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Intestinal microbiota and functional characteristics of black soldier fly larvae (*Hermetia illucens*)

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Abstract

Purpose: Black soldier fly transforms organic waste into insect protein and fat, which makes it valuable for ecological utilization. This process is associated with the intestinal microbiota. This research was developed to determine the type and functional characteristics of intestinal microbiota present in black soldier fly larvae.

Methods: In this research, metagenomics has been used to study black soldier fly larvae gut bacteria, which involves the high abundance of the gut microbe advantage bacterium group, the impact, and the physiological functions of the microbiota. Furthermore, intestinal bacteria and their related functions were investigated by bioinformatics analysis to evaluate potential microbial strains that may be used to improve feed utilization efficiency in factory farming.

Result: The results showed that black soldier fly larvae's intestine contains more than 11,000 bacteria. The high relative abundance of group W (larvae fed with 75% wheat bran and 25% soybean powder) may promote feed utilization efficiency, whereas high relative abundance of group T microbiota (larvae fed with 75% wheat bran and 25% soybean powder supplemented with 1% tetracycline) may play an important role in black soldier fly larvae survival.

Conclusion: The gut bacteria in black soldier fly larvae were involved in polysaccharide biosynthesis and metabolism, translation, membrane transport, energy metabolism, cytoskeleton, extracellular structures, inorganic ion transport and metabolism, nucleotide metabolism, and coenzyme transport physiological processes. The 35 significant differential microbes in group W may have a positive impact on feed utilization and physiological process.

Keywords: *Hermetia illucens*, Intestinal bacteria, Utilization efficiency, Metagenomics

Background

Black soldier fly *Hermetia illucens* (Diptera: Stratiomyidae) larvae are commonly used to recycle organic waste (van Huis 2013). They feed on organic waste including livestock manure (Rehman et al. 2017; Beskin et al. 2018), food waste (Nguyen et al. 2015), and organic waste (Li et al. 2011). The black soldier fly is currently used as a tool for waste transformation and utilization, but few researches consider diverse protocols to improve its waste utilization efficiency. Intestinal microorganisms are involved in several insect functions, including nutritional coordination (Douglas 1998), defense against plant toxins (Hammer and Bowers 2015), physiological response (Basset et al. 2000; Li et al. 2016), life span increase (Hoyt et al. 1971), influence on the development and reproductive potential (Gavriel et al. 2011; Prado and Almeida 2009), and detoxifying of specific foods (Hehemann et al. 2010), among others. Collectively, insect intestinal microorganisms play a crucial role in the growth and development of insects. Other studies

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have shown that bacteria in insects' gut play an important role in promoting food utilization. In this regard, gut bacteria of wood-feeding higher termite promote cellulose and xylan hydrolysis (Warnecke et al. 2007), whereas *Odontotaenius disjunctus* intestinal microbiota contributes to lignocellulose decomposition (Ceja-Navarro et al. 2019). Studies of black soldier fly larvae gut microbes include antibacterial peptide active substances extraction (Park et al. 2015), intestinal specific microbiota (Xie 2010), conserved microbiota analysis (Shelomi et al. 2020), and active enzymes analysis and identification (Kim et al. 2011; Lee et al. 2014, 2016). A recent review evaluated black soldier fly larva microbial community and prospected its feasibility to utilization improvement (De Smet et al. 2018). Furthermore, a relevant research finished an in-depth study to classify the positive influence of the gut microbiota of black soldier fly larvae (Bruno et al. 2019). However, there is scarce research focused on biological function analysis of black soldier fly larvae's gut microbiota. In addition, no research has been performed to search for microbiota that enhance feed utilization efficiency. It then becomes necessary to develop research to better understand the black soldier fly larvae's microbiota characteristics and functions.

Metagenomics research is a useful tool in gut microbe research, whose information is based on sequencing data (Furrie 2006). Based on the development of this technology, many unculturable microbes have been studied and analyzed (Handelsman 2004). In this concern, metagenomics research has expanded the mining of microbial community structure and function in the environment. To evaluate the black soldier fly larvae's intestinal bacteria biological function and screen the potential microbial strain that may be exploited to improve feed utilization efficiency in factory farming, such bacteria were systematically analyzed through metagenomics.

Results

Data quality assessment

Raw reads obtained from contig sequencing were assembled with the software MEGAHIT (Gurevich et al. 2013) in default parameter. Contig sequences shorter than 300 bp were discarded, and the assembly results were evaluated by QUAST (Zhu et al. 2010) with the default parameter. The results demonstrated that the number of contig in different experimental groups was about 600, 000 and greatly varied in length. The N50 length was about 1000–1200 bp with the alignment rate exceeding 95% (Table 1).

