MICROBIAL DYNAMICS DURING INDUSTRIAL REARING AND PROCESSING OF INSECTS

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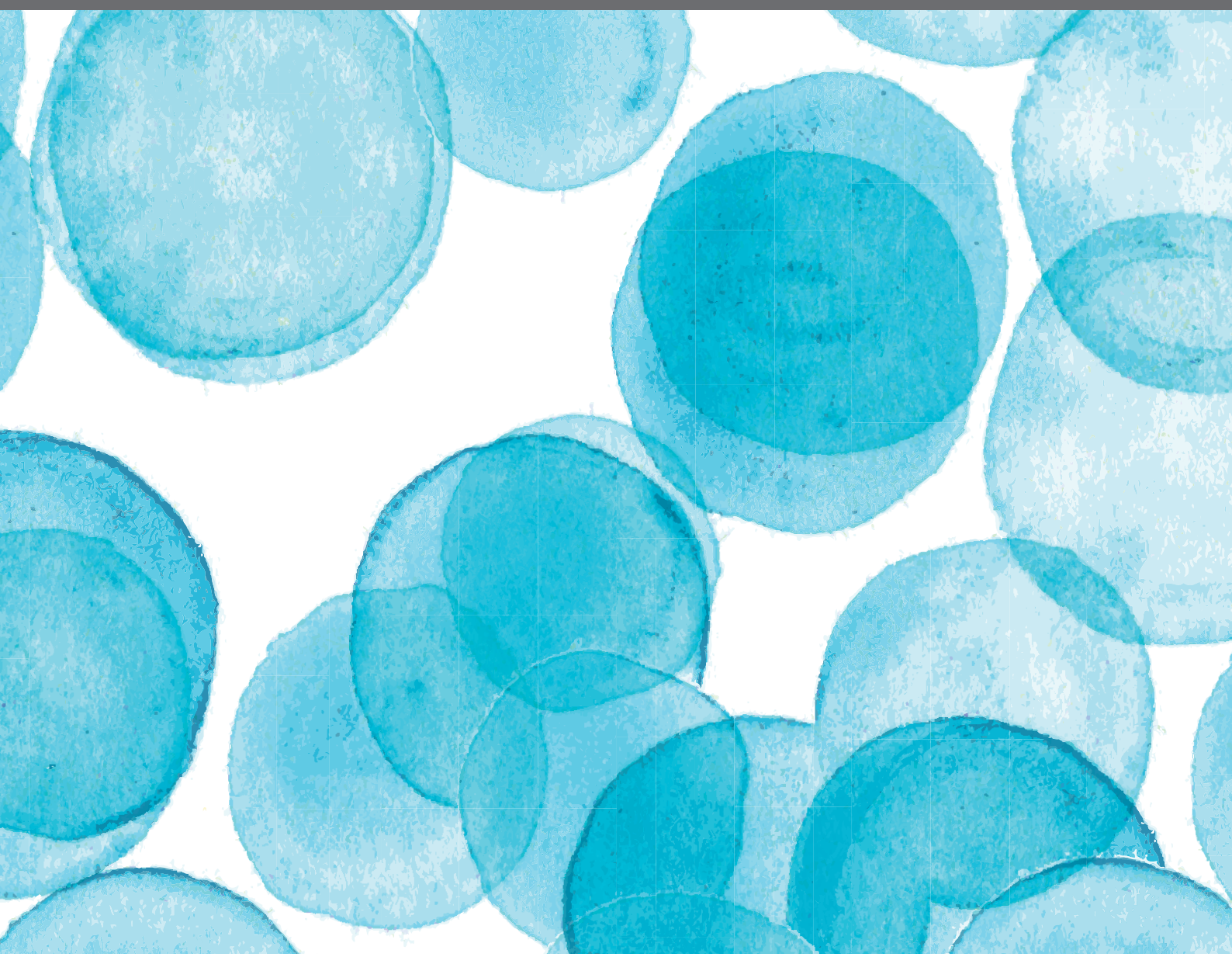

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MICROBIAL DYNAMICS DURING INDUSTRIAL REARING AND PROCESSING OF INSECTS

Topic Editors:
Leen Van Campenhout, KU Leuven, Belgium
Jørgen Eilenberg, University of Copenhagen, Denmark

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EDITORIAL
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Editorial: Microbial Dynamics During Industrial Rearing and Processing of Insects

Leen Van Campenhout[1,2]* *and Jørgen Eilenberg*[3]

[1] Research Group for Insect Production and Processing, Department of Microbial and Molecular Systems (M2S), Geel Campus, KU Leuven, Geel, Belgium, [2] Leuven Food Science and Nutrition Research Centre (LFoRCe), KU Leuven, Leuven, Belgium, [3] Department of Plant and Environmental Sciences, Faculty of Science, University of Copenhagen, Copenhagen, Denmark

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Editorial on the Research Topic

Microbial Dynamics During Industrial Rearing and Processing of Insects

Today, a number of insect species are farmed at a large scale. The major aim of mass production is insect biomass, which is then processed into food, animal feed, or pet food. The insect biomass can eventually be fragmented and purified to deliver biochemicals. Alternatively, the major aim of insect rearing can rather be waste conversion, with the resulting insect biomass as a useful byproduct improving the circularity of waste processing. Whatever the objective of the industrial cultivation and processing of insects, microbiological questions and challenges arise on many occasions in any insect production and processing project. Insects naturally harbor a rich microbiota, containing organisms that in some way contribute to, or conversely partially or fully obstruct the application. As industrial applications for insects make progress in versatility and scale, so does the research, and this is well-reflected in this Research Topic. The Topic covers microbiological questions and challenges related to the rearing, processing, and storage of insects, and it also addresses insect-related final products.

The major part of the papers relates to the rearing phase, and the share of papers envisaging processing and end products is smaller. Most of the research presented considers the dynamics of the whole microbiota in the insects (and the related environment), while only a few papers (additionally) focus on the role of specific microbial species.

The papers in the Topic related to microbial dynamics during the rearing phase all focus on black soldier fly larvae (BSFL), *Hermetia illucens*. The range of substrates BSFL can feed on is extensive, and a fascinating question is whether this variety in substrates also leads to a corresponding diversity in the microbiota. The papers in this Topic do not allow a profound meta-analysis necessary to formulate general conclusions. Nevertheless, the presented studies on the endogenous microbiota of BSFL during rearing cover a range of substrates, such as cottonseed press cake (Tegtmeier et al.), brewers' spent grain, kitchen food waste, poultry manure and rabbit manure (Tanga et al.), canteen waste and oil separator waste (Klammsteiner et al.), chicken manure and chicken waste (Shumo et al.). They all used (at least) amplicon sequencing of the whole microbiota to obtain the relative abundances of genera present.

In addition to the microbiota as it occurs during the routine rearing of insects, a contribution to the Topic by Yang et al. described the substantial impact of interventions during rearing, i.e. starvation, on the composition as well as the predicted metabolic functions of the microbiota. Studying the reaction of the microbiota on such interventions can help to optimize rearing practices toward maximal biomass yield or conversion efficiency. Whereas Yang et al. used the well-defined

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Edited by:
Aldo Corsetti,
University of Teramo, Italy

Reviewed by:
Christos Rumbos,
University of Thessaly, Greece

***Correspondence:**
Leen Van Campenhout
leen.vancampenhout@kuleuven.be

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and research-oriented Gainesville diet for their starvation study, the effect of such interference when using industrial waste streams is a subject for future research.

Another dimension in the research on the insect microbiome during rearing relates to the addition and monitoring of a particular species of microorganisms, either an unwanted one or a potentially beneficial one. To monitor food pathogens during rearing in order to evaluate microbiological safety, they are typically inoculated under controlled conditions and then kept track of using selective media. In this way, Lopes et al. inoculated *Salmonella* and *E. coli* during fish waste processing by BSFL. They found these bacteria to be reduced by the larvae, yet the reduction was dependent on the feeding regime. Jensen et al. exposed mealworm larvae (*Tenebrio molitor*) to a flour-based substrate contaminated with *Salmonellla* at several inoculation levels. Except at the lowest contamination level, *Salmonella* remained present in the larvae throughout the 14-days rearing period. This indicated that using substrates free of *Salmonella* is a key measure in the production of microbiologically safe mealworms. When investigating the fate of food pathogens during insect rearing, the implications on the microbiological end product safety are the main concern, and not the impact of the food pathogens on insect growth performance. This is in contrast with studies investigating the effect of the addition of potential probiotic bacteria during rearing, such as that of Kooienga et al. They tested the effect of *Arthrobacter*, *Bifidobacterium breve* and *Rhodococcus rhodochrous* cultures on growth performance, conversion efficiency, and microbial population structure and function of BSFL grown on the Gainesville diet at laboratory and industrial scales. At both scales, the performance of the larvae was improved, but the authors mentioned that care should be taken towards the appropriate bacterial supplement and more trials are needed for proper confirmation of the probiotic effect.

Reared insects reside in an environment with a high microbial load and hence they tend to contain a high number and a wide diversity of microorganisms. Typical processes to transform the insects into a stable end product suitable for a certain application include a large reduction in numbers and/or a change in microbial composition. This is illustrated in this Topic by Fröhling et al. in the flour production process from crickets (*Acheta domesticus*), consisting of freezing, thawing, washing, heating, drying, and pulverization steps. Evidently, microbial numbers were highly reduced after this processing chain, but a small microbiota was still present and grew on selective media for a range of food pathogens, indicating that some microbiological risk possibly remained. Borremans et al. studied the stabilization of blanched and pulverized mealworms. Fermentation of the paste appeared to be a better approach to control the residual microbiota in the paste than applying traditional meat preservatives. These two Topic contributions show that the microbial dynamics during insect processing are highly dependent on the insect species in combination with the processing technologies (and the concomitant process parameters) applied. Generalizing and extrapolating conclusions from one insect species to another and from one processing technique to another is difficult or even impossible.

Finally, the topic also includes work on the microbiology of insect-based end products. Obviously, the insect biomass is the main end product, but the residue (feces, unconsumed substrate, and/or exuviae) is another end product. Mao et al. explored for the first time the microbiota of tea prepared from the feces of meal moths (*Pyralis farinalis*) reared on a specific plant-based diet. The microorganisms mainly originated from the insects and not from the plants they were reared on, and they appeared to survive the roasting and the brewing of the tea. Residues (frass) from BSFL rearing are increasingly being investigated and applied as soil improvers. Some microorganisms are thought to play a role here, but research so far is mainly directed to unraveling the composition of the microbiota of the frass. As BSFL can be grown on a wide variety of substrates, the microbiological quality of BSFL frass can also be different. In this Topic, Gold et al. described the microbiota of frass of BSFL produced using canteen food waste and household food waste. Some typical members of the BSFL core microbiome, such as *Providencia, Dysgonomonas, Morganella,* and *Proteus*, appeared also to be abundant in the frass. Research on the microbiology of insect frass is only in its infancy now, but knowledge about the composition and role of microorganisms in frass may contribute to upcycling frass as a side stream of insect production, improving its economic value and giving it a significant place in a circular economy.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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Microbiome-Guided Exploration of the Microbial Assemblage of the Exotic Beverage "Insect Tea" Native to Southwestern China

Xin Mao[1,2,3], Peter Kusstatscher[4], Haoxi Li[3,5], Xiaoyulong Chen[3,5], Gabriele Berg[4], Maofa Yang[2,3,5] and Tomislav Cernava[3,4,5]**

[1] College of Forestry, Guizhou University, Guiyang, China, [2] Institute of Entomology, Guizhou University, Guiyang, China, [3] Guizhou Provincial Key Laboratory for Agricultural Pest Management of the Mountainous Region, Guiyang, China, [4] Institute of Environmental Biotechnology, Graz University of Technology, Graz, Austria, [5] College of Tobacco Science, Guizhou University, Guiyang, China

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Edited by:
Leen Van Campenhout,
KU Leuven, Belgium

Reviewed by:
Andrea Osimani,
Marche Polytechnic University, Italy
Lucia Aquilanti,
Marche Polytechnic University, Italy

***Correspondence:**
Maofa Yang
gdgdly@126.com
Tomislav Cernava
tomislav.cernava@tugraz.at

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Insect tea is a unique beverage that is native to Southwestern China and traditionally produced by local farmers in an elaborate process. It consists of insect larvae excrements that are commonly obtained from meal moths (*Pyralis farinalis* Linnaeus 1758) reared on a specific plant-based diet. We have reconstructed the whole production process under laboratory conditions in order to obtain microbiome-level insights into this uncommon beverage and to trace back the origin of the prevalent bacteria in the final product. The bacterial community composition was specific for each production stage, with a high proportion of *Streptomycetacea*, *Pseudonocaridaceae*, *Enterococcaceae*, and *Enterobacteriaceae* in the insect tea. A large proportion of the constituents was traced back to the producing insect (13.2%) and its excrements (43.8%), while the initial plant-based substrate for tea production was found to contribute only 0.6% of the traceable bacteria in the final product. Moreover, an enrichment of *Enterobactericeae* was observed during the analyzed process steps and verified with complementary analyses. The cultivation experiments indicated a high occurrence of viable bacteria in the tea at $2.7 \times 10^5 \pm 1.2 \times 10^5$ cfu g^{-1}. The isolated bacteria included *Bordetella petrii* and *Enterococcus* spp. that were recovered from a commercial product. By implementing an integrative approach, the insect tea was shown to harbor a species-rich bacterial community that can be traced back to certain plant and insect microbiome constituents from distinct production steps. Moreover, the microbial profile of the insect tea was found to be unique for a food product so far and contained several bacterial groups that are considered from the current perspective as food contaminants or yet unreported in other beverages. Due to the high number of viable bacteria, the tea harbors a so far undescribed dynamic component that might have implications for human health.

