

A peer-reviewed version of this preprint was published in PeerJ on 28 August 2018.

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Hu Z, Chen X, Chang J, Yu J, Tong Q, Li S, Niu H. 2018. Compositional and predicted functional analysis of the gut microbiota of *Radix auricularia* (Linnaeus) via high-throughput Illumina sequencing. PeerJ 6:e5537 <https://doi.org/10.7717/peerj.5537>

Compositional and functional analysis of the Gut Microbiota of *Radix auricularia* (Linnaeus) via high-throughput Illumina sequencing

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ABSTRACT

Widely distributed across the world, the freshwater snail *Radix auricularia* plays an important role in freshwater systems. In this study, gut bacterial communities of *R. auricularia* were characterized using 16S rRNA amplicon sequencing, then intestinal bacteria were compared at different growth stages: adult snails (AS) (with complete gonadal development) and juvenile snails (JS) (with incomplete gonadal development). We obtained 251,072 high quality sequences which were clustered into 1,196 operational taxonomic units (OTUs) with 97% sequence identity. The predominant phyla were Proteobacteria and Cyanobacteria, followed by Chloroflexi, Firmicutes, and Actinobacteria. Other bacterial species such as Tenericutes, Bacteroidetes, Fusobacteria and Verrucomicrobia were present to a lesser extent. 52 bacterial families and 55 genera were found in > 1% of each sample. A large number of species could not be successfully identified. 469 core OTUs were found to make up 39.38% of all OTUs and 88.38% of all sequences. Samples obtained from juvenile organisms possessed higher ratios of Ruminococcaceae, *Subdoligranulum*, and *Faecalibacterium* than adult species. Furthermore, 16S

rRNA gene data was used to predict function, showing that genes related to metabolism and environmental information processing were rich in snail samples.

Keywords: *Radix auricularia*, Intestinal bacterial communities, 16S rRNA gene, Illumina Miseq sequencing.

INTRODUCTION

Radix auricularia (Linnaeus, 1758) is a Pulmonata Mollusca is naturally distributed in freshwater systems across Europe and Asia (Stift *et al.*, 2004; Vasileva, 2012). As a primary consumer, snails are common in freshwater systems, and their energy and biomass can be transferred to fish, turtles, water birds and mammals (Dewitt *et al.*, 1999; Eckblad J. 2010). Along with their ecosystem roles, *R. auricularia* are intermediate hosts for many parasites (e.g. flukes), which can harm cattle, birds, and humans. Therefore, these organisms are generally considered to be noxious animals (Soldánová *et al.*, 2010; Bargues *et al.*, 2001). Previous studies have demonstrated that this gut bacteria could assist in digestion of lignocellulosic plant biomass. This is important for degradation of vascular plant and fallen leaves, and in biofuel production (Cardoso *et al.*, 2012b; Dar *et al.*, 2015). However, our understanding of the intestinal microbiota of snails is very limited.

Gut bacteria in snails are involved in multiple physiological processes, assisting their hosts with digestion, disease resistance, and environmental resistance (Nicolai *et al.*, 2015). Previous studies have shown that many carbohydrases such as cellulase and chitinase in the digestive tracts of the snail were of bacterial origin (Strasdiné *et al.*, 1963; Pinheiro *et al.*, 2015). In addition, some symbiotic bacteria have the ability to nitrogen-fix, or can assist with sterol and vitamin supply (Salem *et al.*, 2014). It has been proposed that the cellulolytic bacteria isolated

from these snails may be applied in various industrial processes, including the production of biofuels from plant feedstocks (Li et al., 2009). It is therefore important to identify and determine the function of the bacteria in *R. auricularia*.

Typically *R. auricularia* live in ponds and other small water-bodies. They are often faced with habitat-level changes due to seasonal variation, including freezing in the winter, and desiccation in the summer and autumn. Other environmental and anthropogenic factors can also affect the water quality and food availability in their aquatic habitats. The diets of *R. auricularia* vary from single celled algae and aquatic plants, to terrestrial plants and fallen leaves (Schamp et al., 2010). The influence of gut bacteria on snail diet has been previously reported for the land snail *Achatina fulica* (Cardoso et al., 2012a). Furthermore, bacterial community structure in *A. fulica* gut has been found to change as the snails enter estivation state (Pawar et al., 2012a).

Generally speaking, snails have weak locomotive skills and live in restricted regions (Brown et al. 2012), so their diet is often region-specific. This can influence gut bacterial communities in snails (Nicolai et al., 2015). Furthermore, gut bacterial communities can differ depending on location within the digestive tract. Indeed, a previous study has shown that this can occur in the land snail, (Pawar et al, 2012a).

Previous research on the gut bacterial community in Pulmonata has mainly focused on terrestrial snails (Pinheiro et al., 2015; Cardoso et al., 2012; Pawar et al., 2012; Nicolai et al., 2015; Charrier et al., 1998). As found in other animals, gut microbiome could also be influenced by host development and growth (Jami et al., 2013; Nistal E et al., 2012; Stephens et al., 2016; Llewellyn et al., 2015). Indeed, study of the gut microbiome in freshwater snails has been limited. Furthermore, in most of studies researchers have focused on the isolation of cellulolytic bacteria (Pinheiro et al, 2015; Wijanarka et al., 2016) but not diversity of gut bacterial communities.

