Gut bacterial communities in the freshwater snail *Planorbella trivolvis* and their modification by a non-herbivorous diet

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The freshwater pulmonate snail *Planorbella trivolvis* is a common species in various bodies of water but is not native to China. *Planorbella trivolvis* usually live on diets with high fibre content, such as water grasses, algae and fallen leaves. These snails can attach to the wall of a water tank or to water grass and can be transported overseas to China through the ornamental fish trade. There are few studies investigating the intestinal microbiota of freshwater snails. In this study, using culture-independent molecular analysis, we assessed for the first time the complexity of bacterial communities in the intestines of reared snails. The intestinal microbiota in the snails fed different diets, that is, herbivorous feed (HV) with high cellulose and non-herbivorous feed (NHV) with low cellulose, were analysed by Illumina sequencing. The results showed that the NHV-based diet significantly increased the body mass, shell diameter and specific growth rate of the snails after 60 days of rearing ($P < 0.05$). Histological experiments showed that the fat droplets in the epithelium columnar cells of the intestines of the NHV snails increased, and the cilia on these cells fell off. The sequencing results identified 486 and 195 OTUs in HV and NHV, respectively. Lots of bacteria were not reported previously in snails. The intestinal microbiota diversity index (Shannon, Simpson, Ace and Chao) in the NHV snails was significantly lower than that in the HV snails. The gut microbiota in the HV snails were predominantly Proteobacteria (52.97%) and Bacteroidetes (28.75%), while the gut microbiota in NHV snails were predominantly Proteobacteria (95.23%). At the genus level, *Cloacibacterium* (24.60%), *Pseudomonas* (4.47%), OM6ON (6.12%), and *Rhodobacter* (5.79%) were observed to be abundant in HV snails. However, *Aeromonas* (85.4%) was determined to be predominant in NHV snails. Functional prediction of the gut microbiome in snails by PICRUSt demonstrated a significant difference between the two groups, and the HV snails exhibited higher lignocellulose enzyme activity than did the NHV snails. This study represents a first step in characterizing the gut microbiota of the freshwater snail. Most of these microbes can process plant biomass and digest cellulose and lignocellulose, and the enzymes of these

bacteria may have potential biotechnological applications in a variety of industrial processes.

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ABSTRACT:

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analysed by Illumina sequencing. The results showed that the NHV-based diet significantly increased the body mass, shell diameter and specific growth rate of the snails after 60 days of rearing ($P < 0.05$). Histological experiments showed that the fat droplets in the epithelium columnar cells of the intestines of the NHV snails increased, and the cilia on these cells fell off. The sequencing results identified 486 and 195 OTUs in HV and NHV, respectively. Lots of bacteria were not reported previously in snails. The intestinal microbiota diversity index (Shannon, Simpson, Ace and Chao) in the NHV snails was significantly lower than that in the HV snails. The gut microbiota in the HV snails were predominantly Proteobacteria (52.97%) and Bacteroidetes (28.75%), while the gut microbiota in NHV snails were predominantly Proteobacteria (95.23%). At the genus level, *Cloacibacterium* (24.60%), *Pseudomonas* (4.47%), OM6ON (6.12%), and *Rhodobacter* (5.79%) were observed to be abundant in HV snails. However, *Aeromonas* (85.4%) was determined to be predominant in NHV snails. Functional prediction of the gut microbiome in snails by PICRUSt demonstrated a significant difference between the two groups, and the HV snails exhibited higher lignocellulose enzyme activity than did the NHV snails. This study represents a first step in characterizing the gut microbiota of the freshwater snail. Most of these microbes can process plant biomass and digest cellulose and lignocellulose, and the enzymes of these bacteria

may have potential biotechnological applications in a variety of industrial processes.

Keywords: *Planorbella trivolvis*, freshwater snails, cellulose, gut microbiota, functional prediction, intestine histology

INTRODUCTION

Planorbella trivolvis belongs to the family Plamorbidae and is primarily distributed in Africa, Europe and North America. (*Hunter, 1990*). *Planorbella trivolvis* is one of the most abundant taxa in the subclass Pulmonata, with more than 250 species in 40 genera being recorded worldwide. These species can serve as intermediate hosts for a variety of trematodes, such as *Ribeiroia ondatrae* and echinostome, thereby contributing to the global disease burden (*Klockars et al., 2007*; *Peterson & Nathan, 2007*). Snails are common in ponds, lakes and marshes and feed on algae, aquatic plants, the fallen leaves of terrestrial plants and various types of detritus (*Lombardo & Cooke, 2002*). Therefore, the ability of snails to digest lignocellulose is notable, and they are thought to contain a gut microbiome that is specialized in the rapid hydrolysis and fermentation of lignocellulosic plant biomass (*Pinheiro et al., 2015; Wijanarka, et al., 2016*).

There is considerable bacterial diversity in the intestinal tracts of animals. These bacteria can interact with host animals, enhance animal immunity, help to

digest nutrients, and play important roles in inhibiting harmful foreign bacteria (*Lee et al., 2013*; *Carnevali et al, 2017*; *Guarner et al., 2003*). Up to now, the gut microbiota in herbivores have been well studied in many animals, such as cows, ants, and pandas (*Bergmann et al., 2015*; *Jami et al., 2013*; *Russell et al., 2009*; *Tun et al., 2013*).

However, the gut microbial communities of snails remain largely unstudied (*Joynson et al., 2017*; *Van Horn et al., 2012*). The early study of intestinal microbiota in snails employed culture-based methods, and a limited number of bacteria were identified, such as *Enterobacter*, *Enterococcus*, *Lactococcus*, and *Clostridium* (*Charrier et al., 2006*). Next, molecular biology techniques were used to study the snail gut microbiota, such as DGGE fingerprint analysis, which has been utilized to obtain the structural composition of the dominant bacterial community in the snail *Helix pomatia*, and the number of identified bacteria was also limited (*Nicolai et al., 2015*). Recently, high-throughput sequencing based on parts of 16S rRNA gene, which can reveal more kinds of microbiota even that with low abundance, were used to study the gut microbiomes in freshwater snails *Biomphalaria pfeifferi*, *Bulinus africanus*, *Helisoma duryi* and *Radix ovata* (*Van Horn et al., 2012*; *Hu et al., 2018*). Furthermore, the intestinal microorganisms of snail play an important role in cellulose decomposition. Some studies have isolated cellulolytic bacteria from the intestine of snails by carboxymethylcellulose (CMC)

plates (*Charrier et al., 2006*; *Pinheiro et al., 2015*). Other studies, using metagenomic, have identified a great number of genes involved in lignocellulosic breakdown in intestinal microorganisms of snails *Achatina fulica*, *Arion ater* (*Cardoso et al. 2012*; *Joynson et al., 2017*) .

The main focus of this work was to characterize intestinal microbiomes of *Planorbella trivolvis* shaped by diets. Previous studies showed that high-fibre or low-fibre diets shape the intestinal microbiota and microbial metabolites and affect the growth performance and intestinal morphology in various animals, such as pigs and rats (*Stark et al., 1996*; *Heinritz et al., 2016*; *Coble et al., 2018*). Therefore, the variation in fibre content in diets may affect the intestinal microbiota in snails or affect the growth and intestinal status of snails. However, the effects of specific dietary modulation on the intestinal microbiota and growth of snail have not been fully elucidated.

In this study, a culture-independent molecular analysis based on the 16S rRNA gene was performed (1) to demonstrate the effect of the diet (high-cellulose and low-cellulose) on gut microbiota in *Planorbella trivolvis* and (2) to examine functional differences in the microbiomes of snails fed different diets. The effects of diet on snail growth and intestinal morphology were also examined in this study.

MATERIALS AND METHODS

100 **Rearing of snails**

101 The snails were originally purchased from the ornamental fish market, and a
102 breeding colony were formed in the laboratory. When a sufficient number of
103 newborn snails were reared to about 5-7 mm of shell diameter, a total of 120
104 juvenile snails with uniform size (5.88 ± 0.14 mm of shell diameter) were selected
105 and randomly distributed into two groups by their diets: herbivorous groups (HV)
106 (60 snails) and non-herbivorous groups (NHV) (60 snails). Each group had three
107 replicate aquariums with 20 snails in each repetition. HV group was fed dry alfalfa
108 contain a high content of cellulose (26%), and NHV group was fed market-
109 purchased pellet feed (Takara sakana-ii, for ornamental fish feeding), which
110 contained low cellulose (5%) (Table 1). The aquaculture water temperature was
111 maintained at 24~25 °C, with a pH of 6.7 ± 0.4. The snails were reared for 60
112 days and no snails died during rearing.

113 **Sample collection and processing**

114 After 60 days of rearing, the body length and weight of snails were
115 determined first. Next, the intestines were sampled. When the intestines were
116 collected, the snails were cleaned with tap water, and the surface of the shell was
117 subsequently wiped with 70% alcohol. The shells were carefully broken and
118 disassembled from the snail body. After that step, the intestines were extracted
119 carefully to avoid rupturing the gut wall. Next, the intestines were sampled and

contained their contents. All dissections were conducted under aseptic conditions. For histological examination, the collected intestines were fixed in 4% formaldehyde for histological examination. Because the intestines of individual snails were too small to meet the sampling requirements, the intestines and contents of the 5-8 snails were packed into a centrifuge tube to form a repeat sample for sequencing.