MetaGeneMark (Fu et al. 2012) was used to identify coding regions in the genome with default parameters. Gene prediction was conducted according to the assembled contigs. The number of genes in different samples was about 500,000. The average gene length measured in each sample was in the range of 260 to 340 bp. The Cd-hit software (version 4.6.6) was used to remove the redundancy with default parameters; the similarity threshold was set at 95% and the coverage threshold at 90% (Fu et al. 2012). It was concluded that there were 2, 168,041 non-redundant genes obtained in this sequence process, with an average length of 280 bp (Table 2).

Species information statistics

The species composition and relative abundance of the samples were obtained by comparing the above non-redundant genes with the species information of the sequence in the Nr database (Ashburner et al. 2000). The intestinal microbial species of the black soldier fly larvae in different groups were counted in taxa of the kingdom, phylum, class, order, family, genus, and species. The gut larvae microbiota was abundant, reaching more than 2300 genera and 11,000 species. The number of microbial species on different taxa in different groups was relatively similar, but a significant difference was

Table 1 Assembled data assessment

Sample	Total length (bp)	Contig number	Largest length (bp)	GC (%)	N50 (bp)	Mapped (%)
F1	713,374,913	679,554	332,933	41.78	1115	97.07
F2	601,410,151	594,324	264,723	41.58	1061	95.68
F3	621,800,601	613,040	315,309	41.63	1065	95.84
T1	573,217,820	519,415	184,590	41.85	1194	96.05
T2	716,521,041	613,618	721,453	41.86	1290	97.17
T3	649,395,570	557,814	186,681	42.01	1287	96.87
W1	604,749,023	581,793	363,719	42.28	1072	95.93
W2	712,670,354	668,870	399,518	42.11	1116	96.38
W3	680,722,887	639,170	744,843	42.37	1108	96.35

Sample is the sample number; total length is the sum of the base numbers of all contigs; contig numbers are the numbers of contigs after assembly; largest length is the number of bases in the longest contig; N50 are contigs that were sorted from long to short, and the cumulative length was counted. When a contig was added and the cumulative length was equal to half of the sum of the lengths of all contigs, the length of the contig was N50; Mapped is the alignment rate between sequencing reads and assembled contigs

Table 2 Predicted genes overview

Sample ID	Gene number	Total length (bp)	Average (bp)	Max length (bp)	Min length (bp)
F1	550,158	152,574,384	277	7536	102
F2	464,889	125,423,319	269	9972	102
F3	485,384	132,226,104	272	7536	102
T1	426,498	117,609,837	275	8286	102
T2	531,807	152,545,194	286	8175	102
T3	472,169	132,783,018	281	8904	102
W1	506,309	164,800,824	325	12,033	102
W2	579,598	184,957,656	319	10,866	102
W3	576,423	195,543,927	339	11,607	102
Gene set	2,168,041	608,038,053	280	12,033	102

Samples is the sample number; gene numbers is the number of predicted genes; total length is the base sum of predicted genes; average length is the average bases of predicted genes

observed in genus between groups W and T. In general, the intestinal microbe species of group W were higher, but not in a significant level than those of groups F and T (Table 3).

Analysis of high-abundant bacteria in the intestinal tract of black soldier fly larvae

The resulting top 15 high abundance bacteria were selected for comparison. The results showed that the relatively high abundance of bacteria at the level of phylum and genus was highly comparable (Fig. 1). The highest relative abundance of bacteria belonged to Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria phyla. The highest abundance of bacteria at the species level were *Enterococcus*, *Acinetobacter*, *Providencia*, *Enterobacter*, and *Myroides*.

Similarity analysis of microbial and functional genes between groups

The microbiota and functional gene composition of the samples were hierarchically clustered through R and the unweighted paired average method (UPGMA). Based on this, the similarity of species composition and functional gene composition of each sample was determined. The sample distance in the sample hierarchy clustering indicates the similarity of the species composition of the two samples. The results showed that samples in the same groups are closer and the branches are shorter; therefore, the microbial species structure and functional gene composition of the samples between the same treatment groups were comparable (Fig. 2a, c).

Principal component analysis (PCA) decomposes the differences of multiple sets of data on the two-dimensional coordinate chart through processing complex data into two eigenvalues. Composition analysis of different samples (97% similarity) reflects differences and distances between samples. The composition of the species in the two samples is similar, when they come closer on a PCA diagram. The results showed that the same sample in the same group was closer to each other, which demonstrated that the microbial composition and functional gene composition were comparable. The sum of the first dimension (98.4%) and the second dimension (1.13%) reached 99.53%, which explains the difference between the different groups to a great extent (Fig. 2b, d).