The aim of this study was to characterize the whole profile of the gut bacterial community in *R. auricularia* and also to determine how growth stage influences gut bacterial community composition. This was accomplished by comparing gut microbiome between juvenile (immature) and adult (mature) snail populations. Furthermore, we wanted to explore the environmental cleaning functions of these snails using metagenomic data to obtain functional predictions of gut bacterial action.

MATERIALS AND METHODS

Research permits were provided by the Forestry Bureau in Tongliao (TL218) and by the Inner Mongolia University for Nationalities' Institutional Animal Use and Care Committee (2016-IMUN-029).

Sample collection

Forty *R. auricularia* snails were sampled from a pond in Tongliao, Inner Mongolia, China (43°38'2.184"N; 122°15'43.9632"E). The depth of pond was approximately 0.5 m. We selected four mature snails (with adequately developed gonads) and four juvenile snails (without developed gonads). The adult snails had the aged of over the last winter (e.g. > 1 year old) and the juvenile snails were born in current year (e.g. < 1 year old).

After being transported to lab, the snails were measured and grouped. The average shell heights of juvenile snails were 12 mm ± 0.2 mm, and the average shell height of adult snails were 20 mm ± 0.2 mm. Snail guts were about 7-15 mm long (not including stomach). The crop and esophagus were not considered in this study.

Snails were washed first in tap and then in sterile water. 70% ethyl alcohol was used to scrub the surface of the snails. The snails were then anaesthetized with MS-222 (Sigma, St. Louis, MO, USA), crushed, and then the gut was removed. The gut and its contents were

carefully collected into plastic cryo-tubes, flash frozen in liquid nitrogen, and then stored at -80°C until the time of analysis.

DNA extraction and PCR amplification

Genomic DNA was extracted using the FastDNA® Spin Kit for Soil (MP Biomedical, U.S.) according to the manufacturer's instructions. The 338F/806R primer set, targeting V3-4 region of the bacterial 16S rRNA gene, was used for PCR amplification as described in *Dennis et al (2013)*. The sequences of each sample were recognized through 12-bp barcodes on primers. The PCR amplification was performed using the TransStart® FastPfu system (Transgen biotech company) (*Ma et al., 2013*).

16S rRNA gene library Sequencing

A NEB Next® Ultra™ DNA Library Prep Kit for Illumina (New England Biolabs Inc., Ipswich, MA, USA) was used to generate sequence libraries according to the manufacturer's instructions. The library quality was assessed by spectrophotometry and fully sequenced on an Illumina Miseq PE300 platform (Illumina Corporation, San Diego, USA) at the Shanghai Majorbio Bio-Pharm Technology co., Ltd. (Shanghai, China).

Data analysis

The generated sequences were processed using the QIIME (version 1.17) pipeline (*Lai et al., 2014*). We eliminated low quality sequences (e.g. those with > 200 bp, > 6 bp of homopolymers, primer mismatches, or quality scores lower than 25). Chimeric sequences were also removed by HCHIME. Using Usearch (vsesion 7.0 <http://drive5.com/uparse/>) operational taxonomic units (OTUs) were clustered and defined at a 97% similarity threshold. Community composition was analyzed using indices of community diversity (Shannon, Simpson) and community richness

(Chao, Sobs) using Mothur software (<http://www.mothur.org/>) (*Schloss et al., 2009*). Phylogenetic affiliations of representative sequences were analyzed by using RDP Classifier against the silva (SSU115) 16S rRNA database with confidence threshold of 70%. Metagenomic functional composition was predicted from the latest Kyoto Encyclopedia of Genes and Genomes (KEGG) database using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) approach (*Langille et al., 2013*). The similarity of bacterial communities in different samples was calculated by using weighted UniFrac and unweighted UniFrac principal co-ordinates analysis (PcoA) analysis. Welch's *t*-test (confidence interval method: Welch's inverted, $P < 0.05$) was performed to compare differences in species abundance between the two groups.

RESULTS

Bacterial complexity of snail gut flora

A total of 576,400 raw reads were generated using the Illumina Miseq sequence platform, and 251,072 high quality sequences were obtained (following quality control and sequence filtration). The mean (\pm standard deviation) number of sequences per sample was $31,384 \pm 4,292$ (Table S1), and the average length of each sequence was 434 bp. Sequences were clustered into 1,196 OTUs (mean number in each sample: 890.75 ± 43.80), with 1,130 and 1,125 OTUs in juvenile and adult snails, respectively. The representative sequences for all OTUs are available in Data S1. The plateau status of the rarefaction curves indicated that the depth of sequencing was sufficient (Fig. 1). Ace, Chao, Shannon, Simpson and Sobs indices indicate that there were no significant

differences in diversity between juvenile population and adult population ($p > 0.05$, student's T test) (Table 1).

Taxonomic composition of gut bacterial community

Abundances of bacterial taxa that were found in the snails is illustrated in Figure 2. The OTUs that could not be assigned to a genus are displayed using the highest taxonomic level that could be assigned (order, class, or phyla).