Growth index

The growth performance of snails after 60 days of rearing was characterized by their weight gain rate (WGR) and specific growth rate (SGR). The WGR was calculated by comparing their weight gain (WG) with their initial average weight (IW). The SGRs were obtained by dividing their weight gain by 60 days. The formulas of WGR and SGR are as follows:

$$WGR\ (\%) = [(TW - IW)/IW] \times 100$$

$$SGR\ (\%/d) = [(TW - IW)/d] \times 100$$

WGR: Weight gain rate; SGR: Specific growth rate; TW: terminal weight; IW: initial weight; d: rearing days

Intestinal morphology analysis

To examine the effect of diets on the intestinal morphology of snails, the intestinal samples were washed in saline solution and fixed in 4% paraformaldehyde solution

above 48 h. Next, the fixed samples were successively treated as follows: dehydrated by an alcohol gradient (50%, 70%, 90% and 100%), xylene transparency, and paraffin embedding. Three paraffin sections from each sample were cut and stained by the haematoxylin-eosin (HE) method. Last, the stained slice was sealed by neutral gum and subjected to observation and scenery shooting using a panoramic scanner (3DHISTECH, Pannoramic MIDI, Hungary).

Determination of intestinal microbiota diversity

The intestine samples obtained as described above contained the contents used for genome extraction. Total DNA was extracted using the FAST DNA™ Spin Kit for soil (MP-BIO, Santa Ana, CA, USA) following the protocol of the manufacturer. The extracted DNA was subjected to 1.5% agarose-gel electrophoresis, and the DNA concentration and OD260 nm/OD280 nm (OD, optical density) value were determined with a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, USA).

To detect intestinal bacteria, the V3~V4 regions of the bacterial 16S rRNA gene were amplified with DNA primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) (*Mori et al., 2013*). The 12 bp barcodes on both ends of the primers were used to identify the sequences of different samples. Next, PCR amplification was conducted, and the procedure was as follows: initial

159 denaturation at 95 °C for 3 min followed by 27 cycles of denaturing at 95 °C for 30
160 s, annealing at 55 °C for 30 s and extension at 72 °C for 45 s, single extension at
161 72 °C for 10 min, and ending at 4 °C. The amplified products were subjected to
162 electrophoresis and extracted from a 2% agarose gel. Next, the products were
163 purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union
164 City, CA, USA). After Qubit quantification and detection, amplicons from each
165 PCR sample were normalized to equimolar amounts, and high-throughput
166 sequencing was performed using the 2 x 300 bp protocol on an Illumina MiSeq
167 PE300 platform (Illumina, San Diego, CA, USA).

168 The raw reads were deposited into the NCBI Sequence Read Archive (SRA)
169 database (Accession Number: PRJNA640745).

170 Raw fastq files were demultiplexed, quality-filtered by Trimmomatic (*Bolger*
171 *et al., 2014*) and merged by FLASH. UPARSE (*Edgar et al., 2013*) was utilized to
172 conduct operational taxonomic unit (OTU) clustering analysis at 97% identity, and
173 chimaeric sequences were identified and removed using UCHIME (*Edgar et al.,*
174 *2011*). Next, the representative OTUs were analysed on the Qiime platform
175 (*Caporaso et al., 2010*) against the Silva_132 16S Database (http://www.arb-
176 silva.de) to determine taxonomy.

177 The heatmap analysis was performed in the heatmap package in R. Venn and
178 PCoA analyses were performed in the vegan package in R. The alpha -diversity of

179 the gut bacterial community, containing indices of Sobs, Shannon, Chao, Simpson,
180 Ace, and coverage, was analysed by Mothur (*Schloss et al., 2009*). Biomarker
181 discovery was performed on the linear discriminant analysis (LDA) effect size
182 (LEfSe) to identify the specific organisms whose relative abundances differ
183 between two groups of samples (*Segata et al., 2011*).

184 To examine functional differences in the microbiome of snails, the functional
185 attributes of metabolic genes from the snail gut microbiota (KEGG Orthologs, KOs)
186 were predicted by a phylogenetic investigation of communities by reconstruction
187 of unobserved states (PICRUSt), which were obtained by macthing the sequencing
188 data against the genomic KEGG database (*Langille et al., 2013*). As a
189 computational approach, PICRUSt can predict the functional composition of an
190 obtained metagenome using marker gene data and a database of reference genomes
191 (*Langille et al., 2013*).

192 **Statistical analysis**

193 SPSS 19.0 software was employed for a two-tailed Student's t-test on the
194 growth data of snails. P values < 0.05 were considered to indicate significant
195 differences, and descriptive statistics were expressed as the mean ± SD.

196

197 **RESULTS**

198 **Snail growth**

199 The initial size and weight did not differ between the two groups ($P > 0.05$) (Table
200 2). After 60 days of rearing, the diameter of the final shell of the HV snails was
201 about 10.53 mm, representing an increase of 176.08% compared with the initial
202 shell diameter (Table 2). The final shell diameter of NHV snails was about 11.86
203 mm, representing an increase of 205.19% compared with the initial shell diameter;
204 thus, and the final shell diameter of snails in the NHV group was significantly
205 larger than that of the HV group ($P < 0.05$). The endpoint weight of HV group was
206 0.219 g, and that of NHV group was 0.432 g. The weight gain rate of the NHV
207 group (566.90%) was significantly higher than that of the HV group (321.40%) (P
208 < 0.05). Also, the specific growth rate of snails in the NHV group (3.16%) was
209 significantly higher than that in the HV group (1.94%) ($P < 0.05$) (Table 2).

210 **Intestinal morphology**

211 To examine the effect of feed on the intestinal morphology of snails, histological
212 examination were conducted (Figure 1). The differences between treatment groups
213 were clearly shown in the simple columnar epithelium cells (SCEC). For the HV
214 snails, the intestinal structure was intact, and the SCEC arrangement was regulated.
215 The microvilli were tightly attached to the SCEC. For the NHV snail, the intestinal
216 SCECs were filled with fat droplets and thus appear transparent in this image
217 (Figure 1C, D). The fat droplets occupied the cytoplasm of the SCEC, and the
218 nucleus was observed to be indented (Figure 1C, D), indicating that low fibre

feeding leads to increased fat deposition in intestinal epithelial cells. The microvilli were detached to a certain extent from the SCEC.

Alpha diversity based on 16S rRNA gene sequencing

A total of 737,284 raw reads were generated using the Illumina MiSeq sequence platform and 485,342 high quality sequences were obtained (following quality control and sequence filtration) with an average length of 459.9 bp. The mean number of sequences per sample was 54,277.60 ± 6,298.01 in HV snails and 39,606.60 ± 6,846.06 in NHV snails (Table 3). The 31,486 rarefied sequences were clustered into 525 OTUs at 97% identity, with 486 and 195 OTUs being identified in HV and NHV, respectively.

The obtained 525 OTUs include members of 419 species, 304 genera, 184 families, 108 orders, 37 classes and 19 phyla. There were 18 phyla, 286 genera, 108 orders, 36 classes, 486 OTUs belonging to HV, and there were 13 phyla, 52 orders, 21 classes, 132 genera, and 195 OTUs belonging to NHV. All of the alpha-diversity indices (Sobs, Shannon, Simpson, Ace, Chao) were significantly different between HV and NHV populations ($P < 0.05$) (student's t-test) (Table 3). The Good's coverage for the observed OTUs was above 99.70%, and the rarefaction curves showed clear asymptotes (Fig. 2A), which, taken together indicated that the given level of sampling effort was sufficient to capture the bacterial communities in snails. Notably, there were clear differences for the rarefaction and rank-

abundance curves between the treatment groups (Fig. 2A, 2B). The abundance analysis showed that there were only 5 OTUs in the NHV snails with abundances greater than 1%, while there were as many as 22 OTUs in the HV snails (Fig. 2B). The total abundance of OTUs with abundance greater than 1% reached 79.68% in the HV snails and 83.39% in the NHV snails. The number of core OTUs identified in all samples was 22 (Fig. 2C).

Venn analysis showed that the bacteria belonging to 12 phyla, 77 families, and 114 genera were shared by two groups: 6 phyla, 97 families, and 172 genera were unique in HV snails, and 1 phylum, 10 families, and 18 genera were unique in NHV snails (Fig. 2D-F).

Composition of snail intestinal microbiota

The phyla Proteobacteria (52.97%) and Bacteroidetes (28.75%) were predominant among the bacteria in the HV group (Fig. 3A). The phyla with abundances above 1% were Actinobacteria, Verrucomicrobia, Chloroflexi, Cyanobacteria, and Chlamydiae. However, only Proteobacteria (95.23%) was predominant among the bacteria in NHV snails. Bacteroidetes was the other phylum with an abundance above 1%.

At the family level, there were 20 families and 8 families with abundances above 1% in the HV and NHV snails, respectively. Weeksellaceae (24.6%) and

Rhodobacteraceae (12.38%) showed high abundance in HV snails. Aeromonadaceae (85.40%) dominated the microbiota in NHV snails (Fig. 3B).

At the genus level, the composition of intestinal microbiota differed between the HV group and the NHV group (Fig. 3C). In the HV group, there were 22 genera with abundances above 1%. *Cloacibacterium* (24.60%) showed the highest abundance in the HV groups. Others, such as *Pseudomonas* (4.47%), *OM6ON* (6.12%), and *Rhodobacter* (3.78%) were also abundant. In the NHV snail, there were 7 bacteria with abundances higher than 1%. *Aeromonas*, exhibited the highest abundance, accounting for 85.40%.

