Metagenomics Analysis of the Gut Microbiome Structure and Function in Black Soldier Fly (Hermetia illucens) Larvae Reared With Different Types of Diet[1]

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Abstract Black soldier fly, *Hermetia illucens* (Diptera: Stratiomyidae), larvae are renowned for their bioconversion of organic waste into nutrient-rich supplements for various applications. In previous studies, the predominant genera of gut bacteria show a large variability among fly larvae, likely due to variability in diets. The ability of catabolic degradation by black soldier fly larvae might be ascribed to intestinal microorganisms. Diets can influence the gut microbiota of *H. illucens.* However, the effect of distinct foods on bacterial communities of gut bacteria is poorly understood. For this purpose, we undertook this study to assess the impacts of diet on the structure and function of the microbial communities in the gut of black soldier fly larvae fed with representative types of diets. We found that the most abundant bacteria in the black soldier fly larvae gut metagenome were *Morganella* (17.02%), *Enterococcus* (10.27%), *Paenibacillus* (9.50%), *Klebsiella* (7.29%), and *Enterobacteriaceae* (10.27%) and, thus, represent the core microbiome. Our results provide insights into the bacterial genes in *H. illucens* the larval gut, and we concluded that the microbiota structure and function could be shaped by the edible mushroom residue diet. Characterizing the interplay between the gut microbiome and black soldier fly larvae diets helps to clarify the underlying degradation processes and may contribute to improved large-scale black soldier fly larvae rearing. These data sets help to exploit the microbiological optimization of *H. illucens* as a sustainable insect for industrial rearing and the microbiome for novel biotechnological applications.

Key Words metagenomics, gut microbiome, black soldier fly, *Hermetia illucens*

Hermetia illucens (L.) (Diptera: Stratiomyidae), commonly called the black soldier fly, is one of the most promising insects being mass reared globally, due to its ability to recycle many types of organic wastes effectively. Furthermore, it is not recognized as a pest (Jeon et al. 2011, Zhan et al. 2020). Black soldier fly larvae can thrive on diverse substrates including animal manures, human excreta, fruit and vegetable wastes, carrion, and even food waste (Gold et al. 2018). For most animals, microbial communities play an particularly important role in the digestive tract, where they should be the key mediators for many survival types of insect hosts (Engel and Moran 2013). The high-complexity gut microbiome plays an essential role in insect digestion and performance, host physiology and immunity, and health

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(Cifuentes et al. 2020, De Smet et al. 2018). Therefore, an in-depth characterization of black soldier fly larval microbiota and the factors that influence its composition is particularly important.

In recent years, the literature available on the composition of the black soldier fly larval gut microbiota, which is possibly related to its substrate and other rearing factors, increased substantially, with examples of relevant studies (Gorrens et al. 2021, IJdema et al. 2022, Wynants et al. 2019). Whereas, the studies mentioned focused on the identification of members of the black soldier fly larval microbial community, knowledge on rearing substrate impact on the composition and the functions of the gut microbiota have been only preliminarily understood. The influencing factors and underlying mechanisms still need to be revealed. Nevertheless, one of the challenges of the utilization of some food substrates that are exploited by black soldier fly larvae (e.g., dairy manure, soybean curd residue, rice straw, brewer grains) is that they contain various indigestible fibers (e.g., cellulose, hemicelluloses, lignin), starches, and sugars that limit the performance of the larvae (ur Rehman et al. 2017). T\he role of symbiotic microorganisms and cellulolytic enzymes in digestive processes in insects that successfully feed on lignocellulosic biomass is widely recognized (Gorrens et al. 2021). Bacteria that are better adapted to digest a specific compound are expected to have greater fitness in exploiting specific diets. However, few investigations have been undertaken on the connection between fiber diet and larval gut microbiota. It is necessary to develop relevant studies in order to better understand the relationship between diet, microbiota characteristics, and function of black soldier fly larvae.

Some studies suggest that the rearing substrate is an important factor shaping the gut microbiota in black soldier fly larvae (Gorrens et al. 2021). We hypothesized that the ability of catabolic degradation of high-fiber food waste by black soldier fly larvae might be due to gut microbes. Therefore, the effect was evaluated by rearing black soldier fly larvae on 4 representative diets, including edible mushroom residue and soybean curd residue, concentrated feed and concentrated feed with cellulose, on the microbial community composition and function.