Analysis of differential microbes

About 100 species were selected ($p < 0.05$) to draw differential heatmaps. According to the heatmap, there exists 35 high relative abundance species in group W (*Faecalicatena contorta*, *Stenotrophomonas acidaminiphila*, *Sphingobacterium cellulitidis*, *Salana multivorans*, *Enterococcus* sp. Gos25–1, *Lachnospiraceae bacterium* OF09-33XD, *Pusillimonas caeni*, *Hungatella hathewayi*, *Sphingobacterium* sp. 30C10-4-7, *Stenotrophomonas* sp. Leaf70, *Pusillimonas* sp. T7-7, uncultured *Stenotrophomonas* sp., *Frischella perrara*, *Myroides* sp. N17-2, *Microbacterium* sp. CH12i, *Ochrobactrum* sp. A44, *Microbacterium ginsengiterrae*, *Kluyvera*

Table 3 Microbial species statistics

Group	Kingdom	Phylum	Class	Order	Family	Genus	Species
F	6	115.33 ± 1.15	124.67 ± 1.15	269.67 ± 1.53	623.33 ± 0.58	2309.67 ± 9.61 ab	11,510.33 ± 25.50
T	6	115.33 ± 1.53	125.33 ± 0.58	269.33 ± 2.08	619 ± 5.20	2283 ± 7.81 a	11,432.33 ± 68.72
W	6	111.67 ± 1.15	125.33 ± 1.15	269.67 ± 0.58	622 ± 3.46	2321.67 ± 10.11 b	11,880 ± 17.09

Data are indicated ad means ± standard error. Non-parametric ANOVA was used to analyze the data in the same column. Different letters in the same column indicate a significant difference($p < 0.05$) between the groups

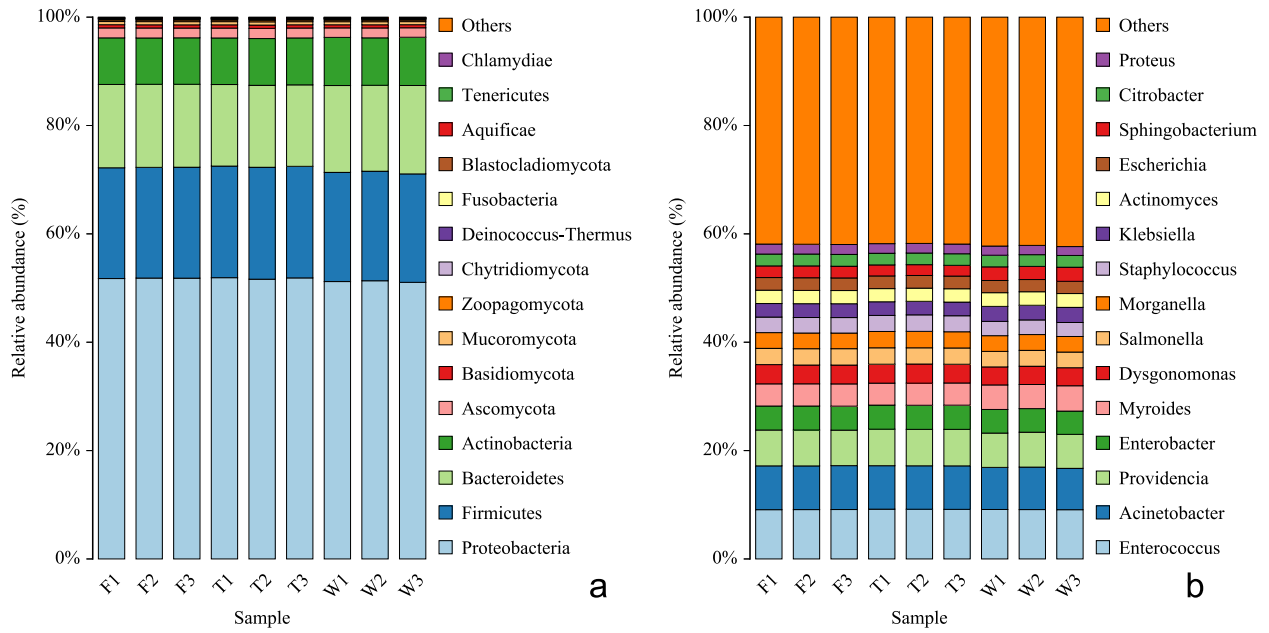

Fig. 1 Relative abundance microbiota analysis in the phylum and genus. **a** Relative abundance microbiota analysis in the phylum. **b** Relative abundance microbiota analysis in the genus. F1, F2, and F3 are replicates of group F; T1, T2, and T3 are replicates of group T; W1, W2, and W3 are replicates of group W

