

## Research Article

# The Effects of Rearing Media on the Growth and Microbiome Diversity in the Digestive Tract of Black Soldier Fly (*Hermetia illucens*) Larvae

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### ABSTRACT

This study aimed to determine the nutritional content and rearing substrate and identify types of microbiota in the digestive tracts of BSF larvae. The substrates used include BSFB (fruit waste), BSFC (fermented bread waste), BSFP (bread waste), BSFS (palm kernel meal), and BSFSO (organic waste). Data on the nutritional content of the substrates and body, the length of the intestinal epithelium of BSF larvae, and the diversity of 32 microbiomes in the digestive tract of BSF larvae were analyzed statistically using analysis of variance with a significance level of 95%. The high and low nutrient content of BSF larvae is influenced by the substrate used. BSF larvae utilized the protein in the substrate to form their body proteins. BSF larvae grown on BSFS substrate showed the highest increase in length of digestive tract epithelium compared to other substrates. Differences in substrate types are one of the factors that affect the diversity of bacterial communities. The most dominant phylum in BSFB, BSFC, BSFS and BSFSO was Proteobacteria, with relative abundances of 62.53 %, 59.63 %, 56.35 %, and 61.35 %, respectively. The most dominant genus in BSFP was *Dysgonomonas* (69.04 %). Differences in substrate type are one of the factors influencing the diversity of bacterial communities within the digestive tract of BSF larvae. These results provide information for formulating specific substrates that promote beneficial gut bacteria, optimize nutrient conversion, and reduce rearing costs.

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## INTRODUCTION

Aquaculture production is highly dependent on the quantity and quality of feeds. Feed contributes largely to the overall operational cost, accounting for 60-70 % of the total aquaculture production cost. The biggest portion of feed is the protein source. Traditionally, the popular source of protein for aquaculture feeds is fishmeal. However, recent developments have shown that the use of fishmeal in feed production has decreased due to depletion of many fishery stocks, increase costs, and environmental concerns (Mohan et al. 2022). Fortunately, various alternative protein sources have been found to equally or more effectively improve feed utilisation and fish growth, among which is the black soldier fly.

The black soldier fly (BSF) (*Hermetia illucens* L.) larvae have been used as a component of fish feed formulations and have received special attention from fish farmers. According to Henry et al. (2015), BSF larvae have a complete nutrient profile that is suitable for fish. BSF larvae contain 10-13 % ash, 38-42 % protein, and 28-35 % fat, as well as amino acids and fatty acids. However, studies have shown that the nutrient profile of BSF larvae is affected by the composition of the media or substrates used in the production (Mirwandhono et al. 2022). BSF larvae can be cultivated using organic waste or agro-industrial products. BSF larvae act as natural bio-converters, which can convert organic waste for growth into nutrient-rich biomass efficiently. Bioconversion using insects is a promising strategy to convert organic waste into biomass that can be used for various applications and converted into high value-added products, thereby overcoming environmental, social, and economic problems (Eke et al. 2023).

According to Allegretti et al. (2017), BSF larvae are capable of extracting energy from animal and plant waste, and converting them to biomass (Zarantoniello et al. 2019). The use of BSF larvae has gained popularity in Indonesia and many parts of the world as a sustainable and cost-effective alternative to traditional livestock feed. This is driven by the high protein content of maggots, which makes them an excellent nutritional supplement for poultry, fish, and other livestock (Sumbung 2020).

The rearing substrate and the culture conditions have been shown to influence the development process of BSF larvae (Yuwono & Mentari 2018). Furthermore, it has also been shown to significantly affect the overall composition of microbial community in the gut of the larvae. The maintenance media and gut microbiota in BSF larvae can contribute to a deeper understanding of how substrate composition affects insect physiology and microbial ecology. The quantity and quality of the substrate have also been shown to affect the survival and nutrient content of BSF larvae (Wardhana 2016). However, studies have also shown that not all substrates can guarantee the provision of appropriate nutrients for BSF larval development (Wang & Shelomi 2017). Studies have optimized the mass rearing of BSF larvae on several organic substrates for maximum biomass yields (Bruno et al. 2019). According to Lestari et al. (2020), a mixture of 25 % fermented rice bran, 25 % fermented coconut dregs, 25 % fermented tofu dregs, and 25 % fermented palm kernel cake was the best culture medium for BSF larvae. Osimani et al. (2021) showed that BSF larvae reared on silverskin coffee substrate enriched with *Schizochytrium limacinum* or *Isochrysis galbana* harbours, *Lactobacillus*, *Leuconostoc* and *Weissella* as the main component of its microbiota. Many previous studies investigated the digestive system of different developmental stages of the BSF (Bruno et al. 2019). However, no studies have examined the microbiota histology of the BSF larvae's digestive system and its growth in relation to different media/types of feed with varying proximate compositions. The present study aimed to determine the effect of the nutrient content of rearing substrate on BSF larval growth, the condition of the larval epithelium, and the

types of microbiota present in the larval gut.

## **MATERIALS AND METHODS**

### **Materials**

The materials used in this study included substrates of BSFB (fruit waste), BSFC (fermented bread waste), BSFP (bread waste), BSFS (palm kernel meal), BSFSO (organic waste), BSF eggs, 10 % NBF, ethanol (70 %, 75 %, 96 % and absolute ethanol), alcoholic acid, xylol, paraffin, distilled water, sterile water, haematoxylin eosin dye, tissue paper, Canada balsam, DNA purification kit, and agarose gel electrophoresis.

### **Methods**

The substrates used in this study included fruit waste (BSFB), fermented bread waste (BSFC), bread waste (BSFP), palm kernel meal (BSFS), and organic waste (BSFSO). These substrates were subjected to proximate analysis prior to use. The moisture content was determined using the thermogravimetric method, ash content using the ashing method, crude protein using micro-Kjeldahl method, crude fat using the Soxhlet method, and crude fibre using strong acid and base methods.

### **BSF larvae rearing**

The rearing substrate was weighed at approximately 120 g per a clean plastic container measuring 32 x 19 x 11 cm<sup>3</sup> (L x W x H) and added with 120 ml of sterile distilled water, stirred, and evenly distributed throughout the container. The BSF eggs were obtained from PT Tribumi Yogyakarta. The BSF eggs were then sprinkled on the substrate and kept until they hatched after 7 days. After hatching, the larvae were kept for 11 days.

### **Proximate and amino acid analysis of BSF larvae**

A total of 55 g of BSF larvae were prepared for proximate analysis. The BSF larvae were ground until smooth using a grinder and then prepared for analysis. The proximate analysis was conducted as described for proximate analysis for substrates. Amino acid analysis was conducted using HPLC, UPLC, and LC-MS/MS methods.

### **Histologically analysis of digestive tract of BSF larvae**

Histological analysis of the digestive tract of the BSF was conducted using the Haematoxylin-Eosin method described by (Bruno et al. 2019). Briefly, the intestinal organs of the BSF larvae were soaked in 10 % NBF for 3 hours, followed by washing with distilled water. First, the fixed intestinal organs were cut to approximately 3 mm and placed into embedding cassettes. Next, the fixed specimens were dehydrated by first removing excess water using clean tissue paper and then soaking them in a series of ethanol 70 % solutions. After that, the dehydrated fixed tissues were cleared of remaining alcohol by soaking them. After complete clearing, the fixed tissues were then impregnated in Toluol:paraffin (3:1) for 30 minutes, then in Toluol:paraffin (2:2), (1:3), and finally in 100 % paraffin for 50 minutes each. After the paraffin impregnation, the tissues were then embedded in paraffin and sectioned at a thickness 5 µm using a rotary microtome (Microm HM 310). The tissue sections were floated in warm distilled water (50 °C) to flatten them before being placed on glass slides. The slides were stained using the haematoxylin and eosin staining method described by Bancroft and Layton (2019). After staining, slides were mounted with Canada balsam and covered with a cover glass. Finally, the slides were examined under a compound microscope (Leica, ICC 50) at 40x and 100x magnifications.

