

Original paper

Volatile fatty acids and microflora composition in the digestive tract of the East European vole (*Microtus levis*)

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Abstract: Herbivorous rodents are essentially hindgut fermenters, but some rodents have a compartmentalized stomach (forestomach and glandular stomach) in addition to a cecum. To elucidate the digestive mechanisms of such grass-eating rodents, we evaluated the production of volatile fatty acids (VFAs) as well as lactic acid by the forestomach and cecum of the East European vole (*Microtus levis*). In addition, we compared the microflora of both forestomach and cecum contents using 16S rRNA V3-V4 region amplicon analyses. We detected similar levels of VFAs and lactic acid from both the forestomach and cecum of the vole; acetic acid was most abundant (3,687–6,441 μg/g), and propionic acid (0–1,228 μg/g), butyric acid (619–2,124 μg/g), and lactic acid (71–1,613 μg/g) were also detected. No significant differences were observed between the contents of the forestomach and cecum. On the other hand, bacterial microflora differed between these portions of the intestine, even at the phylum level. Firmicutes was predominantly detected in the forestomach (93.3%), whereas Bacteroidetes (33.4%) and Firmicutes (59.0%) were dominant in the cecum. These results indicate that fermentation occurred in both the forestomach and cecum but was accomplished by different communities of microbiota.

I. Introduction

Herbivores are the most abundant trophic level of mammals in both number and species richness. The success of mammalian herbivores is due in part to an extremely efficient masticatory apparatus and several major adaptations of the gastrointestinal tract (Stevens & Hume 1995). Herbivorous mammals are typically equipped with one or more fermentative chambers in their gastrointestinal tract, and they use microbial fermentation to acquire nutrition and energy from plant materials. Based on their gastrointestinal tract characteristics, herbivorous mammals can be divided into four major groups: foregut fermenters (e.g., ruminants), large hindgut fermenters (e.g., horses), small hindgut fermenters (e.g., rabbits and herbivorous rodents), and others (e.g., "carnivorous" herbivores such as the giant panda) (Stevens & Hume 1995). Foregut fermenter mammals have voluminous forestomaches for decomposing plant fibers to yield volatile fatty acids (VFAs) with the aid of enzymes produced by symbiotic microbes (Stevens & Hume 1995, 1998). The VFAs are then absorbed as energy sources. The large hindgut fermenters use the voluminous cecum or colon for similar purposes as foregut fermenters. Small hindgut fermenters also have relatively large ceca, with a complex structure composed of sacculations, haustrations, and spiral folds. VFAs produced are primarily absorbed in the cecum; however, the surplus of bacteria and many of the available nutrients (e.g., vitamins and proteins) produced are lost in the feces, as these mammals do not possess the ability or adequate length of the large intestine to use these byproducts. Therefore, such small hindgut fermenters recover these microbial substances via coprophagy (eating feces), passing them through the gut a second time (Chivers & Langer 1994).

The voles (Rodentia; Cricetidae; Arvicolinae) are grass eaters (Wilson et al. 2017) and are essentially small hindgut fermenters. Interestingly, voles often have a compartmentalized stomach in addition to a large cecum (Vorontsov 1962, Chivers & Langer 1994, Stevens & Hume 1995). The proximal portion (forestomach) is usually constituted by non-glandular stratified squamous epithelium. The division of the stomach into two (or more) partially separated chambers and the reduction in the amount of glandular mucosa presumably maintain a higher pH in the fornix

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ventricularis or forestomach, which allows cellulolytic fermentation to proceed (Chivers & Langer 1994). Therefore, it is notable that voles might use both foregut and hindgut fermentation. Hindgut fermentation is essential for rodents; however, the role of the forestomach in rodents remains poorly understood, despite continuous discussion for over a century (Toepfer 1891, Kunstýř 1974, Ehle & Warner 1978, Sakaguchi et al. 1991, Chivers & Langer 1994, Shichijo et al. 2013, Shinohara et al. 2016). Traditionally, it has been widely hypothesized that the forestomach in rodents plays a role in food digestion and preservation via microbial fermentation (review in Gärtner 2001). Interestingly, the forestomach has also been shown to function in microbial detoxification of plant secondary compounds in the desert woodrat (*Neotoma* spp.), which eats creosote (Kohl et al. 2014). Furthermore, several reports have examined the microflora and/or amounts of VFAs in the forestomach of Cricetidae rodents (Kohl et al. 2011, 2014, 2016, Shinohara et al. 2016, Allan et al. 2018). These results suggest that foregut microbial diversity differs among species and is affected by diet and captivity status. However, the number of taxa analyzed is limited; thus the means by which herbivorous rodents use microbial fermentation in the forestomach remains unclear.