Keywords: insect tea, tea microbiome, food microbiome, *Pyralis farinalis*, *Enterobacteriaceae*, Chong Cha

INTRODUCTION

The so-called insect tea (Chinese: 虫茶; pinyin: Chong Cha) is a traditional beverage of the ethnic minorities in Southern China and mainly produced in the mountainous region south of the Changjiang river (Xu et al., 2013). This tea is a highly exceptional beverage because it solely consists of excrements obtained from insect larvae that were reared on a specific plant leaf diet (Bai, 2010). Nowadays, various commercial products are available in the Chinese market, yet it is a costly niche product that is only consumed by a small group of the population. The traditional procedure to obtain this unconventional product starts with the collection of fresh leaves which are then air-dried and stacked in baskets made of bamboo or other wooden containers. Various plant species can be used for the production of insect tea, including *Cyclocarya paliurus* (wheel wingnut, Juglandaceae) (Wen and Guo, 1997; Xiang and Lu, 1998; Wang et al., 2010). In analogy to the broad substrate range, many insect species from the order Lepidoptera can be utilized to produce insect tea (Qin, 1983; Bai, 2010; Liu et al., 2014). Interestingly, *Pyralis farinalis*, which is commonly known as the meal moth and famous as cosmopolitan pest of stored grain, is also often used for insect tea production (Shang et al., 2011). After rearing the larvae on leaf stacks for several months, their excrements are collected and roasted before they are packed and marketed (Bai, 2010). The quality grade of the insect tea depends on particle diameter, leaf harvesting time, and processing methods; the microbiological parameters are currently not assessed.

Similar to other eukaryotic organisms, insects are colonized by microbial communities of varying complexity that primarily occur in the insect's gut (Engel and Moran, 2013) and the exoskeleton (Skovgaard et al., 2015); however, some specifically adapted endophytes can also colonize other internal tissues (Eleftherianos et al., 2013). Recent findings related to the insect gut microbiome have shown that it generally harbors a lower microbial diversity as compared to the mammalian guts (Engel and Moran, 2013). The most abundant phyla in various insect species were shown to be *Proteobacteria* and *Firmicutes* (Alonso-Pernas et al., 2017; Kaczmarczyk et al., 2018; van Schooten et al., 2018). Similar to other organisms, the insect-associated microbial communities generally show differences across the life stages of their hosts (Alonso-Pernas et al., 2017; Kaczmarczyk et al., 2018). Moreover, various microbes were shown to be involved in insect growth and development (Shukla et al., 2018). The transmission of the microbiome among insect individuals can be horizontal (Qi et al., 2018) as well as vertical (McManus et al., 2018). In terms of other possible transmission paths, the insect gut microbiome was so far not assessed in connection with its contribution to a food product. While the morphological, biological, and ecological characteristics of meal moths were investigated systematically (Shang et al., 2011, 2013b), their gut microbiome remained uncharacterized. Studies addressing insect tea are rather scarce and focus on its nutritional value and classic microbiological analyses. Recent evaluations of its composition and a biosafety assessment were conducted with products based on *Litsea coreana* and *P. farinalis* larvae (Shang et al., 2013a; Wang et al., 2017). The microbial constituents as well as the contamination of food products are still mostly assessed with standardized cultivation-dependent methods. However, there are various applications that benefit from more detailed community-level assessments of prevailing microorganisms. Examples include the monitoring of fermentation processes (De Filippis et al., 2017), the profiling of microbial communities in ripened products (Quigley et al., 2012), and the linking of product properties with the presence of specific microorganisms (Lattanzi et al., 2013). Due to the broad applicability of microbiome studies in food microbiology, they will likely contribute to the improvement of various food products, which is not feasible with the conventional methods (De Filippis et al., 2018). The pioneering studies addressing the microbial constituents of edible insects with next-generation sequencing delivered community-level insights that will serve as a basis to improve this sustainable protein source in the future (Garofalo et al., 2017, 2019; Vandeweyer et al., 2018). We expected that the insect tea would have a complex bacterial profile due to its origin and the traditional processing steps. Therefore, we implemented it as a model in a systematic approach to show how potential contaminants can be tracked during a production process by using microbiome-based approaches. The objectives of this study were to (1) provide a complete bacterial profile of a representative product, (2) link the plant leaf and insect gut microbiome with the bacteria present in the insect tea, and (3) track selected target bacteria along the whole production process. In a complementary approach, the aerobic mesophilic bacterial fraction of commercial products was cultivated in order to evaluate if they contain living microorganisms that could potentially have implications for human health. *P. farinalis* (insect) and *C. paliurus* (substrate for the plant-based diet of the larvae) were implemented in all of the experiments conducted because they are most commonly used for insect tea production in Southwestern China. The obtained results provide a first insight into the bacterial community composition of a unique product and a basis to further explore its active ingredients.

MATERIALS AND METHODS

Larvae Rearing and Sample Preparation

The *P. farinalis* larvae were obtained from a large colony maintained at the Institute of Entomology, Guizhou University, Guiyang, China. Only larvae that are commonly used for the insect tea production (approximately fourth to sixth instar) were selected for the experiments. Transparent plastic boxes (11 × 11 × 9 cm) were sterilized with 75% ethanol and then placed under a UV lamp (Taisite Instrumental Company; Tianjin, China) for 12 h. Small holes on the top of the containers ensured sufficient aeration during the insect rearing. Six replications were prepared under the same conditions. Each of the containers was equipped with five *C. paliurus* leaves and 20 larvae from the initial colony. The containers were kept at 22°C, RH 80%, and 14-h/10-h day/night cycles for 5 days in a growth chamber (Jiangnan Instrumental Company, Ningbo, Zhejiang, China). This initial step was included for acclimatization and renewal of the gut content. Then, the larvae from each container were transferred into new containers with the same amount of fresh

C. paliurus leaves. The leaf samples for the subsequent DNA extractions were obtained from these containers before the larvae were added. After another 7 days, the excrements were collected separately from each container. At the same time, the midguts from the larvae were separated with sterile forceps and scalpels under a dissecting microscope. All insect larvae were freeze-killed at -20°C according to current research and industry standards before the midgut was removed (Larouche et al., 2019). All samples were stored on ice during processing and subsequently used for the total community DNA extractions. A commercial insect tea product (Chishui Chong Cha, Chishui, Guizhou, China) that is produced by the same insect larvae species and a plant-based diet that is not strictly defined was implemented for comparative analyses. The product was stored at room temperature in sealed sachets before it was transferred to extraction tubes under sterile conditions.

Total Community DNA Extractions

All samples were processed with a DNA extraction kit (FastDNA SPIN Kit for Soil; MP Biomedicals, Solon, OH, United States) to extract the total community DNA. Leaf, excrement, midgut, as well as commercial insect tea product (Chishui Chong Cha, Chishui, Guizhou, China) samples were directly transferred into the extraction vials provided in the aforementioned kit in order to avoid contaminations. The sample types and the experiment procedures are schematically shown in **Supplementary Figure S1**. For the leaf and excrement samples, approximately 150 mg was used for each replicate. The insect midgut samples were pooled for each replicate. They were obtained from 13 larvae reared in the same container, with a total weight of approximately 150 mg for each of the six samples. The samples of the commercial product (155 mg per replicate) were obtained from separate sachets. The total community DNA extractions were conducted according to the manufacturers' protocol, and the extraction efficiency was confirmed by photometric determinations of the DNA concentration (NanoDrop 2000, Thermo Fisher Scientific, Wilmington, DE, United States). For each sample type, six biological replicates were obtained. The total community DNA extracts were stored at -20°C until further processing.

Barcoding and High-Throughput Sequencing of 16S rRNA Gene Fragment Amplicons

The aforementioned DNA samples were transferred to a sequencing company (Novogene Co., Ltd., Beijing, China) for next-generation sequencing that targeted the 16S rDNA Hypervariable Region 4 (V4). The following PCR program (95°C for 5 min to denature the DNA, 30 cycles at 96°C for 60 s, 78°C for 5 s, 54°C for 60 s, 74°C for 60 s, and 10 min at 72°C for a final extension) was used for the generation of amplicons according to the standardized protocol provided by the Earth Microbiome Project[1]. During the PCR amplification, 1 μl of mPNA and pPNA were added to all samples in order to block the amplification of plastid and mitochondrial 16S rDNA sequences (Lundberg et al., 2013). The PCR blockers were obtained from PNA Bio Inc. (Newbury Park, CA, United States). In addition, bovine serum albumin (BBI Solutions, Shanghai, China) was used to increase the efficiency of the PCR reactions. The next-generation sequencing was conducted by Novogene (Beijing, China) on the Illumina PE250 platform that produces 2 × 250-bp paired-end reads.

Bioinformatic Analyses

The reads were assigned to samples by their unique barcode sequences and the barcode which were truncated by the sequencing company (Novogene Co., Ltd., Beijing, China). Demultiplexed paired-end reads were imported into QIIME 2 (2019.1 release; Caporaso et al., 2010), and the DADA2 algorithm (Callahan et al., 2016) was applied to summarize sequence variants (SVs) and to generate a filtered feature table as well as representative sequences. The chimeras were filtered from the table, and the features were classified using a Naïve–Bayes classifier trained on the SILVA 132 release (Quast et al., 2013), with 99% similarity and a confidence level of 0.7 (default value). The mitochondria and the chloroplasts were further excluded from the table, resulting in a total of 3,260,703 reads which are assigned to 8,465 features. The determination of alpha and beta diversity was performed using the QIIME 2 core diversity metrics and group significance tests. The dataset was rarefied to a depth of 42,300 reads per sample. The feature table was split into four tables according to the sample group (leaf, midgut, excrement, and insect tea). Subsequently, the core microbiome was generated by filtering the features present in four out of the six samples (66.6%). All core microbiomes were unified again, resulting in a total of 1,082 core features that were retained. The table was exported from QIIME 2, and barplots were generated with a cutoff of 1% abundance. For network rendering, the features with less than 1,000 reads were further filtered from the core feature table (296 features retained) and collapsed on the genus level. Using the make_out_network.py script in QIIME 1.9.1, this collapsed feature table was exported for visualization in Cytoscape 3.6.1 (Shannon et al., 2003). The SourceTracker 0.9.5 software embedded in QIIME was used to predict the source of the microbial communities in the different sample types.

Verification of *Enterobacteriaceae* Enrichment in the Samples

In order to confirm the accumulation of *Enterobacteriaceae* during the production process of the insect tea, a qPCR-based approach was employed. The same DNA extracts that were used for the amplicon sequencing of the 16S rRNA gene fragments were adjusted to a concentration of 2 ng/μl in order to account for the differences in the extraction efficiency. The primers *rplP* 1F (5′-ATG TTA CAA CCA AAG CGT ACA-3′) and *rplP* 185R (5′-TTA CCY TGA CGC TTA ACT GC-3′) were used according to the method described by Takahashi et al. (2017) to quantify the *Enterobacteriaceae*-specific markers. All quantifications were performed with the PowerUp SYBR Green Master Mix (Applied Biosystems; Vilnius, Lithuania) and the

[1] www.earthmicrobiome.org

CFX96 Real-Time System (Bio-Rad Laboratories, Hercules, CA, United States). Each sample was analyzed with six technical replicates and two independent runs. The mean fluorescence intensity of the leaf samples was used as a reference to determine increases in fluorescence intensity during the production process as well as in the final product.

Isolation and Quantification of Aerobic Mesophilic Bacteria in Commercial Insect Tea

In order to isolate bacteria from a commercial insect tea product (Chishui Chong Cha, Chishui, Guizhou, China), a total of 40 tea sachets, containing 3 g of insect tea each, from at least two production batches were homogenized in 30 ml 0.85% NaCl on a shaker (Jinghong Co., Ltd., Shanghai, China) at 120 rpm for 10 min. Subsequently, a dilution series (1:10 steps) was prepared and plated in triplicate on LB agar (non-selective complex growth medium; Saigon Biotech Co., Ltd., Shanghai, China), MacConkey agar (selective medium for Gram-negative bacteria; Lianmai Bioengineering Co., Ltd., Shanghai, China), and chromogenic coliform agar (selective medium for coliform bacteria; Hope Bio-Technology Co., Ltd., Qingdao, China). The plates were incubated at 30°C and used for the cfu determination after 48 h (only LB plates) and for the isolation of bacterial cultures (all plates) during a period of 7 days with daily inspection of the plates. For the isolation approach, the bacterial colonies were selected according to unique morphological features from all of the implemented cultivation media that included visible colonies. The colonies were purified by streaking and sub-cultivation on LB agar. The taxonomic identity of the 14 isolated bacteria (11 from LB agar and three from MacConkey agar) was assessed by DNA extraction and the subsequent amplification of a 16S rRNA gene fragment with the primer pair 27F and 1492r (Lane, 1991). The fragments were analyzed with Sanger sequencing (Saigon Biotech Co., Ltd., Shanghai, China) and aligned with entries in NCBI's nucleotide database[2] using the BLASTn tool.

Statistical Analyses

The statistical tests within the microbiome datasets were performed in the QIIME 2 and the QIIME 1.9.1 software packages. The significance of differences in the alpha diversity was tested with the implemented Kruskal–Wallis test and in the beta diversity with the anosim test in the QIIME 2 pipeline. The differential occurrences of specific features in the different sample groups were statistically verified with the Kruskal–Wallis test with Bonferroni correction ($p < 0.05$) in QIIME 1.9.1 (group_significane.py script). The statistical analysis of the qPCR data was conducted with the non-parametric Mann–Whitney *U*-test.

Availability of Data and Material

The datasets used and/or analyzed during the current study are available in the ENA repository[3] under the accession number PRJEB32315. The 16S rRNA gene sequences used for the identification of bacterial isolates were deposited at GenBank[4] under the accession numbers MK796111–MK796124.