georgiana, *Sphingobacterium gobiense*, *Myroides odoratus*, *Klebsiella pneumoniae*, *Enterococcus pallens*, *Sphingobacterium lactis*, *Candidatus Schmidhempelia bombi*, *Aequorivita soesokkakensis*, *Vitellibacter aquimaris*, *Enterococcus* sp. 9D6 DIV0238, *Providencia stuartii*, *Miniimonas* sp. PCH200, *Gilliamella apicola*, *Orbus hercynius*, *Sphingobacterium mizutaii*, *Sphingobacterium* sp. 1.A.4, *Microbacterium profundi*, *Thermus filiformis*), 34 in group T (*Dysgonomonas capnocytophagoides*, *Allomyces macrogynus*, *Enterococcus* sp. 9E7 DIV0242, *Candidatus Erwinia dacicola*, *Enterococcus* sp. 4G2 DIV0659, *Propionibacteriaceae bacterium* 16Sb5-5, *Desulfovibrio* sp. DS-1, *Marinifilum breve*, *Tatumella* sp. UCD-D *suzukii*, *Bacillus velezensis*, *Staphylococcus gallinarum*, *Staphylococcus sciuri*, *Dysgonomonas* sp. Marseille-P4361, *Klebsiella aerogenes*, *Providencia rettgeri*, *Mycolicibacterium mucogenicum*, *Corynebacterium nuruki*, *Ruaniaceae bacterium* KH17, *Corynebacterium stationis*, *Enterococcus* sp. 6C8 DIV0013, *Enterococcus faecium*, *Providencia rustigianii*, *Wohlfahrtiimonas populi*, *Weissella jogaejeotgali*, *Bacillus amyloliquefaciens*, *Enterococcus casseliflavus*, *Bacteroides thetaiotaomicron* CAG:40, *Weissella thailandensis*, *Enterobacter hormaechei*, *Staphylococcus xylosus*, *Enterococcus saccharolyticus*, *Carnobacterium maltaromaticum*, *Spizellomyces punctatus*), and 22 in group F (*Citrobacter* sp. MH181794, *Schaalia canis*, *Metarhizium* majus, *Candida maltose*, *Nosocomiicoccus massiliensis*, *Lactobacillus* sp. 54-5, *Dysgonomonas gadei*, *Dorea longicatena*, *Prevotella* sp. 10(H), *Leminorella grimontii*, *Bacteroides caccae*, *Variovorax* sp. EL159, *Solibacillus isronensis*, *Proteus mirabilis*, *Actinomyces* sp., *Staphylococcus lentus*, *Rozella allomycis*, *Ignatzschineria* sp. F8392, *Leucobacter triazinivorans*, *Campylobacter concisus*, *Bacteroides* sp. 1 1 14, *Azospira oryzae*). The relatively high abundance species of the different groups concentrate on different species without obvious overlap in the heatmap. Although the relatively high abundance species of the differential species in group F was the lowest, the relatively low abundance species were also less than the other two groups (Fig. 3).

Analysis of differential functional gene

The heatmap of different functional genes was obtained through a parametric test. The heatmap of the Kegg (Kanehisa et al. 2004) metabolic pathways in differential abundance gene shows that groups possess differences in polysaccharide biosynthesis and metabolism, translation, membrane transport, and energy metabolism. Group W results showed the highest relative abundance in four biological processes, group F maintained middle-level abundance in those four-biological process, and group T showed low abundance in those four biological processes (Fig. 4a). The EggNOG (Powell et al. 2014) heatmap evidences the differences of the cytoskeleton, extracellular structures, inorganic ion transport and metabolism, nucleotide transport and metabolism, and coenzyme transport and metabolism in different groups. Group W showed a high abundance in extracellular