The heat map analysis constructed from the top 35 abundant genera, reflected that the intestinal microbiota in the two groups was different (Fig. 4). The Wilcoxon rank-sum test showed that the abundance of 26 genera was significantly higher in HV snails than in NHV snails ($P < 0.05$). Only two genera (*Aeromonas* and *Comamonas*) with significantly higher abundances were observed in NHV snails.

Clustering of the snail gut bacterial community

Clustering analysis of intestinal microbiota associated with diet were performed. Principal coordinates analysis (PCoA) of sequencing data using pairwise weighted and unweighted UniFrac distances showed that the bacterial community structure of HV was different from that of NHV (Fig. 5). UniFrac clearly separated different

278 microbiota efficiently by diet, demonstrating the importance of food as a driver of
279 community composition in this freshwater snail.

280 LEfSe analysis (the LDA threshold was 4) was used to screen out
281 microorganisms with significant differences in the snail intestines of the two
282 groups fed different diets. There were 11 bacterial genera that were significantly
283 enriched in the intestinal samples of the HV group, and there was one genus
284 (*Aeomonas*) that was enriched in the NHV group (Fig. S1). When the LDA
285 threshold was set to 2, 114 bacterial genera were significantly distributed in the
286 intestinal samples of the HV group, while only 4 bacterial genera were
287 significantly distributed in the NHV group (Table S1).

288 Although the intestinal microbiota of snails was influenced by food type, we
289 found that 27 genera existed as core microbiota of all samples in both groups.
290 Among these genera, 19 were affiliated with Proteobacteria, 4 were affiliated with
291 Bacteroidetes, 3 were affiliated with Actinobacteria, 2 were affiliated with
292 Verrucomicrobia and 1 was affiliated with Chloroflexi (Table 4).

293 **Predictive Metagenomic Profiling**

294 PICRUSt analysis was performed to generate a predictive functional profile to gain
295 insight into the metabolic capacity of the enteric microbiome (Fig. 6). Based on
296 this analysis, the relative abundance of several COG functions was significantly
297 different between feeding regimens, such as the enrichment of genes involved in

298 energy production and conversion, lipid transport and metabolism in HV, and the
299 enrichment of genes involved in amino acid transport and metabolism, signal
300 transduction mechanisms in NHV. No significant differences were observed for the
301 relative abundance of genes involved in carbohydrate transport and metabolism,
302 inorganic ion transport and metabolism, and transcription.

303 Because plant fibre degradation requires a diverse suite of enzymes for
304 complete hydrolysis, the abundance of genes for several lignocellulose-active
305 enzymes was also examined (Fig. 7). Eighteen enzymes determined to be more
306 enriched in HV snails than in NHV snails, acting upon cellulose, hemicellulose,
307 lignin, and cello-oligosaccharides, respectively. Six enzymes were more enriched
308 in NHV than HV snails, acting upon hemicellulose, and cello-oligosaccharides,
309 respectively (Fig. 7). Enzymes related to protein degradation were also analyzed.
310 The result showed that a large number of genes related to aminopeptidase,
311 dipeptidase, carboxypeptidase and endopeptidase were more enriched in HV snails
312 than in NHV snails (Fig. 8).

313

314 **DISCUSSION**

315 For invertebrates, the study of gut microbes remains an open question and warrants
316 further research to characterize roles played by bacteria. In this work, we
317 investigated the effect of diets, containing a high (herbivorous diets, HV) or low

(non-herbivorous diets, NHV) cellulose, on gut bacterial community structure in *Planorbella trivolvis*. We also compared the effect of these diets on the growth and gut histological morphology. NHV significantly promoted the body length and body weight growth of snails, indicating that snails obtained more crude protein and energy and less crude fibre when feeding on pellet feed. However, for herbivorous feed, namely, alfalfa, the snails needs to use their radulas to scrape the plant ingredients; therefore, the feeding efficiency is considerably lower.

The two diets were observed to influence the intestinal morphology of snails. As the results showed, there were no fat droplets in the simple columnar epithelium cells (SCECs) of HV snails. While in NHV snails, the intestinal SCECs are filled with fat droplets. The reason for this phenomenon may lie in diets in NHV with low dietary fibre, which contain more soluble carbohydrates and would be easily digested and absorbed by snails. Furthermore, dietary soluble carbohydrates can promote intestinal lipid accumulation in aquatic organisms (*Zhao et al., 2020*; *Castro et al., 2019*). Therefore, the fat droplets occupied the cytoplasm of the SCECs, and the nucleus was indented, which may be related to a disturbed state of the normal metabolism of the cell and caused the detachment of microvilli from SCEC in NHV snails.

Bacteria in the digestive tract may have the ability to ferment cellulose and chitin to produce substances that snails can easily absorb, such as small molecules

of organic acids and sugars (*Speiser, 2001*; *Charrier et al., 2006*). In this study, the bacterial community in HV snails was dominated by Proteobacteria, Bacteroidetes and Actinobacteria, indicating which bacteria may be related to plant digestion. In fact, previous studies on herbivores have observed that Proteobacteria are the dominant species in snails (*Cardoso et al., 2012*; *Joynson et al., 2017*; *Nicolai et al., 2015*). In this study, we found that Bacteroidetes and Actinobacteria were also associated with plant-eating gastropods. At the phylum level, the research results of Van Horn et al. (*2012*) on the three Planorbidae have some similarities with ours. Their study showed that Proteobacteria, Bacteroidetes, and Acidobacteria were present and dominant in the intestines of these snails. A large number of bacterial species belonging to Proteobacteria, Bacteroidetes, and Acidobacteria are also observed in the gut of termites, which eat lignocellulose entirely (*Mikaelyan et al., 2017*). However, for the American bison, a herbivore, the gut microbiota was very different from that of the freshwater gastropods, with the order of abundance from most to least being Firmicutes (53%), Bacteroidetes (33%) and Tenericutes (4%) (*Bergmann et al., 2015*).

Core microbes are microbes that are consistently present in a particular habitat, such as intestines of animals, despite the high variable conditions in that habitat. In this study, 27 genera were observed as core microbiota between the two dietary groups, among which *Cloacibacteria*, *Aeromonas* and *Pseudomonas* had the

358 abundance above 1% in both groups (HV and NHV), and *Aeromonas*,
359 *Pseudomonas*, *Flavobacterium*, and Enterobacteriaceae families have been
360 characterized as common in terrestrial or aquatic gastropods by previous studies
361 (*Cardoso et al., 2012*; *Joynson et al., 2017*). Although the role of the core
362 microbiota in the gut microbiota of animals is not well understood, it is likely
363 critical and needs further study (*Shade and Handelsman, 2012*; *Kokou et al.,2019*).

364 Some bacteria can be found both in the gut microbiota of snails and free-living
365 in the natural water environment. *Cloacibacterium*, which is abundant in HV snails,
366 is a facultative anaerobic and gram-positive bacterium and can be isolated from
367 freshwater sediments (*Allen et al., 2016*). *Rhodobacter* and *Pseudomonas*, widely
368 distributed in seawater and fresh water, were also found in the intestinal microbiota
369 of *Potamopyrgus antipodarum*, a freshwater snail from New Zealand (*Vesbach et*
370 *al., 2016*). Vesbach *et al*. (*2016*) determined that *Rhodobacter* colonized the
371 intestinal tract of *Potamopyrgus antipodarum* and formed symbionts with the host.
372 Symbiosis of *Rhodobacter* with host was also observed in sponges (*Halichondria*
373 *Panicea*) and Daphnia (*Althoff et al., 1998*； *Qi et al., 2009*).

374 There were 26 genera significantly increased in HV snails compared to NHV
375 snails, which were potentially plant-related intestinal microbiota, such as
376 *Cloacibacteria*, *Pseudomonas*, *Flavobacterium*, *Mycobacterium*, and *Rhodobacter*.
377 *Pseudomonas* has been identified as a cellulolytic species in invertebrates (*Huang*

et al., 2012). In fact, most plant-related bacteria varied with the snail species. Cardoso *et al*. (2012) reported that the bacterial taxa closely related to herbivores are *Pseudomonas*, Clostridiaceae, *Lactococcus*, *Bacteroides*, Flavobacteriaceae, *Mucilaginibacter*, *Citrobacter*, *Klebsiella*, *Aeromonas*, *Acinetobacter*, and *Comamonas*. Among these bacteria, *Pseudomonas*, Flavobacteriaceae and *Aeromonas* were found in our study. *Microbacterium*, *Cellulosimicrobium*, *Nocardiopsis*, *Aeromonas*, *Flavobacterium*, *Klebsiella* were isolated from the intestines of *Achatina fulica* with cellulose degradation activity (*Pinheiro et al. 2015*), and in which only *Aeromonas* and *Flavobacterium* were found in our study.

In this study, the intestinal microbiota of freshwater snails was modified by diet, which was also reported in other animals, such as mammals (*Muegge et al., 2011*) and fish (*Kokou et al., 2019*). A similar study reported that the sugarcane-based diet altered the gut microbiota of snail *Achatina fulica* (*Cardoso et al., 2012*). Although *Aeromonas* was abundant (exhibiting an abundance of 85.40%) in NHV and was scarce in HV (1.78%), this result does not mean that *Aeromonas* was incapable of digesting plant fibre. Pinheiro *et al*. (2015) found that the 6 *Aeromonas* isolated from snail *Achatina fulica* all had the ability to hydrolyse lignocellulose. Similar conditions were found in the genus *Comamonas*, which also exhibited significantly higher abundance in NHV than in HV snails, but relevant

397 studies also indicate that it may be related to herbivorous species (*Cardoso et al.,*
398 *2012*).