Ten phyla represented the core members of the snails gut microbiota in adult and juvenile snails, accounting for 98.88% of total sequences (Fig. 2A). Proteobacteria (JS: 35.98%, AS: 31.57%), Cyanobacteria (JS: 16.30%, AS: 19.48%), Chloroflexi (JS: 9.73%, AS: 13.14%), Firmicutes (JS: 14.39%, AS: 6.73%) and Actinobacteria (JS: 8.15%, AS: 12.58%) were the dominant phyla. Other phyla with lower abundance were Tenericutes (JS: 7.25%, AS: 6.17%), Bacteroidetes (JS: 3.37%, AS: 2.03%), Fusobacteria (JS: 1.32%, AS: 1.15%), Verrucomicrobia (JS: 0.73%, AS: 1.62%). Proteobacteria contained the largest number of OTUs, followed by Firmicutes, Cyanobacteria, Actinobacteria, Bacteroidetes, and Chloroflexi.

The prominent phylum, Proteobacteria, showed high diversity. 464 OTUs of Proteobacteria belonged to the following classes: gamma-, delta-, beta- and alpha-Proteobacteria (Fig. S1). Among them, alpha-proteobacteria ranked first in terms of their sequence numbers (51.41%), followed by gamma-proteobacteria (36.60%). Delta- and epsilon-proteobacteria made up only a few of the sequences.

There were 53 identifiable bacterial families with >1% abundance in each sample (Fig. 2B). Abundance was as follows: FamilyI_o_SubsectionIII (c_Cyanobacteria), *Rhodobacteraceae*, *Chloroflexaceae*, *Mycoplasmataceae*, *Chromatiaceae*, FamilyII_o_SubsectionII

(c__Cyanobacteria), Cyanobacteria, Lachnospiraceae, Ruminococcaceae, Caldilineaceae, Nocardiodaceae, Acetobacteraceae, Leptotrichiaceae, and MNG7.

Genera with <1% abundance in each sample made up abundance of 44.39% (on average). Of the 54 genera with >1% abundance in each sample, 36 genera were identified (Fig. 2C), the classified genera with relative high abundance were *Paracoccus*, *Pleurocapsa*, *Microcoleus*, *Thiodictyon*, *Leptolyngbya*, *Eubacterium*, *Subdoligranulum*, *Nocardioideis*, *Pseudomonas*, *Faecalibacterium*, *Chroococcidiopsis*, *Kluyvera*, *Rhodobacter*, *Lemprocystis*, *Gemmobacter*, *Hyphomicrobium*, and *Cryobacterium*.

Microbial community analysis

Principal coordinate analysis (PcoA) was used to determine the similarities in gut microbial communities between juvenile and adult snails. Although unweighted UniFrac distance PcoA showed that juvenile snail samples formed a distinct cluster and were separated from adult snail samples, weighted UniFrac distance PcoA indicated that the samples did not cluster into two groups (Fig. 3).

We also assessed the differences in species abundance between the JS and AS populations. We found that at both phyla and genera level, there was no difference in abundance of the vast majority of bacteria (Fig. 4A and C). LEfSe analysis (threshold: 3.5) showed that three genera of bacteria were significantly associated with juvenile snails, *Ruminococcaceae*, *Subdoligranulum* and *Faecalibacterium* (Fig. 4B).

Bacterial community differences and similarities

Venn analysis found that a large proportion of OTUs (1060) were shared between juvenile and adult snails, composing 88.70% of all OTUs numbers. In fact, 70 unique OTUs were found in juvenile snails and 65 were found in adult snails (Fig. S2). The core number of OTUs found in

all snail samples was 469, representing 39.38% of all OTUs and 88.38% of OTU sequences (Table S2). Among them, 15 core OTUs had a mean abundance > 1%, and made up 33.87% of all OTU sequences. The most abundant core bacterial genera were *Mycoplasmataceae*, *Chloroflexaceae*, *Paracoccus*, *Microcoleus*, *Pleurocapsa*, *Thiodictyon*, *Caldilineaceae*, *leptolyngbya*, *Eubacterium*, *Subdoligranulum*, and *Nocardioidea* (Table S2).

16S rRNA gene function prediction

Genomic functional prediction, carried out using PICRUSt, is shown in Figure 5. The level 1 KEGG pathway indicated that there were a high abundance of genes related to metabolic pathways, environmental information processing, and genetic information processing. Sequences related to metabolism accounted for 50.82% of total sequences (Fig. S3).

From the level 2 KEGG pathway data (Fig. 5), we found that the sequences related to membrane transport, amino acid metabolism and carbohydrate metabolism were rich in both JS and AS samples, with average abundances of 12.36%, 10.47% and 10.04%, respectively. The KEGG pathway of energy metabolism, cell motility and transcription showed significant differences between JS and AS groups ($P < 0.05$). The sequences related to human diseases (e.g. infectious and neurodegenerative diseases) were found to have low abundances.

Further examination of carbohydrate metabolism revealed an abundance (6.72%) of genes related to starch and sucrose metabolism, including genes that code for cellulose degradation enzymes (Fig. S4).

According to KEGG mapping using the KO system (Fig. S5), genetic pathways associated with xenobiotic biodegradation and metabolism were drawn. Results showed that sequences related to organic contaminant metabolism were abundant. This included contaminants typically

metabolized by the cytochrome p450 family, including benzoate, toluene, aminobenzoate, naphthalene, polycyclic aromatic hydrocarbons and other similar xenobiotics. Furthermore, some genes with that are typically associated with the degradation of highly toxic matter were also present in relatively high abundance, including those associated with degradation of dioxins, atrazine, xylene, bisphenol A, and ethylbenzene. This indicated that gut bacteria in snails may help degrade anthropogenic pollutants which could be harmful to animals and humans.