The edible mushroom residue is that organic material remaining after harvesting mushrooms and is a substantial source of nutritious organic waste (high fiber) that is a promising candidate as a food source for rearing black soldier fly larvae for recycling (Li et al. 2021, Moon et al. 2012). Soybean curd residue, also known as okara, is the main product from processed soy products (Li et al. 2013). This residue is also rich in cellulose and dietary fiber, which can be fermented by microbes in the gut (Li et al. 2013). Edible mushroom residue and soybean curd residue contain nonstarchy carbohydrates commonly found in plant materials, such as cellulose, hemicellulose, and pectin. Currently, optimizing *H. illucens* for recycling particular types of high-fiber waste is especially challenging because little is known about its effect on gut microbiota composition. However, whether the changes in the gut microbiota of black soldier fly larvae on substrates of different types of fiber can be used to characterize the microbial function remains unclear. We, thus, proposed to feed black soldier fly larvae with concentrated feed as a control experimental group. Cellulose fibers were added to the concentrated feed to compare the effect of natural cellulose on intestinal flora.

Metagenomics provide new opportunities and insights for further analysis of the relationship between insect microbial community structure and function. Thus, in

this study, black soldier fly larvae were reared on diets supplemented with 4 representative forms of organic wastes, including edible mushroom residue, soybean curd residue, concentrated feed, and concentrated feed with cellulose. The microbial community structure and function were analyzed by using metagenomics.

Materials and Methods

Insect maintenance and diet preparation. Eggs of the black soldier fly were obtained from Younong Environmental Protection Industry Technology Co., Ltd. (Taizhou, Jiangsu Province, China). Black soldier fly larvae 6 d after hatching were fed a standard diet of 75 g of bran, 75 g of corn flour, and 350 ml of water) as per Somroo et al. (2019). Larvae were then transferred to 1 of 4 treatment diets. The diet containing soybean curd residue was prepared with residue obtained from local farmers' Zhuquan markets in Nanjing. The edible mushroom residue was purchased from Jiangsu Hualvbio Co., Ltd. (Suqian, Jiangsu, China), and the concentrated feed was purchased from Qingdao Kangda Jiahui Fodder Co., Ltd. (Qingdao, Shandong, China). Specifics of the pig concentrated feed (on a dry matter basis) was as follows: crude protein 17.60%, crude fiber 2.26%, crude fat 3.76%, crude fat 5.96%. The cellulose added to the concentrated feed was purchased from Sinopharm Group Co., Ltd. (Beijing, China). All diets were sterilized with heat and pressure to avoid the impact on the intrinsic food bacteria. The physico-chemical properties of the diets are listed in Table 1.

Dry mass was measured by drying (105°C) under atmospheric pressure according to Chinese National Standard GB 5009.3-2010. The ash content was determined following National Standard GB 5009.4-2010. Crude protein (CP) was measured using the Kjeldahl method and a conversion factor of 6.25 was calculated by using the method in GB/T 5009.5-2010. Crude fat (CF) of larvae and feedstock was determined using Soxhlet extraction to GB/T 5009.6-2003. Crude fiber was determined according to the standard determination of crude fiber (GB/T 5009.10-2003).

Gut removal. During these feeding experiments, we extracted in triplicates at day 12 a total of 36 guts from black soldier fly larvae. These were subsequently submitted for sequencing. The surfaces of the 12-d-old black soldier fly larvae were disinfected with 75% alcohol, rinsed with sterile water several times, and then surgically dissected. The entire gut was removed under a stereomicroscope on sterile glass slides. The individual guts were pulled out using sterile forceps, crushed using a mortar, transferred into a sterile microcentrifuge tube, cleaned to remove fat, and frozen until further use. Guts from 3 larvae per sample were pooled for DNA extraction, then homogenized in 4 groups, resulting in a total of 3 replicates per treatment.