399 However, more species in NHV snails (154 genera) disappeared or decreased
400 their abundance significantly compared to HV snails, indicating that these species
401 were better adapted to the intestinal environment of herbivorous diet than the
402 intestinal environment of non-herbivorous diet. Little is known about the role of
403 this microbiota in, for instance, assisting the host with food digestion or immunity.
404 The variation of these gut bacteria will provide better insights into the interaction
405 of snails and their gut microbiota.

406 PICRUSt analysis showed that different feed treatments had a significant
407 effect on the function of snail intestinal microbiota. Most COG functions had
408 significant differences between the HV and the NHV snails. Further KO analysis
409 showed a significant difference in the gene abundance of lignocellulose-active
410 enzymes between the two groups. The abundance of these lignocellulose-related
411 genes was mostly higher in the HV group than in the NHV group, indicating that
412 the intestinal microbiota in herbivorous snails were more effective in at
413 lignocellulosic metabolism than those in non-herbivorous snails. A large number of
414 genes related to protein degradation were higher in HV than in NHV snails,
415 indicating a more efficient protein utilization in herbivorous feeding snails, and
416 this will be helpful to develop microbial proteases from animal intestine.

417

418 **CONCLUSIONS**

419 Our study provides the first characterization of gut community diversity of
420 *Planorbella trivolvis*, providing insight into gut community structure within
421 freshwater snails. Compared with herbivorous feeding (HV), although non-
422 herbivorous feeding (NHV) promoted the growth of snails, it caused an
423 accumulation of lipid droplets in intestinal mucosal cells. A set of 22 core bacteria
424 was determined to be consistently associated in relatively high abundance (>1%)
425 with the diversity of *Planorbella trivolvis*. Compared with herbivorous feeding,
426 non-herbivorous feeding reduced the alpha-diversity of the intestinal microbiota of
427 snails and changed the composition of intestinal bacterial communities. For
428 example, the abundance of many bacteria decreased or disappeared. Functional
429 prediction showed that the abundance of cellulose degradation genes in the
430 intestinal microbiota of HV snails was considerably higher than that of NHV snails.
431 In summary, our findings showed novel snail-microbe associations and,
432 furthermore, suggest that the variety of bacteria within the gut might promote a
433 better digestion ability of the host to different diets.

434

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595

596

Figure 1

Figure 1 Effects of different diets on intestinal histology of *Planorbella trivolvis.*

A: Intestinal morphology of HV snails (200 μm); B: Intestinal morphology of HV snails (50 μm); C: Intestinal morphology of NHV snails (200 μm); D: Intestinal morphology of NHV snails (50 μm).

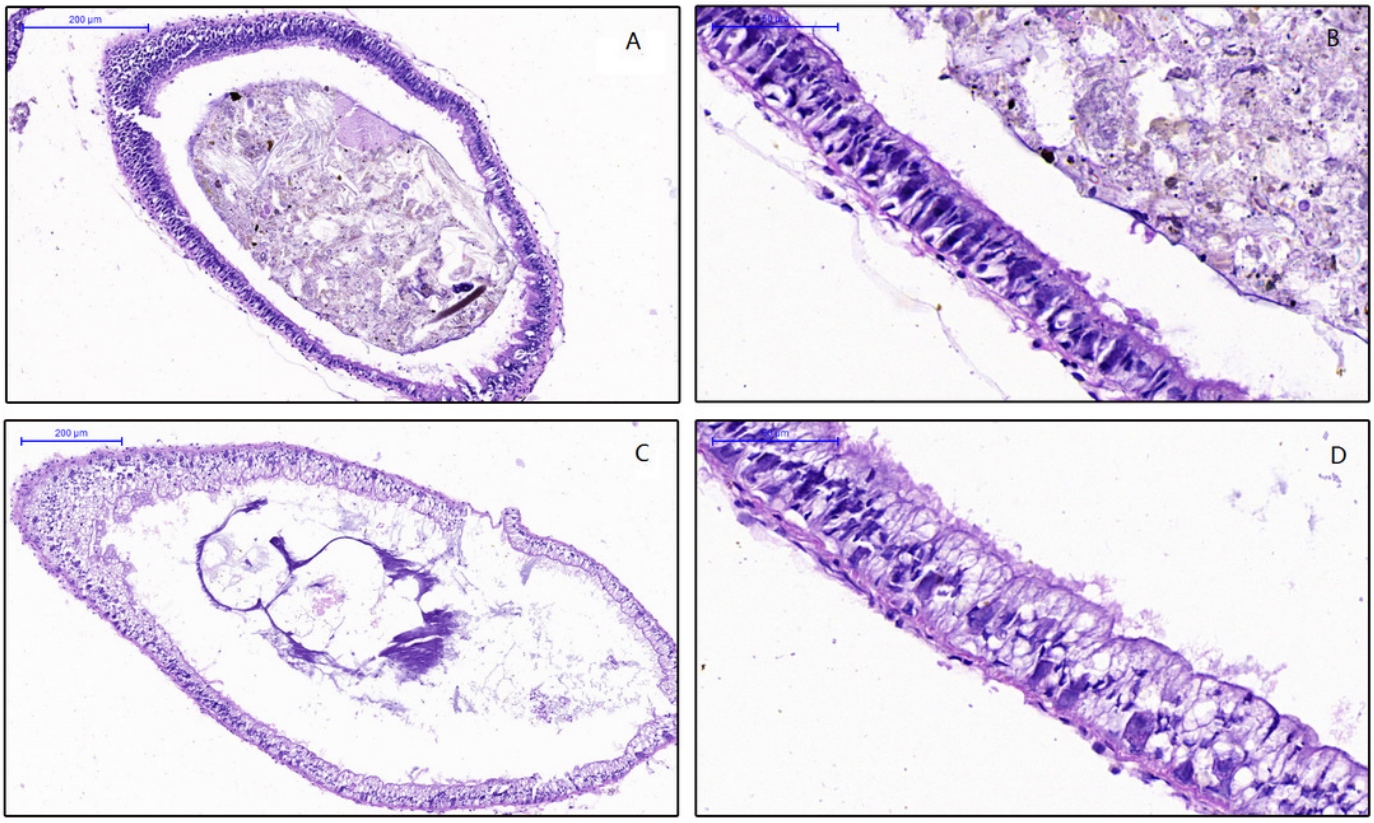


Figure 2

Figure 2 Diversity of gut community in snails fed with diiferent diets types.

A-C: Rarefaction curves (A), Rank-abundance curves (B), and core analysis (C) of intestinal microbial flora at OTU level; D-F: VENN analysis of gut community at phylum (D), family (E), and genus (F) level.

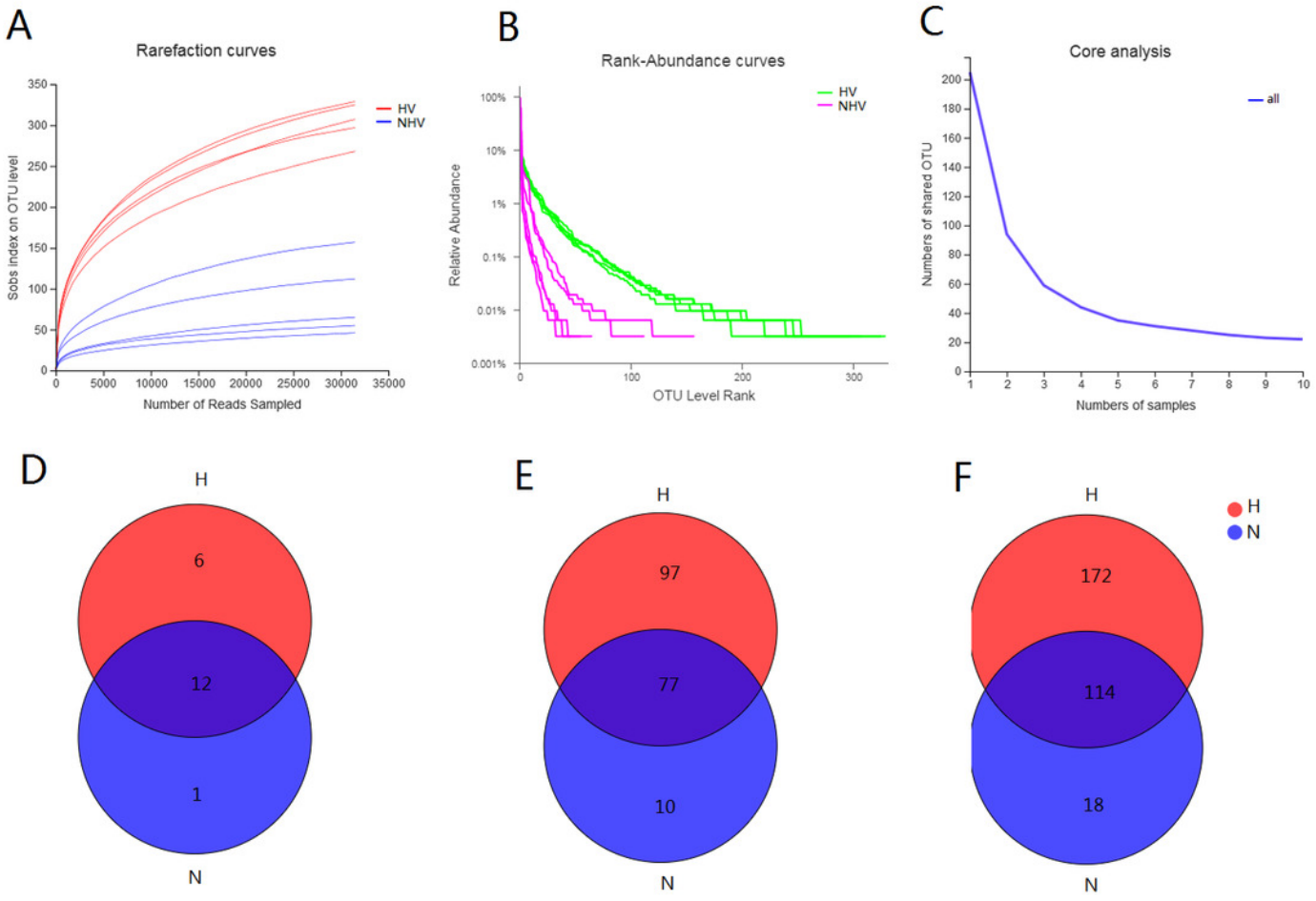


Figure 3

Figure 3 Composition of intestinal microbial flora at phylum(A), family (B) and genus (C) level.