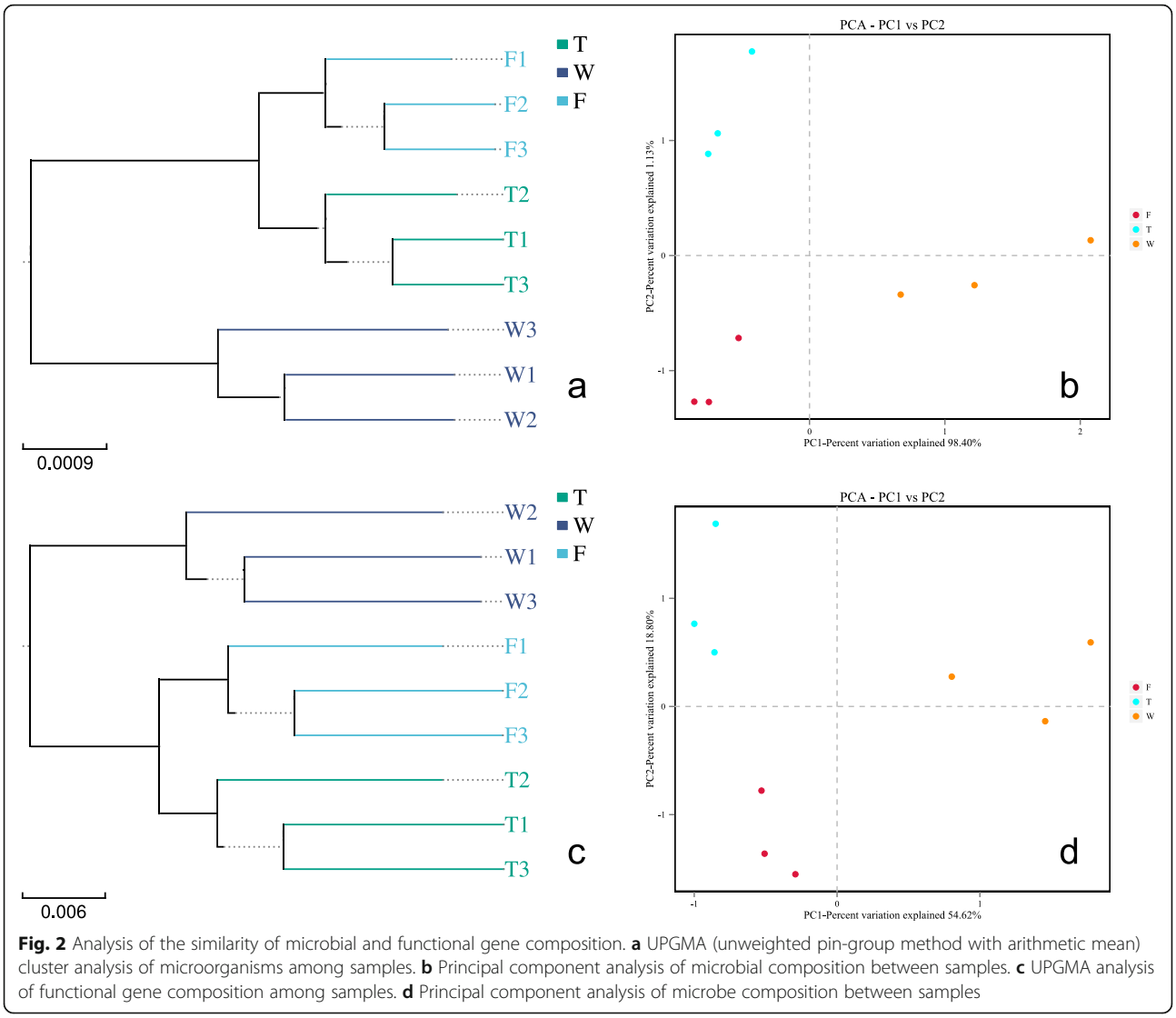

Fig. 2 Analysis of the similarity of microbial and functional gene composition. **a** UPGMA (unweighted pin-group method with arithmetic mean) cluster analysis of microorganisms among samples. **b** Principal component analysis of microbial composition between samples. **c** UPGMA analysis of functional gene composition among samples. **d** Principal component analysis of microbe composition between samples

structure, inorganic ion transport and metabolism, nucleotide metabolism and transport, and coenzyme transport and metabolism, whereas the related functional genes of the cytoskeleton in group T showed relatively high abundance. The extracellular structures of group F were comparable to those of group W, whereas the other categories were less than those of group W (Fig. 4b).

Discussion

The N50 length of assembly sequence was about 1100 bp, with an alignment rate exceeding 95%, whereas the contig number of a single sample reached 600,000, which means that this data assembly quality meets the requirement of research and analysis. Gene number of a single sample up to 550,000 suggests that the sequencing depth is enough for subsequent microbial biological analysis. Moreover, the results of intestinal microbial statistics showed that there were over 11,000 intestinal bacteria in the black soldier fly larvae gut, indicating the presence of highly abundant microbes in the gut. It is relatively similar among different groups in each taxonomic order, indicating that different feeds had a limited influence on the overall microbial community. The top 15 high abundance microbes in the class and genus analysis results demonstrated that there was no apparent difference between the groups. The results indicate that feed played a slight role in the core gut microbiota of black soldier fly larvae, which was consistent with published research (Klammsteiner et al. 2020). The highest relative abundance bacteria belonged to the Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria phyla. It showed some differences with Jeon et al.'s (2011) results, which included Bacteroidetes, Proteobacteria, Firmicutes, Fusobacteria, and Actinobacteria. Among those bacteria, Proteobacteria was observed as a potential microbial signature of disease, Firmicutes was