The East European vole (or the Southern vole) *Microtus levis*, formerly classified as *M. rossiaemeridionalis*, is a member of the *M. arvalis* (Musser & Carleton 2005) or *M. mystacinus* species group (Wilson et al. 2017). This species was domesticated as a laboratory animal by the Russian Academy of Science in the 1990s from wild individuals collected from Sankt Peterburg, Leningrad Obalst, Russia (see Widayati et al. 2003) and has been widely used in a variety of disciplines of the life sciences. *Microtus levis* was imported into Japan as a laboratory animal in 2000 (Widayati et al. 2003). This vole is mostly herbivorous; hence, it serves as an ideal animal experimental model of herbivorous rodents (e.g. Naumova et al. 2001, Chistova et al. 2007, Manaeva et al. 2012). Interestingly, this vole species does not often engage in coprophagy, unlike hamsters, which also have both a forestomach and cecum (Ebino 1993), suggesting that the forestomach does not function in the second passing of feces. In addition, previous studies have suggested that cellobiohydrolase activity as well as nitrogen fixation occur in the gastrointestinal tract of *M. levis* (Manaeva et al. 2012, Varshavskiy et al. 2014). These activities are likely due to symbiotic bacterial fermentation; however, the precise microflora community remains unknown.

In the present study, we compared the VFA components and microflora between the forestomach and cecum of the East European vole to document the role of forestomach fermentation in herbivorous rodents.

II. Materials and Methods

Animals

East European voles maintained at the Department of Zoology, Okayama University of Science, were used for the study. Voles were fed a commercial pellet diet formulated for mice and rats (Labo MR Breeder, Nosan Corporation, Yokohama, Japan) and timothy hay (Super-premium Timothy, Leaf Corporation, Sano, Japan), with tap water ad libitum. The temperature of the breeding room was kept at approximately 25°C without humidity control. The light–dark cycle was 12L:12D (photophase 8:00–20:00). All animal experiments were conducted in accordance with the regulations for animal experiments of the Okayama University of Science. Our experimental protocols were approved by the Animal Experiments Committee of the Okayama University of Science (No. 20150604–01).

VFA measurements

Four adult males (2 months of old) were used for quantitative measurements of VFAs and lactic acid in the forestomach and cecum. The animals were euthanized, and digestive tracts were immediately removed from the body. The forestomach and cecum were ligated to keep them separate and avoid mixing the contents from adjacent intestinal parts; the contents were then collected separately. Each aliquot (0.1 g) was suspended in 10 ml of H_2O, vortexed, and centrifuged at 4ºC, 10,000 rpm, 30 min; 1 ml of supernatant was analyzed for VFAs (acetic acid, propionic acid, and butyric acid). An additional 0.5 ml of the supernatant was mixed well with 0.5 ml of ADAM acetone (0.01 vol%), placed in the dark at room temperature for 1 h, and then analyzed for lactic acid. VFA concentrations were determined using the Agilent 7890A gas chromatograph system (Agilent Technologies, Santa Clara, CA, USA), and lactic acid was measured using Agilent 1260 Infinity HPLC (Agilent Technologies). The Wilcoxon rank sum test was applied using Rcmdr package version 2.5-1 (Fox 2005) with R version 3.5.1 (R Core Team 2018) for comparisons between groups.