RESULTS

Assessment of Bacterial Diversity and Composition Among Different Samples

For an overall characterization of the bacterial communities, the alpha diversity was calculated for each of the sample types. The Shannon index (*H*) was used to compare the bacterial diversity among the different samples: insect tea ($H = 8.0 \pm 0.6$) as well as larvae excrements ($H = 8.0 \pm 0.5$) had the highest alpha diversity. In contrast, the alpha diversity in the leaf ($H = 6.6 \pm 0.2$) and in the midgut ($H = 5.1 \pm 0.6$) samples was statistically significantly lower ($p < 0.05$). The beta diversity assessments showed that the bacterial community composition was significantly different ($p < 0.05$) in each of the analyzed sample types. When a principal coordinate analysis was conducted, a close clustering of the insect tea and the excrement samples was observed (**Figure 1**).

The leaves of *C. paliurus* were primarily colonized by *Proteobacteria* (average abundance 66.2%), followed by *Firmicutes* (22.1%) and *Actinobacteria* (5.7%). *Bacteroidetes* (2.3%) and a group of unassigned bacteria (1.5%) were less abundant. The taxonomic assignments at the class level showed a predominance of *Alphaproteobacteria* (61.2%), *Bacilli* (21.8%), *Actinobacteria* (class, 5.5%), and *Gammaproteobacteria* (4.6%). The prevalent bacterial orders were identified as *Sphingomonadales* (35.3%), *Rhizobiales* (21.4%), *Lactobacillales* (13.5%), *Bacillales* (8.2%), and *Micrococcales* (3.1%). On the family level (**Figure 2**), *Sphingomonadaceae* (35.3%), *Beijerinckiaceae* (13.8%), *Enterococcaceae* (11.5%), *Staphylococcaceae* (8.0%), *Rhizobiaceae* (5.4%), and *Microbacteriaceae* (3.0%) were the prevalent taxa. *Sphingomonas* (32.5%), *Methylobacterium* (12.9%), *Enterococcus* (11.5%), *Staphylococcus* (8.0%), and *Aureimonas* (3.0%) were the most common genera in the leaf samples.

The midguts of the *P. farinalis* larvae were mainly colonized by the phyla *Firmicutes* (67.4%), *Proteobacteria* (20.8%), and *Actinobacteria* (9.4%). *Bacteroidetes* (0.9%) and other phyla accounted only for a minor proportion of the community. The prevalent bacterial classes were identified as *Bacilli* (66.4%), *Alphaproteobacteria* (16.4%), *Actinobacteria* (9.2%), and *Gammaproteobacteria* (4.2%). *Bacteroidia* (0.9%) and *Clostridia* (0.6%) were less abundant. On the order level, *Lactobacillales* (44.2%), followed by *Bacillales* (22.2%), *Sphingomonadales* (8.8%), and *Rhizobiales* (7.1%), was the most abundant representative. *Micrococcales* (3.8%), *Pseudonocardiales* (2.1%), *Enterobacteriales* (1.5%), and *Clostridiales* (0.6%) were less abundant. On the family level, *Enterococcaceae* (43.9%), *Staphylococcaceae* (21.8%), *Sphingomonadaceae* (8.8%), and *Beijerinckiaceae* (4.7%) were the prevalent taxonomic groups. *Enterococcus* (44.0%) was

[2] www.ncbi.nlm.nih.gov/nucleotide

[3] https://www.ebi.ac.uk/ena

[4] https://www.ncbi.nlm.nih.gov/genbank

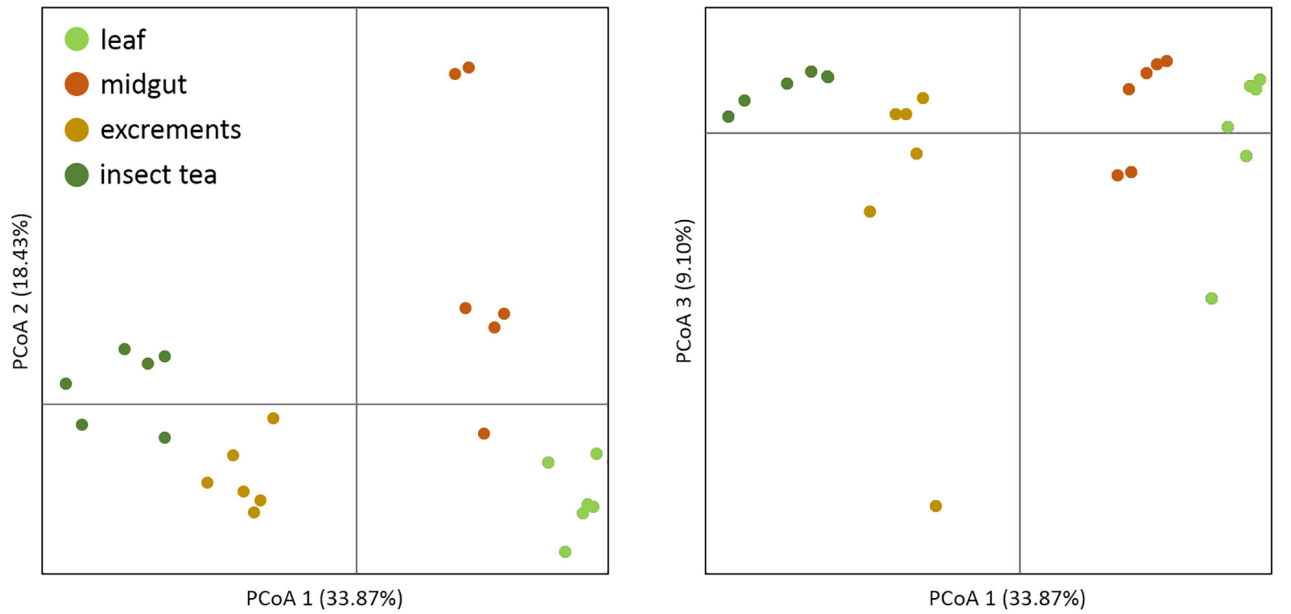

FIGURE 1 | Beta diversity assessment of different sample types. A principal coordinate analysis was conducted in order to visualize the clustering and similarity of the analyzed sample types. The three dimensions which explain the highest degree of variance were included in the visualization. Different colors of the dots indicate distinct sample types. The significance of dissimilarity between the different bacterial communities was tested with anosim in QIIME 2.

the predominant genus in the midgut samples, followed by *Staphylococcus* (21.8%) and *Sphingomonas* (8.0%). The genera *Methylobacterium* (4.4%), *Streptomyces* (1.3%), as well as two genera not further classified but belonging to *Pseudonocardiaceae* (1.0%), and *Enterobacteriaceae* (0.9%) were less prevalent.

The excrement samples produced under laboratory conditions and the commercial tea had a similar bacterial community composition; however, differences in the abundance of the most prevalent groups were observed. The prevalent phylum was either *Proteobacteria* (excrements [*e*] = 39.0%, tea [*t*] = 22.8%) or *Actinobacteria* (*e* = 22.4%, *t* = 50.1%) followed by *Firmicutes* (*e* = 13.4%, *t* = 20.3%) and *Bacteroidetes* (*e* = 20.2%, *t* = 5.2%). On the class level, high abundances of *Actinobacteria* (*e* = 21.9%; *t* = 49.4%), *Alphaproteobacteria* (*e* = 22.8%; *t* = 6.5%), *Bacilli* (*e* = 9.9%; *t* = 17.2%), *Gammaproteobacteria* (*e* = 15.8%; *t* = 16.0%), and *Bacteroidia* (*e* = 20.2%; *t* = 5.2%) were observed. The prevalent bacterial classes were represented by *Bacteroidales* (*e* = 18.7%; *t* = 3.3%), *Lactobacillales* (*e* = 8.2%; *t* = 12.4%), *Streptomycetales* (*e* = 6.2%; *t* = 16.9%), *Pseudonocardiales* (*e* = 5.8%; *t* = 15.7%), *Sphingomonadales* (*e* = 11.0%; *t* = 2.1%), *Enterobacteriales* (*e* = 8.0%; *t* = 10.1%), *Rhizobiales* (*e* = 8.9%; *t* = 3.3%), and *Micrococcales* (*e* = 4.8%; *t* = 6.6%). On the family level, *Streptomycetacea* (*e* = 6.2%; *t* = 16.9%), *Pseudonocaridaceae* (*e* = 5.8%; *t* = 15.7%), *Enterococcaceae* (*e* = 5.4%; *t* = 11.2%), *Sphingomonadacea* (*e* = 11.0%; *t* = 2.1%), *Enterobacteriaceae* (*e* = 8.0%; *t* = 10.1%), *Muribaculaceae* (*e* = 10.4%; *t* = 1.9%), and *Prevotellaceae* (*e* = 6.3%; *t* = 0.6%) were prevalent. The most abundant genera were assigned to *Streptomyces* (*e* = 5.9%; *t* = 16.7%), *Enterococcus* (*e* = 5.4%; *t* = 11.2%), and *Sphingomonas* (*e* = 9.9%; *t* = 1.6%). Two abundant representatives of *Enterobacteriacea* (*e* = 6.2%; *t* = 9.2%) and *Pseudonocardiaceae* (*e* = 3.5%; *t* = 10.5%) remained unassigned at the genus level.

Common Occurrence of Distinct Taxa in the Insect Tea Production Process

A network based on the core microbiomes of each sample type was generated in order to visualize bacterial genera that are either specific for one of the production steps or such that are shared by at least two sample types (**Figure 3**). The taxonomic features shared by all sample types were primarily identified as members of the phyla *Proteobacteria* and *Firmicutes*. In total, 48 features were present in all sample types, including *Enterococcus*, *Sphingomonas*, *Staphylococcus*, *Streptomyces*, *Enterobacteriaceae*, *Pseudonocardiaceae*, *Methylobacterium*, *Phylobacterium*, *Saccharopolyspora*, *Lactobacillus*, *Aureimonas*, and *Pseudomonas*. Moreover, 25 of those core features occurred with a total abundance of at least 10,000 reads. The samples from the excrements and the tea shared the highest number of features. They shared 36 taxonomic groups that were not present in the other two sample types. These features included 14 members of *Actinobacteria* and eight members of *Proteobacteria*. The bacterial phylum *Spirochetes* was only present in these two groups and not present in the core microbiome shared by all sample types. The midgut, the excrement, and the tea samples shared 16 features, while the midgut, the leaf, and the excrement samples shared 12 unique bacterial genera. The leaves, the excrements, and the insect tea had seven distinct features in common that were not present in the midgut. Only four genera were found to be unique for one of the sample types. A member of *Betaproteobacteria* and an unidentified SV with the

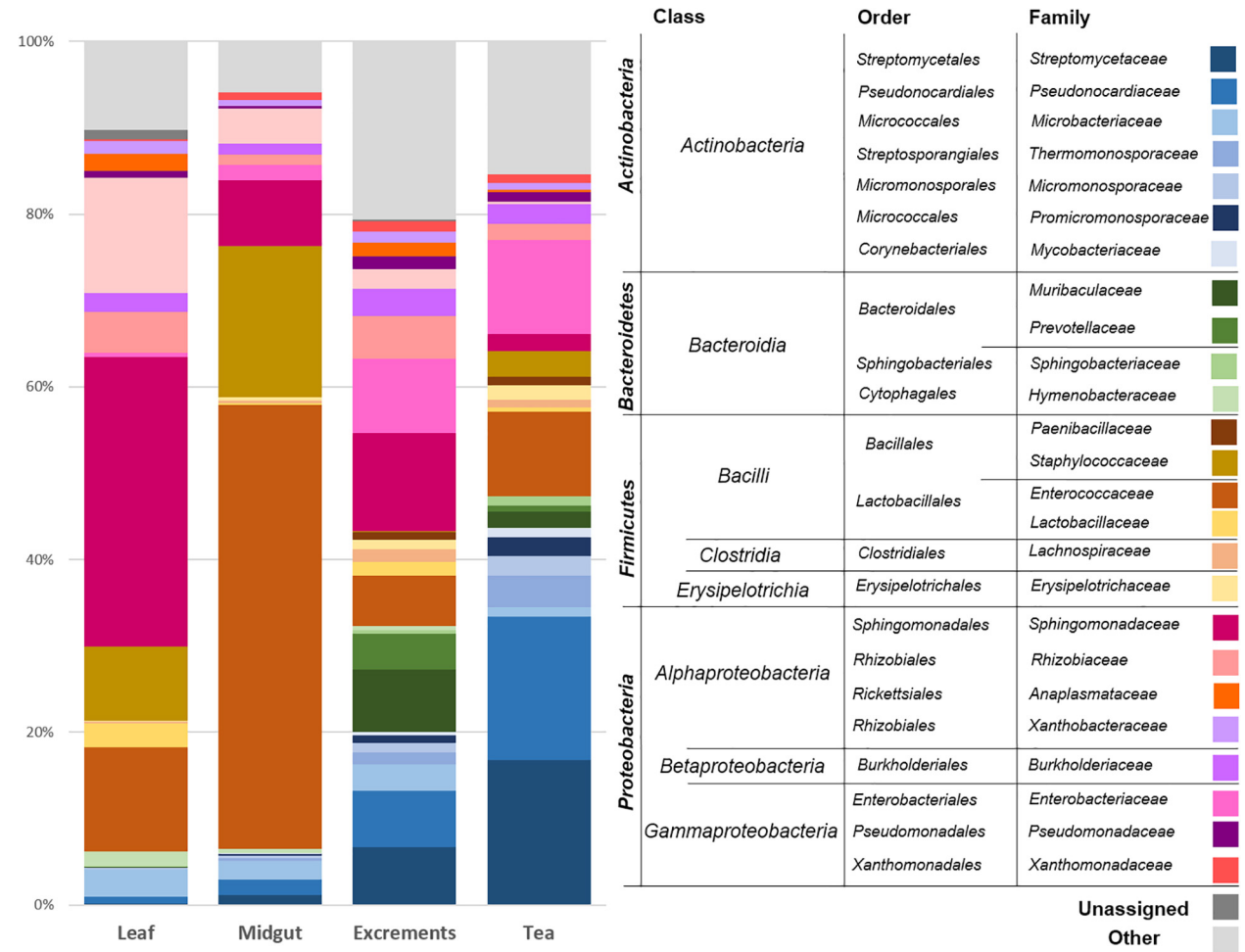

FIGURE 2 | Bacterial community structure of the different sample types. The bacterial community structure was visualized up to family level for each sample type. Taxonomic assignments at higher levels are provided and clustered in the legend. The average abundance of each taxon in six replicates for each sample type (leaf: *C. paliurus* leaves, midgut: *P. farinalis* midguts, excrements: *P. farinalis* excrements, tea: commercial insect tea product) is shown. All taxa with an occurrence of ≥1% were included in the legend. Taxa with a lower abundance were summarized as "other."

closest match to the eukaryotic SAR (Stramenopiles, Alveolata, Rhizaria) supergroup were only found in the larvae midguts, while bacteria from the class *Erysipelotrichia* were only present in the larvae excrements, and the genus *Phycicoccus* was only found in the commercial tea product.

