeces compared with other gut regions. At the genus level, *Oscillospira* was the most dominant identified genus of the woodrat gut. Although the functional capabilities of the uncultivable genus *Oscillospira* have not been determined, it is likely that it plays a role in fibre fermentation due to its presence in numerous rumen systems and its greater abundance in hosts that are fed fresh forage (Mackie *et al.*, 2003). This genus along with another cellulolytic genus, *Ruminococcus*, was present throughout the gut, but had low abundance in the small intestine. *Lactobacillus* comprised a substantial portion of the foregut community (~12.5%), but was not as abundant in other regions (< 4%). The dominance of *Lactobacillus* in the woodrat foregut has been documented for two other woodrat species, *Neotoma bryanti* and *Neotoma lepida* (Kohl and Dearing, 2012), and thus may be a common pattern in the gut communities of *Neotoma*. The genus

Lactobacillus does not perform extensive fibre fermentation, but may be fermenting the simple sugars present in the foregut.

An immense amount of diversity existed in the woodrat gut in the form of rare and unidentified taxa. Approximately 50 identified genera collectively comprised less than 8% of the community of any gut region. We were unable to identify roughly 75–90% of sequences in any gut region at the genus level, and these sequences contained thousands of OTUs. Thus, the woodrat gut represents an extensive source of novel bacterial genera and species. This finding supports previous studies showing a high amount of novel sequences from the faecal microbial community of *N. bryanti* (Kohl *et al.*, 2011). Across mammals, herbivores exhibit the highest microbial diversity, and different mammalian clades harbour distinct communities (Ley *et al.*, 2008; Pope *et al.*, 2010). Other herbivorous rodents have not been
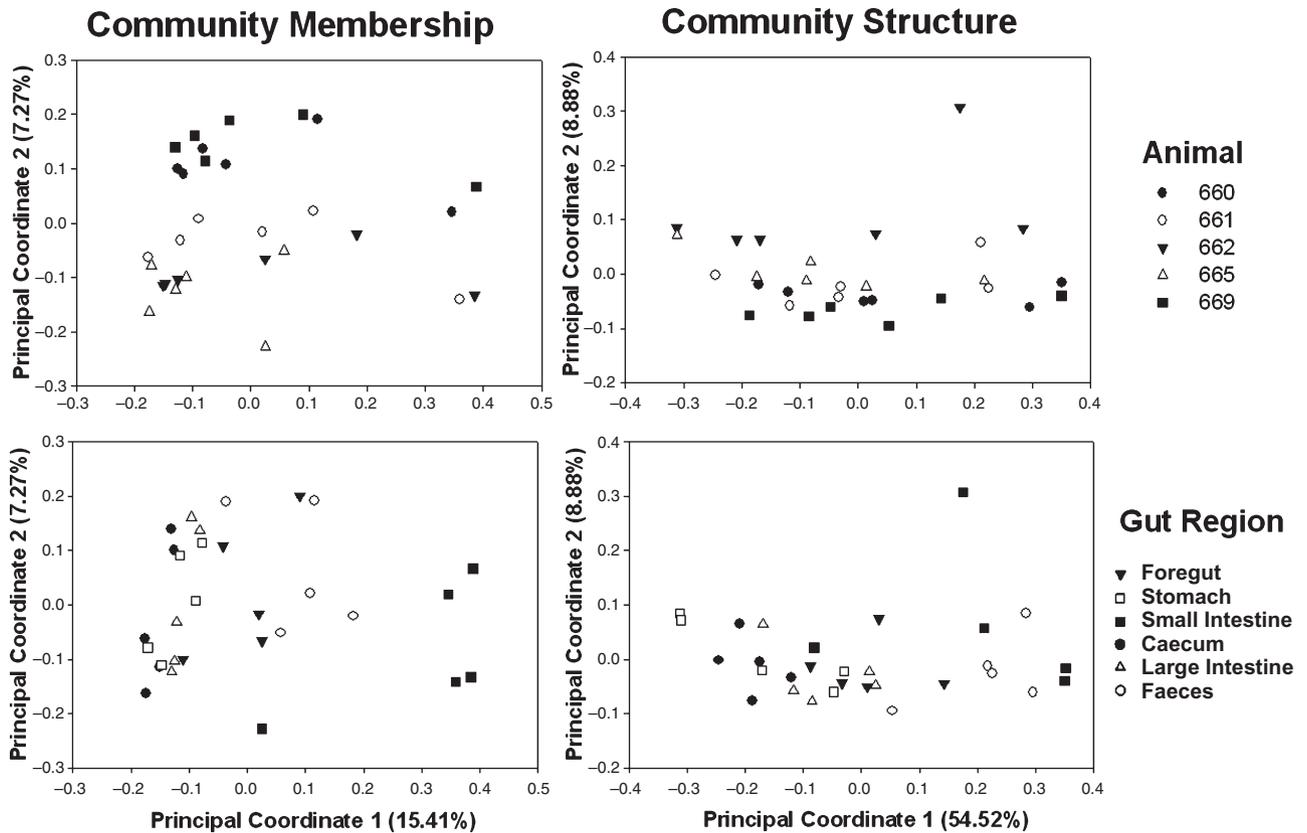
Fig. 5. Principal coordinate analysis of samples from *N. albigula*, grouped by either individual animal or woodrat gut region. Community membership, or the presence and absence of bacterial lineages, was primarily driven by gut region (adonis: $R^2 = 0.28$; $P = < 0.001$) and also by the individual animal ($R^2 = 0.05$; $P = 0.02$). Community structure, which takes relative abundances of taxa into account, was only driven by gut region ($R^2 = 0.55$; $P = < 0.001$) and not by individual animal ($R^2 = 0.04$; $P = 0.27$).