Microflora analysis

Samples from the forestomach and cecum were collected from one individual of the East European vole using the same method as that for the VFA measurements described above. An Extrap Soil DNA Kit Plus version 2 (Nippon Steel &

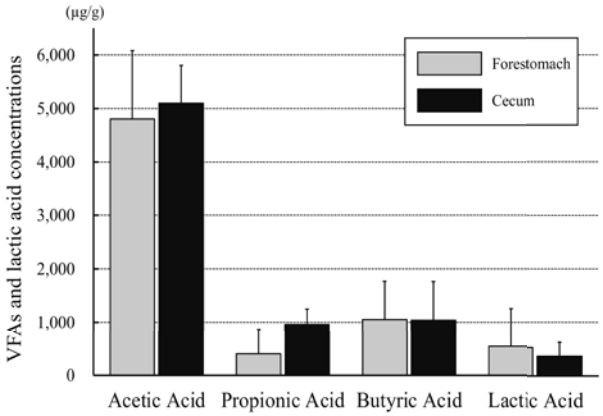

Fig. 1. Concentrations (average ± SD, μg/g) of volatile fatty acids (VFAs) and lactic acid in the forestomach and cecum contents from four adult males of the East European vole (*Microtus levis*).

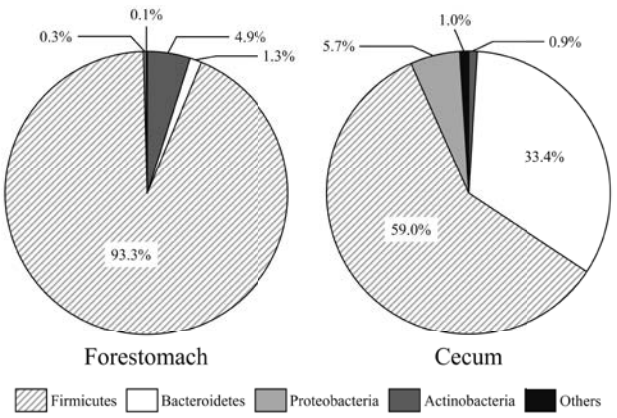

Fig. 2. Relative abundances (%) of obtained bacterial 16S rRNA gene sequences isolated from the forestomach and cecum contents of the East European vole (*Microtus levis*) classified at the phylum level.

Sumikin Eco-Tech Corporation, Tokyo, Japan) was used for DNA extraction. Primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 802R-mix (5′-TACNVGGGTATCTAATCC-3′, 5′-TACCAGAGTATCTAATTC-3′) were used for polymerase chain reaction (PCR) amplification of the hypervariable V3–V4 regions in 16S rRNA. After purification, the PCR products were analyzed using a 2100 Bio-analyzer (Agilent Technologies, Santa Clara, CA, USA) to confirm the absence of nonspecific amplification products, and their concentrations were measured using a PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Concentration-adjusted PCR products were sequenced using a MiSeq sequencer (Illumina, San Diego, CA, USA). Paired-end sequencing was performed on the PCR products with an interval of approximately 250 bp from both ends. The sequences were overlapped to obtain sequence data from a length of about 430 bp. These experiments were conducted at the Nippon Steel & Sumikin Eco-Tech Corporation (Tokyo, Japan). The data obtained in this study were deposited into the DDBJ sequence read archive (DRA) with accession numbers PRJDB8382.

The sequence data sets were then analyzed using Mothur v.1.40.3 (Schloss et al. 2009), following the standard operational protocol (SOP) for MiSeq 16S rRNA amplicon data analyses (Kozich et al. 2013, MiSeq SOP; https://www.mothur.org/wiki/MiSeq_SOP). In brief, the quality of the sequences was checked using make.contigs (default settings) and screen.seqs (maxambig = 0, maxlength = 550, minlength = 350, maxhomop = 8), and chimeras were detected with chimera.vsearch (Rognes et al. 2016) using reference data of the Silva Small Subunit rRNA Database (Quast et al. 2013) release 132. After eliminating low-quality and/or putative chimera reads, the resulting sequence data were aligned and classified using SILVA release 132, and then clustered into operational taxonomic units (OTUs) sharing over 97% homology following MiSeq SOP. Subsequently, we calculated the inverted Simpson index (Simpson 1949) and the Shannon index (Shannon 1948) to estimate alpha diversity indices of each bacterial community.