### Measurement of the length of the digestive tract epithelium

The length of the digestive tract (epithelium) of the BSF larvae was measured using ImageJ software. Briefly, the ImageJ was first calibrated to the magnification used when capturing the microscope images. Then, a ruler image matching the photo resolution and magnification was inserted into ImageJ and used to set the scale. This involved drawing a straight line from one point to another on the ruler image, ensuring proper alignment and setting the known distance. Once calibrated, ImageJ was used to measure epithelial length of the digestive tract of BSF larvae by drawing a line across the epithelium and selecting the measurement options.

### Gut microbiome analysis of BSF larvae

BSF larvae were fasted for 24 hours, washed with sterile distilled water, and then stunned using cold water at  $-20\text{ }^{\circ}\text{C}$ . The BSF larvae were surface sterilised with 75 % alcohol and washed with sterile distilled water. The guts of BSF larvae were then aseptically removed for use in DNA extraction using the Promega's Wizard® Genomic DNA Purification Kit. The presence of DNA was verified on 0.8 % agarose gel electrophoresis whereas the DNA purity was determined spectrophotometrically using a Nanodrop spectrophotometer at wavelengths of 260 nm and 280 nm, respectively. In this study, only DNA samples with purity and concentration of 1.8~2.0 (ratio of absorbance at wavelengths of 260 nm and 280 nm) and  $\geq 20\text{ ng }\mu\text{L}^{-1}$ , respectively were proceeded with PCR amplification and sequencing. The PCR amplification was achieved using universal bacterial/archaeal primers 515f (G T G C C A G C M G C C G C G G T A A ) and 806r (GGACTACHVGGGTWTCTAAT) that target the variable regions (V3 and V4) of the 16S rRNA.

This PCR amplification and amplicon sequencing were carried out by a service company (NovogeneAIT) in Singapore. The same amount of PCR products from each sample was pooled, end-repaired, A-tailed, and further ligated with Illumina adapters. Libraries were sequenced on a paired-end Illumina MiSeq platform to generate 250bp paired-end raw reads. Sequencing libraries were generated using NEBNext® Ultra™ II FS DNA PCR-free Library Prep Kit (New England Biolabs, USA). The library was checked with Qubit and real-time PCR for quantification and Bioanalyzer for size distribution detection. Quantified libraries were pooled and sequenced on Illumina MiSeq platforms according to the effective library concentration and the required data output. The bioinformatics analysis pipeline included sequence assembly using FLASH (V1.2.11, <http://ccb.jhu.edu/software/FLASH/>) (Magoc & Slazberg 2011). Data filtering was performed using the fastp (Version 0.23.1) software to obtain high-quality Clean Tags (Bokulich et al. 2012). Chimeras were removed using VSEARCH (V2.16.0) package (Edgar 2013). OTU production was performed using UPARSE software (v7.0.1001, <http://drive5.com/uparse/>) (Edgar 2013). Species Annotation 16S was conducted using SILVA database (<http://www.arb-silva.de/>) (Quast et al. 2012). Top 10 taxa were selected to plot a histogram of relative abundance in Perl with SVG output. Heatmap visualisations of abundance were generated using R; Venn and flower diagrams were produced in R and in Perl using SVG functions. Alpha diversity was calculated with QIIME (Version 1.9.1) and displayed using R (version 4.0.3). PCoA analysis was performed and visualised using the ade4 package and ggplot2 packages in R software (version 4.0.3). The entire analysis was conducted using Python 3.6.13, R 4.0.3, and Perl 5.26.2.

### Data analysis

Data on nutrient contents of substrates of BSF larvae, amino acid composition, length and weight of BSF larvae, and the length of gut epithelium were

statistically analysed using analysis of variance (ANOVA) at a 95 % significance level. The significant difference between treatments was tested using the Duncan Multiple Range Test. The gut microbiome diversity in BSF larvae was determined using the next-generation sequencing (NGS) method and statistically analysed using analysis of variance at a 95 % significance level.

**RESULTS**

The feed or rearing substrates used in this study included BSFB (fruit waste), BSFC (fermented bread waste), BSFP (bread waste), BSFS (palm kernel meal), and BSFSO (organic waste). These substrates have different proximate compositions, including protein, moisture, ash, carbohydrate, lipid, and energy, as shown in Table 1.

Identification of the condition of the BSF larvae rearing medium was necessary to improve its productivity. According to Table 1, the nutritious essential substance of the rearing media of BSF larvae includes protein, moisture, ash, carbohydrate, lipid, and energy ranging from  $5.42 \pm 0.33$  to  $17.85 \pm 0.17$  %,  $8.30 \pm 0.17$  to  $46.84 \pm 0.23$  %,  $1.95 \pm 0.08$  to  $7.39 \pm 0.05$  %,  $35.44 \pm 0.01$  to  $83.40 \pm 0.17$  %,  $0.00 \pm 0.00$  to  $7.13 \pm 0.06$  %, and  $202.32 \pm 0.36$  to  $317.34 \pm 0.01$  kcal g<sup>-1</sup>, respectively.

Substrates for BSF larvae have different nutrient contents. The high and low nutrient content of BSF larvae is influenced by the substrate used. BSF larvae utilized the protein in the substrate to form their body proteins. According to Table 2, the nutrient content of BSF larvae include protein, moisture, ash, carbohydrate, lipid, and energy ranging between  $35.15 \pm 1.48$  to  $49.25 \pm 0.49$  %,  $23.90 \pm 0.57$  to  $33.76 \pm 0.20$  %,  $1.85 \pm 0.07$  to  $6.39 \pm 0.24$  %,  $8.60 \pm 0.42$  to  $27.85 \pm 0.49$  %,  $3.04 \pm 0.49$  to  $7.85 \pm 0.07$  %, and  $302.45 \pm 0.09$  to  $321.30 \pm 0.02$  kcal g<sup>-1</sup>, respectively.

Measuring the amino acid compositions in BSF larvae was crucial for ensuring their effectiveness and reliability as a high-quality protein source, optimizing their production, and enhancing our understanding of their nutritional and biological characteristics. Therefore, the levels of amino acids like alanine, arginine, aspartate, glycine, glutamate, histidine, isoleucine, cysteine, leucine, lysine, methionine, tryptophan, valine, phenylalanine, proline, serine, threonine, and tyrosine in BSF larvae were measured in this study.

The profiles of both essential and non-essential amino acids in BSF larvae are listed in Table 3. The most abundant essential amino acids are leucine, lysine, and valine, whereas the most abundant non-essential amino acids are alanine, glutamate, and proline. BSFP produced the highest total amino acid content, followed by BSFSO, BSFB, BSFC, and BSFS.