DISCUSSION

R. auricularia is a freshwater herbivorous snail of great environmental and ecological importance (AL-Sultan et al., 2017; Gerard et al., 2008). In this study, snails were sampled in the summer, during a time the snails typically undergo rapid growth due to suitable temperatures and adequate food supplies (Guo et al., 2016; Zhang et al., 2016). To compare gut microbial communities during different growth stages, adult and juvenile snails were captured. To limit environmental condition differences, these organisms were all sampled from the same aquatic area.

Juvenile and adult snail populations we collected all had a well-developed digestive system, so diet was highly similar between sample groups. Furthermore, as they were from the same water area, the dietary composition for snails could be assumed to be similar. The main difference between the two sampled populations was gonadal development, body size, and growth rate. These factors may have influenced the gut bacterial composition and due to differences in colonization conditions (e.g. the more mature snails provided bacteria with a longer time to colonize). In this study, we characterized the gut bacterial community of snail *R. auricularia* using new generation sequencing technology. The large number of OTUs (1196)

251 obtained indicated rich bacterial resources in snails. These species belong to over ten phyla taxa
 252 (predominantly Proteobacteria followed by Cyanobacteria, Chloroflexi and Firmicutes).
 253 Bacterial composition showed some similarity with other snails. For example, in *A. fulica*
 254 (*Pawar et al., 2012*), *H. pomatia* (*Nicolai et al., 2015*), *Biomphalaria pfeifferi*, *Bulinus africanus*,
 255 and *Helisoma duryi* (*Horn et al., 2011*) Proteobacteria were the prominent phylum. However, the
 256 bacterial composition of *H. pomatia* as determined by *Cardoso et al. (2012)* was also different
 257 than what is reported here. They found that the dominant gut bacteria phylum was bacteroidetes
 258 (and the second and third most dominant bacterial phyla were Proteobacteria and Firmicutes),
 259 while Proteobacteria was very dominant in nearby crops. However, the bacteria present in our
 260 study (e.g. *Pseudomonas*, *Clostridiaceae*, *Lactococcus*, *Bacteroides*, *Flavobacteriaceae*,
 261 *Mucilaginibacter*, *Citrobacter*, *Klebsiella*, *Aeromonas*, *Acinetobacter*, and *Sulforosprillum*) were
 262 also previously found to exist in the gut of *A. fulica* (*Cardoso et al., 2012*), which indicates that
 263 these gut microbiota can exist in herbivorous snails. The genera *Klebsiella* and *Enterobacter* are
 264 also often found in *A. fulica* gut (associated with carboxymethyl cellulase (CMCase) activity) but
 265 were not found in our study (*Pawar et al., 2010*). Community diversity, obtained using Ace,
 266 Chao, Shannon and Sobs diversity indices, was fairly similar between the juvenile and adult
 267 snails. Juvenile and adult snails also had a high proportion of shared OTUs and similarities
 268 between the most abundant bacterial taxa. This indicated that adult and juvenile snails likely
 269 have similar gut bacterial structures. In fact, using unweighted abundance (unweighted UniFrac)
 270 PCoA analysis, samples were clustered according to their growth stage (juvenile-stage and adult-
 271 stage clusters), indicating that the development of stage had some effect on gut bacterial
 272 community.

Cyanobacteria are widespread in aquatic areas and are a main source of energy for snails (Qiao *et al.*, 2018). Cyanobacteria were the second most dominant bacterial taxa in our study. There were 147 OTUs assigned to Cyanobacteria, which represented 12.29% of total OTUs and 17.89% of total abundance. Like in other herbivores, the high abundance of Cyanobacteria was likely the result of incomplete digestion of exogenous plants (Lin *et al.*, 2014). Among this phylum, FamilyI (order SubsectionIII) was the most dominant among all measured snail samples (Fig. 2B), suggesting that FamilyI (order SubsectionIII) may be an important dietary resource for *R. auricularia*. Many of the Cyanobacteria bacteria found in snail gut are of environmental origin. Typically these bacteria come from freshwater and soils, and often include *Leptolyngbya*, *Nostoc*, and *pleurocapsa*, *Microcoleus*, *Gemmobacter*, *Exiguobacterium*, and *Rubrobacter*. The incomplete digestion of these species in snail diets may be due to the short-residence time of consumed food in digestive tract.

The gut bacterial communities play important roles in the digestion of cell walls and plant lignocelluloses into glycoside hydrolases (Flint *et al.*, 2008; King *et al.*, 2010; Morrison *et al.*, 2009; Prasad *et al.*, 2018). Many of bacteria found in our study are related to cellulolytic species. Huang *et al* (2012) found that 70% of the isolated cellulolytic bacteria from the gut of *Holotrichia parallela* larvae were Proteobacteria, and some of cellulolytic bacteria belonged to Actinobacteria, Firmicutes and Bacteroidetes. Similarly, the genera found in our study (e.g. *Paracoccus*, *Pseudomonas*, *Aeromonas*, *Stenotrophomonas* and *Citrobacter*, *Bacillus*, and *Micrococcus*) have previously been identified as cellulolytic species (Huang *et al.*, 2012; Shil *et al.*, 2014; Saha *et al.*, 2006).