III. Results

Analysis of VFAs

The quantification of VFAs and lactic acid indicated that the forestomach contents contained 4,804.7 ± 1,276.9 μg/g (3,687–6,441 μg/g) acetic acid, 395.3 ± 471.4 μg/g (0–1,080 μg/g) propionic acid, 1,051.1 ± 717.5 μg/g (619–2,124 μg/g) butyric acid, and 534.1 ± 724.4 μg/g (97–1,613 μg/g) lactic acid. The cecum contents contained 5,100.5 ± 703.3 μg/g (4,204–5,910 μg/g) acetic acid, 961.7 ± 288.0 μg/g (604–1,228 μg/g) propionic acid, 1,042.6 ± 721.0 μg/g (659–2,123 μg/g) butyric acid, and 369.2 ± 263.3 μg/g (71–636 μg/g) lactic acid (Fig. 1). No significant differences were detected between the forestomach and cecum in the amounts of any of these components (acetic acid: $p = 0.69$, propionic acid: $p = 0.11$, butyric acid: $p = 0.89$, lactic acid: $p = 1.00$).

Microflora analysis

More than 50,000 reads were obtained, and 41,457 and 38,562 reads were extracted as cleaned chimera-free sequences from the forestomach and cecum contents, respectively (Table 1).

Of these, 93% of the reads in the forestomach were classified as Firmicutes, whereas 59% of reads in the cecum were classified as Firmicutes (Fig. 2). Both the ratio and the constituent members of the sequences classified as Firmicutes dif-

Table 1. Microbial diversity and species richness of obtained bacterial 16S rRNA gene sequences isolated from the forestomach and cecum contents of the East European vole (*Microtus levis*).

	Forestomach	Cecum
Library information		
Number of reads obtained	58,601	52,265
Number of reads purified	41,457	38,562
Number of OTUs (97%)	1,259	1,889
Coverage (%)	97.3	96.7
Diversity estimates*		
Shannon index	2.40	4.96
Inverse Simpson index	5.55	52.71
Number of OTUs shared	124	

* Alpha level diversities were obtained from 38,562 subsampled reads from the contents of both portions of the intestine for accurate comparison.

Table 2. Classification at the family level of obtained bacterial 16S rRNA gene sequences classified into Firmicutes from the forestomach and cecum contents of the East European vole (*Microtus levis*).

	Forestomach	Cecum
Planococcaceae	13	0
Staphylococcaceae	4,987	0
Bacilli unclassified	31	0
Aerococcaceae	14,588	0
Carnobacteriaceae	1,702	0
Lactobacillaceae	10,787	74
Lactobacillales unclassified	234	0
Streptococcaceae	142	0
Clostridiales unclassified	2	19
Clostridiales vadinBB60 group	0	442
Family XIII	0	148
Lachnospiraceae	1	14,375
Peptococcaceae	0	54
Ruminococcaceae	33	6,895
Erysipelotrichaceae	6,121	737
Others *	12	9
unclassified	46	9
total	38,699	22,762

* Others include fewer than 10 reads in each familial group; these are Bacillaceae, Bacillales unclassified, Paenibacillaceae, Clostridia unclassified, Christensenellaceae, Clostridiaceae 1, and Eubacteriaceae.

Table 3. Classification at the family level of obtained bacterial 16S rRNA gene sequences classified into Bacteroidetes from the forestomach and cecum contents of the East European vole (*Microtus levis*).

	Forestomach	Cecum
Bacteroidales unclassified	7	148
Marinifilaceae	0	133
Muribaculaceae	549	12,004
Rikenellaceae	0	379
Rs-E47 termite group	0	151
Bacteroidia unclassified	2	47
unclassified	0	1
total	558	12,862

fered between the forestomach and cecum (Table 2). In the forestomach, Aerococcaceae (37.7%), Lactobacillaceae (27.9%), Erysipelotrichaceae (15.8%), Staphylococcaceae (12.9%), and Carnobacteriaceae (4.4%) were the five most abundant members of the Firmicutes. Of these, most of the Aerococcaceae were classified as *Facklamia* spp. (12,745 reads, 30.7% of total reads), the most abundant genus detected in the forestomach, followed by *Lactobacillus* spp. (10,786 reads, 26.0% of total reads) of the Lactobacillaceae. On the other hand, in the cecum, most reads were classified as Lachnospiraceae (63.2%) and Ruminococcaceae (30.3%). Unfortunately, we were unable to identify the most abundant group in the cecum to the genus level; the unclassified bacterium group of the in Lachnospiraceae accounted for 7,681 reads (19.9% of total reads). Interestingly, sequences identified as Ruminococcaceae from the cecum were classified into many genera (more than 30 generic-level groups), including *Oscillibacter* spp. and *Ruminococcus* spp. (data not shown).