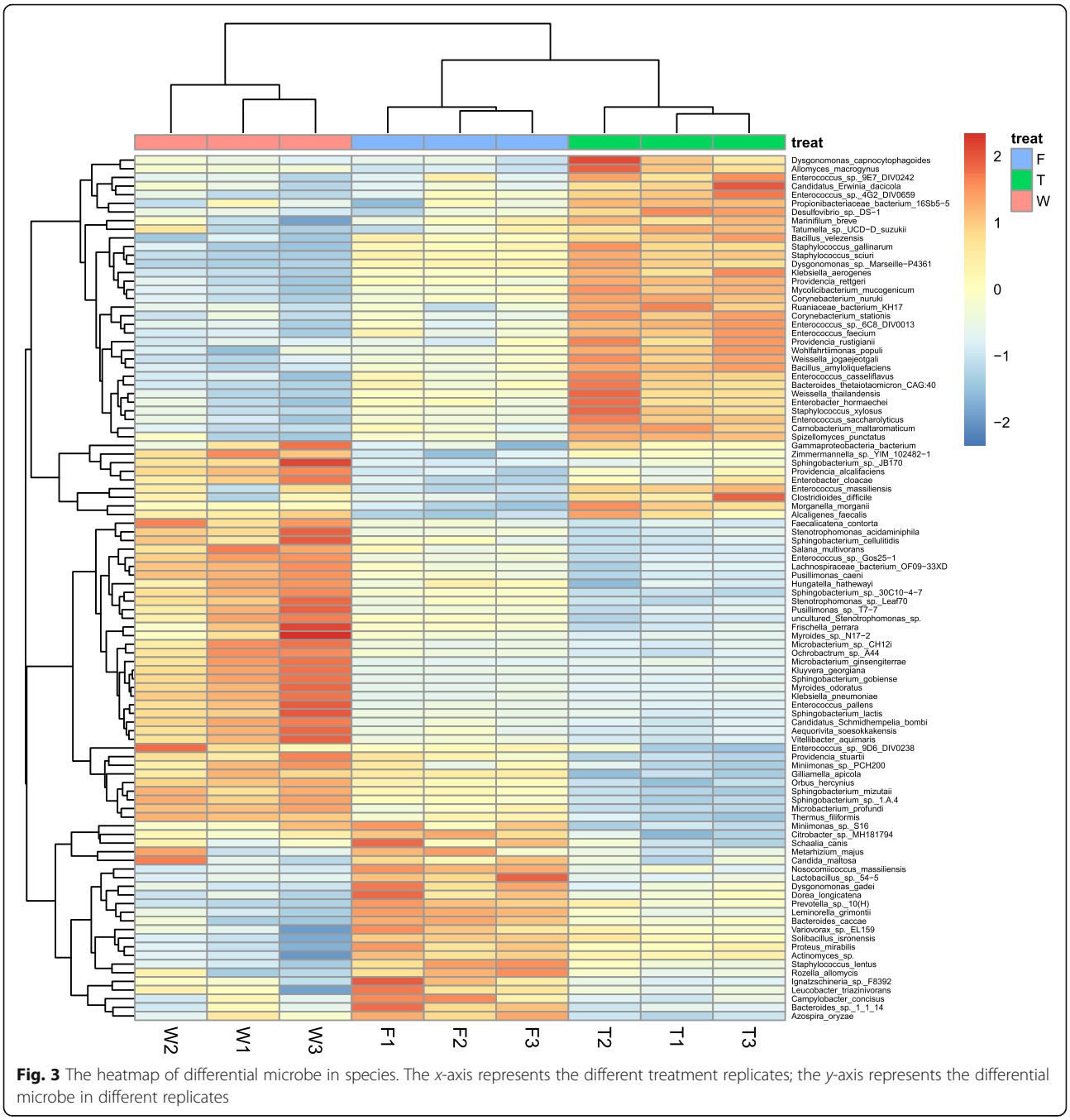

Fig. 3 The heatmap of differential microbe in species. The *x*-axis represents the different treatment replicates; the *y*-axis represents the differential microbe in different replicates

considered to play an important role in the digestion of animal manure, and Bacteroidetes were shown to degrade high-molecular weight organic matter (Zhan et al. 2020). Taken together, the larvae gut core microbiota facilitated degradation of animal manure and organic matter, which may provide microbial evidence for Kim et al.'s (2011) research. However, we should be more cautious to adopt black soldier larvae protein as edible for Proteobacteria in its gut (Wang and Shelomi 2017).