DNA extraction, MiSeq sequencing and data analysis. After thawing the gut samples, DNA was extracted using a DNA extraction Kit (E.Z.N.A.® Soil DNA Kit, Omega Bio-Tek, VWR, Radnor, PA). DNA quality was examined after electrophoresis on agarose gel (1%, w/v). DNA concentration was quantified using a Nano-drop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). To minimize DNA extraction bias, 3 replicated DNA isolates from each sample were pooled. Subsequently, genomic DNA was sheared into DNA fragments of about 500 bp by using a Covaris S220 focused-ultrasonicator (Covaris Inc., Woburn, MA). An Illumina library was then constructed by using NEB Next® Ultra™ DNA Library Prep Kit for Illumina® (Illumina, San Diego) following the manufacturer's protocol. Finally, the library preparations were

Table 1. Compositional analysis of specimens reared on 1 of 4 diets for *Hermetia illucens* larvae (% dry matter).

Nutrient (%)	Soybean Curd Residue	Edible Mushroom Residue	Concentrated Feed	Concentrated Feed With Cellulose Fibers
Crude ash (%)	4.33 ± 0.16	5.82 ± 0.35	5.96 ± 0.75	5.96 ± 0.75
Crude fat (g/kg)	9.19 ± 0.43	2.56 ± 0.28	3.76 ± 0.34	3.76 ± 0.34
Crude fiber (g/kg)	22.18 ± 0.89	21.42 ± 0.67	2.26 ± 0.54	21.80 ± 0.54
Crude protein (%)	23.34 ± 0.34	15.58 ± 0.13	17.60 ± 0.20	17.60 ± 0.20

sequenced on an Illumina Hiseq X Ten platform (Hiseq PE150, Illumina) at Sangon Co., Ltd. (Shanghai, China).

Raw data were checked for quality assessment with FastQC, adapter, and quality trimming was carried out using Trimmomatic (Bolger et al. 2014). High-quality reads were integrated and assembled using the IDBA-UD (Peng et al. 2012) algorithm that is based on the De Bruijn graph. Contigs were obtained according to overlap between reads, evaluated by using Kmer overlap information, ensuring improved results.

Results

Overview of the metagenome data. To understand whether distinct foods affect the microbial community that colonizes the digestive tract of black soldier fly larvae, the gut metagenomes of the larvae were sequenced using the Illumina platform. Metagenomic assembling was accomplished using the prominent assembler IDBA-UD. The quality checked (QC) library was taken for sequencing. After quality control filtering, a total of 18,762,958 reads was maintained for further analysis, with an average of 1,563,580 reads per sample. The metagenomic sequence data from this study have been submitted to the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) (accession numbers SRR21686212, SRR21686213, SRR21686214, and SRR21686215). The predicted open reading frames (ORFs) with a length larger than 100 bp were selected and translated into amino acid sequence using the software Prodigal version 2.6.3 with the -p meta options (Hyatt et al. 2012). The sequencing statistics are listed in Table 2. A total of 101,253, 303,004, 273,568, and 143,979 ORFs were found in soybean curd residue, edible mushroom residue, concentrated feed, and concentrated feed with cellulose, respectively. These ORFs were predicted for functional annotation, and approximately 50% of these ORFs were classified using KEGG and COG databases.

Taxonomic composition of the microbial communities. Taxonomic annotation based on SSU rRNA annotation implied that bacteria, fungi, viruses, and archaea accounted for approximately 97.39%, 2.29%, 0.28%, and 0.04% of the sequences, respectively. At phylum level, 17 bacterial groups, 3 fungal groups, 1 virus, and 4 archaeal groups were observed in all the samples. Metagenomic sequencing showed that Proteobacteria and Firmicutes were the dominant phyla in all black soldier fly larvae gut samples with an average abundance of 56.17% and 32.47%, respectively, followed by Actinobacteria (4.82%), Bacteroidetes (3.37%), and Ascomycota (1.44%) (Fig. 1A).

COG function annotation. These predicted genes were classified by aligning them to the COG protein database that was derived from those proteins encoded by the genomes of bacteria, archaea, and unicellular eukaryotes. COG ID-based functional genes were annotated using Diamond runs against the eggNOG database for COG annotation. The functional potential genes were subsequently classified via COG analysis. A total of 212,685, 79,514, 56,963, and 56,422 COG IDs were identified in the soybean curd residue, edible mushroom residue, concentrated feed, and concentrated feed with cellulose, respectively. Function unknown (S), amino acid transport and metabolism (E), carbohydrate transport and metabolism (G), transcription (K), and inorganic ion transport and metabolism (P) were the dominant functions among the 25 categories (Fig. 2).