Origin Tracking and Sample-Specific Accumulations of Bacterial Groups

When the origin of the microbial communities was tracked at different production steps, it was shown that the leaf microbiome of *C. paliurus* contributes only to a minor part of the bacterial composition of the insect tea (**Figure 4**). While the microbial community of the larvae excrements produced under controlled conditions still contained 14.6% constituents that can be tracked back to the leaves, the commercial product only contained 0.6%. When source tracking was applied to the insect tea and the larvae excrements produced under laboratory conditions, 43.8% of the bacterial population was assignable. The bacteria originating from the larvae midgut were better established in the insect tea (13.2%) than in the excrement samples (7.2%). When the prevalent bacterial families were followed through the production process, each stage showed a distinctive accumulation of a specific lineage (**Figure 5**). The insect tea samples had the highest prevalence of *Enterobacteriaceae* (10.8%) and *Streptomycetaceae* (16.8%), which were both shown to gradually increase during the production process. The leaf and the midgut samples were characterized by *Sphingomonadacea* (33.4%) and *Enterococcaceae* (51.4%), respectively, which decreased during the subsequent production steps.

Complementary Molecular and Cultivation-Dependent Assessments

A qPCR-based approach was used to verify the accumulation of *Enterobacteriaceae* during the production of the insect tea. The results indicated a substantial increase of this taxonomic group in the commercial tea product (**Figure 6**). It was 3.6-fold more

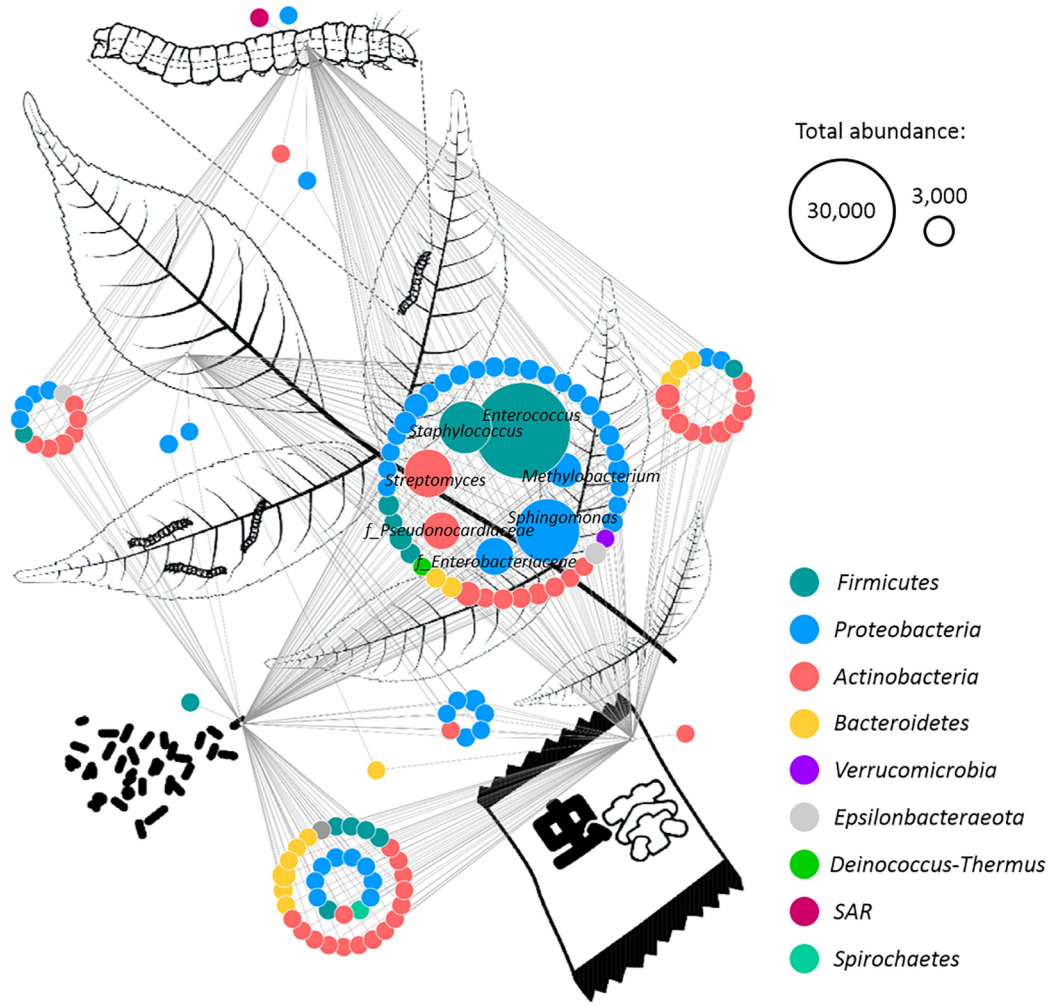

FIGURE 3 | Identification of common microbial components in different sample types. A network was rendered with Cytoscape 3.6.1 in order to identify shared bacterial taxa among different samples. The different sample types (plant leaves, insect midgut, excrements, and insect tea) are schematically visualized. Edges connect each feature with its origin, while multiple edges indicate occurrence in different sample types. The predominant features were labeled with their taxonomic assignment at family (indicated with "f") or genus level. Node colors indicate the assignment to distinct bacterial phyla, and their size correlates with the abundance of each feature. Two representative node sizes are shown in the legend.

abundant in the insect tea than in the initial substrate (*C. paliurus* leaves). In contrast, neither the midgut nor the excrement samples showed a notable increase in fluorescence intensity when compared to the leaves, indicating a similar prevalence of *Enterobactericeae* in these sample types. Cultivation-dependent assessments have shown that the commercial tea can contain a high number of viable bacteria, which were determined at $2.7 \times 10^5 \pm 1.2 \times 10^5$ cfu g^{-1}. No cell growth was observed on the selective chromogenic coliform agar. The isolation approaches of the morphologically distinct samples have resulted in the detection of *Enterobacter* spp. (seven isolates), *Bordetella* spp. (three isolates), *Bacillus* sp. (three isolates), and *Mixta* sp. (one isolate) as potential contaminants of the commercial tea product. The isolates that were assignable at the species level were identified as *Bordetella petrii* and *Enterobacter hormaechei*.

DISCUSSION

The findings of the present study show that the insect tea harbors a rich bacterial community that originates from the different steps of the production process. While several health-promoting effects are attested to the insect tea (Xu et al., 2013), it remains unknown which constituents confer these effects. One potential contribution of the insect tea to human health could be the enrichment of the human gut microbiome with the bacteria from this unique beverage similar to fecal transplants that are an efficient treatment for specific inflammatory bowel diseases (Suskind et al., 2015).

We could show that the insect tea harbors a high number of viable bacteria that can be recovered from commercial products. Although only a low number of isolates with distinct morphological features was further analyzed and is therefore not

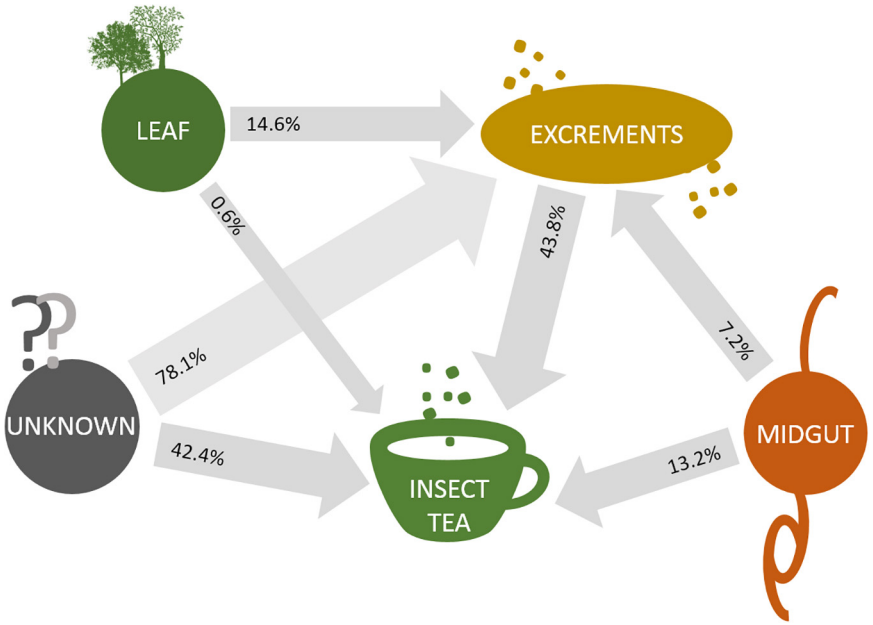

FIGURE 4 | Source tracking of bacterial constituents in insect tea. SourceTracker 0.9.5 was employed in order to identify the source of the bacterial communities that are transferred between different sample types. Transfer routes are visualized with arrows, and the percentage of assignable transferred constituents is provided for each sample pair that was analyzed. Routes were exclusively constructed to follow the direction of the production steps.

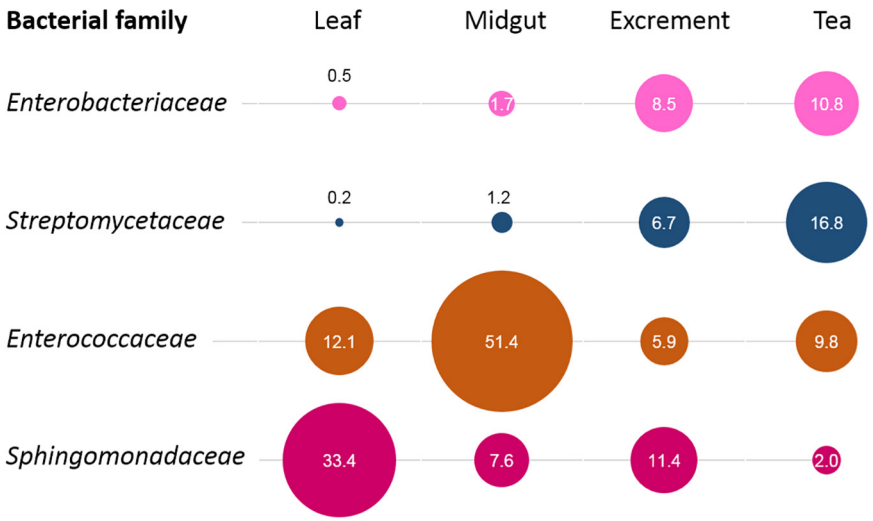

FIGURE 5 | Process-step-specific enrichments of distinct bacterial families. For each process step, the enrichment of distinct bacterial families was observed. The identified families were unevenly distributed among sample types and showed a clear predominance at a distinct production step. The bubble sizes correlate with their relative abundance in the respective sample type, while the numbers indicate their exact percentage.

representing the whole cultivable fraction, we found potentially health-relevant bacteria to be present in the insect tea. These microorganisms can be primarily attributed to the digestive system of the tea-producing insect larvae. The occurring members of *Enterobacteriaceae* and *Enterococcaceae* are also considered as classic contaminants in food production (Giraffa, 2002; Takahashi et al., 2017). In the context of the insect tea, it is not clear if the living microorganisms in the final product constitute contaminants or should be considered as active ingredients similar to those in the probiotics or the fermented foods. This is mainly due to the fact that, although the larval excrements are roasted, there is no standardized heat deactivation step involved in the production process that would warranty a contamination-free product. The insect tea and the kopi luwak coffee produced by the Asian palm civet (*Paradoxurus hermaphroditus*) are both unconventional beverages that consist

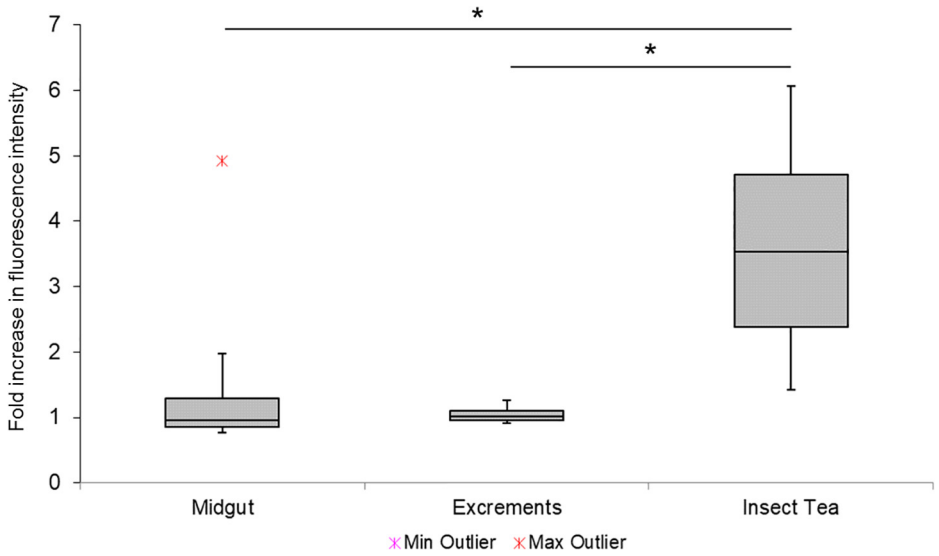

FIGURE 6 | Complementary assessment of *Enterobacteriaceae* during different process steps. The enrichment of *Enterobacteriacea* was confirmed with a complementary qPCR-based approach. The relative fluorescence signal increase in different samples was assessed with group-specific primers, and the fold increase was calculated by using the initial substrate (*C. paliurus* leaves) as an internal reference. Minimum and maximum values are indicated by the whiskers, while outliers are separately shown. Statistical significance ($p < 0.05$) is indicated with a black asterisk.

of animal excrements and thus linked to initially high microbial loads. However, the kopi luwak coffee is generally heat-treated at > 200°C (Jumhawan et al., 2013), which is sufficient to deactivate a large proportion of prevalent microorganisms. A study that compared microbial counts in the kopi luwak coffee before and after the beans were roasted showed substantially higher microbial counts before the heat treatment (Marcone, 2004). The counts for aerobic, mesophilic bacteria before the beans were roasted were in the same range as those for the commercial tea product in our study. We therefore assume that a large proportion of the present bacteria survived the roasting process in the insect tea or that the surviving fraction repopulates the product during storage. Although the insect tea is brewed with hot water, preferably at a temperature of 100°C, a high proportion of the content remains at the surface of the water or sticks to the edge of the cup that is used for its preparation. It can be therefore excluded that tea preparation by the final consumers can replace a standardized heat deactivation process that will eliminate all microorganisms.