**Table 1.** Nutrient content of BSF larvae rearing substrates.

Nutrient content	Media				
	BSFB	BSFC	BSFP	BSFS	BSFSO
Protein (%)	$5.42 \pm 0.33^e$	$12.57 \pm 0.08^c$	$11.91 \pm 0.29^d$	$17.85 \pm 0.17^a$	$15.01 \pm 0.01^b$
Moisture (%)	$8.30 \pm 0.17^e$	$23.09 \pm 0.07^b$	$21.24 \pm 0.05^d$	$21.69 \pm 0.14^c$	$46.84 \pm 0.23^a$
Ash (%)	$2.86 \pm 0.16^c$	$3.37 \pm 0.04^b$	$2.44 \pm 0.01^d$	$7.39 \pm 0.05^a$	$1.95 \pm 0.08^e$
Carbohydrate by different (%)	$83.40 \pm 0.17^a$	$53.82 \pm 0.07^c$	$57.52 \pm 0.05^b$	$50.35 \pm 0.47^d$	$35.44 \pm 0.01^e$
Lipid (%)	$0.00 \pm 0.00^d$	$7.13 \pm 0.06^a$	$6.86 \pm 0.25^a$	$2.73 \pm 0.10^b$	$1.18 \pm 0.02^c$
Energy (kcal g <sup>-1</sup> )	$316.29 \pm 0.00^a$	$309.81 \pm 0.08^b$	$317.34 \pm 0.01^a$	$281.39 \pm 0.35^c$	$202.32 \pm 0.36^d$

Notes: BSFB (fruit waste), BSFC (fermented bread waste), BSFP (bread waste), BSFS (palm kernel meal), and BSFSO (organic waste); Energy = {(protein x 4.5 kcal g<sup>-1</sup>) + (lipid x 9.1 kcal g<sup>-1</sup>) + (carbohydrate x 3.5 kcal g<sup>-1</sup>)} / 100 (NRC 1993). All values are presented in average value ± standard deviation. The superscript letters in each line show a significant difference (P<0.05).

**Table 2.** Nutrient content of BSF larvae.

Nutrient content	Substrates				
	BSFB	BSFC	BSFP	BSFS	BSFSO
Protein (%)	35.15 ± 1.48 <sup>d</sup>	49.25 ± 0.49 <sup>a</sup>	44.60 ± 0.14 <sup>b</sup>	44.83 ± 0.33 <sup>b</sup>	39.95 ± 1.20 <sup>c</sup>
Moisture (%)	30.93 ± 0.18 <sup>b</sup>	33.76 ± 0.20 <sup>a</sup>	31.20 ± 0.23 <sup>b</sup>	23.90 ± 0.57 <sup>c</sup>	22.73 ± 0.95 <sup>c</sup>
Ash (%)	2.85 ± 0.16 <sup>d</sup>	1.85 ± 0.07 <sup>e</sup>	4.75 ± 0.21 <sup>b</sup>	3.87 ± 0.08 <sup>c</sup>	6.39 ± 0.24 <sup>a</sup>
Carbohydrate by different (%)	24.74 ± 1.26 <sup>b</sup>	8.60 ± 0.42 <sup>d</sup>	11.55 ± 0.21 <sup>c</sup>	23.19 ± 1.38 <sup>b</sup>	27.85 ± 0.49 <sup>a</sup>
Lipid (%)	6.34 ± 0.21 <sup>b</sup>	6.50 ± 0.14 <sup>b</sup>	7.85 ± 0.07 <sup>a</sup>	4.22 ± 0.57 <sup>c</sup>	3.04 ± 0.49 <sup>d</sup>
Energy (kcal g <sup>-1</sup> )	302.45 ± 0.09 <sup>c</sup>	310.87 ± 0.05 <sup>b</sup>	312.56 ± 0.00 <sup>b</sup>	321.30 ± 0.02 <sup>a</sup>	304.91 ± 0.04 <sup>c</sup>

Notes: BSFB (fruit waste), BSFC (fermented bread waste), BSFP (bread waste), BSFS (palm kernel meal), and BSFSO (organic waste). Energy = {(protein x 4.5 kcal g<sup>-1</sup>) + (lipid x 9.1 kcal g<sup>-1</sup>) + (carbohydrate x 3.5 kcal g<sup>-1</sup>)} / 100 (NRC 1993). All values are presented in average value ± standard deviation. The superscript letters in each line show a significant difference (P<0.05).

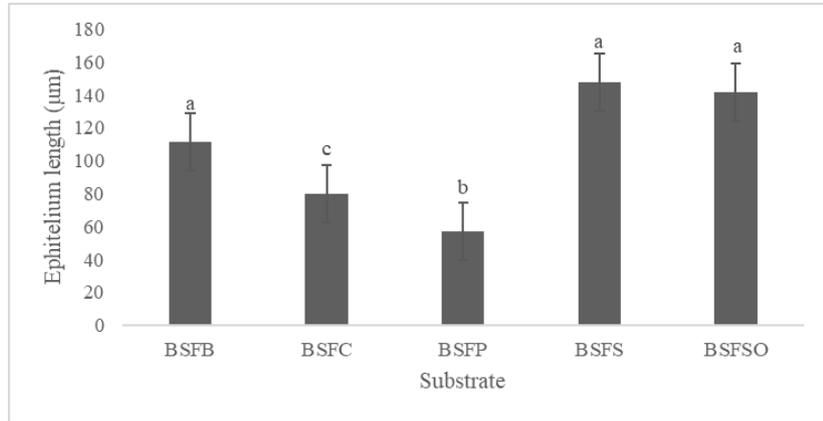
**Table 3.** Amino acid composition of BSF larvae.