Table 2. Summary of the metagenomic sequencing.

Category	Soybean Curd Residue	Edible Mushroom Residue	Concentrated Feed	Concentrated Feed With Cellulose Fibers
Raw reads	37,558,180	45,263,940	54,422,674	37,513,472
Clean reads	34,623,440	43,286,152	51,263,608	35,596,210
Assembled contigs number	28,998	131,148	251,194	55,208
Largest contig length (bp)	1,328,220	524,693	625,017	226,222
Average Length (bp)	3,046.25	1,848.51	994.28	2,343.62
Contig_N50 length (bp)	17,333	3,412	909	5,090
Contig_N90 length (bp)	813	659	553	772
Predicted ORFs	101,253	303,004	273,568	143,979
GC content	48.23%	46.82%	41.76%	51.45%

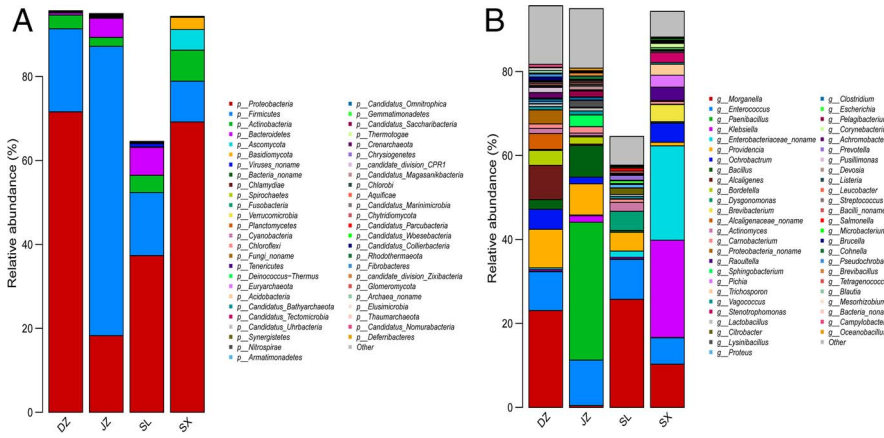

Fig. 1. Relative abundance analyses at phylum (A) and genus (B) levels in 4 diets (group). Different colored bars represent different bacterial species. The legends are shown at the right of the figure. Soybean curd residue (DZ), edible mushroom residue (JZ), concentrated feed (SL), and concentrated feed with cellulose (SX).

KEGG function annotation. KEGG can be used to infer the functional and taxonomic association of protein groups based on the biochemical function of the orthologous groups (KEGG orthology group [KO] terms] using Ghost KOALA (Kanehisa et al. 2016). The differential function genes showed the relative functional gene abundance of various diets. Based on KEGG pathways at levels 1 and 2 (Fig. 3), metabolism was the largest group, with higher proportions of carbohydrate metabolism, amino acid metabolism, overview, nucleotide metabolism, metabolism of cofactors, and vitamin and energy metabolism. Collectively, the 13 functional categories in the metabolism group accounted for 59.72% of the hits. Carbohydrate metabolism and amino acid metabolism were the most abundant pathways and functional categories, representing 11.17% and 9.44% of the black soldier fly larvae gut metagenome, respectively. It is noteworthy that the relative abundance of these main metabolism in edible mushroom residue diet was much higher compared with that under the other 3 diets. Furthermore, the differential microbiota in different groups are found to play an important role in such biological processes; based on the KO (KEGG Orthology) pathways system for functional orthologs (Fig. 4A), the predicted function associated with 8 of 30 highest-expressed genes in edible mushroom residue diet. These statistical differences indicating the edible mushroom residue diet significantly affected these KEGG pathways in the *H. illucens* microorganisms, which warranted further investigation. The top 30 functional modules (level 4) were detected in the microbial communities of the 4 diets (Fig. 4A).