The bacterial taxon with abundance below 1% in phylum, 4% in family, and 3% in genus level was merged and shows as “others”.

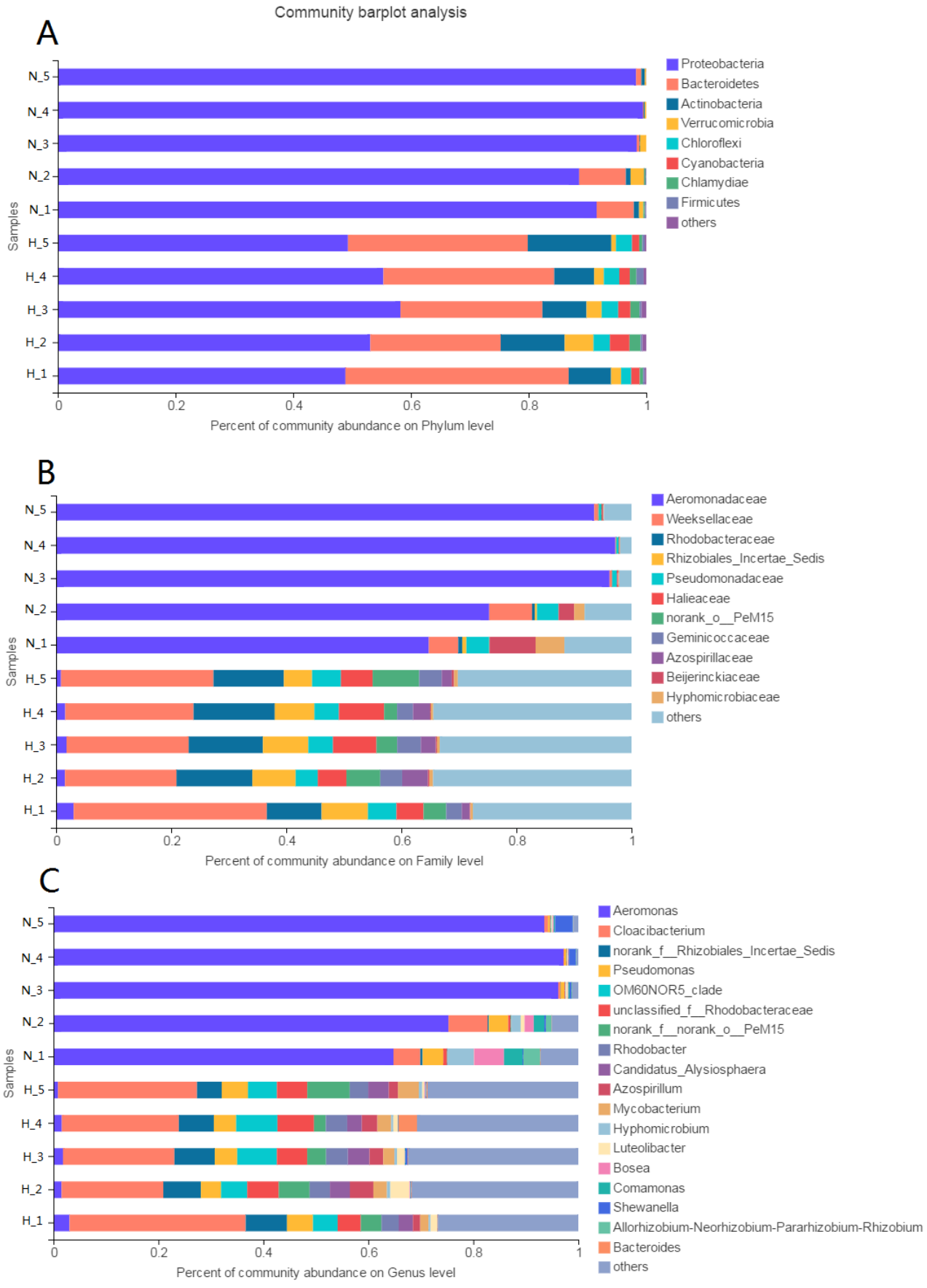

Community barplot analysis
A
N_5
N_4
N_3
N_2
N_1
H_5
H_4
H_3
H_2
H_1
Samples
0
0.2
0.4
0.6
0.8
1
Percent of community abundance on Phylum level
Proteobacteria
Bacteroidetes
Actinobacteria
Verrucomicrobia
Chloroflexi
Cyanobacteria
Chlamydiae
Firmicutes
others
B
Percent of community abundance on Family level
Aeromonadaceae
Weeksellaceae
Rhodobacteraceae
Rhizobiales_Incertae_Sedis
Pseudomonadaceae
Halieaceae
norank_o__PeM15
Geminicoccaceae
Azospirillaceae
Beijerinckiaceae
Hyphomicrobiaceae
others
C
Percent of community abundance on Genus level
Aeromonas
Cloacibacterium
norank_f__Rhizobiales_Incertae_Sedis
Pseudomonas
OM60NOR5_clade
unclassified_f__Rhodobacteraceae
norank_f__norank_o__PeM15
Rhodobacter
Candidatus_Alysiosphaera
Azospirillum
Mycobacterium
Hyphomicrobium
Luteolibacter
Bosea
Comamonas
Shewanella
Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium
Bacteroides
others

Figure 4

Figure 4 Heat map showing the relative abundance of the 35 bacterial genus in snail.

The HV snails are shown by H1, H2, H3, H4, H5 and NHV snails are shown by N1, N2, N3, A4, N5. The red asterisk represented the significantly different genera among two groups, based on wilcoxon rank-sum test, “*” represent $P < 0.05$, “**””represent $P < 0.01$.