In order to assess the representativeness of the reconstructed production process, the bacterial compositions of the larvae excrements and of the commercial insect tea were compared. Both sample types showed a similar microbial community composition, although substantial variations in the abundance of distinct groups were evident. These sample types also shared the highest number of common taxonomic groups, indicating a high representativeness of the reconstructed production process. The observed differences might be due to the potential variations of the plant leaves used for insect rearing by the commercial producer. Another important factor is the roasting process that was not included for the laboratory-produced larvae excrements and thus favorable for distinct members of *Proteobacteria*, which were prevalent in the excrements. Various members of this large taxonomic group are generally known to be highly susceptible to heat deactivation (van der Voort et al., 2016). The *Enterobacteriaceae* constitute one of the bacterial signatures of the insect tea; they were enriched during the production process, which was confirmed with complementary analyses. The qPCR-based analyses indicated a substantial enrichment in the product, while the midgut and the excrement samples harbored comparatively lower amounts of quantifiable *Enterobacteriaceae*. We assume that the assessed enterobacterial populations of these samples harbor less-known species that might not be targeted with the implemented primers. While they were identified as a major constituent of all insect-derived samples, they were not identifiable at higher taxonomic resolutions in the 16S rRNA gene fragment datasets nor were they cultivable under the tested conditions. Recent findings related to the plant microbiome and more specifically the leafy green vegetables have shown that the *Enterobacteriaceae* often constitute indigenous members (Rastogi et al., 2013; Erlacher et al., 2014; Cernava et al., 2019b). This makes them a suitable habitat for closely related pathogens that can survive and spread when present (Brandl and Amundson, 2008). However, the direct as well as the indirect implications of the non-pathogenic *Enterobacteriaceae* for human health remain mostly unknown. This led to the natural vaccination hypothesis by Berg et al. (2015) which states that the naturally occurring plant-associated enterobacteria might positively affect our immune system. We have also found that a substantial fraction of the bacterial community present in the final product was assigned to members of *Streptomycetacea*. This bacterial family is primarily known for its antibiotic-producing *Streptomyces* spp. that have been studied for many decades. The highly diverse antibiotic spectrum makes these

bacteria very competitive and has resulted in the development of therapeutic antibiotics (Mahajan and Balachandran, 2012) as well as in the common application of these microorganisms for biological control in agriculture (Fravel, 2005). It is likely that the insect tea includes active amounts of bacterial metabolites in addition to its earth-like taste (as assessed by the authors in the frame of conventional tastings) due to the high occurrence of *Streptomycetacea*. In terms of biosafety and human consumption, the *Streptomyces* members rarely cause disease symptoms in humans who are not immunocompromized (Riviere et al., 2012). Therefore, this constituent of the insect tea can be regarded as safe as long as the consumer has a functioning immune system. Another remarkable constituent was identified as *Bordetella petrii* and could be recovered as an isolate from the commercial product that was used in this study. This bacterial species was already found in the gut of related insects (Grabowski and Klein, 2017). While many *Bordetella* species are obligate human pathogens (Weiss and Hewlett, 1986), *B. petrii* was so far primarily isolated from natural environments, although it can also have clinical relevance (Gross et al., 2008). Only a smaller fraction of the identified microorganisms in the product samples originates from the initial substrate (*C. paliurus* leaves). The leaves were shown to harbor a bacterial community that is similar to the phyllosphere microbiomes of the various plant species and especially to those of the perennial plants (e.g., Durand et al., 2018; Cernava et al., 2019a). Although feed is an important factor that shapes the gut microbiome of insects (Lewis and Lizé, 2015), it can be anticipated that only a smaller fraction can adapt to the varying conditions during the formation process of the insect tea. Previous studies focusing on *Drosophila melanogaster* have shown that changes in the diet also result in changes of its gut microbiome (Sharon et al., 2010). We would expect certain effects on the final product when leaves from different plant species are used for insect tea production. However, it remains to be elucidated how pronounced these effects are and if they can be standardized in order to obtain a consistent bacterial community composition. Although *P. farinalis* is a cosmopolitan pest, its gut microbiome was not assessed so far. In the present study we could show that its midgut is mainly colonized by *Firmicutes* and *Proteobacteria*, which corresponds to the findings on other insects (Alonso-Pernas et al., 2017; Kaczmarczyk et al., 2018; van Schooten et al., 2018). While it contributed to the bacterial composition of the insect tea, the final composition is likely affected by handling, processing, and potentially also by storage since not all bacteria are equipped with mechanisms to survive under these conditions, e.g., transition into a dormant stage.

In summary, our microbiome-guided assessment of the bacterial communities in the insect tea has shown that this unconventional beverage contains diverse microbial populations. The reconstruction of the production process and the tracking of bacterial constituents provided a deepening insight into the assemblage of this exotic beverage. The microbial profile is so far unique for an unspoiled food product and contains several bacterial groups that are considered as contaminants in the field of food microbiology. In this context, we could show that a high number of the prevalent microorganisms is viable and can be recovered from the commercial products. The assemblage of the community can be tracked back to specific production steps of the tea but still underlies the dynamics after excretion by the larvae. Although we found a highly diverse bacterial community in the various production steps and recovered a substantial number of living bacteria from a commercial product, deepening analyses of certain taxa would be required to specifically identify human pathogens. While this study confirmed that viable bacteria are a component of insect tea, it remains to be further elucidated if these microbial constituents have any positive or negative implications on human health.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the ENA repository (PRJEB32315) and Genbank (MK796111–MK796124).

AUTHOR CONTRIBUTIONS

TC and MY conceived the idea and developed the study design. XM and HL performed all laboratory experiments under the supervision of TC and XC. PK performed the general bioinformatics analyses. PK and TC interpreted the bioinformatic data and prepared the final visualizations. GB provided valuable inputs related to microbial ecology and the interpretation of the results. TC, MY, XC, and GB wrote the manuscript. All authors reviewed the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.03087/full#supplementary-material

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Thermal Impact on the Culturable Microbial Diversity Along the Processing Chain of Flour From Crickets (*Acheta domesticus*)

Antje Fröhling[1], Sara Bußler[1,2], Julia Durek[1] and Oliver K. Schlüter[1,2]*

[1] Quality and Safety of Food and Feed, Leibniz Institute for Agricultural Engineering and Bioeconomy (ATB), Potsdam, Germany, [2] Food4Future, Leibniz Institute of Vegetable and Ornamental Crops (IGZ), Großbeeren, Germany

The role of insects for human consumption has lately increased in interest and in order to deliver safe and high-quality raw materials and ingredients for food and feed applications, processing of insects is a major pre-requisite. For edible insects a thermal treatment and appropriate storage conditions are recommended to minimize the microbiological risk and the impact of processing methods on the microbial contamination needs to be considered and determined. Based on standard process conditions for the production of *Acheta domesticus* flour, different heating treatments were used to reduce the microbial load of *A. domesticus*. In addition, the drying temperature and drying time were varied to determine whether the required residual moisture of <5% can be achieved more quickly with consistent microbial quality. The influence of the process conditions on the microbial community of *A. domesticus* along the processing chain was finally investigated under optimized process conditions. The total viable count was reduced from 9.24 $\log_{10}$ CFU/g_{DM} to 1.98 $\log_{10}$ CFU/g_{DM} along the entire processing chain. While Bacillaceae, Enterobacteriaceae, Enterococcaceae, and yeast and molds were no longer detectable in the *A. domesticus* flour, Staphylococcaceae and mesophilic spore forming bacteria were still found in the flour. The results indicate that the steaming process is essential for effectively increasing microbial safety since this processing step showed the highest inactivation. It is recommended to not only evaluate the total viable count but also to monitor changes in microbial diversity during processing to ensure microbial safety of the final product.

Keywords: MALDI-ToF MS, steaming, product quality, edible insects, process water

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****Correspondence:***
Antje Fröhling
afroehling@atb-potsdam.de

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INTRODUCTION

The use of insects as food poses potential microbiological risks, as insects can be vectors for human, animal, or plant pathogenic microorganisms. In 2015, the European Food Safety Authority (EFSA) estimated in its risk profile the microbiological risk for the production and consumption of insects as food and feed to be comparable to other unprocessed animal protein sources when conventionally authorized feed is used as insect substrate. In addition to insect microbiota and feed microbiota, microbial contamination of insects may also occur through production and processing conditions and during storage (FASFC, 2014; EFSA, 2015). Despite the insect species itself, the

substrate and hygienic conditions during farming and processing influence the microbiological risk of edible insects (van der Fels-Klerx et al., 2018). For example, a thermal treatment and appropriate storage conditions are strongly recommended for edible insects before placing them on the market (FASFC, 2014; NVWA, 2014). Extensive studies on the presence of pathogenic microorganisms in insects used as food and feed are lacking and to protect consumers against potential health hazards these data are required, especially since no specific microbiological criteria for edible insects exists up to now in the EU (Fernandez-Cassi et al., 2019).

The microbial flora of insects in general consists of *Staphylococcus*, *Streptococcus*, *Bacillus*, *Proteus*, *Pseudomonas*, *Escherichia*, *Micrococcus*, *Lactobacillus*, and *Acinetobacter* (EFSA, 2015). Among the different edible insect species, the house cricket (*Acheta domesticus*) is a promising insect species since its protein and lipid content is comparable to beef or chicken and it is an indigenous species in many European countries (Fernandez-Cassi et al., 2019). A total aerobic mesophilic count of 10^5–10^6 colony forming units per gram (CFU/g) was determined for *A. domesticus*. The total bacterial count consisted mainly of Gram-negative bacteria (fecal and other coliform bacteria). Gram-positive bacteria were represented by *Micrococcus* spp., *Lactobacillus* spp., and *Staphylococcus* spp. (Belluco et al., 2013). Garofalo et al. (2017) investigated different commercially available edible insects [e.g., *A. domesticus* (whole and powder)] using cultivation-dependent and cultivation-independent methods. They found a total aerobic mesophilic bacterial count of 4.1 log CFU/g and 4.5 log CFU/g for whole *A. domesticus* and 4.8 log CFU/g and 3.9 log CFU/g for *A. domesticus* powder. Enterobacteriaceae and *Clostridium perfringens* were below the detection limit of 2 log CFU/g and *Salmonella* as well as *Listeria monocytogenes* were not detectable in 25 g. Yeasts were present in whole *A. domesticus* with 4–5 log CFU/g and in the powder <2 log CFU/g. The opposite was determined for molds. The whole *A. domesticus* showed mold counts <2 log CFU/g and the powder was between 2.9 and 3.1 log CFU/g. It has been shown that there are large variations in the microbial composition between insect species.

Cricket rearing has nowadays shifted from harvesting in the wild to mass rearing (especially in Thailand) and therefore, the establishment of good farming practices and good hygiene habits as well as the implementation of standard operating procedures and HACCP concepts in the production systems of edible insects is required to ensure high level of food safety (Fernandez-Cassi et al., 2019; Melgar-Lalanne et al., 2019). In European countries the acceptance to consume insects might be enhanced if the insects are used as flours or powders, i.e., in an unrecognizable form. To overcome consumer's concerns and to ensure product safety, adequate post-harvest technologies have to be established in the processing of edible insects (Melgar-Lalanne et al., 2019). Different processing technologies and storage conditions were tested to improve acceptability of insects and insect products as well as to increase the shelf-life of these products.

