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Original Investigation

Microbial diversity in forestomach and caecum contents of the greater long-tailed hamster *Tscherskia triton* (Rodentia: Cricetidae)

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ABSTRACT

The mammalian gastrointestinal tract has evolved to facilitate food utilisation. Hamsters within the subfamily Cricetinae (Rodentia, Cricetidae) have a compartmentalised stomach consisting of a forestomach and a glandular stomach. The role of the forestomach in hamsters has long been discussed. In this study, we evaluated the microflora and volatile fatty acid contents of the large forestomach and caecum of a greater long-tailed hamster (*Tscherskia triton*). The estimated bacterial biodiversity in the forestomach based on 16S rRNA library sequencing analyses was low (sequence $n = 226$, Shannon index $H' = 2.12$) compared to that in the rumen of ruminants. In contrast, the bacterial diversity in the caecum was very high ($n = 259$, $H' = 4.45$), and comparable to that of other hindgut fermenters. The forestomach bacterial flora was dominated by *Lactobacillus* spp. (179/226 clones), and high concentrations of lactic acid were observed in the forestomach. These results indicate that the forestomach does not function like the rumen of ruminants, but that lactic acid fermentation does take place in this compartment. The caecum is thought to play a more important role in food digestion via fermentation by symbiotic microbes than the forestomach. When all cloned 16S rRNA gene sequences ($n = 485$) were grouped into operational taxonomic units (OTUs) with 97% similarity, the majority of clones isolated from the forestomach contents were also present in the caecum (85.4%), although fewer clones isolated from the caecum contents shared OTUs with clones from the forestomach (19.3%). Based on these results, we hypothesise that bacteria were transferred from the caecum to the forestomach by coprophagy.

Introduction

Mammalian food habits can be grouped into four major categories; insectivore, carnivore, herbivore, and omnivore (Feldhamer et al., 2007). Of these categories, insectivorous and carnivorous mammals typically have a simple gastrointestinal tract, while herbivorous mammals have a complex digestive tract (Chivers and Langer, 1994; Stevens and Hume, 1995). The digestion of dietary plant cell walls necessitates a more complex gastrointestinal tract because a chamber is required where microbes can ferment fibre. Depending on the location of the fermentation chamber, herbivorous mammals are further divided into two groups: foregut and hindgut fermenters (Feldhamer et al., 2007). The foregut fermenters (for example ruminants) have voluminous and highly compartmentalised stomachs (Stevens and Hume, 1995). In contrast, the hindgut fermenters (for example equids or lagomorphs) have a simple stomach but usually a long intestine and/or caecum as a fermentation chamber (Stevens and Hume, 1995). In both cases, various microbes inhabit the fermentation chambers and contribute to food digestion and nutrient supply (Chivers and Langer, 1994; Stevens and Hume, 1995; Ley et al., 2008).

The order Rodentia is the most successful taxon in terms of ecological distribution and food utilisation. Rodents are generally herbivores, although some are omnivores or carnivores (Landry, 1970; Stevens and Hume, 1995; Feldhamer et al., 2007; Wilman et al., 2014). The gastrointestinal tract of rodents shows similar features to that of hindgut fermenters; the hindgut (colon and caecum)

Abbreviations: HPLC, high-performance liquid chromatography; OTU, operational taxonomic unit; VFA, volatile fatty acid.

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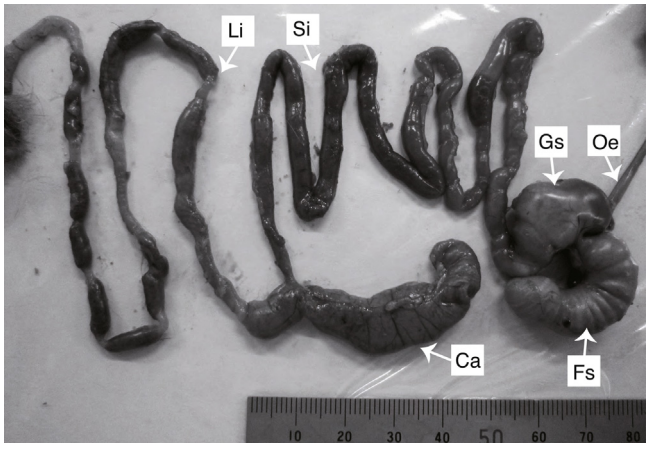

Fig. 1. Photograph of the forestomach and caecum of the greater long-tailed hamster (*Tscherskia triton*). Oe, oesophagus; Fs, forestomach; Gs, glandular stomach; Si, small intestine; Ca, caecum; Li, large intestine.

is complex with many spiral folds, recesses, and saclike expansions to facilitate efficient microbial fermentation (Chivers and Langer, 1994; Björnhag and Snipes, 1999; Sakaguchi, 2003). In contrast to the complexity of the hindgut, the stomach of rodents is usually simple; however, some rodents have compartmentalised stomachs consisting of a forestomach and a glandular stomach (Vorontsov, 1962; Carleton, 1973; Chivers and Langer, 1994; Stevens and Hume, 1995; Kohl et al., 2011, 2013). Compartmentalised stomachs are often found in herbivorous or omnivorous muroid rodents, such as voles and hamsters (family Cricetidae), although these animals also have a well-developed caecum that may function as a fermentation chamber.