The intergroup similarity analysis of microbial species and functional genes demonstrates that both feeds and tetracycline had an impact on the intestinal microbial community structure of the black soldier fly larvae. The intestinal microbial community structure of larvae fed under a similar environment was comparable, which agrees with other studies (Jiang 2018; Tanaka et al. 2006; Liang et al. 2014; Bruno et al. 2019). Furthermore, our results agreed with (Engel et al. 2013) in that the diet

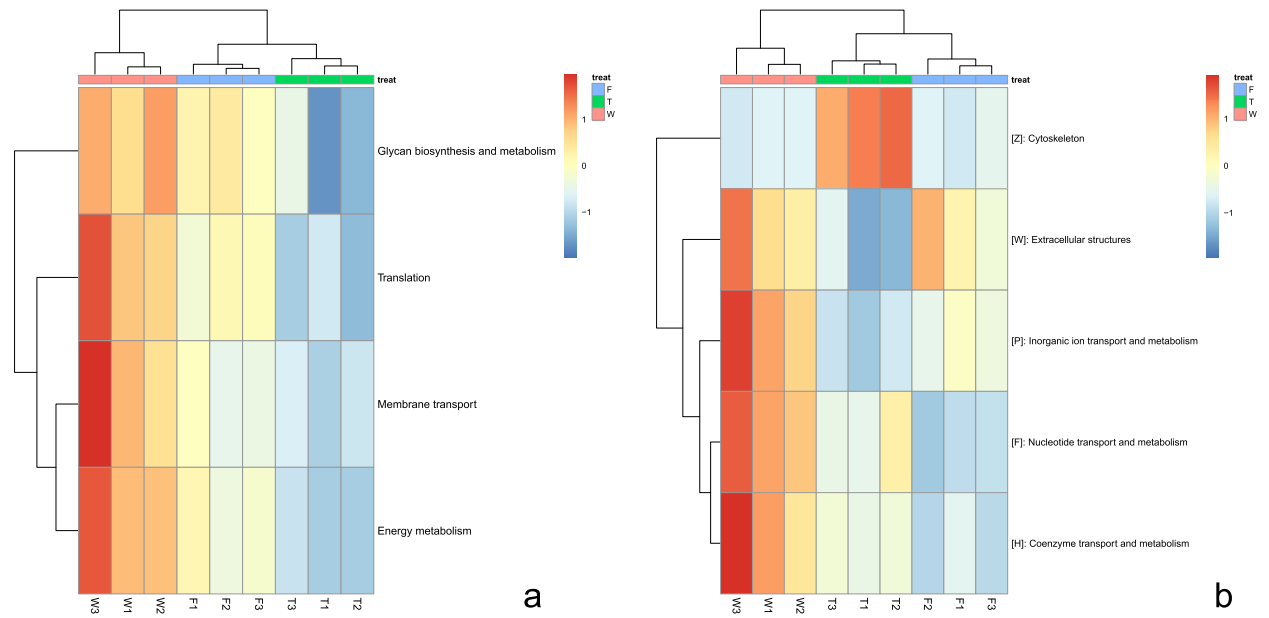

Fig. 4 Analysis of difference groups between functional genes. **a** Heatmap of differentially functional gene abundance in the Kegg metabolic pathway. **b** Heatmap of differentially functional genes in the eggNOG metabolic pathway. Genes shown have *p* values < 0.05, after metagenome sequencing analysis

shapes the composition and activity of the gut microbiota. Heatmap analysis results demonstrated that different treatments influence some microbes in the black soldier fly larvae. A similar trend shown in function genes indicates a relation between differential microbes and function genes. We can then infer that those microbes play a crucial role in black soldier fly larvae growth and development, which agrees with De Smet et al.'s (2018) conclusion. From these results, we may conclude the relatively high abundance microbiota in group W (35) and group F (22) play an important role in growth and development, whereas the relatively high abundance microbiota in group T (34) are relevant for black soldier fly larvae to survive in harsh conditions. However, further research is required to address such assumptions. Combining heatmap of Kegg and eggNOG analysis in differential function genes showed that relative function gene abundance of groups W, F, and T gradually reduces polysaccharide biosynthesis and metabolism, translation, and membrane transport and energy metabolism which associate with feeding. Hereby, we inferred that the 35 differential microbiota in group W were closely related to feeding. Furthermore, we believe that the differential microbiota in different groups play an important role in such biological process. Group T has a relatively high abundance of functional genes in the cytoskeleton indicating that the relatively high abundance of differential microbiota in group T was related to the cytoskeleton, which may be due to the inhibitory effect of tetracycline on some essential microbes involved in the development and feeding (Cai et al. 2018; Cifuentes et al. 2020), showing a great difference with mycotoxins and pesticides (Purschke et al. 2017). However, naval approaches are necessary to investigate this issue. The functional gene in group W displayed a high level in cell structure, inorganic ion transport and metabolism, nucleotide metabolic process, and coenzyme transport and metabolism, which indicate that the relatively high abundance of differential microbiota in group W is essential in these biological processes. Based on the aforementioned result, we conclude that the 35 differential microbes in group W were mainly associated with energy metabolism which influences growth a lot, whereas the 34 differential microbes in group T were related to survival in harsh environments. The present study provided a better understanding of the intestinal microbiota of black soldier fly larvae; however, it did not fully address the relation between microbes and function. We have research underway to elucidate such a relationship.