CAZy function annotation. Enzymes are produced by black soldier fly larvae or by related microbes located inside or outside the digestive tract of the larvae. These enzymes hydrolyze complex carbohydrates into simple sugars (mono and disaccharides). To further understand the complex carbohydrate and glycoconjugate enzymes present in the black soldier fly larvae gut microbiome, we submitted the samples to

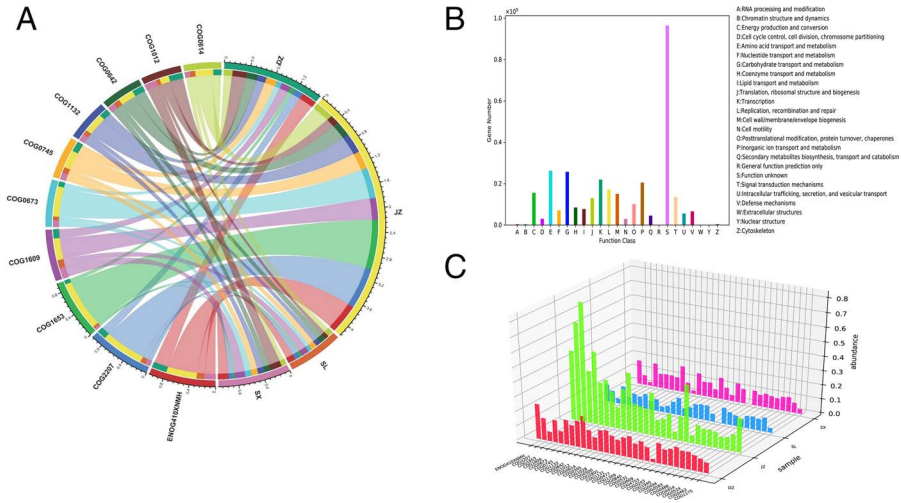

Fig. 2. (A) Circos analysis displays the corresponding abundance relationship between groups and COG function annotation. (B) COG functional classification statistics bar chart. The genes were annotated and classified into 25 COG functional categories. (C) 3-d bar-plot of COG function annotation abundance. Soybean curd residue (DZ), edible mushroom residue (JZ), concentrated feed (SL), and concentrated feed with cellulose (SX).

the carbohydrate-active enzymes database (CAZy) (Cantarel et al. 2009). The variations of carbohydrate active enzyme gene numbers and abundance ranked in decreasing order as (Fig. 5): glycoside hydrolases (GHs, 97, 44.97%), glycosyl transferases (GTs, 53, 23.65%), carbohydrate esterases (CEs, 16, 21.86%), carbohydrate-binding modules (CBMs, 32, 2.54%), polysaccharide lyases (PLs, 19, 3.83%), and auxiliary activities (AAs, 10, 3.15%). The abundant enzymes can be classified according to their main functions as follows: glycosidase (GH109, GH13, GH3, GH1, GH43, GH41, GH2), glycosyltransferase (GT51, GT41, GT9), esterase (CE1, CE9, CE10, CE4), and synthase (GT2, GT4).

Discussion

The core microbiota Firmicutes was considered to play an essential role in the digestion of animal manure (Zhan et al. 2020). A finer resolution on genus level is illustated in Fig. 1B. The most abundant ones (average presence across the 4 rearing diets) were *Morganella* (17.02%)*, Enterococcus* (10.27%), *Paenibacillus* (9.50%), *Klebsiella* (7.29%), and *Enterobacteriaceae* (10.27%) in black soldier fly larvae gut metagenome. These dominant bacteria reflect largely the bacterial profiles of black soldier fly larvae already described in the literature with respect to the most important phyla and genera in the gut community (Gorrens et al. 2021). It can then be inferred that those microbes play a crucial role in black soldier fly larval growth and development; however, some differences can be noticed depending on diet. The more