Small or middle scale cricket production facilities include in most cases the following general processing steps of the crickets. Prior to harvest, the crickets are fasting for 24 h and after harvest they are killed either by freezing, heating, or drowning. The killing step is followed by rinsing, boiling, fast cooling, freeze-drying, and storage of the packaged crickets (Fernandez-Cassi et al., 2019). Drying is the most common used technology to increase the shelf-life of foods. Whole edible insects are preferably sun-dried (Manditsera et al., 2018), freeze-dried or oven-dried (Fombong et al., 2017), and microwave-dried (Vandeweyer et al., 2017b) and subsequently crushed or pulverized to increase consumer's acceptance (Melgar-Lalanne et al., 2019). Besides the water reduction during drying, the color and protein functionality are changed as well as lipid oxidation can occur to different extend depending on the applied drying technique. Thus, the drying method has to be chosen with respect to the final consumption form of the insects, e.g., as whole insect or as flour. The quality of mealworms in terms of protein, fat, and chitin extractability was similar for freeze-drying and oven-drying, so that the most preferred industrial drying method is oven-drying (Purschke et al., 2018). Grabowski and Klein (2017) recommended species-specific drying procedures as well as a reheating of the insects before consumption to ensure food safety, since microorganisms may only slowed down in growth during drying and may start growing again at appropriate water conditions.

This study aimed to evaluate appropriate process conditions for the production of cricket flour. Based on standard process conditions for the production of cricket flour, which includes cooking as a heating step and drying at 110°C for 8 h, different heating steps such as boiling, steaming, and autoclaving were used to reduce the microbial load of the crickets. In addition, the drying temperature and drying time were varied to determine whether the required residual moisture of <5% can be achieved more quickly with consistent microbial quality. The influence of the process conditions on the microbial community of the crickets along the processing chain was finally investigated under optimized process conditions aiming to gain knowledge about possible resistant microorganisms and possible pathways of cross-contaminations during processing. Additionally, the microbial loads of the cricket process water as well as the protein content were evaluated to monitor the contamination of the process water.

MATERIALS AND METHODS

Processing Chain Cricket Flour

House crickets (*A. domesticus*) were purchased from Terra-Discount (Germany) and inactivated by freezing at −20°C directly after delivery. Prior to the experiments, the crickets were thawed at room temperature for 1 h. Each process chain was conducted in three independent experiments. For better comparability a single batch of approx. 2000 g was used for all experiments.

Washing

Washing was conducted in three steps to remove adherent dirt and feed residues. Crickets were washed with tap water in a ratio of 7:20 for 5 min under stirring. The crickets were removed from

the water using a sieve and after draining for 1 min washed in a second bath (cricket to water ratio was 7:20) for 5 min under stirring. After draining for 1 min another washing step was conducted as described before. The temperature of the used tap water before washing was 22.8 ± 0.1°C. During the first washing step the water temperature decreased to 11.6 ± 0.7°C due to the low temperature of the crickets and during washing step two and three the water temperature was 20.5 ± 0.6°C and 22.2 ± 0.5°C, respectively.

Thermal Treatments

After washing different thermal treatment steps (autoclaving, boiling, and steaming) were tested to reduce the microbial load of the crickets. The different thermal treatments as well as the considered treatment parameters were chosen because they were reported as conventionally applied thermal treatments (on industrial scale) for crickets in Thailand (personal communication).

Autoclaving was performed in a laboratory autoclave (Systec VX-75, Systec GmbH, Germany) for 15 min at 121°C and 2.1 bar. 100 g crickets were placed in a 250 ml beaker. The dwell time at 121°C for 15 min was ensured by placing the temperature sensor of the autoclave in the middle of the cricket samples. Three beaker containing 100 g crickets each were autoclaved at the same time.

For the boiling step, tap water was heated to 100°C in a cooking pot and crickets were then added for 10 min in a cricket to water ratio of 1:5. The temperature of the water and the crickets during boiling was recorded using a thermocouple (Model PDT300, VWR International GmbH, Germany). The crickets were cooled on ice after boiling. The boiling treatment was conducted in triplicate.

For the steaming step, crickets were placed on a gauze cloth covering a steaming insert in a cooking pot after boiling of the water. During the thermal treatment by steaming, the crickets did not lie in one layer in the steaming insert but on top of each other. After 10 min of steaming the crickets were removed and cooled on ice. The temperature of the water and the crickets during steaming was recorded using a thermocouple (Model PDT300, VWR International GmbH, Germany). Each steaming step was performed in triplicate. The statistical significance of differences between thermal treated samples was evaluated using Welch's unequal variances *t*-test with significance levels of 0.05, 0.01, and 0.001.

Recording of Drying Kinetics

Drying kinetics were recorded during oven-drying (Heratherm OMH180, Thermo Electron LED GmbH, Germany) at different temperature time profiles. The temperature of the crickets during drying was recorded using a thermocouple (Model PDT300, VWR International GmbH, Germany). A drying time of 8 h at 110°C was reported as standard drying procedure for crickets in Thailand (personal communication) to ensure microbial safety and to obtain a residual moisture content <5%. In comparison to this standard drying procedure it was evaluated if residual moisture content <5% could be realized within a shorter drying time at 110°C or at lower temperatures (90°C) while ensuring microbial safety. Therefore, crickets were dried up to 4 h at 110°C and at 90°C. In addition, it was investigated whether the drying time is strongly influenced by a single-layer or multi-layer position of the crickets. After 0.5, 1, 2, 3, and 4 h, the moisture content was gravimetrically determined by differential weighing during drying at 110°C for 24 h. The TVC count was evaluated as described in section "Microbiological Analyses." The statistical significance of differences between samples was evaluated using Welch's unequal variances *t*-test with significance levels of 0.05, 0.01, and 0.001.

Pulverization

Dried crickets were pulverized to flour using a mill (Retsch Grindomix, Retsch GmbH, Germany) for 5 s at 10000 rpm.

Color Measurements

The color of food products is an important quality parameter for consumer's acceptance of the product. Therefore, the color of the cricket flour was determined after drying at 90°C and 110°C using a Minolta spectrophotometer (CM-2600D, Konica Minolta Inc., Japan) with CIELab system, illuminant D65, SCE (specular component excluded) mode, and 10° observer angle. The browning index (BI) was calculated using the following equations (Saricoban and Yilmaz, 2010):

$$BI = \frac{(100 \times (x - 0.31))}{0.17} \quad (1)$$

$$x = \frac{a^* + 1.75 \times L^*}{5.645 \times L^* + a^* - 3.012 \times b^*} \quad (2)$$

Additionally, the hue angle was recorded with 0°, red; 90°, yellow; 180°, green; and 270°, blue (McLellan et al., 1995) allowing to distinguish between intermediate colors between adjacent pairs of basic colors. All measurements were performed at three different places on the bags surface. The statistical significance of differences between samples was evaluated using Welch's unequal variances *t*-test with significance levels of 0.05, 0.01, and 0.001.

Microbiological Analyses

Microbiological analyses of the crickets were conducted using European standard methods. Untreated crickets, washed crickets, heat treated crickets, dried crickets, and cricket flour were prepared according to EN ISO 6887-4:2017 with slight modifications. Before homogenization of the samples using a bag mixer (BagMixer® 400 CC®, Interscience, France) at speed 2 for 2 min and buffered peptone water as dilution medium (1:10), the crickets were coarsely crushed in the homogenization bag. In preliminary experiments the microbial load of untreated (frozen) crickets and dried crickets using a resuscitation step or without a resuscitation step was compared. As well as for untreated crickets and dried crickets no significant differences were found between the two preparation steps (data not shown). However, since the European Standard EN ISO 6887-4:2017 recommends the resuscitation step for dried samples, dried crickets and cricket flour were left in buffered peptone water for 30 min at room temperature before homogenization in the bag mixer. The aerobic mesophilic total viable count (TVC) was conducted for all samples in duplicate from each initial

dilution. To evaluate the culturable microbial diversity changes during processing of cricket flour, the viable count of mesophilic spore-forming bacteria, *E. coli*, Enterobacteriaceae, *Enterococcus*, *Staphylococcus*, *Bacillus cereus*, *Clostridium perfringens*, and yeasts and molds using the same initial dilution as for the TVC as well as the presence of *Salmonella*, *Listeria monocytogenes*, and *Campylobacter* was determined along the optimized processing chain. The determination of the presence of *Salmonella*, *Listeria monocytogenes*, and *Campylobacter* required specific enrichment steps and were conducted according to European standard methods. **Table 1** summarizes the applied microbiological methods. The viable count is expressed as $\log_{10}$ colony forming units per gram dry matter [$\log_{10}$ CFU/g_{DM}] and each value is the average of six single values. The dry mass and the moisture content of the crickets were gravimetrically determined by drying at 110°C for 24 h.

In addition, the TVC of the tap water, the process water from all washing steps as well as the steaming water was examined in order to obtain a statement on the degree of contamination. Therefore, the water was serially diluted using a peptone salt solution and the dilutions were spread on plate count agar. After incubation for 72 h at 30°C the colony forming units per ml ($\log_{10}$ CFU/ml) were determined. The statistical significance of differences between samples was evaluated using Welch's unequal variances *t*-test with significance levels of 0.05, 0.01, and 0.001.

MALDI-ToF MS Analysis

For both the crickets and the process water, the microorganisms grown on the culture media were identified by MALDI-ToF MS (Matrix assisted laser desorption/ionization time of flight mass spectrometry) to evaluate the microbial diversity of the most abundant microorganisms. Due to the high number of different growth media and the resulting large number of grown colonies, an attempt was made to provide the best possible image of the different colonies to obtain the best possible summary of the microbial diversity. It was ensured that all colonies with different appearance were selected for further analyses. In the case of the selective media, all typical colonies were additionally examined using MALDI-ToF MS. Colonies were designated as typical if they complied with the descriptions of the European standards or the manufacturer's specifications for the growth media. For the analyses, cell material of the colonies was suspended in 300 µl purified water and incubated for 5 min after addition of 900 µl ethanol (96%). Afterward the samples were centrifuged at 12400 × *g* for 3 min. The supernatant was removed and the samples were centrifuged again at 12400 × *g* for 1 min and the pelleted material was dried below the laminar flow and stored at −80°C until further analysis. After thawing of the samples, the pelleted material was suspended in 7.5 µl formic acid and 7.5 µl acetonitrile. Again, the samples were centrifuged at 12400 × *g* for 3 min. One µl supernatant was transferred to a target and after drying overlaid with 1 µl α-cyano-4-hydroxy cinnamic acid (CHCA) matrix (RIPAC-LABOR GmbH, Germany) and analyzed by MALDI-ToF MS (Axima Confidence, Shimadzu Deutschland GmbH, Germany) after drying. The spectra were measured using the linear mode in a mass range between 3000 and 20000 m/z and a laser repetition rate of 50 Hz. Calibration was conducted using *E. coli* ribosomal proteins. Reference mass spectra from the AnagnosTec SARAMIS^{TM}

TABLE 1 | European and German standards applied for the evaluation of viable cell counts with corresponding growth parameters.

Target	Norm resp. analytical method	Culture medium	Growth temperature [°C]	Growth time [h]	Growth conditions
Aerobic mesophilic total viable count (TVC)	DIN EN ISO 4833-2:2013	Plate count agar (PCA)	30	72	Aerobic
Spore-forming bacteria	DIN EN ISO 6887-1:1999	Nutrient agar (NA)	37	24	Aerobic
E. coli	ISO 16649-2:2001:2001-04	Tryptone bilex X-glucurronide (TBX)	37 + 44	4 + 20	Aerobic
Enterobacteriaceae	EN ISO 21528-2:2009-12	Violet red bile dextrose agar (VRBG)	37	24	Aerobic
Enterococcus spp.	BVL L 06.00-32:1992-06	Kanamycin asculin azide agar (KAA)	37 + RT	24 + 24	Aerobic
Staphylococcus spp.	IS0 6888-1:1999/Amd.l:2003 (E)	Baird-Parker agar (BP)	37	48	Aerobic
B. cereus	EN ISO 7932:2005-03	Polymyxin pyruvate egg-yolk mannitol–bromothymol blue agar (PEMBA)	30	24	Aerobic
C. perfringens	ISO 7937:2004-08	Tryptose sulfite cycloserine agar (TSC)	37	20	Anaerobic
Yeasts and molds	ISO 21527-1:2008-07	Dichloran-bengal red-chloramphenicol agar (DRBC)	25	120	Aerobic
Salmonella spp.	EN ISO 6579:2007-10	Xylose-lysine deoxycholate agar (XLD); Brilliant green agar (BGA)	37	24	Aerobic
L. monocytogenes	EN ISO 11290-1:2005-01	Polymyxin acriflavine lithium chloride ceftazidime aesculin mannitol agar (PALCAM), Brilliance^{TM} Listeria	37	24–48	Aerobic
Campylobacter spp.	DIN EN ISO 10272-1:2017-09	Campylobacter selective agar (CCDA); Skirrow agar	42	44	Microaerophilic

database (Spectral Archive And Microbial Identification System, bioMérieux Deutschland GmbH, Germany) were used for the identification of the obtained mass spectra. UPGMA clustering was conducted using the peak based similarity coefficient 'Dice' within the BioNumerics software (Version 7.6, Applied Maths NV, Belgium) using the following parameters: linear tolerance = 500 ppm and constant tolerance = 3 m/z. Reliable and unreliable clusters were separated using the cophenetic correlation. A match of the measured mass spectrum with the reference mass spectrum and a confidence level >90% led to identified clusters whereas a confidence level between 75 and 89.9% only allowed a cluster identification to the family level and a confidence level below 75% resulted in 'not identified' clusters. The relative abundance of microorganisms was only calculated for the identified microorganisms.

Soluble Protein Content

The amount of soluble proteins contained in the wash and steaming water was determined via Bradford assay (Bradford, 1976) using the Roti®-Nanoquant Protein quantitation microassay (CarlK880, Carl Roth GmbH, Germany). Bovine serum albumin (Fluka Chemie AG, Switzerland) was used as the calibrating protein at a concentration of 1–20 μg/ml in 2 mg/ml intervals in DI water. The assay consisted of 800 μl of the sample solutions reacting with 200 μl of Bradford reagent. OD_{595} (BioPhotometer plus, Eppendorf AG, Germany) of the standard solutions and samples was measured after 20 min (20°C) against the zero value. Bradford assay was conducted in triplicates for each wash and steaming water sample. The statistical significance of differences between samples was evaluated using Welch's unequal variances *t*-test with significance levels of 0.05, 0.01, and 0.001.