Amino acid	Substrate				
	BSFB	BSFC	BSFP	BSFS	BSFSO
Alanine	7758.94 ± 18.42 <sup>d</sup>	11431.61 ± 30.72 <sup>b</sup>	14497.46 ± 30.85 <sup>a</sup>	5379.41 ± 15.64 <sup>e</sup>	10126.3 ± 16.49 <sup>c</sup>
Arginine	3478.54 ± 0.47 <sup>c</sup>	2566.80 ± 1.20 <sup>d</sup>	4752.37 ± 7.62 <sup>a</sup>	1790.05 ± 1.20 <sup>e</sup>	4127.51 ± 5.24 <sup>b</sup>
Aspartate	4706.28 ± 2.47 <sup>d</sup>	5263.71 ± 8.42 <sup>c</sup>	9019.08 ± 13.59 <sup>a</sup>	1063.57 ± 0.36 <sup>e</sup>	5758.66 ± 6.41 <sup>b</sup>
Glisin	5722.79 ± 16.23 <sup>c</sup>	5489.31 ± 12.17 <sup>d</sup>	8421.25 ± 18 <sup>a</sup>	4009.99 ± 13.17 <sup>e</sup>	7087.68 ± 7.80 <sup>b</sup>
Glutamate	9516.67 ± 5.25 <sup>c</sup>	7512.52 ± 15.97 <sup>d</sup>	14012.09 ± 29.19 <sup>a</sup>	3712.53 ± 4.67 <sup>e</sup>	11928.04 ± 5.25 <sup>b</sup>
Histidine	946.61 ± 0.45 <sup>d</sup>	1446.47 ± 1.59 <sup>b</sup>	2615.78 ± 4.87 <sup>a</sup>	ND	1052.79 ± 1.37 <sup>c</sup>
Isoleusine	3211.86 ± 1.30 <sup>c</sup>	2083.59 ± 2.08 <sup>d</sup>	3590.42 ± 0.82 <sup>a</sup>	2075.75 ± 7.28 <sup>d</sup>	3282.51 ± 25.78 <sup>b</sup>
Cystein	718.78 ± 1.40 <sup>e</sup>	2365.97 ± 1.85 <sup>c</sup>	4465.76 ± 2.49 <sup>a</sup>	1462.97 ± 0.50 <sup>d</sup>	3728.57 ± 3.83 <sup>b</sup>
Leucin	5577.33 ± 10.85 <sup>c</sup>	4226.68 ± 5.23 <sup>d</sup>	7405.18 ± 11.72 <sup>a</sup>	3515.13 ± 12.44 <sup>e</sup>	6984.11 ± 13.51 <sup>b</sup>
Lysine	4602.71 ± 6.44 <sup>c</sup>	4297.13 ± 1.94 <sup>d</sup>	7031.22 ± 14.04 <sup>a</sup>	2567.39 ± 6.67 <sup>e</sup>	5494.71 ± 9.60 <sup>b</sup>
Methionine	75.08 ± 0.04 <sup>a</sup>	88.8 ± 0.014 <sup>a</sup>	547.34 ± 27.06 <sup>a</sup>	91.58 ± 0.035 <sup>a</sup>	123.61 ± 0.27 <sup>a</sup>
Tryptophan	486.35 ± 2.36 <sup>d</sup>	671.44 ± 5.81 <sup>c</sup>	991.36 ± 0.87 <sup>a</sup>	907.94 ± 1.83 <sup>b</sup>	999.13 ± 2.83 <sup>a</sup>
Valine	4945.97 ± 7.36 <sup>c</sup>	3931.11 ± 2.7 <sup>d</sup>	6328.97 ± 2.48 <sup>a</sup>	3565.23 ± 4.41 <sup>e</sup>	5482.99 ± 0.77 <sup>b</sup>
Phenylalanin	2420.72 ± 3.88 <sup>d</sup>	2660.59 ± 4.37 <sup>c</sup>	4400.95 ± 7.07 <sup>a</sup>	929.75 ± 0.47 <sup>e</sup>	3289.81 ± 8.54 <sup>b</sup>
Proline	6568.85 ± 11.00 <sup>d</sup>	4127.37 ± 10.71 <sup>e</sup>	7474.07 ± 7.16 <sup>b</sup>	7035.82 ± 23.34 <sup>c</sup>	8447.56 ± 11.32 <sup>a</sup>
Serine	4375.89 ± 5.77 <sup>c</sup>	4204.41 ± 5.79 <sup>d</sup>	6585.47 ± 0.48 <sup>a</sup>	2609.18 ± 4.7 <sup>e</sup>	5358.67 ± 3.65 <sup>b</sup>
Treonine	2587.15 ± 4.95 <sup>c</sup>	2059.23 ± 2.14 <sup>d</sup>	3883.52 ± 6.75 <sup>a</sup>	610.19 ± 1.06 <sup>e</sup>	2587.15 ± 4.95 <sup>b</sup>
Tyrosin	2336.63 ± 0.46 <sup>d</sup>	3399.03 ± 4.70 <sup>b</sup>	5992.48 ± 10.55 <sup>a</sup>	ND	3288.2 ± 2.63 <sup>c</sup>
Total (mg kg <sup>-1</sup> )	70037.15 ± 2592.89	67825 ± 2632.73	112014.8 ± 3750.83	41326.48 ± 1951.50	89148 ± 3143.34

Notes: BSFB (fruit waste), BSFC (fermented bread waste), BSFP (bread waste), BSFS (palm kernel meal), and BSFSO (organic waste). ND = not detected. All values are presented in average value ± standard deviation. The superscript letters in each line show a significant difference (P<0.05).

Based on Figure 1, the provision of BSFS substrate showed the highest length of digestive tract epithelium in BSF larvae. Additionally, the administration of a substrate in the form of BSFSO also showed that the length of the digestive tract epithelium was not much different from the results shown by the BSFS substrate.

Based on the results obtained in this study, the administration of BSFS substrate resulted in the highest intestinal epithelium length in the BSF larvae (Figure 2). In addition, the BSFSO substrate produced a gut epithelium length comparable to that of BSFS, followed by BSFB, BSFC, and BSFP substrates. Increased epithelium lengths indicate the addition of the absorption surface as a tissue response to the high fiber and cellulose content in BSFS and BSFSO substrates.



**Figure 1.** Length of digestive tract epithelium of BSF larvae. Remarks: BSFB (fruit waste), BSFC (fermented bread waste), BSFP (bread waste), BSFS (palm kernel meal), and BSFSO (organic waste).

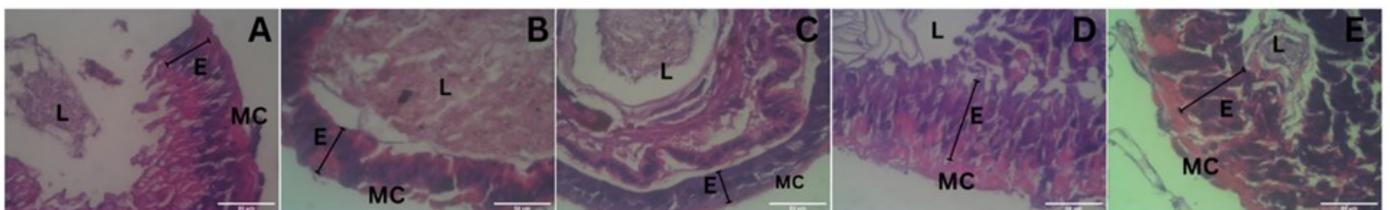
In this study, the bacterial community in the BSF larvae digestive tract was observed using amplicon sequencing. The dominance of bacterial communities among the five groups of BSF larvae fed with different substrates directly influenced bacterial diversity. The bacterial community phyla in the digestive tract of BSF larvae reared on several media are listed in Table 4.