Bacteroidetes were not rich in the forestomach (1.3%) but were abundant in the cecum (33%); most were classified as members of the Muribaculaceae (Table 3), although we were unable to identify them to the genus level. Actinobacteria was the third most abundant group in the forestomach (4.9%; Fig. 2), and most were classified into the Corynebacteriaceae. In contrast, Proteobacteria were the third most common group in the cecum (5.7%; Fig. 2), although we were unable to identify them to the family level (data not shown).

Using a 97% similarity threshold, 1,259 and 1,889 OTUs were found from the forestomach and cecum contents, respectively. Alpha diversity levels (Shannon index and inverse Simpson index) were higher in the cecum compared to the forestomach (Table 1). We found 124 only OTUs (around 10%) that were shared between the forestomach and cecum contents (Table 1).

IV. Discussion

In the present study, we detected similar levels of VFAs and lactic acid from the forestomach and cecum of East European voles (Fig. 1). In contrast, bacterial community composition differed between these portions of the intestine, even at the phylum level (Fig. 2), indicating that fermentation occurred in both the forestomach and cecum but was driven by different consortia of microbiota.

Microbial fermentation in the cecum is essential in most rodents for extracting nutrients from the diets. In our study, VFAs and lactic acid were

detected from cecum contents (Fig. 1). Previous studies have also detected VFAs from caecum contents in Cricetinae rodents (Hoover et al. 1969, Obara & Goto 1980, Sugawara & Oki 1982, Shinohara et al. 2016), and many studies of various rodents have further demonstrated the energy contribution of VFAs from the cecum (e.g. Bergman 1990, Hume et al. 1993, Stevens & Hume 1998). In the present study, acetic acid was the most abundant VFA, whereas propionic and butyric acids were detected at lesser but nearly equal proportions (Fig. 1). Hume et al. (1993) reported that a small percentage of the acetate absorbed at concentrations normally present in the hindgut of voles may contribute to a rich acetic acid environment. Similarly abundant acetic acid environments have also been reported from the fermentative chambers of many herbivorous mammals (Bergman 1990); such high concentrations of acetic acid promote cellulolytic flora (Stevens & Hume 1998). Indeed, our study demonstrated an abundance of Lachnospiraceae and Ruminococcaceae bacteria in the cecum of *M. levis*. Both bacterial groups are anaerobic and are commonly found in the gastrointestinal tract of ruminants. Several strains of Lachnospiraceae are pectinolytic, whereas Ruminococcaceae are cellulolytic. Together, these results indicate that the East European vole utilizes VFAs resulting from symbiotic microbial fermentation of plant materials in the cecum.

We detected similar levels of VFAs in the forestomach of East European voles as were found in the cecum. Obara & Goto (1980) and Sugawara & Oki (1982) also reported high concentrations of acetic acid in the forestomach of Japanese field voles (*M. montebelli*). Moreover, several studies have reported similar results in Cricetinae rodents (e.g. Kohl et al. 2014, Shinohara et al. 2016), suggesting that rodents with forestomaches utilize VFAs from forestomach fermentation in a similar manner as they do those produced in the cecum, as noted above. Although Ehle & Warner (1978) concluded that microbial fermentation in the pre-gastric chamber in rodents would be of limited nutritional benefit due to the short residence time of ingesta in the forestomach, Obara & Goto (1980) reported that VFAs were expended in the forestomach and glandular stomach in voles. Cumulatively, these results suggest that, at least for voles, VFAs are produced in the forestomach by microbial fermentation and then absorbed as energy. Recently, Kirat & Kato (2006) reported that monocarboxylate transporter 1 (MCT1) mediates the transport of VFAs. Further studies using the MCT1 gene will provide additional details.