Our results show that the most abundant OTUs (OTU585) were affiliated with *Mycoplasmataceae* which belongs to phylum Tenericute. In our study, Tenericute was one of

the most abundant bacterial taxa in *R. auricularia*. It has also been found to be abundant in *A. fulica* (Pawar et al., 2010). *Mycoplasma* is believed to be an infectious species that can colonize in humans and a wide range of animal species, and cause diseases in the hosts (Biondi et al., 2014). Another commonly detected bacterium was Chloroflexaceae, which is considered to be a photosynthetic bacteria (Gupta, 2013). The appearance of Chloroflexaceae in the snail gut may have a dietary origin. *Aeromonas* were also found in the gut of snails. As a member of the Enterobacteriaceae family, *Aeromonas* are associated with human diseases that may lead to serious illnesses (Parker et al., 2010; Parker and Shaw, 2011).

Although previous research has confirmed that bacterial communities vary during host development and in growth (from birth to adulthood) (Amato et al., 2014; Stephens et al., 2016), some studies have shown that the bacteria communities are relatively similar between juvenile and adult stages in some animal hosts (Xue et al., 2015; Hird et al., 2014). Our study showed the gut bacterial flora were similar between juvenile and adult snail populations when taking taxon abundance into account (Fig. 3). Few biomarkers were found in JS and AS populations. *Faecalibacterium* and *Subdoligranulum* (both belonging to the family Ruminococcaceae) were enriched in JS and poor in AS. This is commonly found in many animals (GU et al., 2013; Dethlefsena & Relman, 2011). These bacterium have been found to be highly beneficial to their hosts, by producing butyrate and other short-chain fatty acids through the fermentation of dietary fiber (Miquel et al., 2013; Flint et al., 2012). These biomarkers may be important for juvenile snails in terms of improvement of digestive ability, boosting of immune system, and other similar physiological functions.

To reveal the role of gut bacterial community in snails, we explored the function of gut bacteria using PICRUSt (based on the 16rRNA gene data). Results indicated that the microbiome

genes are related to many physiological functions which may help bacterial hosts (*Sommer and Bäckhed, 2013; Zhao et al., 2013*). Previous studies with the snail *A. fulica* showed that many particular functional genes in gut microbiota (e.g. genes associated with the production of amino acids, fatty acids, cofactors, vitamins, and enzymes) are needed by the hosts for plant fiber degradation (*Cardoso et al., 2012*). Most other genes found in our study were also demonstrated to be necessary for many physiological functions. In fact, the richness of cellulolytic genes could lead researchers to be very interested in the isolation of cellulose enzyme bacteria from snails (*Pinheiro et al., 2015*). Furthermore, the discovery of sequences related to human diseases (e.g. infectious and neurodegenerative diseases) illustrate the role of snails in spreading disease between animals and humans.

CONCLUSIONS

In this study, the composition of gut bacteria of snail *R. auricularia* were described and their metagenomic functions were predicted using PICRUSt. The predominant phyla were Proteobacteria, Cyanobacteria, Chloroflexi, Firmicutes, and Actinobacteria. High diversity was showed in OTUs and at the genera level. Growth and gonadal development influenced the taxonomic character of the gut bacterial community to some extent. For *R. auricularia*, potential for isolation of cellulolytic bacteria, and future use in environmental cleaning efforts were demonstrated using metagenomic functional composition predictions. Further research is needed to better characterize the interaction between gut flora and their hosts in snails like *R. auricularia*.

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Table 1(on next page)

Alpha-diversity calculations for each sample of of juvenile snails and adult snails

Student's t test were used to detect if there were significant differences between two groups(* were used to represent significant differences).

Table 1 Alpha-diversity index of juvenile snails and adult snails

Sample\Estimators	chao	shannon	simpson	sobs
Adult snails	969.22±46.64	5.12±0.22	58.46±22.53	879±58.04
Juvenile snails	1017.3±46.51	5.00±0.22	46.73±15.91	905.25±23.04
P-value	0.19	0.48	0.65	0.43

Figure 1

Rarefaction analysis of observed richness of the snails bacterial communities.

There were eight samples collected and all were successfully sequenced. Four samples collected from juvenile snails (JS) and four samples from adult snails (AS). Operational taxonomic units (defined at 97% sequence similarity) identified by Illumina Miseq sequencing of 16S rRNA genes V3-4 region.