**Table 4.** The complexity of bacteria community phyla in the digestive tract of BSF larvae.

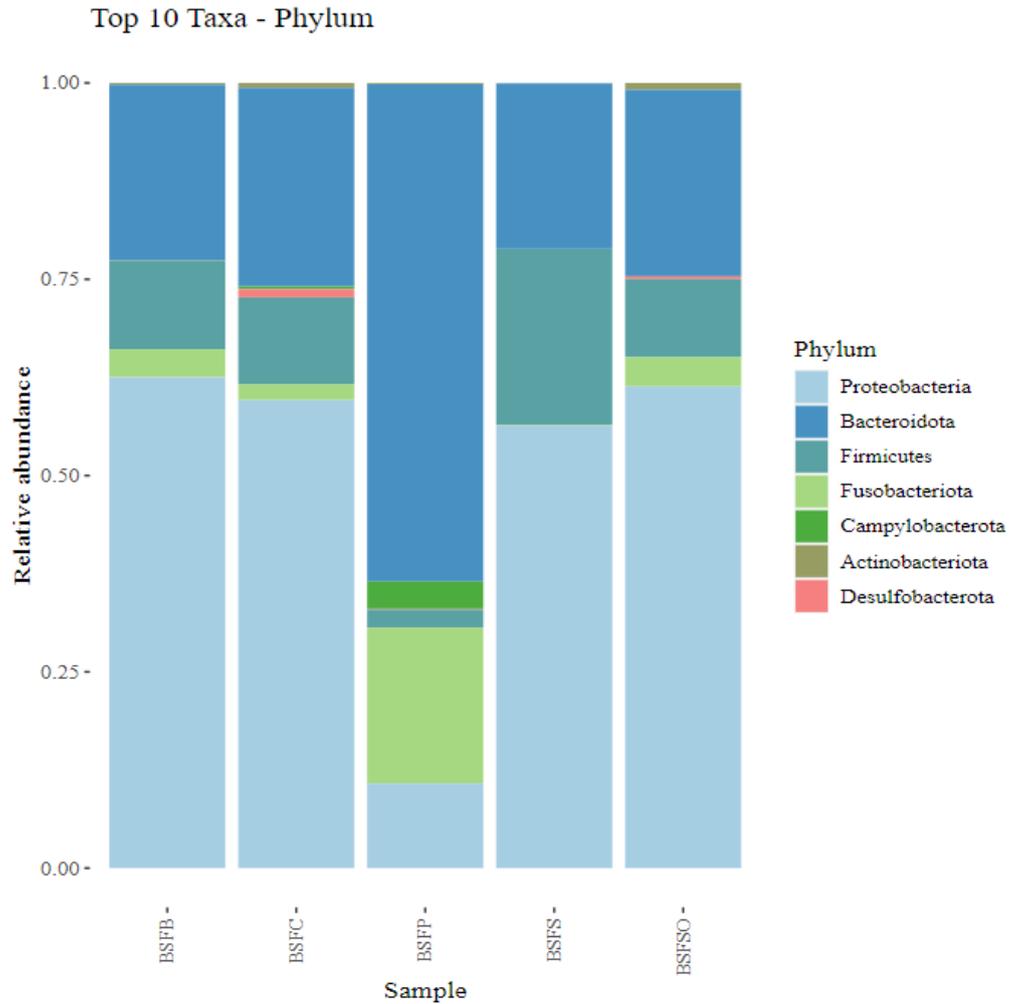
Phyla	Substrate				
	BSFB	BSFC	BSFP	BSFS	BSFSO
Actinobacteriota (%)	0.23	0.62	0.17	0.00	0.85
Bacteroidota (%)	22.32	25.29	63.33	21.08	23.77
Campylobacterota (%)	0.00	0.36	0.34	0.00	0.00
Desulfobacterota (%)	0.00	1.04	1.00	0.00	0.35
Firmicutes (%)	11.32	11.07	2.28	22.37	9.95
Fusobacteriota (%)	3.56	1.99	19.83	0.13	3.73
Proteobacteria (%)	62.53	59.63	10.8	56.35	61.35

Notes: BSFB (fruit waste), BSFC (fermented bread waste), BSFP (bread waste), BSFS (palm kernel meal), and BSFSO (organic waste).

Bacteroidota, Firmicutes, Fusobacteriota, and Proteobacteria are types of phyla found on all substrates. BSF larvae reared on BSFC and BSFP substrates had greater bacterial species complexity than those reared on other media. Table 4 shows that Proteobacteria is the dominant bacterial phylum in the digestive tract of BSFB (62.53 %), BSFP (59.63 %), BSFS (56.35 %), and BSFSO (61.35 %). Meanwhile, the BSFC substrate was dominated by the phylum Bacteroidota (63.33 %). Based on Figure 3, the most dominant bacterial phylum found in the digestive tract of BSF larvae is Proteobacteria, while the least frequently found is Desulfobacterota.



**Figure 2.** Histology of the BSF larvae digestive tract. Notes: Remarks: L (lumen), E (epithelium), MC (muscle cell), and line (epithelium length). Magnification: 40x. Scale bar: 50 µm. A (BSFB/fruit waste), B (BSFC/fermented bread waste), C (BSFP/bread waste), D (BSFS/palm kernel meal), and E (BSFSO/organic waste).



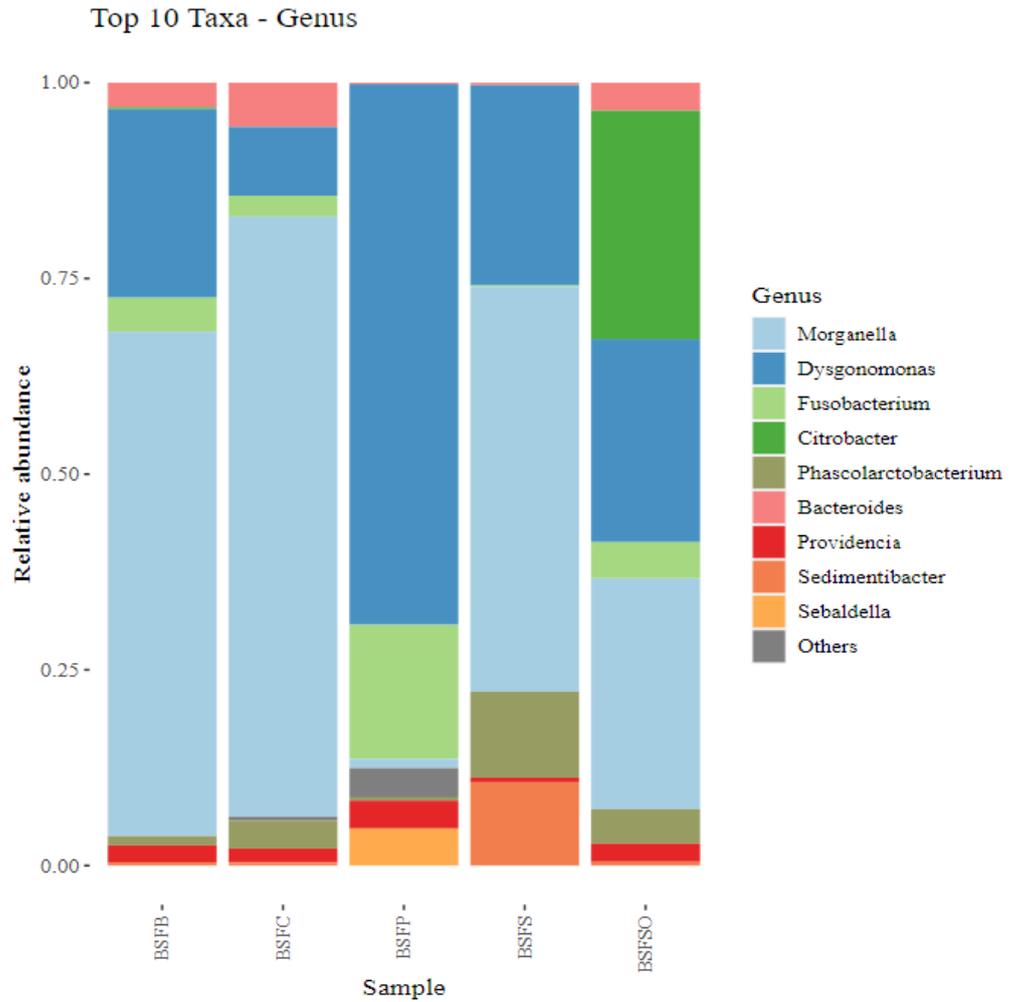
**Figure 3.** Bacterial communities in the digestive tract of BSF larvae based on phyla. Remarks: BSFB (fruit waste); BSFC (fermented bread waste); BSFP (bread waste); BSFS (palm kernel meal); BSFSO (organic waste).