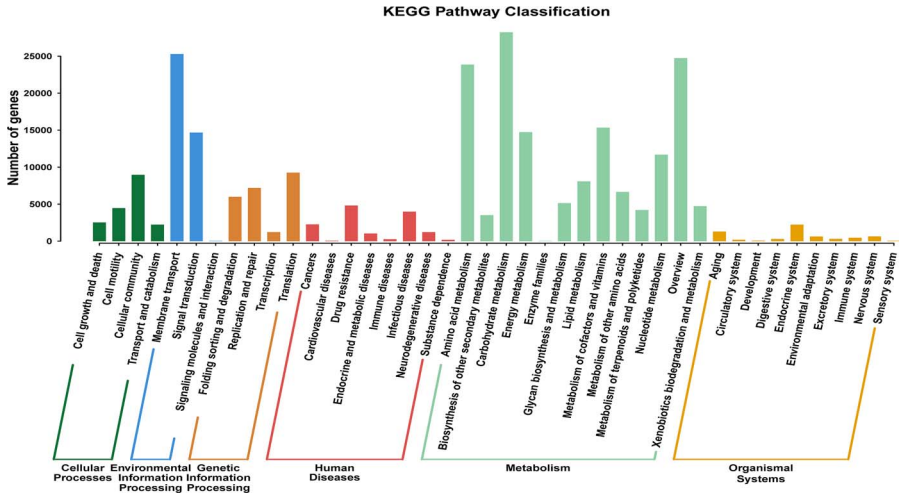

Fig. 3. KEGG pathway function annotation diagram at level 2 for genes in the gut metagenomes of 4 diets using Ghost KOALA.

pronounced differences among the 4 diet groups were as follows: *Morganella* was the most prominent genus in the guts of larvae reared on diet containing soybean curd residue (24.10%) and on the concentrated diet (39.87%), respectively, and was the third most abundant in larvae fed on the concentrated feed with a fiber diet group, but only 0.45% in the larvae fed on the edible mushroom residue diet. In contrast, *Paenibacillus* (34.57%), *Enterococcus* (11.42%), and *Bacillus* (7.95%) were

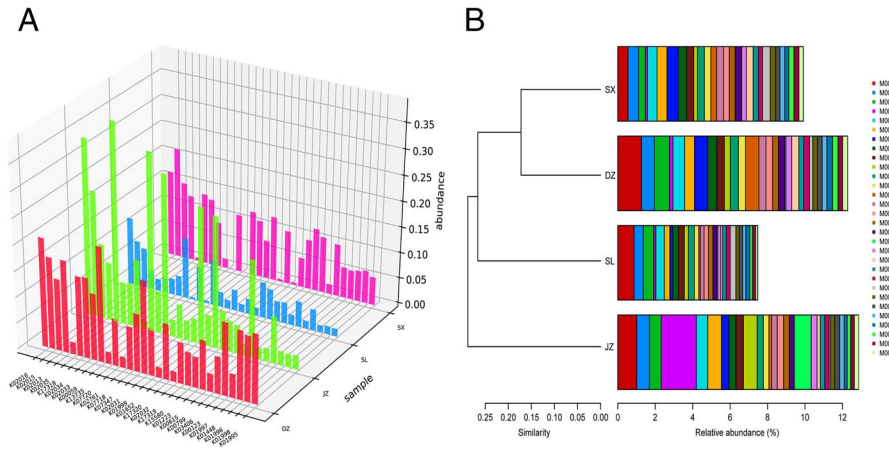

Fig. 4. Cluster bar-plot display the relative abundance and similarity of the dominant KEGG pathways in 4 diets. (A) KO; (B) module level 4. Soybean curd residue (DZ), edible mushroom residue (JZ), concentrated feed (SL), and concentrated feed with cellulose (SX).

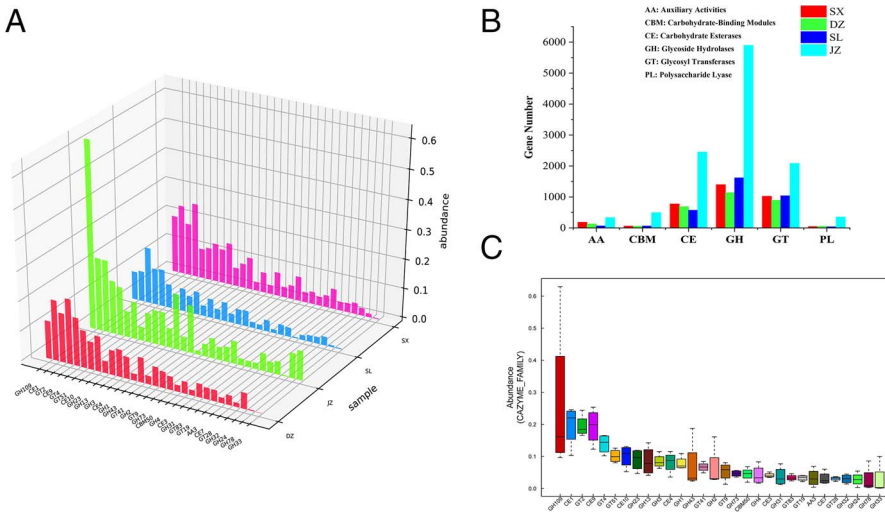

Fig. 5. (A) 3-d bar-plot of CAZ gene abundance and functional subclasses in each group. (B) The variation of CAZ gene numbers in 4 diets. (C) Boxplot representing the abundance of the CAZyme family (top-30). Soybean curd residue (DZ), edible mushroom residue (JZ), concentrated feed (SL), and concentrated feed with cellulose (SX).