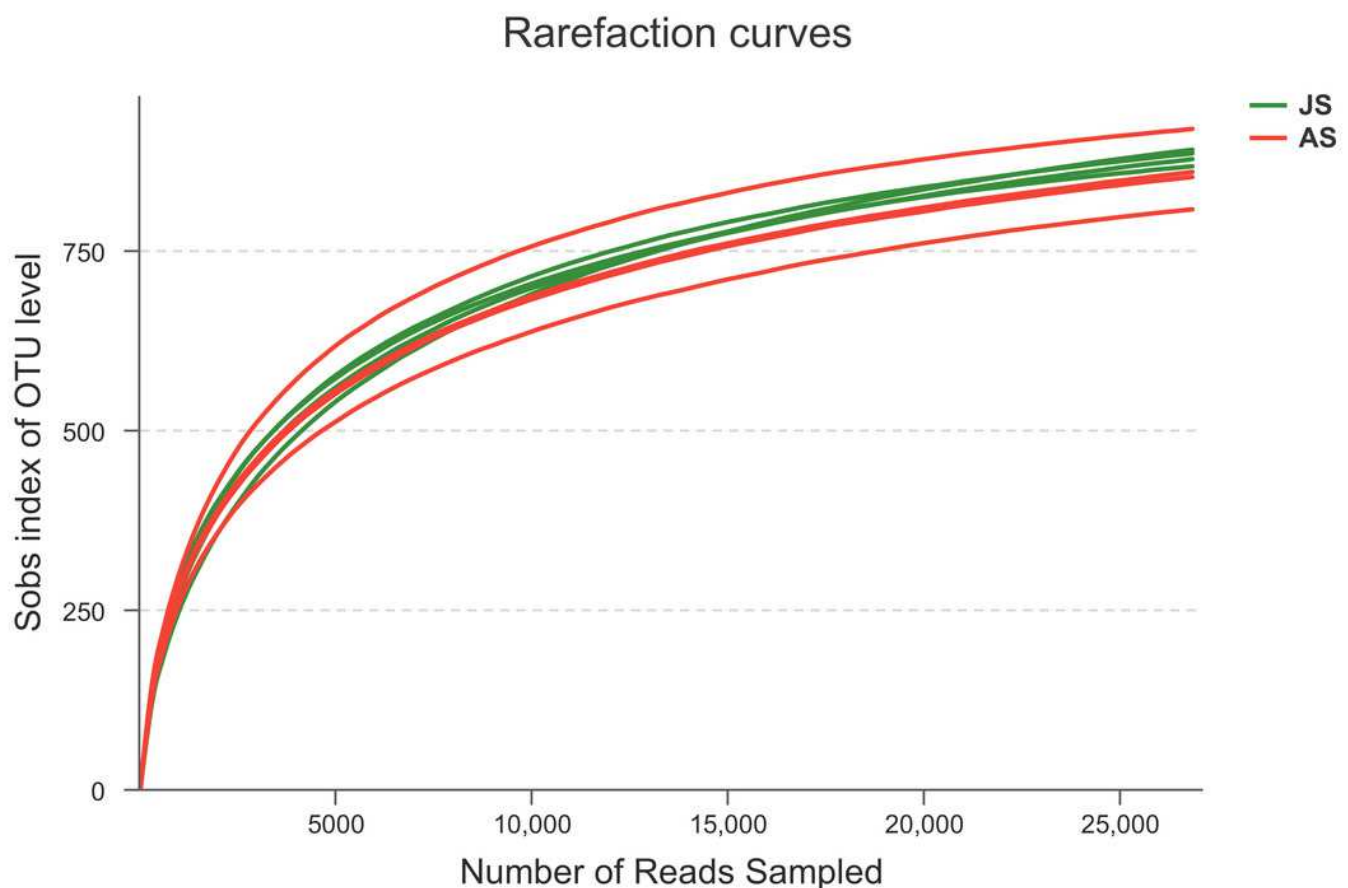


Figure 2

Relative abundance of bacterial communities in *R. auricularia* samples.

(A) phylum level. All remaining taxa that with abundance $<0.01\%$ are summarized as “Other”. (B) family level (or the nearest identifiable phylogenetic level) . All remaining taxa that with abundance $<1\%$ are summarized as “Other”. (C) genus level (or the nearest identifiable phylogenetic level).