RESULTS AND DISCUSSION

Thermal Treatment

The processing of cricket flour as ingredient for food products requires the reduction of microorganisms to ensure the microbial safety of the final product. A cricket core temperature of 100°C was reached after 2 min of boiling whereas during steaming the core temperature of the crickets did not reach 100°C within 10 min of steaming time. Core temperatures >95°C were reached after ∼6 min during steaming. Caparros Megido et al. (2017) achieved a core temperature of 100°C in house crickets after 3.16 ± 1.04 min of boiling and therefore set their blanching time to 4 min for house crickets. With this treatment they achieved a TVC reduction of ∼3.6 log CFU/g. It was shown that fresh and also smoked insects need a cooking step before consumption with species-specific treatment times (Caparros Megido et al., 2017). In our study, the TVCs of the thermal treated crickets were significantly reduced from ∼8 $\log_{10}$ CFU/g_{DM} to < 2 $\log_{10}$ CFU/g_{DM} by boiling, steaming, and autoclaving (**Table 2**). Similar results were obtained by Klunder et al. (2012) with a TVC reduction from 7.2 log CFU/g to 1.7 log CFU/g after boiling of crickets for 5 min. Comparing the TVC after boiling with the TVC after steaming or autoclaving revealed a significantly higher TVC for the boiled crickets. The moisture content of the crickets before thermal treatment was between 69 and 70% (**Table 2**). Autoclaving did not change the moisture content whereas steaming and boiling increased the moisture content of the crickets. An increase in moisture was also reported for mealworms after blanching (Azzollini et al., 2016). The water uptake of mealworms after blanching was attributed to water absorption and entrapment below the chitinous exoskeleton (Melgar-Lalanne et al., 2019). However, boiling led to higher moisture content in comparison to steaming. Higher moisture content might enhance the required drying time of the crickets. Since the moisture content as well as the TVC were not significantly different between autoclaved and steamed crickets, steaming was chosen as preferred method for microbial inactivation before drying and milling because steaming is easier to implement industrially than autoclaving.

Optimization of Cricket Oven-Drying

During processing of cricket flour including steaming as inactivation process and subsequent oven-drying at 110°C for 8 h the moisture content was reduced from 72% to <1% (**Table 3**). Kamau et al. (2018) showed that cricket flour with moisture content <5% can be stored for 7 month at 25°C. The TVC of the crickets was reduced to 1.55 $\log_{10}$ CFU/g_{DM} by steaming and further decreased to 1 $\log_{10}$ CFU/g_{DM} after drying. After milling, the TVC slightly increased to 1.39 $\log_{10}$ CFU/g_{DM} but the increase was not significant. Higher TVC values after pulverizing of crickets were also found by Fernandez-Cassi et al. (2019) and these higher values were attributed to the better extraction of microbes from the insect guts due to the pulverizing process. According to Adámek et al. (2018) mealworm, lesser mealworm, field cricket, and migratory locust should be boiled, dried at 103°C for 12 h and hermetically packaged to achieve a long-term storage of the product. Lower temperatures might lead to increased drying times and higher temperatures might decrease the required drying time.

To reduce the process time, in particular the drying time, crickets were dried in single-layer and multi-layer and the moisture content, TVC, and color of the crickets were recorded during drying at 90°C and 110°C for 4 h. As expected, the temperature profile of the crickets revealed that the core temperature of the crickets increased much slower if the crickets were dried in multi-layer in comparison to the drying of crickets in single-layer (**Figure 1A**). At 90°C, the temperature of the crickets reached 86°C and at 110°C a core temperature of 107°C was reached after 4 h of drying crickets in multi-layer. In contrast, drying of crickets in single-layer increased the core temperature of the crickets within 1.8 h to 107°C. A moisture content of 4.7% and 3.0% was reached after 4 h multi-layer drying at 90 and 110°C, respectively (**Figure 1B**). Especially the multi-layer drying at 110°C showed high standard deviations revealing inhomogeneous drying of the crickets. At 110°C and drying of crickets as a single-layer, a moisture content of 4.7% was already achieved after 2 h and after 3 h the moisture content was reduced to 1.7%. Since the applied steaming process effectively reduced the microbial load of the crickets, microbial contamination

TABLE 2 | TVC and moisture content of crickets after different thermal inactivation steps.

Thermal treatment	TVC of untreated crickets [Log_{10} CFU/g_{DM}]	TVC of treated crickets [Log_{10} CFU/g_{DM}]	Moisture content of untreated crickets [%]	Moisture content of treated crickets [%]
Boiling	8.42 ± 0.17^{a}	1.69 ± 0.04^{a}	70.38 ± 1.03^{a}	79.68 ± 1.74^{a}
Autoclaving	8.55 ± 0.08^{a}	$<1.52^{*b}$	70.35 ± 1.96^{a}	69.68 ± 1.08^{b}
Steaming	8.79 ± 0.52^{a}	1.56 ± 0.02^{b}	68.83 ± 1.71^{a}	72.39 ± 1.39^{b}

**Below the detection limit. Letters in the columns represent significant differences at the significance level $p < 0.001$.*

TABLE 3 | TVC and moisture content of crickets along the processing chain of cricket flour (thermal treatment: steaming; oven-drying: 110°C, 8 h).

Sample	TVC [Log_{10} CFU/g_{DM}]	Moisture content [%]
Untreated crickets	8.96 ± 0.80^{a}	74.25 ± 7.49^{a}
Washed crickets	8.27 ± 0.13^{a}	72.29 ± 7.74^{a}
Steamed crickets	1.55 ± 0.09^{b}	71.57 ± 5.72^{a}
Dried crickets	1.00 ± 0.00^{c}	0.68 ± 0.49^{b}
Cricket flour	$1.39 \pm 0.60^{b,c}$	0.72 ± 0.24^{b}

Letters in the columns represent significant differences at the significance level $p < 0.001$.

of the crickets was monitored during drying, especially to detect recontamination during the process. Traditionally applied sun-drying processes for edible insects showed a recontamination of the insects due to contact with soil as well as poor hygiene and storage conditions (Caparros Megido et al., 2017). Only minor changes in the TVC of the crickets were determined during drying at 90°C and 110°C (**Figure 2A**) indicating that no recontamination occurred during the drying process. With the exception of 1 h drying, no significant differences ($p < 0.01$) in the TVC were found between drying at 90°C and 110°C indicating that both temperatures are suitable for maintaining the achieved microbial reduction during steaming.

Since color changes are the most visible quality parameters during drying (Melgar-Lalanne et al., 2019), the L*a*b* values as well as the hue angle of crickets dried at 90 and 110°C were determined and the browning index was calculated. At both temperatures, the browning index was not significantly changed during drying (**Figure 2B**). Additionally, there was no significant difference ($p < 0.05$) between the browning index at 90 and 110°C with the exception for the longest drying time of 4 h. At 110°C and 4 h drying time the browning index was significantly higher than at 90°C and the same drying time. The increase in the browning index can be attributed rather to non-enzymatic browning reactions than to enzymatic browning reactions (Azzollini et al., 2016). The results imply that at higher temperatures non-enzymatic browning is more pronounced than at lower temperatures. Hue angles of ∼70° were recorded for the crickets dried at 90 and 110°C whereas the drying time did not influence the hue angle (**Figure 2C**). However, similar to the browning index, a drying time of 4 h showed a significant lower hue angle of the crickets dried at 90°C in comparison to 110°C indicating again a higher browning of the crickets.

The results reveal that the drying time of crickets in single-layer can be reduced to 3 h at 110°C and still moisture content below 5% is realized while maintaining the achieved microbial inactivation during steaming and without further affecting the color of the crickets.

Microbial Community Structure Along the Processing Chain of Cricket Flour

Based on the results of the thermal treatment experiments and the optimization of cricket oven-drying an experimental process chain for the production of cricket flour was defined. The production chain of cricket meal comprises a three-stage washing step, followed by thermal treatment by steaming for 10 min and oven drying at 110°C for 3 h before the crickets are ground. A health threat to consumers results in particular from the contamination and proliferation of unexpected pathogens in the food supply chain. Food batches are often sampled for specific microorganisms, so that unexpected pathogens can go undetected. Fernandez-Cassi et al. (2019) introduced a risk profile for house crickets as a novel food and identified data gaps on the impact of thermal processing of the products. Since no specific microbiological criteria are available in the EU up to now, it is proposed to use microbiological criteria of minced meat for edible insects (Caparros Megido et al., 2017). However, the consumption of whole insects, including the intestinal tract, makes it difficult to comply with the microbiological criteria for minced meat (Fernandez-Cassi et al., 2019). Garofalo et al. (2019) conducted a literature review about the microbiota of both fresh or processed edible insects and revealed market variations in microbial load and diversity as well as species-specific microbiota of the edible insects. However, most of the studies are dealing either with fresh or with processed insects and only few studies are available on the microbial dynamics during production (Stoops et al., 2017; Vandeweyer et al., 2018; Wynants et al., 2018). Appropriate HACCP systems for the supply chain of edible insects have to be established (Garofalo et al., 2019). The investigation of microbial changes along the processing chain could play an important role in the implementation of microbiological criteria. But the development of bacterial contaminants in interaction with various process sequences is currently not sufficiently investigated. The question of whether an inadequate inactivation measure can also lead to an increased proliferation of human pathogens in the further course of the product chain and thus cause serious cases of illness when the infectious dose is taken up is also not answered yet. For this reason, a characterization of the microbial diversity on the surface of crickets during processing was carried out. **Table 4** summarizes the viable count obtained on the

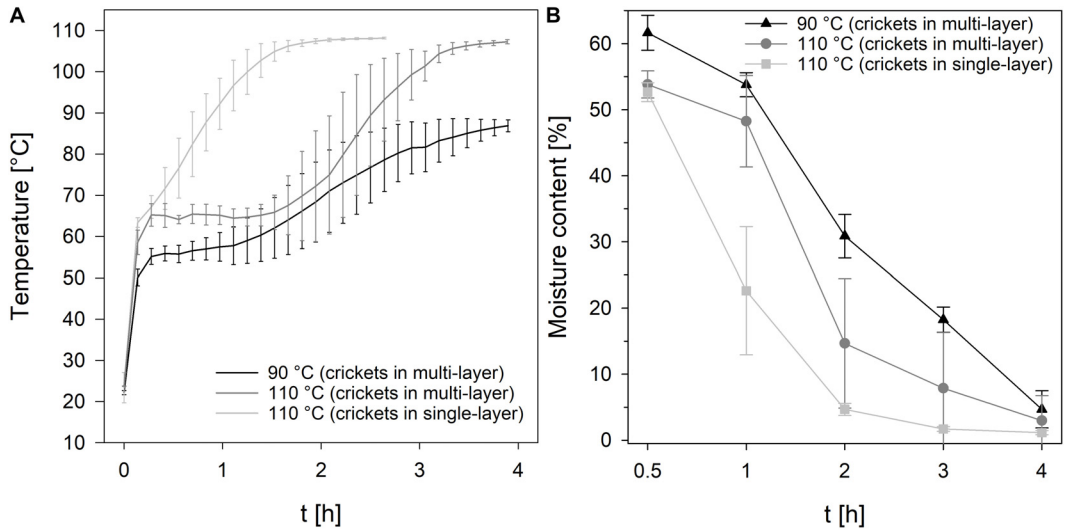

FIGURE 1 | Temperature profiles **(A)** and moisture content **(B)** of crickets during oven-drying at 90°C and 110°C.

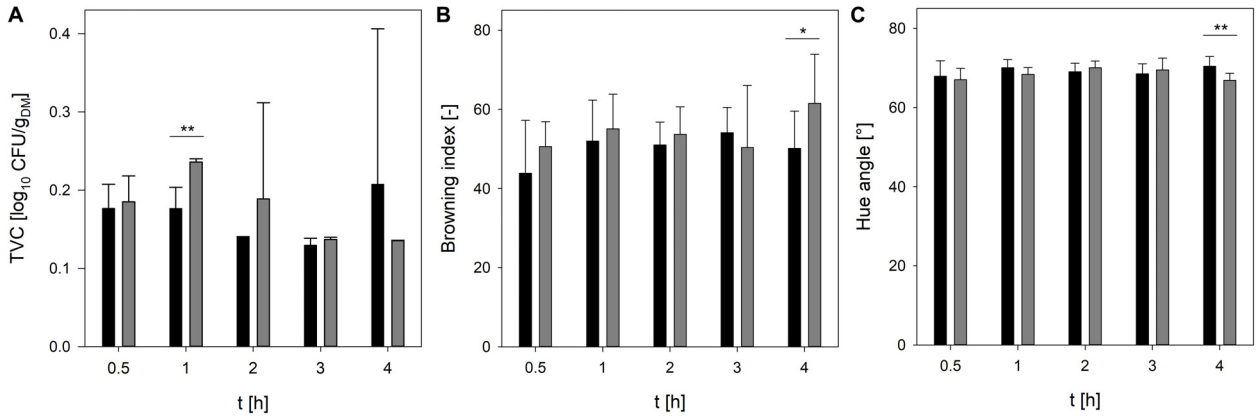

FIGURE 2 | TVC **(A)**, browning index **(B)**, and hue angle **(C)** of crickets during oven-drying at 90°C (black bars) and 110°C (gray bars). Statistical significances were calculated between the different drying temperatures at the different drying times with $*p < 0.05$ and $**p < 0.01$.

different selective and non-selective growth media along the processing chain of cricket flour. Similar viable counts of the untreated crickets on the selective growth media in comparison to the TVC were found on the specific growth media for *Enterococcus*, *Bacillus cereus*, and *Clostridium perfringens* while the specific growth medium for spore-forming bacteria showed the lowest viable count. These results were also obtained after the steaming process but after steaming the viable count of spore-forming bacteria was higher than the TVC. Klunder et al. (2012) also revealed higher counts of spore-forming bacteria after heat treatments and suggested an activation of the spores due to the heat treatment. After drying for 3 h at 110°C growth of microorganisms was only detected on the non-selective growth medium and on the selective growth media for *Staphylococcus* and *B. cereus*. After pulverization, growth of microorganisms was again detected on the selective growth media for *B. cereus*, *Enterococcus*, *Staphylococcus*, *C. perfringens*, spore-forming bacteria, and yeasts and molds. The increased microbial load after pulverization is in accordance with other studies and is attributed to the release of gut microbiota (Garofalo et al., 2019).