*Bacteroides*, *Dysgonomonas*, *Fusobacterium*, *Morganella*, *Phascolarctobacterium*, and *Providencia* are some of the genera found on all substrates. Table 5 shows that *Morganella* is the dominant bacterial genus in the digestive tract of BSF larvae reared on BSFB (64.43 %), BSFC (76.65 %), BSFS (51.69 %), and BSFSO (29.47 %). Meanwhile, the BSFP substrate was dominated by the genus *Dysgonomonas* (69.04 %). Based on Figure 4, the most dominant bacterial genus found in the digestive tract of BSF larvae is *Morganella*, while the least frequently found are *Citrobacter* and *Sebaldella*.

**Table 5.** Genus of bacterial community in the digestive tract of BSF larvae.

Genus	Substrates				
	BSFB	BSFC	BSFP	BSFS	BSFSO
Bacteroides (%)	3.14	5.63	0.20	0.31	3.56
Citrobacter (%)	0.25	0.00	0.00	0.00	29.19
Dysgonomonas (%)	24.07	8.8	69.04	25.6	25.93
Fusobacterium (%)	4.34	2.65	17.13	0.19	4.63
Morganella (%)	64.43	76.65	1.17	51.69	29.47
Phascolarctobacterium (%)	1.19	3.58	0.43	10.99	4.42
Providencia (%)	2.16	1.74	3.57	0.56	2.3
Sebaldella (%)	0.00	0.00	4.72	0.00	0.00
Sedimentibacter (%)	0.42	0.39	0.00	10.66	0.48
Others	0.00	0.49	3.74	0.00	0.00

Notes: BSFB (fruit waste), BSFC (fermented bread waste), BSFP (bread waste), BSFS (palm kernel meal), and BSFSO (organic waste).



**Figure 4.** Bacterial communities in the digestive tract of BSF larvae based on genus. Notes: BSFB (fruit waste), BSFC (fermented bread waste), BSFP (bread waste), BSFS (palm kernel meal), and BSFSO (organic waste).

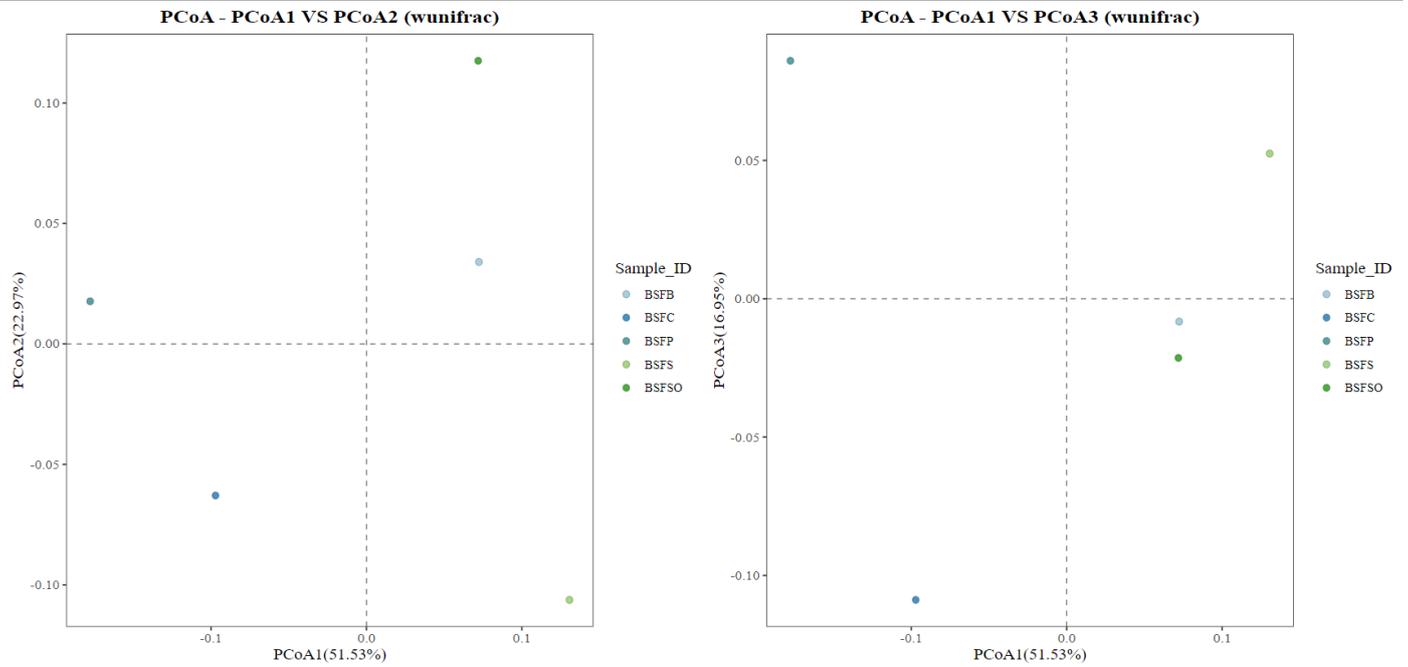
Table 6 shows that the bacterial community in the five BSF larvae gut samples was relatively similar as indicated by the narrow range between the maximum and minimum index values. The bacterial/OTU evenness by Shannon index ranged from 2.803 to 3.528. The dominant bacteria/OTU indicated by Simpson index ranged from 0.800 to 0.949. Meanwhile, the dominant bacteria/OTU in each sample, as indicated by Inverse Simpson index, ranged from 5.00 to 19.90.

**Table 6.** Alpha diversity indices of the bacteria from BSF larvae digestive tract.

Sample	Alpha Diversity Indices		
	Shannon	Simpson	InvSimpson
BSFSO	3.52	0.949	19.988
BSFS	3.03	0.907	10.777
BSFP	2.84	0.891	9.175
BSFB	2.80	0.837	6.140
BSFC	2.91	0.800	5.001

Notes: BSFB (fruit waste), BSFC (fermented bread waste), BSFP (bread waste), BSFS (palm kernel meal), and BSFSO (organic waste).

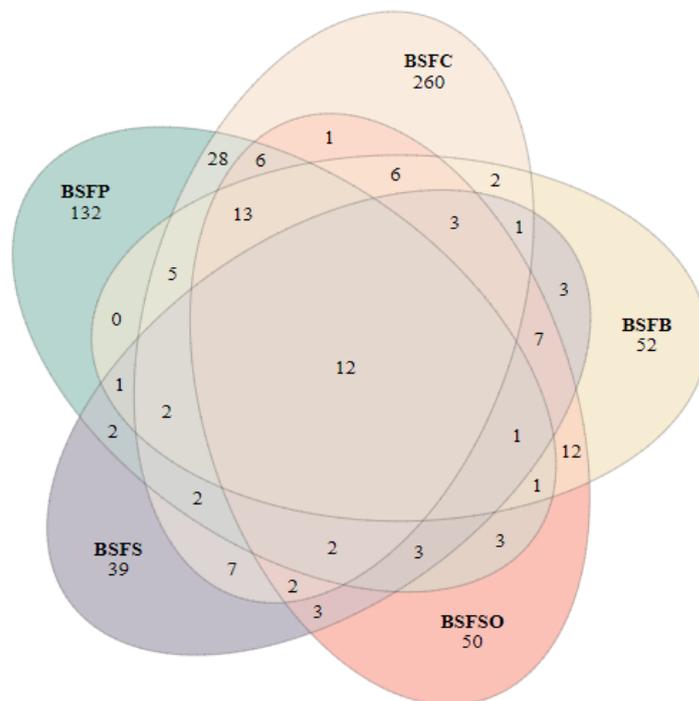
Based on the PCoA diagram (Figure 5), the species composition of the five samples varies. The examination of the composition of various samples (97 % similarity) reflects differences and distances between samples. The results showed that the samples were widely separated. The differences between the groups were explained by the sum of the first dimension (51.53 %)



**Figure 5.** Principal component analysis of microbial composition between samples. Notes: BSFB (fruit waste), BSFC (fermented bread waste), BSFP (bread waste), BSFS (palm kernel meal), and BSFSO (organic waste).