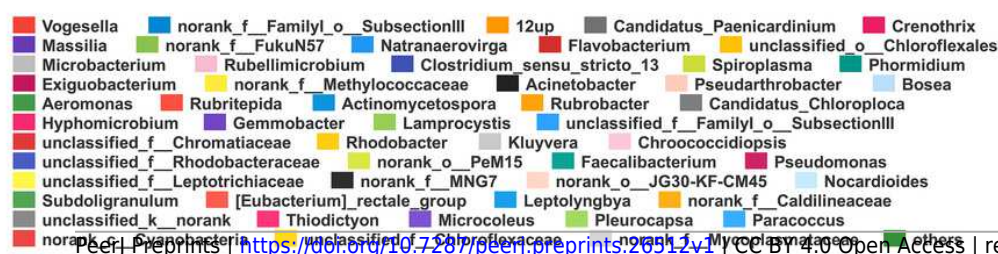
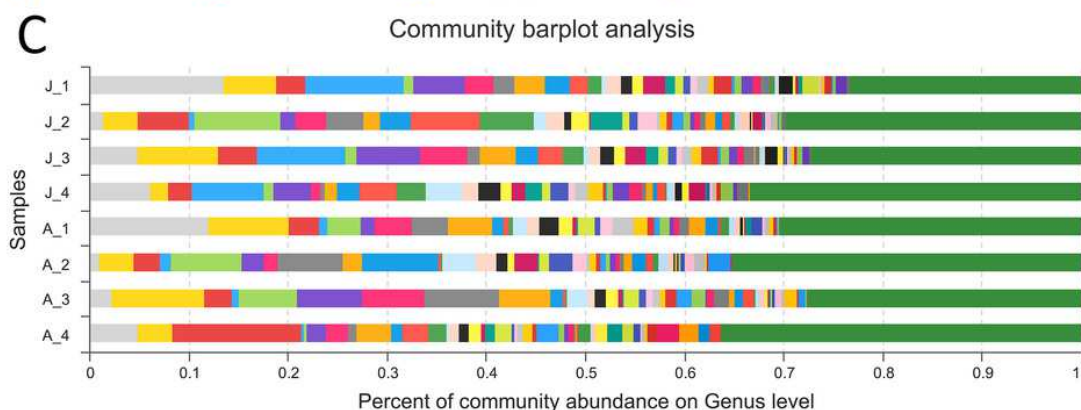
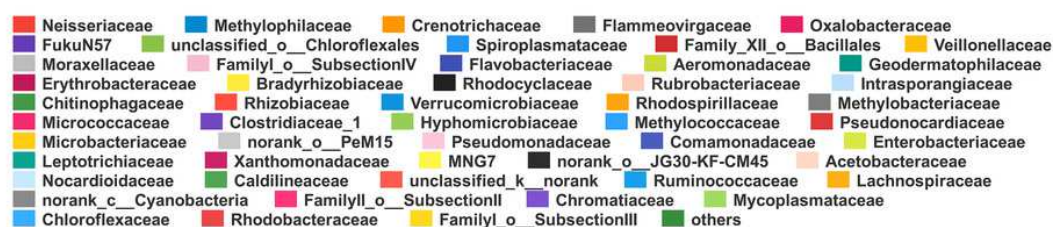
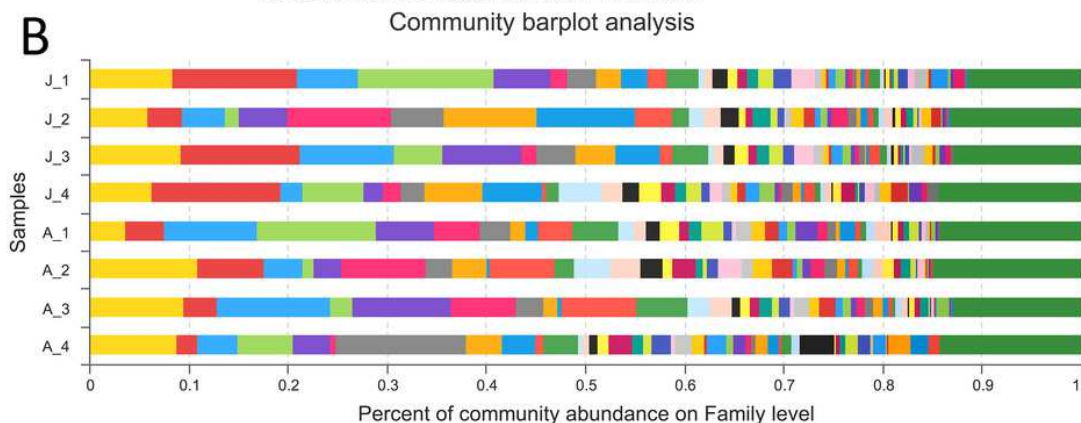
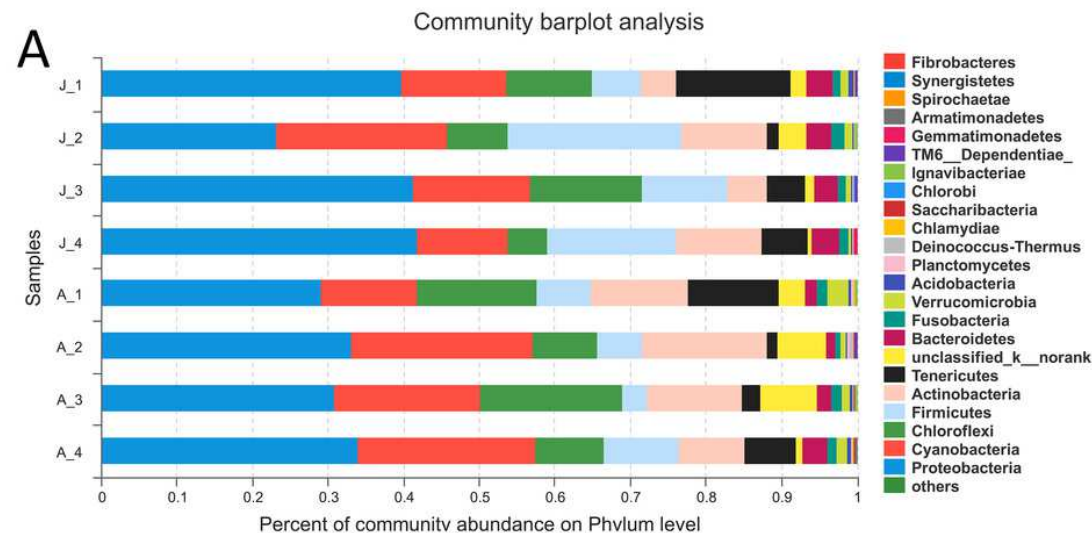


Figure 3

Weighted uniFrac and Unweighted uniFrac principal coordinate analysis of the snails bacterial communities.

(A) Unweighted uniFrac principal coordinate analysis. (B) Weighted uniFrac principal coordinate analysis. The juvenile snails were showed by J1,J2,J3,J4, adult snails were showed by A1, A2, A3, A4.