extensively studied in terms of microbial communities, which may explain this large amount of novelty.

Measurements of diversity varied significantly across gut regions. The small intestine harboured the lowest diversity in all metrics. This trend is likely due to the high activity of the immune system and the high flow rate within the small intestine (Lin, 2004). Surprisingly, the stomach often had the highest estimates of diversity despite its low pH and low density of live cells. However, 16S inventories do not differentiate live cells from dead cells, and so the stomach inventories may combine the resident live population of stomach microbes, as well as sequences from dead foregut microbes found within the stomach. The use of cell sorting based on cell wall integrity and 16S inven-
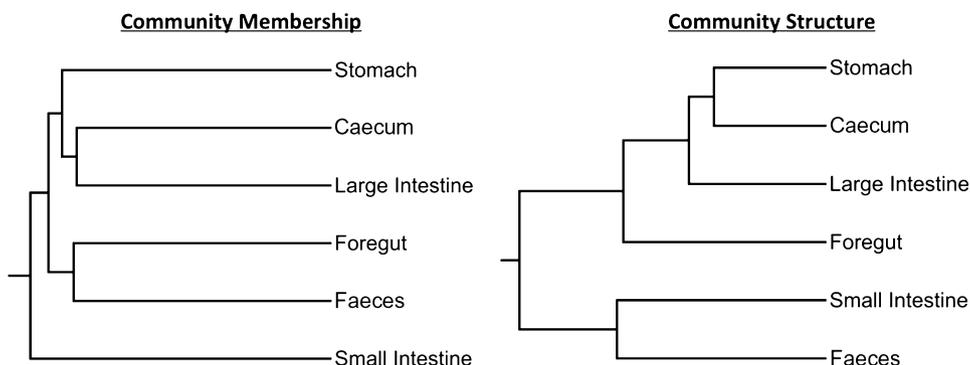

Fig. 6. UPGMA clustering of gut regions from *N. albigula* according to community composition and community structure. All nodes have jackknife support of 1.

tories of resulting 'live' and 'dead' populations would help understand the role of 'dead' cells in biodiversity estimates from whole samples.

Gut chamber influenced both bacterial community membership and structure. Regulation of bacterial communities among gut regions is likely driven by a number of factors, such as nutrient concentrations, immune responses, oxygen concentrations and flow rate. Additionally, the individual animal source determined bacterial community membership, suggesting that individuals have their own unique bacterial communities throughout gut regions. This observation may be driven by genotypic differences between individuals, such as immunity genes (Toivanen *et al.*, 2001) or unique production of glycans, yielding novel energy sources or binding areas (Hooper *et al.*, 2001). Alternatively, individual differences may be a result of founder effects. Mammals obtain their microbial community through contact with maternal faecal and vaginal microbes during the birthing process (Palmer *et al.*, 2007). The importance of this one-time exposure is highlighted by differences in the microbial community structure of conventionally and caesarean-delivered humans from infancy through childhood (Salminen *et al.*, 2004; Dominguez-Bello *et al.*, 2010). These founder effects might determine microbial communities and yield individual variation.

Bacterial communities were rarely similar between adjacent gut regions. Rather, communities were more similar between disparate gut regions. For example, the stomach and caecum have similar bacterial community structure, despite being separated by the small intestine and having radically different pH values. This suggests that there are complex interactions between various environmental characteristics (pH, flow rate, nutrient composition, immunity gene expression, etc.) that determine the community of any gut region.