Conclusion

The present study demonstrated that gut bacteria in black soldier fly larvae were involved in polysaccharide biosynthesis and metabolism, translation, membrane transport, energy metabolism, cytoskeleton, extracellular structures, inorganic ion transport and metabolism, nucleotide metabolism, and coenzyme transport physiological processes. The 35 significant differential microbes in group W have a positive impact on energy metabolism and physiological

process which could be exploited to improve transform efficiency. Subsequent studies will explore the function of specific differential high relative abundance microbes in group W and evaluate the possibility of improving farming efficiency with those microbiotas.

Methods

The research aims at exploring the relation between black soldier fly larvae and its intestinal microbiota. Fourth to 5th stage black soldier fly larvae fed with varied feeds were selected to study the intestinal microbiota. In order to avoid accidental outcomes, we set 3 repeats (10 individuals for 1 repeat) for every treatment. The metagenomic method which is a practical tool was utilized to achieve our goal.

Insect sources and breeding

Black soldier fly eggs and larvae were partly acquired from Guangzhou Anruijie Environmental Protection Technology Limited company, all of them were trapped in a field and reared in 25 °C, 16 h photoperiod, 60–70% relative humidity feed for 30 generations.

After hatching from eggs, black soldier fly larvae were incubating with 75% wheat bran, 25% soybean powder (group W), food waste (group F), or 75% wheat bran and 25% soybean powder supplemented with 1% tetracycline (group T) for 10 days. Then, 10 4–5th stage larvae were selected in each group for intestinal anatomy, performing triplicate determinations.

Intestinal dissection of black soldier fly larvae

Black soldier fly larvae were starved for 24 h, after which they were washed with sterile water and inactivated for 10 min at − 20 °C. Next, they were surface sterilized with 75% alcohol and washed with sterile water. Larva intestines were dissected out with sterile scissors and tweezers on a sterile operating table, eliminating intestinal adhesions. The obtained larvae intestines were preserved at − 80 °C for subsequent sequencing at Beijing Biomarker Technologies.

Metagenomic sequencing and bioinformatics analysis

DNA isolation, sequencing, and bioinformatics analysis were performed by Beijing Biomarker Technologies, acquiring 10 G of sequencing data for every replicate (three replicate determinations were achieved). Filtering and quality controlling were processed to get original clean reads for subsequent analysis. The Trimmomatic software was used for original splicing sequences (raw tags), whereas Bowtie2 was used to sequence alignment with the host genome to remove host contamination. MEGAHIT (Gurevich et al. 2013) was utilized for metagenome assembly, and contig sequences shorter than 300 bp were not considered. QUAST (Zhu et al. 2010) was used to evaluate assembly results, whereas MetaGeneMark (Fu et al. 2012) was used to predict the encoding genes and perform functional annotation on the encoding genes in general database and special database. Those bioinformatics analysis tools were kept in default parameter while analysis performing. Taxonomic analysis was performed based on clean reads data. Furthermore, species composition, abundance information, and function genes of the samples were statistically analyzed.

Statistical analyses

The statistics in this research were analyzed in Prism GraphPad 8; the figures were drawn through R.

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Authors' contributions

All authors contributed to the study conception and design. The first draft of the manuscript was written by Yuan Zhineng, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Declarations

Ethics approval and consent to participate

No approval of research ethics committees was required to accomplish the goals of this study because experimental work was conducted with an unregulated invertebrate species. The larvae were utilized to experiment without abuse or maltreatment. All authors understand that my participation is voluntary and agree to participate in the above research.

Consent for publication

Written informed consent for publication was obtained from all participants.

Competing interests

The authors declare that they have no competing interests.

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