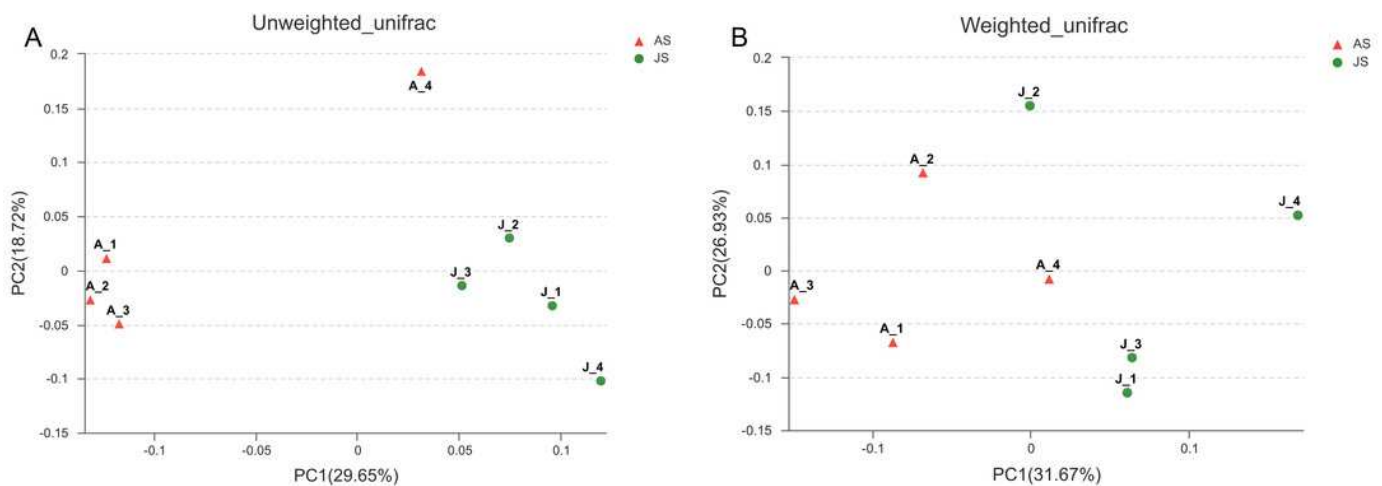


Figure 4

Taxonomic difference between juvenile and adult groups.

(A) Wilcoxon rank-sum test bar plot of bacterial taxon phyla. (B) Wilcoxon rank-sum test bar plot of bacterial taxon core OTUs. (C) Diagram of significant associations between gut bacterial taxon and snail population (linear discrimination algorithm LEFSe, Threshold = 3.5). AS represented adult snails and JS represented juvenile snails.

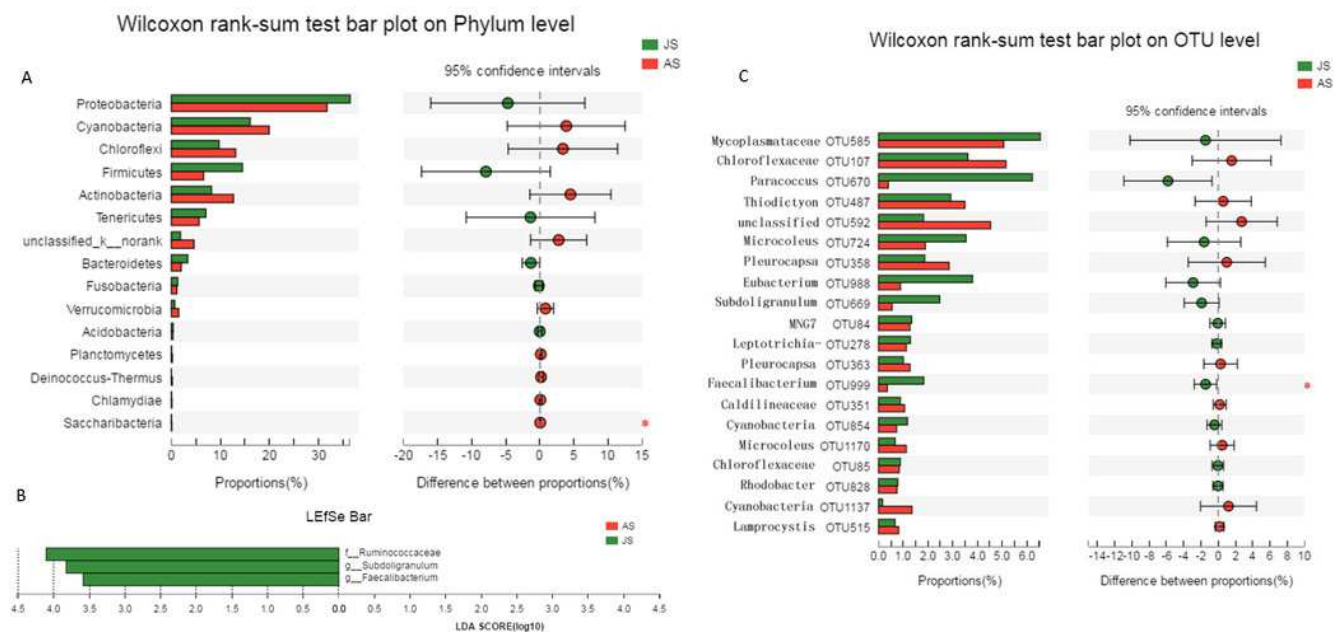


Figure 5

PICRUSt predict the functional composition of snails microbiome.

The left list represent KEGG pathway at level 1, middle list represent KEGG pathway at level 2, the right list represent the abundance of each function pathway. JS: juvenile snails, AS: adult snails.