Interestingly, the community in the faeces was not most similar to that of the large intestine in terms of community composition or structure. This difference could be due to exposure to oxygen upon leaving the gastrointestinal tract. Rather, faecal inventories were most similar to foregut communities in terms of bacterial community membership. We hypothesize that this similarity is driven by woodrats engaging in coprophagy (Kenagy and Hoyt, 1980). This behaviour may alter microbial community structure throughout the gut and may offer a constant source of microbial inoculation.

The results suggest that inventories from faecal material should be interpreted with caution. Researchers should refrain from extrapolating faecal inventories as indicators of microbial diversity of specific gut regions and should instead rely on direct sampling. In woodrats, there is a slight bias in terms of community structure. However, faecal inventories may still be useful to researchers. In woodrats, they seem to be somewhat indicative of the rest of the gut in terms of microbial community membership. Additionally, faecal inventories are informative when comparing treatment groups or species within a study, and provide the opportunity to conduct repeated sampling of an individual or collect non-lethal samples.

These data represent some of the first supporting the hypothesis that the rodent foregut serves to house an active microbial community, an idea that has existed for over a century (Toepfer, 1891; Carleton, 1973). The activity of this microbial population is striking given the small size and short residence time of this chamber. We hypothesize that the rodent foregut microbial community is important for several functions, such as the initial digestion of fibre, recycling of endogenous nitrogen and detoxification of dietary toxins.

Experimental procedures

Animals

Five individuals of *Neotoma albigula* were collected from Castle Valley, UT, on 17 October 2012. Animals were captured using Sherman live traps baited with peanut butter and oats, and were immediately transported back to the University of Utah Animal Facility. Woodrats were given cactus (*Opuntia spp.*) and juniper foliage (*Juniperus osteosperma*) *ad libitum* for one night. These are the dominant plants in Castle Valley and represent the natural diet (Macêdo and Mares, 1988). The following morning, woodrats were euthanized under CO_2 and dissected. We measured the mass of contents found within the foregut, stomach, small intestine, caecum and large intestine. Contents from each of these sections, as well as faecal samples, were collected and divided for various uses described below.

For measurements of gut transit time (described below), we used six individuals of *N. lepida* that were already in our colony, collected in July 2011 from Beaver Dam, Washington County, UT. Using these animals was preferable to collecting additional animals from their natural habitat. *Neotoma lepida* are similar to *N. albigula* in terms of size and gastrointestinal anatomy (Carleton, 1973). Also, congeneric rodents tend to display similar gut transit times (Kostelecka-Myrcha and Myrcha, 1964). The University of Utah Institutional Animal Care and Use Committee approved all experimental techniques under protocol 12–12010.

pH

Fresh contents from *N. albigula* were used immediately following dissection to measure the pH of various gut regions with an Omega Soil pH electrode (PHH-200).

Flow cytometry

Microbial density, as well as the proportion of live, injured and dead cells, were determined with the LIVE/DEAD BacLight Bacterial Viability and Counting Kit (Life Technologies, Grand

Island, NY, USA). Optimal filter settings were determined using a variation of the standard protocol. Woodrat faeces were cultured overnight in heart-brain infusion broth. Microbial cells were pelleted, and a subset was killed with 30 min incubation in isopropyl alcohol. Mixtures of live and dead cells were created to determine boundaries of gates for 'live' and 'dead' cells. During analysis of actual gut contents, cells falling between these regions were assigned as 'injured'.

Immediately following dissection, fresh gut contents from *N. albigula* were diluted with nine parts physiological saline and passed through 50 μm mesh cell strainers into tubes. Ten microlitre of filtrate was further diluted in 990 μl saline and stained with 3 μl of a mixture of components A and B from the LIVE/DEAD *Bac*Light Bacterial Viability and Counting Kit. Mixtures were incubated in the dark at room temperature for 30 min to allow for complete staining. Flow cytometric measurements were performed on a BD Biosciences FACSCanto II Flow Cytometer (San Jose, CA, USA) with 488 nm excitation. SYTO 9 and propidium iodide were measured through 530/30 and 695/40 filters respectively.

Volumetric measurement was determined by calibrating instrument flow rate with Spherotech Accucount Particles (ACFP 70–5; Lake Forest, IL, USA) as per manufacturer's guidelines. Briefly, clearly distinguishable fluorescent beads of a known concentration were acquired using the same flow rate as test samples to determine the volumetric flow rate per minute of the instrumentation.