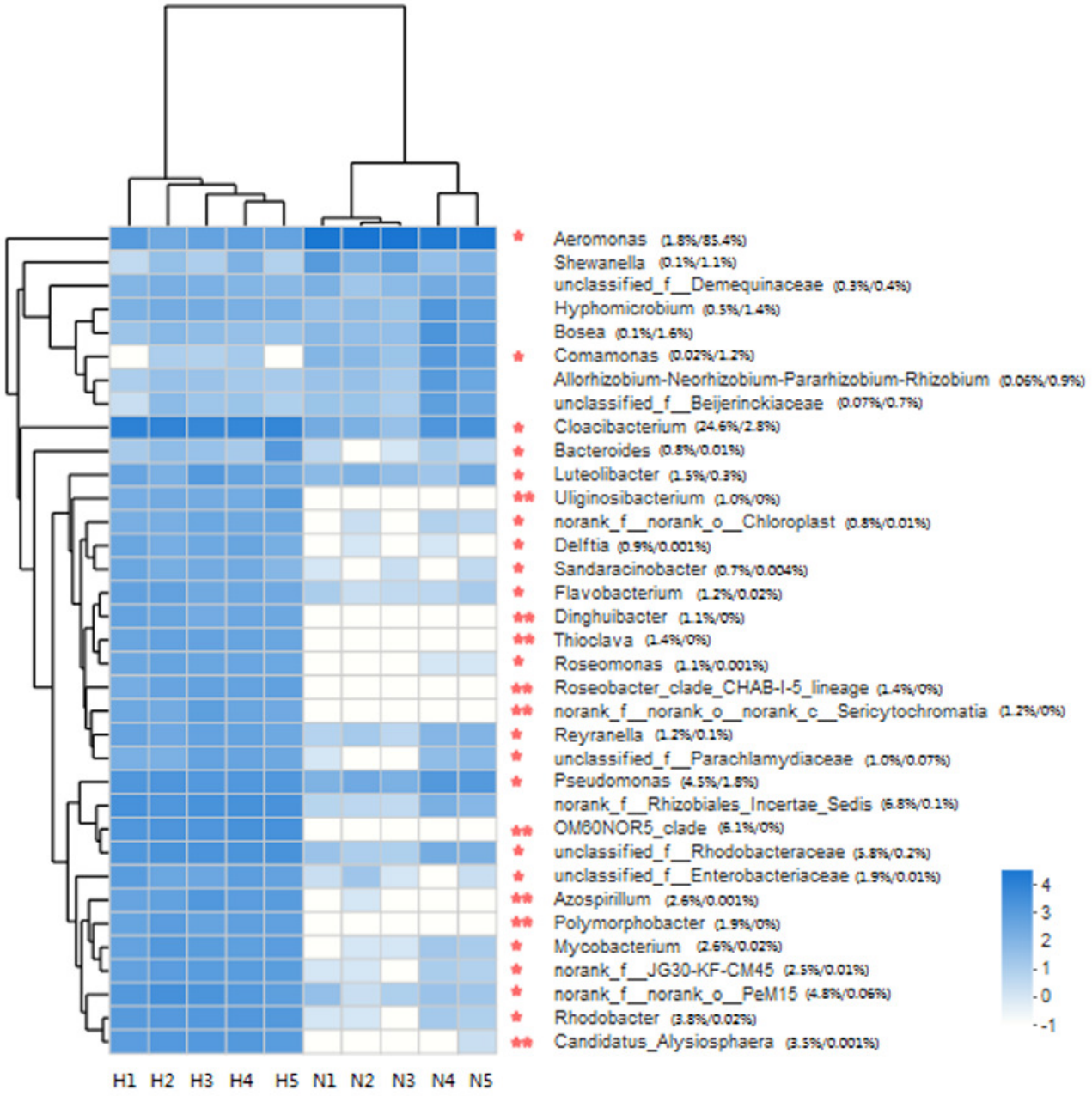

Aeromonas (1.8%/85.4%)
Shewanella (0.1%/1.1%)
unclassified_f__Demequinaceae (0.3%/0.4%)
Hyphomicrobium (0.5%/1.4%)
Bosea (0.1%/1.6%)
Comamonas (0.02%/1.2%)
Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium (0.06%/0.9%)
unclassified_f__Beijerinckiaceae (0.07%/0.7%)
Cloacibacterium (24.6%/2.8%)
Bacteroides (0.8%/0.01%)
Luteolibacter (1.5%/0.3%)
Uliginosibacterium (1.0%/0%)
norank_f__norank_o__Chloroplast (0.8%/0.01%)
Delftia (0.9%/0.001%)
Sandaracinobacter (0.7%/0.004%)
Flavobacterium (1.2%/0.02%)
Dinghuibacter (1.1%/0%)
Thioclava (1.4%/0%)
Roseomonas (1.1%/0.001%)
Roseobacter_clade_CHAB-I-5_lineage (1.4%/0%)
norank_f__norank_o__norank_c__Sericytochromatia (1.2%/0%)
Reyranella (1.2%/0.1%)
unclassified_f__Parachlamydiaceae (1.0%/0.07%)
Pseudomonas (4.5%/1.8%)
norank_f__Rhizobiales_Incertae_Sedis (6.8%/0.1%)
OM60NOR5_clade (6.1%/0%)
unclassified_f__Rhodobacteraceae (5.8%/0.2%)
unclassified_f__Enterobacteriaceae (1.9%/0.01%)
Azospirillum (2.6%/0.001%)
Polymorphobacter (1.9%/0%)
Mycobacterium (2.6%/0.02%)
norank_f__JG30-KF-CM45 (2.5%/0.01%)
norank_f__norank_o__PeM15 (4.8%/0.06%)
Rhodobacter (3.8%/0.02%)
Candidatus_Alysiosphaera (3.5%/0.001%)
4
3
2
1
0
-1
H1 H2 H3 H4 H5 N1 N2 N3 N4 N5

Figure 5

Figure 5 Principal Coordinates Analysis (PCoA) plots in intestinal microbiota of snails at OTU level using pairwise unweighted (A) and weighted UniFrac distances (B).

The HV snails are shown by blue dot and NHV snails are shown by orange triangle. Adonis: p-value = 0.004, R-value = 0.4916 (A); p-value = 0.004, R-value = 0.9170 (B).

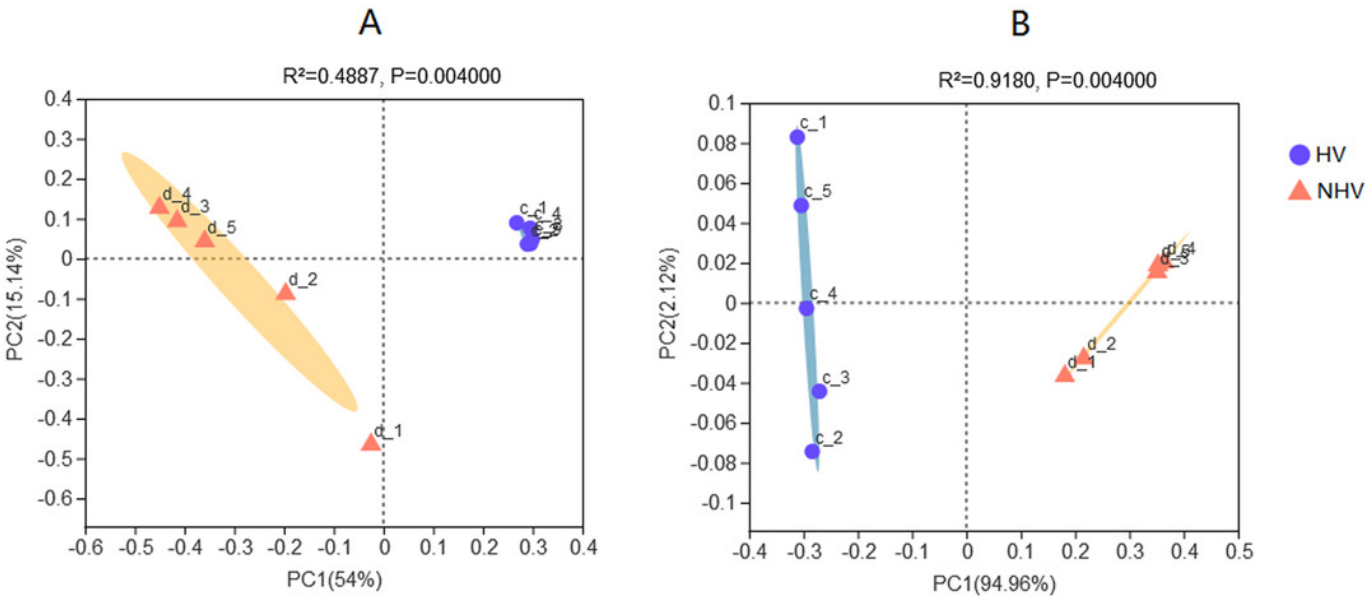


Figure 6

Figure 6 Predictive functional profiles generated from 16S rRNA gene sequences using PICRUSt.

Significant differences between HV and NHV snails were observed for several COG function (two-tailed Student's t-test ; *P < 0.05, **P<0.01). The HV group is represented by green bars (H) and the NHV group by red bars (N).