the most prominent genera in the edible mushroom residue diet microbiota. Diets with soybean curd residue and concentrated feed are associated with an overall similar microbiota composition, both leading to increased levels of *Morganella*, *Enterococcus*, and *Providencia.*On the other hand, the microbiota of a diet composed of concentrated feed with fiber was dominated by *Klebsiella* and *Enterobacteriaceae*. The group analysis of microbial species indicates that diet impacts the intestinal microbial community structure of black soldier fly larvae.

Diet composition plays a major role in shaping the diversity of the gut microbiota (Gorrens et al. 2021). The findings from these analyses are consistent with Bruno et al. (2019), who investigated the effects of different substrates on the microbiota of black soldier fly larvae and concluded that the substrate played a major role in the composition of the microbiota. In our study, the diet with the edible mushroom residue appeared to have the strongest effect on the larval gut microbiota, leading to a higher abundance of *Paenibacillus* taxa. This suggests that this bacterial family may play a key role in metabolism, although further research is required to address such assumption. Meanwhile, other types of diets may play a smaller role in the intestinal tract of black soldier fly larvae and may be related to substrate types (Klammsteiner et al. 2020).

Of the known functions, sequencing data showed that the relative abundance of metabolic genes in edible mushroom residue group was significantly increased, suggesting that the high cellulose content increased the activity of microorganisms. ENOG410XNMH (histidine kinase) was found to be the most abundant COG functional gene category and was involved in signal transduction mechanisms. Functions

of other high abundant COGs are mainly related to COG2207 (transcriptional regulator AraC family) responsible for sugar uptake and metabolism (Nie et al. 2012).

Lee et al. (2014) found that bacteria in the black soldier fly larval gut possess enzymes that can hydrolyze starch, cellulose, proteins, and lipids and, thus, contribute to biowaste decomposition. Interestingly, the number of genes in the CAZy database in the diet containing edible mushroom residue was higher than that in the other groups. This finding suggests that a high-fiber diet may improve the efficacy of cellulose-degrading microbes and carbohydrate degradation potential. Similar studies proved that dietary fiber can promote human gut bacteria to produce a large number of CAZymes and degrade the compounds into metabolisable substances (Tasse et al. 2010). These dominant enzymes were often found to be involved in the biodegradation of organic compounds such as saccharides or lignin. For example, GHs and GTs played key roles in the enzymatic breakdown to polymetric substrates (Roth et al. 2017). GHs are found in almost all organisms and can hydrolyze the glycosidic bonds of various sugar-containing compounds to form monosaccharides, oligosaccharides, or sugar complexes.

Many insects harbor a complex community of microorganisms in their gut. As fly larvae feed on diets high in carbohydrates, gut microbes can metabolize starch, sugars, and fibers into organic acids such as short-chain fatty acids or simple alcohols (Gold et al. 2018). Bacteria in the gut of black soldier fly larvae produce enzymes that hydrolyze starch, cellulose, proteins, and lipids to decompose organic agricultural waste. Intestinal microbial community structure is the result of the coevolution of the microbes, the host animal, and its environment, and intestinal microorganisms influence physiological function. Together, these observations indicate that the intestinal bacterial community is structured to the dietary specialisation of the host.

Our results provide a better understanding of the intestinal microbiota of black soldier fly larvae; however, the results did not fully address the relationship between microbes and their function. We found that the composition of the edible mushroom residue diet significantly affected the composition of the black soldier fly larval gut microbiota. This indicates that distinct diets, particularly a diet containing edible mushroom residue, are capable of shaping the structure and function of gut microbiota of black soldier fly larvae. These findings could be used to explore the potential relationship between the diets and commensal bacteria of *H. illucens*, thereby paving the way for developing novel strategies to promote bioconversion of organic waste and enhancing studies on insect symbiosis.

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