In addition to VFAs, we also detected lactic acid in the forestomach; however, the concentration was relatively low (Fig. 1). These results differ from those recently reported for hamsters, in which high amounts of lactic acid were detected in the forestomach (Shinohara et al. 2016). Most of the bacteria detected in the hamster forestomach were Lactobacilaceae (79%; Shinohara et al. 2016). In the present study, *Lactobacillus* spp. was also a major group in the forestomach of voles, but they only accounted for 26% of the bacterial community. This variation in the abundance of Lactobacilaceae may contribute to the observed differences in the level of acetic acid in the forestomaches of hamsters and voles. In addition, Shinohara et al. (2016) suggested that coprophagy may contribute to the high diversity of microflora in the forestomach of hamsters, as the number of shared OTUs between the forestomach and cecum was high. However, in the present study, only 10% of OTUs were shared between the microflora of the forestomach and cecum in the East European vole. Furthermore, this vole only occasionally eats feces and does not engage in coprophagy as frequently as hamsters (Ebino 1993). These results indicate that the role of the forestomach in voles differs from that in hamsters. Interestingly, Sugawara & Oki (1982) suggested that, in the Japanese field vole (*M. montebelli*), lactic acid is absorbed in the forestomach, transported to the liver, and then used not only as the glyconeogenetic precursor but also as a direct energy source. Therefore, voles likely use lactic acid as a direct energy source as well. In our study, *Facklamia* spp. was the most abundant genus in the forestomach (30.7%), followed by *Lactobacillus* spp. The *Facklamia* genus was designated in 1997 based on clinical specimens. Although the characterization is not yet fully resolved, *Facklamia* has been reported to generate acids from glucose and several other sugars (Collins & Lawson 2009). In the general pathway of lactic acid generation, starch is decomposed into glucose, which is then converted to lactic acid. Thus, lactic acid-producing bacteria such as *Lactobacillus* consume glucose when generating lactic acid, and *Facklamia* also generates acids from glucose, likely resulting in the production of the VFAs detected in our study. To elucidate the role of forestomach fermentation in voles by these two bacterial groups, it is important to identify the bacteria to the species level using the 16S rRNA cloned-library method and to further define their abilities using meta-genomic sequencing.

Acknowledgements

Drs. H. Takasaki at OUS Department of Zoology and F. Ike at RIKEN BioResource Research Center provided the author with help and support

in this study in various ways. Drs. N. Hashikawa at OUS Department of Life Science, and H. Ishibashi at Food and Environment Analysis Center guided part of the experiments in this study. This study was supported by the Sasagawa Scientific Research Grant (fiscal year 2015) to Ikemoto from The Japan Science Society (Grant# 27-534).

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池本眞希・篠原明男・城ヶ原貴通・織田銑一・目加田和之：東ヨーロッパハタネズミ（*Microtus levis*）の消化管における揮発性脂肪酸と微生物相組成

要約

草食性の齧歯類は基本的に後腸発酵動物であるが，一部の齧歯類は盲腸に加え，区分けされた胃（前胃や腺胃）をもつ．このような草食性齧歯類の消化メカニズムを解明するために，我々は東ヨーロッパハタネズミ（*Microtus levis*）の前胃と盲腸の揮発性脂肪酸（VFAs）および乳酸の産生を評価した．さらに，16S rRNA V3-V4領域のアンプリコン解析を用いて，前胃と盲腸の内容物の微生物叢を比較した．結果として，前胃と盲腸の両方で同程度のVFAsと乳酸が検出された．酢酸が最も多く（3,687–6,441 μg/g），プロピオン酸（0–1,228 μg/g）と酪酸（619–2,124 μg/g）に加え，乳酸（71–1,613 μg/g）も検出された．前胃と盲腸の内容物組成の間には大きな違いは見られなかった．一方，細菌叢は部位によって，門レベルで異なることが明らかとなった．前胃では，Firmicutesが主に検出された（93.3%）のに対し，盲腸では，Bacteroidetes（33.4%）とFirmicutes（59.0%）が優勢であった．これらの結果は，本ハタネズミの前胃と盲腸の両方で消化管内発酵が行われており，それぞれの発酵は異なる微生物叢によるものであることを示す．

(Accepted 13 December 2021)