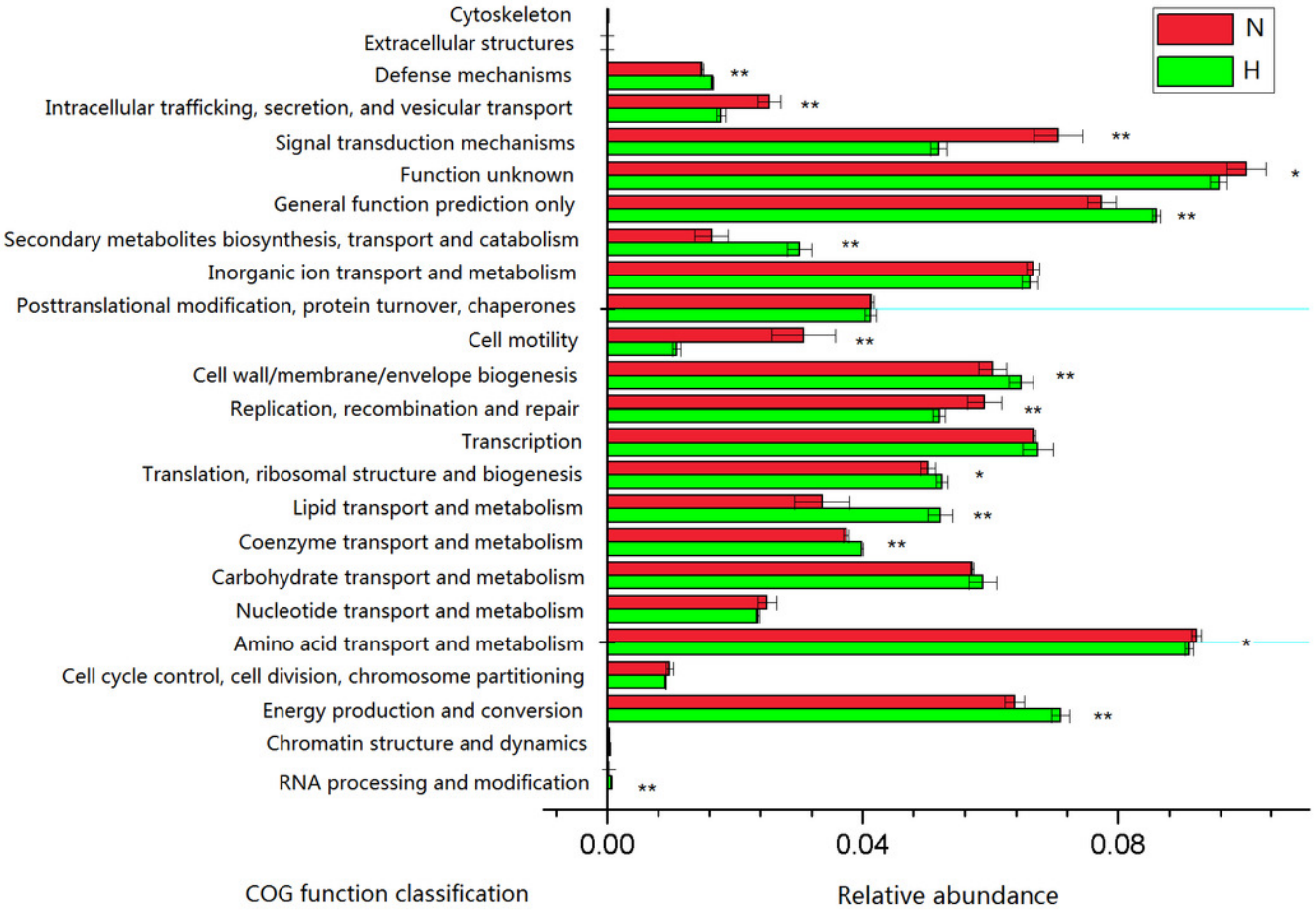
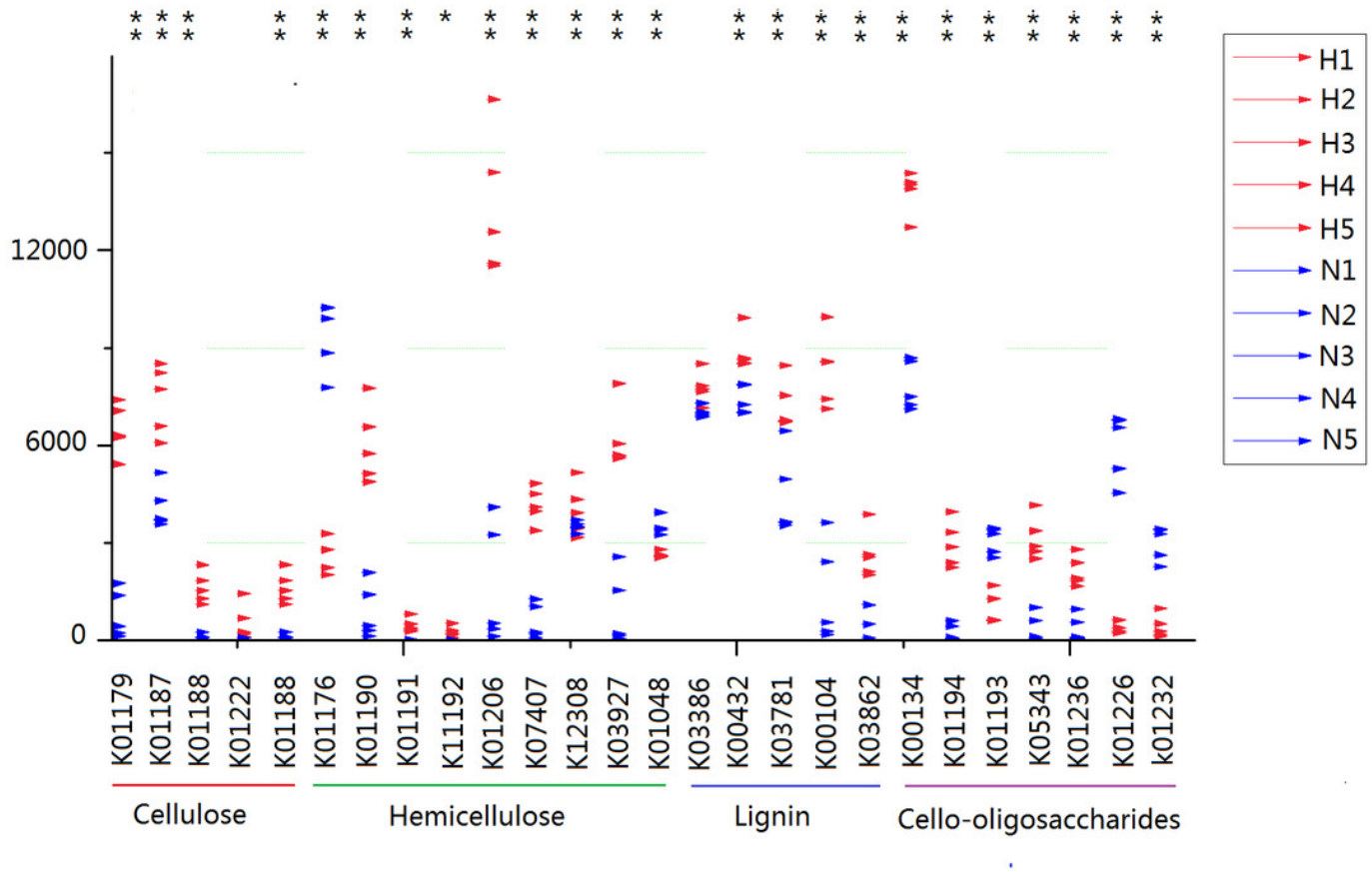


Figure 7

Figure 7 Predictive KEGG Orthology (KO) of lignocellulose-active enzymes across diet types.

H1 to H5 (red color) indicates the sequence number of predicted genes among the HV snails, and N1 to N5 indicates the sequence number of predicted genes among the NHV snails. Each column represents a different entry in the greengene database, each with a unique K-number

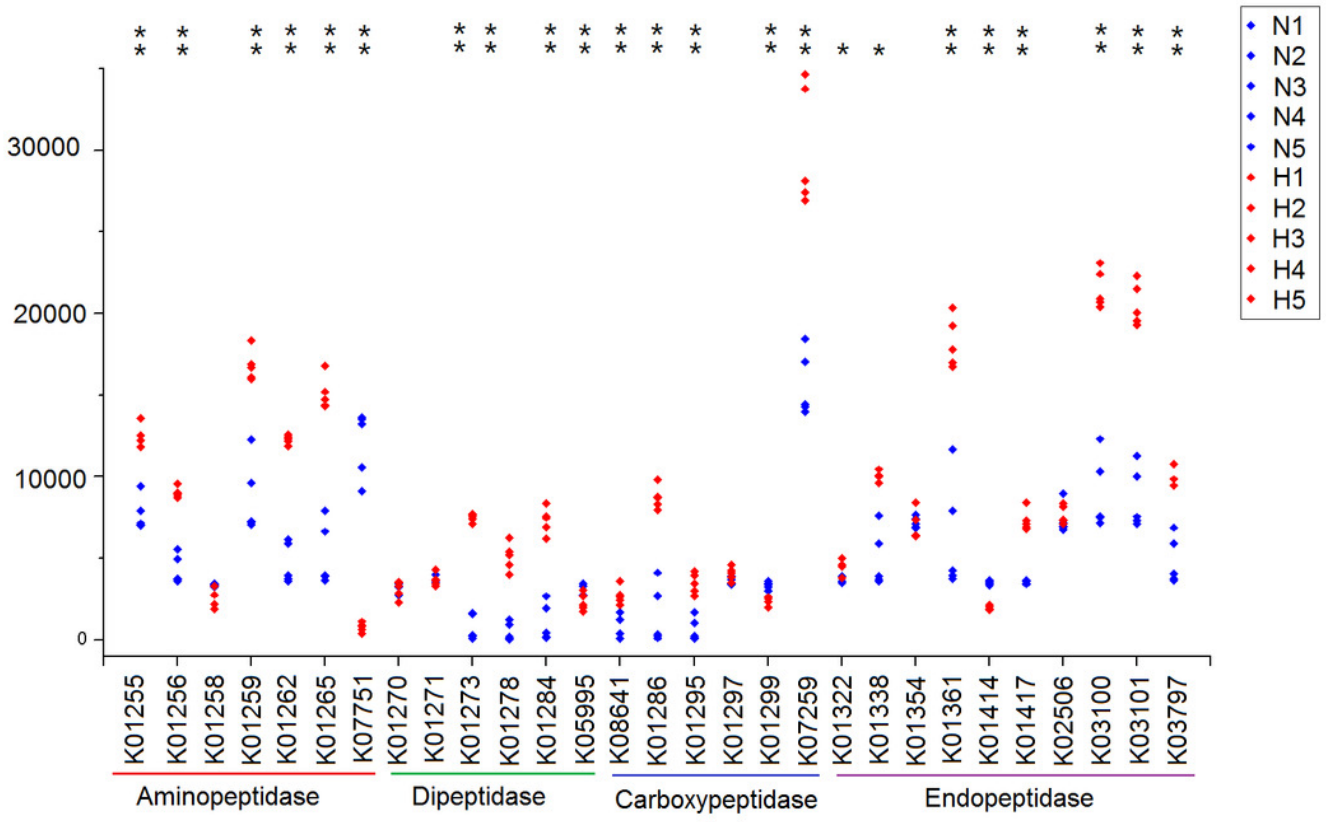


K01179: endoglucanase [EC:3.2.1.4]; K01187: alpha-glucosidase [EC:3.2.1.20]; K01188: beta-glucosidase [EC:3.2.1.21]; K01222: 6-phospho-beta-glucosidase [EC:3.2.1.86]; K01188: beta-glucosidase [EC:3.2.1.21]; K01176: alpha-amylase [EC:3.2.1.1]; K01190: beta-galactosidase [EC:3.2.1.23]; K01191: alpha-mannosidase [EC:3.2.1.24]; K11192: PTS system, N-acetylmuramic acid-specific IIC component; K01206: alpha-L-fucosidase [EC:3.2.1.51]; K07407: alpha-galactosidase [EC:3.2.1.22]; K12308: beta-galactosidase [EC:3.2.1.23]; K03927: carboxylesterase 2 [EC:3.1.1.1]; K01048: lysophospholipase [EC:3.1.1.5]; K03386: peroxiredoxin (alkyl hydroperoxide reductase subunit C) [EC:1.11.1.15]; K00432: glutathione peroxidase [EC:1.11.1.9]; K03781: catalase [EC:1.11.1.6]; K00104: glycolate oxidase [EC:1.1.3.15]; K03862: vanillate monooxygenase [EC:1.14.13.82]; K00134: glyceraldehyde 3-phosphate dehydrogenase [EC:1.2.1.12]; K01194: alpha,alpha-trehalase [EC:3.2.1.28]; K01193: beta-fructofuranosidase [EC:3.2.1.26]; K05343: maltose alpha-D-glucosyltransferase / alpha-amylase [EC:5.4.99.16/3.2.1.1]; K01236: maltooligosyltrehalose trehalohydrolase [EC:3.2.1.141]; K01226: trehalose-6-phosphate hydrolase [EC:3.2.1.93]; K01232: maltose-6'-phosphate glucosidase [EC:3.2.1.122].