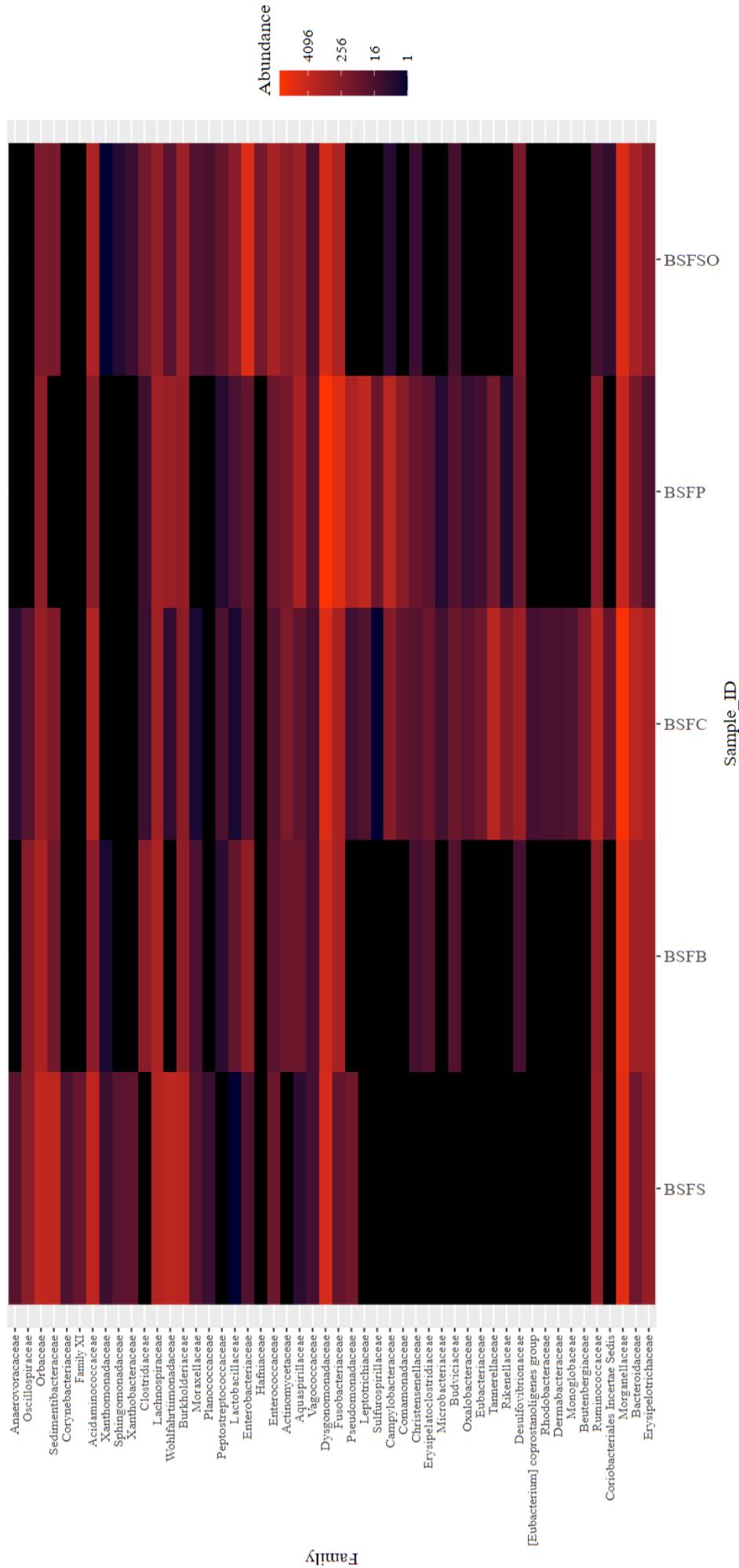
and the second dimension (22.97 %) reaching 74.5 %.

Among 649 identified OTUs, the 12 OTUs (1.85 %) were common core microbiomes in the digestives tracts (guts) of BSF larvae, as they overlapped to all three-colour samples (Figure 6). The 28 OTUs (4.35%) were present in the microbiomes of BSFC and BSFP guts; 2 OTUs (0.38%) in BSFP and BSFS; 3 OTUs (0.51%) in BSFS and BSFSO; 12 OTUs (1.85%) in BSFSO and BSFB; and 2 OTUs (0.38%) in BSFB and BSFC digestive tracts.



**Figure 6.** Venn diagram of the number of OTUs in the digestive tracts of BSF larvae. Notes: BSFB (fruit waste), BSFC (fermented bread waste), BSFP (bread waste), BSFS (palm kernel meal), and BSFSO (organic waste).

Based on Figure 7, the heatmap analysis revealed that the microbiome of BSF larvae had various bacterial communities based on the substrates, as



**Figure 7.** The heatmap of bacterial communities at the family level in the digestive tracts of BSF larvae. Notes: BSFB (fruit waste), BSFC (fermented bread waste), BSFP (bread waste), BSFS (palm kernel meal), and BSFSO (organic waste).

indicated by different dominant families. According to the heatmap, the families Morganellaceae, Dysgomonadaceae, Wolfahrtiimonadaceae, Sedimentibactaceae, Orbaceae, Acidaminococcaceae, Bukholdericeae, and Lachnospiraceae were dominant in BSFS. The families Morganellaceae, Dysgomonadaceae, Orbaceae, Fusobacteriaceae, Orbaceae, Clostridiaceae, Bukholdericeae, and Enterobacteriaceae were dominant in BSFB. The families of Morganellaceae, Dysgomonadaceae, Fusobacteriaceae, Tannerellaceae, Acidaminococcaceae, Orbaceae, Lachnospiraceae, Bacteroidaceae, Ruminococcaceae, and Bukholdericeae were dominant in BSFC. The families of Dysgomonadaceae, Fusobacteriaceae, Leptotrichiaceae, Campylobacteraceae, Morganellaceae, Aquaspirillaceae, and Lachnospiraceae were dominant in BSFP. The families of Morganellaceae, Enterobacteriaceae, Dysgomonadaceae, Fusobacteriaceae, Aquaspirillaceae, and Bacteroidaceae were dominant in BSFO.

## DISCUSSION

Substrates are the primary source of microbial diversity. Microbial communities interact with different diets and impact larval growth and health (Zheng et al. 2013). Additionally, they act as selective barriers that support the formation of microorganisms in the digestive tract to meet the specific nutrient requirements of the host (Eke et al. 2023).

The nutrient contents in BSF larvae vary depending on the substrate used. BSF larvae rely on the protein found in the substrate to develop their own body proteins. It's essential to take into account the substrate's nutrient composition to maximize the growth of BSF larvae. The composition and dynamics of the bacterial community in BSF larvae can be used to improve the performance of larvae assisted by microbiome engineering (Vendemeyer et al. 2023).