The stomach of some hamster species (subfamily Cricetinae) is quite developed and bilocular-hemiglandular (Vorontsov, 1962). Indeed, the forestomach and glandular stomach of some hamsters are clearly compartmentalised with marked constrictions (Fig. 1). The role of the forestomach in hamsters has been discussed for over a century (e.g. Toepfer, 1891; Kunstýř, 1974; Ehle and Warner, 1978; Sakaguchi et al., 1981; Chivers and Langer, 1994; Dehority 1997; Shichijo et al., 2013b). The forestomach and glandular stomach are histologically different (Hoover et al., 1969; Sakata and Tamate, 1976), and their pH values differ significantly (Smith, 1965; Hoover et al., 1969; Kunstýř, 1974; Kararli, 1995). In addition, volatile fatty acids (VFAs) have been detected in the forestomach (Hoover et al., 1969). This accumulated evidence suggests that the forestomach plays a role in food utilisation via microbial fermentation.

Recent molecular techniques using 16S ribosomal RNA (16S rRNA) gene markers have revealed that the symbiotic microbiomes in the mammalian gastrointestinal tract are highly diverse, and many constituent taxa are difficult to detect using traditional culturing methods (e.g. Krause and Russell, 1996; Zoetendal et al., 2008; Tomas et al., 2012). In addition, the bacterial diversity in the gut of domestic and wild large mammals tends to be higher in herbivores than in carnivores and omnivores (Ley et al., 2008). Moreover, 16S rRNA library-based analyses of the microbiomes of ruminants have demonstrated high biodiversity in their rumen, revealing complex ecosystems (Krause and Russell, 1996; Whiteford et al., 1998; Tajima et al., 1999, 2000; Edwards et al., 2004; Yang et al., 2010a,b). Therefore, we hypothesised that if the forestomach of hamsters has an analogue function to the fermentation chambers of larger mammalian herbivores, then the level of bacterial diversity should be similarly high. In this study, we assessed levels of bacterial diversity in the greater long-tailed hamster (*Tscherskia triton*), which has a very large forestomach (Fig. 1), and is classified as a herbivorous/omnivorous (granivorous) species (Wang et al., 1996). We compared bacterial diversity of the forestomach with that of the caecum, as well as with that of the rumen of ruminants. We also compared VFA and lactic acid levels in forestomach and caecum contents.

Material and methods

Animals used in this study

In total, 12 adult greater long-tailed hamsters (*Tscherskia triton*) were used in this study (one for sequencing analyses, and 11 for VFA and lactic acid measurements). The hamsters were obtained from the breeding stock of the Frontier Science Research Center, University of Miyazaki, Japan. The hamsters were provided with standard laboratory rodent chow (CE-2, CLEA Japan Inc., Tokyo, Japan, or Labo MR stock, Nihon Nosan Kogyo, Tokyo, Japan: nutrient contents of these chow are provided by Supplementary Table S1) and tap water *ad libitum*. All experimental procedures in this study were approved by the Committee for the Ethics of Animal Experiments at the University of Miyazaki (Permission Nos. 2008-505 and 2010-512).

16S rRNA library sequencing

To avoid the dilution effect, and to perform a detailed taxonomic comparison of the bacterial communities in the forestomach and caecum contents of the same individual, we sequenced a number of clones with a longer nucleotide sequence from one individual rather than a pool of several samples (Monteils et al., 2008). With careful health monitoring, one healthy adult female hamster was euthanised, the chest was opened immediately, and the border between the forestomach and the glandular stomach was ligated to prevent mixing of their contents. The stomach and caecum (Fig. 1) were removed and the forestomach and caecum contents were transferred to freezer vials. The samples were stored at −80 °C until DNA extraction. To avoid environmental bacterial contamination, all surgical instruments were washed and sterilised before use.

Total DNA was extracted from samples using ISOFECAL for Bead Beating (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. Construction of the 16S rRNA libraries was performed as described by Ley et al. (2008). Bacterial 16S rRNA genes (ca. 1300 bp) were amplified with the universal primer pair 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1391R (5′-GACGGGCGGTGWGTRCA-3′) using the following PCR conditions: 94 °C for 2 min, followed by 20 cycles of 94 °C for 1 min, 55 °C for 45 s, and 72 °C for 2 min, with a final extension period of 20 min at 72 °C. The PCR products were gel-purified using a GENE CLEAN II kit (Funakoshi, Tokyo, Japan), cloned into pGEM-T vector (Promega, Tokyo, Japan), and transformed into *Escherichia coli* JM109 competent cells (Promega). From each library, more than 200 colonies containing cloned amplicon were processed for sequencing. Plasmid inserts were sequenced in both directions with vector-specific primers using a Big Dye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, Foster City, CA, USA) and a genetic analyser (Model 3130, Applied Biosystems).

In silico analyses of cloned 16S rRNA sequences

Obtained sequences (234 and 268 clones from forestomach and caecum samples, respectively) were aligned using the online alignment tool of the ribosomal database project (RDP; Cole et al., 2009; https://rdp.cme.msu.edu). Chimeric sequences were then detected using the UCHIME algorithm (Edgar et al., 2011) implemented in the 16S rRNA analyses software package Mothur v. 1.31.0 (Schloss et al., 2009) with reference to the gold database (downloaded from UCHIME author's HP: http://drive5.com/usearch/manual/uchime_algo.html). To detect chimeric