Figure 8

Predictive KEGG Orthology (KO) of enzymes related to protein degradation in snails.

H1 to H5 (red color) indicates the sequence number of predicted genes among the HV snails, and N1 to N5 indicates the sequence number of predicted genes among the NHV snails. Each column represents a different entry in the greengene database, each with a unique K-number. The differences of each KO genes were showed by “*” at the top of plot. “*” represent $P < 0.05$, “**””represent $P < 0.01$.



K01255: leucyl aminopeptidase [EC:3.4.11.1]; K01256: aminopeptidase N [EC:3.4.11.2]; K01258: tripeptide aminopeptidase [EC:3.4.11.4]; k01259: proline iminopeptidase [EC:3.4.11.5]; K01262: Xaa-Pro aminopeptidase [EC:3.4.11.9]; K01265: methionyl aminopeptidase [EC:3.4.11.18]; k07751: PepB aminopeptidase [EC:3.4.11.23]; K01270: dipeptidase D [EC:3.4.13.-]; K01271: Xaa-Pro dipeptidase [EC:3.4.13.9]; K01273: membrane dipeptidase [EC:3.4.13.19]; K01278: dipeptidyl-peptidase 4 [EC:3.4.14.5]; K01284: peptidyl-dipeptidase Dcp [EC:3.4.15.5]; k05995: dipeptidase E [EC:3.4.13.21]; k08641: D-alanyl-D-alanine dipeptidase [EC:3.4.13.22]; K01286: D-alanyl-D-alanine carboxypeptidase [EC:3.4.16.4]; K01295: glutamate carboxypeptidase [EC:3.4.17.11]; K01297: muramoyltetrapeptide carboxypeptidase [EC:3.4.17.13]; K01299: carboxypeptidase Taq [EC:3.4.17.19]; k07259: D-alanyl-D-alanine carboxypeptidase [EC:3.4.16.4]; K01322: prolyl oligopeptidase [EC:3.4.21.26]; K01338: ATP-dependent Lon protease [EC:3.4.21.53]; K01354: oligopeptidase B [EC:3.4.21.83]; k01361: lactocepin [EC:3.4.21.96]; K01414: oligopeptidase A [EC:3.4.24.70]; K01417: extracellular elastinolytic metalloproteinase [EC:3.4.24.-]; k02506: leader peptidase HopD [EC:3.4.23.43]; k03100: signal peptidase I [EC:3.4.21.89]; k03101: signal peptidase II [EC:3.4.23.36]; k03797: carboxyl-terminal processing protease [EC:3.4.21.102]

Table 1(on next page)

Table 1 Chemical characteristics of alfalfa (g/100g).

HV (alfalfa): the herbivorous snails reared with alflafa; NHV(pellet feed): the non-herbivorous snails reared with pellet feed. The data on the composition of the pellet feed come from the manufacturer (Takara sakana-ii). The composition of the alfalfa was determined in laboratory as follwing methods: (1)The NDF and ADF levels were measured as described by Van Soest et al. (1991). (2) The crude protein (CP) content was measured by the Kjeldahl N method (AOAC 2000). (3) Soxhlet immersion extraction was used for measure crude fat. (4) Crude ash was measured by firing the sample for 30 min under 550 ℃ in muffle furnace.

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Diets type	Protein	Crude fiber	Crude fat	Crude ash
HV(alfalfa)	19.62 ± 0.23	26.75 ± 0.24	4.86 ± 0.11	8.34 ± 0.02
NHV(pellet feed)	27.22 ± 0.09	4.97 ± 0.03	2.17 ± 0.05	9.03 ± 0.06

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Table 2(on next page)

Table 2 Growth performance of *Planorbella trivolvis*.

In the same column, values with no letter or the same letter superscripts mean no significant difference ($P > 0.05$), while with different letter superscripts mean significant difference ($P < 0.05$). HV, the herbivorous snails reared with alflafa; NHV, the non-herbivorous snails reared with pellet feed. SGR: special growth rate; WGR: Weight gain rate.

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Items	Groups	Initial value	difference	Final value	difference
diameter of shell/mm	HV	5.98±0.07	a	10.53±0.45	b
	NHV	5.78±0.13	a	11.86±0.11	a
Weight/g	HV	0.068±0.003	a	0.219±0.003	b
	NHV	0.065±0.005	a	0.432±0.007	a
WGR/%	HV	\		321.40±16.01	b
	NHV	\		566.90±46.28	a
SGR/%	HV	\		1.94±0.08	b
	NHV	\		3.16±0.11	a

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Table 3(on next page)

Table 3 alpha-diversity indices of intestinal microbial flora of *Planorbella trivolvis*.

HV, the herbivorous snails reared with alflafa; NHV, the non-herbivorous snails rearedwith pellet feed. *P*-values were from a t-test.

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Estimators	HV-Mean	HV-Sd	NHV-Mean	NHV-Sd	P-value
OUT number	307.60	25.71	90.00	46.67	0
Sequences number	54277.60	6298.01	39606.6	6846.06	0.008
sobs	305.2	24.56	87.00	46.67	1.513e-05
shannon	3.42	0.19	1.00	0.50	7.253e-06
simpson	0.09	0.03	0.61	0.17	0.000133
ace	379.08	29.34	118.69	47.56	6.243e-06
chao	375.04	26.28	105.4	47.40	3.809e-06
coverage	0.9976	0.0003	0.9992	0.0003	4.689e-05

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Table 4(on next page)

Table 4 Shared core bacteria among two groups.

HV, the herbivorous snails reared with alflafa; NHV, the non-herbivorous snails reared with pellet feed.

1 Shared core flora among two groups

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Phylum	Family	Genus	HV	SD	NHV	SD
Proteobacteria	Rhizobiaceae	Allorhizobium	0.07	0.03	0.91	1.38
Bacteroidetes	Flavobacteriaceae	Flavobacterium	1.17	0.45	0.02	0.01
Proteobacteria	Rhizobiaceae	norank	0.01	0.01	0.12	0.17
Proteobacteria	Shewanellaceae	Shewanella	0.11	0.16	1.11	1.36
Proteobacteria	Rhodobacteraceae	Rhodobacter	3.78	0.37	0.02	0.02
Bacteroidetes	Weeksellaceae	Cloacibacterium	24.6	5.66	2.75	3.33
Proteobacteria	Aeromonadaceae	Aeromonas	1.78	0.8	85.4	14.57
Verrucomicrobia	Rubritaleaceae	Luteolibacter	1.53	1.25	0.3	0.27
Proteobacteria	Reyranellaceae	Reyranella	1.21	0.34	0.13	0.15
Bacteroidetes	Cytophagaceae	Cytophaga	0.07	0.07	0.07	0.08
Proteobacteria	Sphingomonadaceae	Altererythrobacter	0.02	0.01	0.02	0.02
Proteobacteria	Rhizobiales_Incertae_Sedis	unclassified	0.15	0.03	0.06	0.07
Verrucomicrobia	Rubritaleaceae	Haloferula	0.05	0.05	0.6	0.59
Bacteroidetes	Chitinophagaceae	Sediminibacterium	0.11	0.05	0.26	0.37
Actinobacteria	Demequinaceae	unclassified	0.35	0.13	0.41	0.29
Actinobacteria	Mycobacteriaceae	Mycobacterium	2.58	0.88	0.02	0.02
Proteobacteria	Beijerinckiaceae	unclassified	0.07	0.07	0.68	0.98
Proteobacteria	Xanthomonadaceae	Stenotrophomonas	0.06	0.02	0.05	0.08
Proteobacteria	Enterobacteriaceae	Kluyvera	0.56	0.58	0.02	0.04
Proteobacteria	Pseudomonadaceae	Pseudomonas	4.47	0.5	1.84	1.81
Proteobacteria	Enterobacteriaceae	unclassified	1.85	0.82	0.02	0.03
Actinobacteria	norank_o__PeM15	norank	4.75	2.27	0.06	0.05
Chloroflexi	JG30-KF-CM45	norank	2.51	0.45	0.01	0.01
Proteobacteria	Rhodobacteraceae	unclassified	5.79	0.91	0.25	0.28
Proteobacteria	Rhizobiales_Incertae_Sedis	norank	6.81	1.27	0.15	0.2
Proteobacteria	Beijerinckiaceae	Bosea	0.12	0.06	1.58	2.41
Proteobacteria	Hyphomicrobiaceae	Hyphomicrobium	0.51	0.12	1.42	2.12

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