Identifying the conditions of the media for rearing BSF larvae is necessary to enhance their productivity. According to Eke et al. (2023), microbial flora colonizing the rearing substrate, nutrient content of the consumed substrate, and the digestive tract of BSF larvae are parameters influencing their productivity, alongside factors like temperature, lighting conditions, and humidity. Previous studies have shown that the nutritional content of the substrate can affect the growth, development, and nutritional quality of BSF larvae (Albalawneh et al. 2024). For example, substrates rich in protein tend to result in larvae with higher protein content, which are desirable for applications in animal feed and bioconversion of organic waste (Spranghers et al. 2017). Additionally, the moisture content of the substrate can impact larval growth rates and overall biomass production (Frooninckx et al. 2024).

*Hermetia illucens* can use a number of organic materials for its growth. However, not all substrates are able to guarantee the proper provision of nutrients for larval development (Wang & Shelomi 2017). Figure 1 shows that BSF larvae grown on organic substrates (BSFSO) produce the highest increase in length of digestive tract epithelium compared to other substrates. The nutrient content of the substrate greatly influences the length and weight of BSF larvae, because good nutrient content will have a positive effect on increasing the length and weight of BSF larvae (Raharjo et al. 2016). The increase in digestive tract epithelial length indicates an increase in absorption surface as a tissue response to the high fibre and cellulose content in BSFS and BSFSO substrates.

The midgut epithelium of the adult BSF (Figure 2) is primarily composed of columnar cells that are responsible for digestive processes. The activity of this organ is supported by endocrine and stem cells. Additionally, the midgut epithelium is notably thicker in BSF, an adaptation that is believed to enhance its ability to process a wide variety of substrates and protect against potential damage from harsh or contaminated food sources. According to research by Bonelli et al. (2020), the morphology of the digestive tract epitheli-

um, especially the brush border on columnar cells, shows different lengths and depends on the substrate.

The bacterial communities in the digestive tracts of BSF larvae in this study showed differences in the taxonomic diversity and richness between samples. The substrate treatment of BSF larvae showed the highest alpha species diversity and richness according to the Shannon index. The highest diversity was in the guts of BSFSO (black soldier fly fed with organic waste). Differences in substrate types are among the factors that contribute to the diversity of bacterial communities. BSF larvae fed with different feeds had different bacterial communities. This shows that the type of food directly influences the diversity of bacteria in the guts (Jeon et al. 2011). Each substrate has different physicochemical conditions and nutrient content, thereby giving possibility for many microorganisms to grow. According to Cifuentes et al. (2020), bacterial community dynamics are correlated with rearing performance and physicochemical properties and composition of the residue. The dominance of bacterial communities among the groups of BSF larvae given different feed revealed that the type of substrate time could slightly change bacterial diversity. According to the previous research, the dominant species (*Bacteroides*, *Dysgonomonas*, *Morganella*, *Enterococcus*, *Providencia*, *Klebsiella*, and *Bacillus*) showed significant variability in BSF larvae, possibly related to feed variations (Klammsteiner et al. 2020). The composition and number of bacterial communities in insects' guts are influenced not only by nutrition but also by the insect species.

The most dominant phylum in BSFB, BSFP, BSFS, and BSFSO was Proteobacteria with relative abundance of 62.53 %, 59.63 %, 56.35 %, and 61.35 %. Meanwhile, the most dominant phylum in BSFP was Bacteroidota (63.3 %). Bacteroidota was the second most dominant in BSFB, BSFC and BSFSO with relative abundance of 22.32 %, 25.29 % and 23.77 %, respectively. This study showed that the most dominant phylum in BSFC, BSFS, BSFSO and BSFB was Proteobacteria. Other studies showed that Proteobacteria and Bacteroidota were also found as the most dominant phyla of bacterial communities in the gut of BSF larvae fed with chicken manure substrate (Shumo et al. 2021), chicken feed and camelina commercial feed (Schreven et al. 2022), canteen waste and household waste (Gold et al. 2020). The enhanced proportions of these two phyla demonstrate an abundance of such bacteria to regulate hydrolytic enzyme activities for organic breakdown. Bacteroidota was largely responsible for the degradation of high molecular weight organic matter, such as proteins and carbohydrates (Zhang et al. 2014).

Gut bacterial communities of BSF larvae at the phylum level are mostly composed of *Proteobacteria*, *Bacteroidota*, *Firmicutes* and *Fusobacteria*. Jeon et al. (2011) reported some differences, which included *Bacteroidetes*, *Proteobacteria*, *Firmicutes*, *Fusobacteria*, and *Actinobacteria*. Those bacteria have important role in the gut of BSF larvae. *Proteobacteria* was observed as a potential microbial signature of disease and *Firmicutes* was considered to play an important role in the digestion of animal manure (Zhan et al. 2020).

At the genus level, *Morganella* was the most abundant in BSFB (64.43 %), BSFC (76.65 %), BSFS (51.69 %) and BSFSO (29.47 %), whereas the most dominant genus in BSFP was *Dysgonomonas* (69.04 %). The second abundant in BSFB (24.07 %), BSFC (8.8 %), BSFS (25.6 %) and BSFSO (25.93 %) was *Dysgonomonas*. Other studies showed that *Morganella* and *Dysgonomonas* were also found as the most dominant genera of bacterial communities in the gut of BSF larvae fed on fruit, vegetable waste, and supermarket/restaurant waste (Wynants et al. 2019). Symbiotic microorganisms are important for host survival and reproduction as they perform essential metabolic roles such as nutrient digestion (Engel & Moran 2013; Gold et al. 2020). *Morganella morgani*

can express urease which in turn leads to the production of high levels of biogenic amines (Özogul & Özogul 2004; Gold et al. 2020). *Dysgonomonas*, as a major genus in the gut of BSF larvae, could be involved in the digestion, playing a significant role in breaking down complex polysaccharides, proteins and lipids. *Dysgonomonas* could contribute to nutrient decomposition in the residue (Bruno et al. 2019).

The bacterial communities also play a significant role in host growth, digestion and immunity, and the prevention of gut colonisation by pathogens. The characteristics of the bacterial community in the larval gut can be influenced by changing the food (Vogel et al. 2018). Meanwhile, symbiosis with hosts may change surrounding microbial ecosystems (Hammer et al. 2017). Clearly, the gut flora of many insects is an open system where exogenous microorganisms can colonize and interact with the internal gut microbiome.

## CONCLUSION

This research provides valuable insights into the relationship between rearing media and gut microbiota in Black Soldier Fly larvae, contributing to a deeper understanding of how substrate composition influences insect physiology and microbial ecology. By revealing the potential of specific organic waste types to enhance larval development and microbial diversity, the study supports the development of more efficient, sustainable insect farming systems. These findings can inform future efforts to formulate tailored substrates that promote beneficial gut bacteria, optimize nutrient conversion, and reduce rearing costs. Further research should explore the functional roles of dominant microbial taxa and investigate how manipulating the microbiome could enhance BSF resilience, disease resistance, and feed efficiency at larger production scales.

## AUTHOR CONTRIBUTION

S.H. contributed to the research design and was responsible for all research activities and finalising the manuscript. N.I.S. has contributed to histological preparation, measurement of the digestive tract epithelium length, and manuscript improvement. T.P.F. has contributed to the analysis of nutrient content and the types of microbiota in the intestines. J.R. has contributed to the analysis of nutrient content and manuscript improvement.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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