Microbial metabolites

A portion of contents of the foregut and caecum from *N. albigula* were preserved for the analysis of VFAs and ammonia nitrogen (NH_3-N) by placing contents in an equal volume of 1 M NaOH or 1 M HCl respectively. Samples were frozen and transported to the University of Illinois. VFAs were measured using gas chromatography (Erwin *et al.*, 1961), and ammonia was determined by the indophenol method (Chaney and Marbach, 1962).

Gut transit time

To measure movement of digesta through various gut regions, we used six individuals of *N. lepida* fed with powdered rabbit chow (Harlan Teklad 2031, Madison, WI, USA) containing 2% (w/w) of coloured, inert plastic markers (1 mm diameter). Animals were given one colour of diet for a 24 h period, and colours were then switched every 1.5 h for 12 h during the animals' dark cycle. This interval was chosen because woodrats generally consume a meal every 1.5 h (Torregrosa *et al.*, 2012). Animals were then euthanized and dissected. The location of all plastic markers was determined, and we calculated the median, quartile, maximum and minimum locations through the gut for the contents of each meal. These values were averaged across individuals.

Bacterial inventories

Bacterial inventories were conducted on the samples collected from *N. albigula*. Frozen gut contents were thawed and whole DNA was isolated using a QIAamp DNA Stool Mini Kit (Qiagen, Germantown, MD, USA). Extracted DNA was sent to Argonne National Laboratories for sequencing. Bacterial inventories were conducted by amplifying the V4 region of the 16S rRNA gene using primers 515F and 806R, and paired-end sequencing on an Illumina MiSeq platform (Caporaso *et al.*, 2012).

Sequences were analysed using the QIIME software package (Caporaso *et al.*, 2010). Sequences underwent standard quality control and were split into libraries using default parameters in QIIME. Sequences were grouped into OTUs using UCLUST (Edgar, 2010) with a minimum sequence identity of 97%. The most abundant sequences within each OTU were designated as a 'representative sequence', and then aligned against the core set of Greengenes 13_5 (DeSantis *et al.*, 2006) using PyNAST (Caporaso *et al.*, 2009) with default parameters set by QIIME. A PH Lane mask supplied by QIIME was used to remove hypervariable regions from aligned sequences. FASTTREE (Price *et al.*, 2009) was used to create a phylogenetic tree of representative sequences. Sequences were classified using the Ribosomal Database Project (RDP) classifier with a standard minimum support threshold of 80% (Wang *et al.*, 2007). A comparison of taxon assignment by RDP and the Greengenes database showed similar results, with RDP performing slightly better. Sequences identified as chloroplasts or mitochondria were removed from analysis.

Several diversity measurements were calculated for each sample. We calculated the Shannon diversity index, a biodiversity measure that incorporates both richness and evenness. We also calculated an estimate of species richness (Chao1) and evenness, or how similar in abundance the OTUs in a sample are. However, these diversity metrics equally weight all OTUs regardless of phylogenetic relationships. Therefore, we calculated a measurement of phylogenetic diversity (Faith, 1992), which measures the cumulative branch lengths from randomly sampling OTUs from each sample. For each sample, we calculated the mean of 20 iterations for a subsampling of 7700 sequences.

We also compared community membership (presence or absence of bacterial lineages) and community structure (taking into account relative abundance of OTUs) of various gut regions. We calculated unweighted (for community membership) and weighted (for structure) UniFrac scores, and conducted PCoA (Hamady *et al.*, 2010). To compare the similarity of gut communities of different regions, we combined sequences within gut regions (across individuals) and conducted UPGMA hierarchical clustering of both unweighted and weighted UniFrac scores. Jackknife support of nodes in UPGMA trees were determined using default settings within QIIME. All sequences were deposited in NCBI's Sequence Read Archive under accession SRP022360.

Statistics

Microbial metabolites were compared between the foregut and caecum using paired *t* tests. Our sample size was insufficient to conduct repeated-measures analysis of variance (ANOVA) across all six gut regions. Relative abundances of bacterial taxa were compared across gut regions using ANOVA, and *P* values underwent Bonferroni correction. Biodiversity metrics were compared across gut regions within

an individual using the Friedman test, which is a non-parametric test for one-way repeated measures analysis and allows for a more conservative estimate of differences between gut regions. We investigated the effects of individual animal and gut region on bacterial community membership and structure using the adonis function in QIIME with 999 permutations.

Acknowledgements

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Percentage of live, injured and dead microbial cells in each gut region of *N. albigula*.