The occurrence of foodborne pathogens such as *Salmonella*, *Listeria monocytogenes*, and *Campylobacter* in crickets seems to be low (Walia et al., 2018), however, the presence of pathogens should be excluded. Along the processing chain, growth of microorganisms on selective growth media for *Salmonella* and *L. monocytogenes* occurred in all tested samples whereas growth on selective media for *Campylobacter* occurred only in untreated and washed crickets (**Table 5**). The growth of microorganisms on special selective growth media is not necessarily associated with the presence of potentially pathogenic microorganisms. For the verification of suspected pathogenic microorganisms further investigations of the grown microorganisms are necessary. Suspected microorganisms on

TABLE 4 | Total viable counts on non-selective and selective growth media along the processing chain of cricket flour.

Growth media	Untreated crickets [Log_{10} CFU/g_{DM}]	Washed crickets [Log_{10} CFU/g_{DM}]	Steamed crickets [Log_{10} CFU/g_{DM}]	Dried crickets (110°C, 3 h) [Log_{10} CFU/g_{DM}]	Cricket flour [Log_{10} CFU/g_{DM}]
PCA [Aerobic mesophilic total viable count]	9.24 ± 0.38^{a}	8.51 ± 0.48^{b}	2.83 ± 0.42^{c}	1.75 ± 0.25^{d}	1.98 ± 0.28^{d}
TBX [*Escherichia coli*]	6.77 ± 0.25^{a}	5.91 ± 0.34^{b}	$<2.10^{*c}$	$<1.63^{*d}$	$<1.63^{*d}$
VRBG [Enterobacteriaceae]	8.01 ± 0.75^{a}	7.10 ± 0.35^{b}	$<2.10^{*c}$	$<1.63^{*d}$	$<1.63^{*d}$
PEMBA [*Bacillus cereus*]	9.33 ± 0.33^{a}	8.53 ± 0.35^{b}	2.61 ± 0.55^{c}	1.64 ± 0.02^{d}	1.88 ± 0.35^{d}
KAA [*Enterococcus*]	9.01 ± 0.45^{a}	8.20 ± 0.40^{b}	$<2.10^{*c}$	$<1.63^{*d}$	1.65 ± 0.02^{d}
TSC [*Clostridium perfringens*]	9.16 ± 0.44^{a}	8.19 ± 0.65^{a}	2.42 ± 0.38^{a}	$<1.63^{*a}$	1.64 ± 0.01^{a}
BP [*Staphylococcus*]	8.62 ± 0.70^{a}	7.70 ± 0.53^{b}	2.45 ± 0.40^{c}	1.64 ± 0.02^{d}	1.70 ± 0.14^{d}
DRBC [Yeasts and moulds]	6.45 ± 0.33^{a}	5.62 ± 0.48^{b}	$<2.10^{*c}$	$< 1.63^{*d}$	1.64 ± 0.01^{d}
NA [Aerobic mesophilic spore-forming bacteria]	4.14 ± 1.61^{a}	3.91 ± 1.81^{a}	$3.54 \pm 2.09^{a,b}$	$<1.63^{*b}$	1.70 ± 0.14^{b}

**Below the detection limit. Letters in the rows represent significant differences at the significance level $p < 0.05$.*

TABLE 5 | Growth on selective media after enrichment procedures with indication of the growth of suspected microorganisms.

Selective media	Untreated crickets [Growth/Suspected colonies]	Washed crickets [Growth/Suspected colonies]	Steamed crickets [Growth/Suspected colonies]	Dried crickets (110 °C, 3 h) [Growth/Suspected colonies]	Cricket flour [Growth/Suspected colonies]
Selective enrichment for *Salmonella*	+/+	+/+	+/+	+/+	+/+
1st enrichment for *Listeria*	+/+	+/−	+/−	−/−	−/−
2nd enrichment for *Listeria*	+/−	+/−	+/−	+/−	+/−
Selective enrichment for *Campylobacter*	+/+	+/+	−/−	−/−	−/−

selective growth media for *Staphylococcus*, *Salmonella*, and *L. monocytogenes* were found in all samples whereas suspected microorganisms for *Campylobacter* and *C. perfringens* were only found in untreated and washed crickets. *Enterococcus* suspected microorganisms were found in untreated and washed crickets as well as in the cricket flour. *B. cereus* suspected microorganisms were only found in washed crickets and in the cricket flour. The identification of these suspected microorganisms via MALDI-ToF MS analysis revealed that none of them could be clearly identified as the suspected foodborne pathogen, even though the SARAMIS^{TM} database contains 957 *Staphylococcus* spectra, 269 *Salmonella* spectra, 246 *Listeria* spectra, 299 *Campylobacter* spectra, and 697 *Clostridia* spectra. The comparison with the SARAMIS^{TM} database either did not lead to an identification of the microorganisms or they were identified as Enterobacteriaceae, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Citrobacter* sp., *Enterobacter* sp., Staphylococcaceae, or *Kodamaea ohmeri*. Only suspicious *Enterococcus* colonies found in untreated and washed samples as well as in cricket flour using selective media for Enterococcaceae could be identified as *Enterococcus faecalis* (data not shown). While in the untreated and washed crickets about 8 log_{10} CFU/g_{DM} *E. faecalis* were found using the selective media for Enterococcaceae, the amount of *E. faecalis* in cricket flour was reduced to 1.7 log_{10} CFU/g_{DM} (data not shown). Even if the typical colonies on the selective media could not be identified as *Salmonella*, *Listeria monocytogenes*, *Staphylococcus* sp. or *Campylobacter*, potentially human pathogenic microorganisms can be found among the microorganisms identified instead. These results clearly emphasize the necessity to identify grown microorganisms in order to verify the presence of pathogenic microorganisms. The results show that there are not only special groups of microorganisms on the food samples, but that in principle unexpected potential pathogens can also occur. The detection of such unexpected pathogens is of considerable importance, particularly with regard to tailored concepts of hygienic procedures and proper decontamination techniques preventing risks to consumers' health. Traditionally, the identification of the bacterial species after cultivation on the growth media is conducted via morphological, physiological,

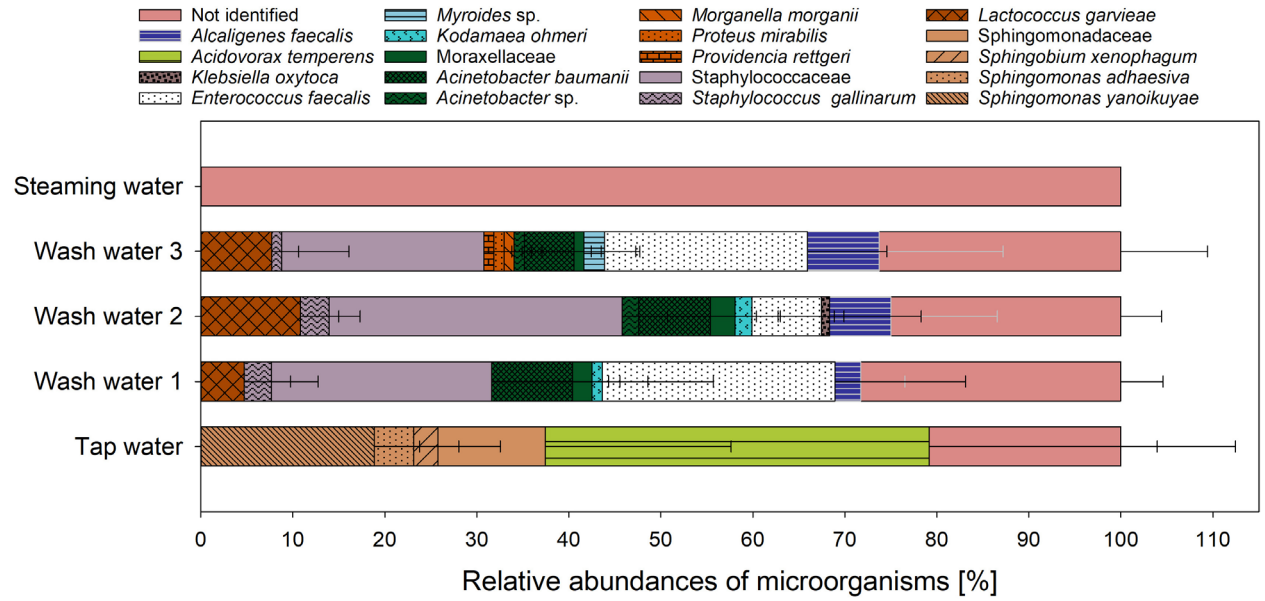

FIGURE 3 | Relative abundances of analyzed microorganisms from crickets along the processing chain of cricket flour.

and biochemical characterization which is very time consuming. In this context, MALDI-ToF MS is a rapid, accurate, and cost-effective technique which has shown to achieve significantly better species identification than conventional biochemical methods or 16S rRNA sequencing (Böhme et al., 2012). To obtain an overview of the culturable microbial community structure of the crickets along the processing chain of cricket flour, grown microorganisms on the selective and non-selective growth media were analyzed by MALDI-ToF MS. Altogether, 1123 grown colonies were analyzed for the untreated crickets, 1091 colonies for the washed crickets, 267 colonies for the steamed crickets, 157 colonies for the dried crickets, and 147 colonies for the cricket flour. The comparison of the obtained mass spectra with the reference mass spectra of the SARAMIS^{TM} database and subsequent cluster analysis led to 35.1 ± 3.4% unidentified microorganisms for the untreated crickets, 41.8 ± 6.1% unidentified microorganisms for the washed crickets, 24.9 ± 24.1% unidentified microorganisms for the steamed crickets, 39.0 ± 38.2% unidentified microorganisms for the dried crickets, and 49 ± 34% unidentified microorganisms for the cricket flour (data not shown). The high number of unidentified microorganisms, which is also reported in other studies for different samples (Hausdorf et al., 2013; Rahi et al., 2016; Fröhling et al., 2018), can be the result of spectra with poor quality or can be caused by the occurrence of unknown microorganisms. Another explanation can be the lack of reference spectra for environmental samples especially for insects since most commercially available databases mainly include clinically relevant microorganisms (Welker and Moore, 2011; Rahi et al., 2016). The relative abundance of analyzed culturable microorganisms is presented in **Figure 3**. Microorganisms belonging to ten different families were identified for the untreated crickets. Members of the family Enterobacteriaceae were the most common microorganisms on the untreated crickets, followed by members of the families Enterococcaceae, Streptococcaceae, Moraxellaceae, Staphylococcaceae, Morganellaceae, Metschnikowiaceae, Pseudomonadaceae, Bacillaceae, and Paenibacillaceae. Vandeweyer et al. (2017a) also found Enterobacteriaceae, Pseudomonadaceae, Enterococcaceae, and Streptococcaceae in whole crickets using metagenetic analysis. After washing, eleven families were detected with relative abundances in descending order: Enterobacteriaceae, Streptococcaceae, Moraxellaceae, Pseudomonadaceae, Enterococcaceae, Morganellaceae, Metschnikowiaceae, Staphylococcaceae, Brucellaceae, Bacillaceae, and Paenibacillaceae. Steaming of the crickets reduced the number of families to five: Enterobacteriaceae, Bacillaceae, Enterococcaceae, Morganellaceae, and Streptococcaceae with relative abundances in descending order. Enterobacteriaceae was also the most predominant family on dried cricket samples followed by Micrococcaceae, Morganellaceae, Staphylococcaceae, Enterococcaceae, Metschnikowiaceae, Moraxellaceae, and Bacillaceae, i.e., the number of detected families increased after drying to eight families. Osimani et al. (2017) also detected Moraxellaceae in dried crickets using PCR-DGGE techniques and Garofalo et al. (2017) found Pseudomonadaceae and Staphylococcaceae in dried crickets using metagenetic analysis. In both studies also Enterobacteriaceae and Bacillaceae were detected in the dried crickets. The cultivable microorganisms from the cricket flour were composed of seven families, whose relative frequencies decreased in increasing order: Enterobacteriaceae, Moraxellaceae, Morganellaceae, Bacillaceae, Enterococcaceae, Staphylococcaceae, and Streptococcaceae. For all tested samples the family Enterobacteriaceae showed the highest diversity with four species identified and no differences

TABLE 6 | Aerobic mesophilic total viable count and protein concentration of the process water along the processing chain of cricket flour.

Sample	Aerobic mesophilic total viable count [Log_{10} CFU/ml]	Protein concentration [μg/ml]
Tap water	2.21 ± 0.22^{a}	0.24 ± 0.27^{a}
Wash water 1	6.22 ± 0.15^{b}	65.56 ± 5.26^{b}
Wash water 2	$6.06 \pm 0.16^{b,c}$	27.69 ± 3.03^{c}
Wash water 3	5.94 ± 0.18^{c}	27.84 ± 2.54^{c}
Steaming water	0.48 ± 0.57^{d}	1.24 ± 1.58^{a}

Letters in the columns represent significant differences at the significance level $p < 0.05$.

were found in the samples during processing of the cricket flour. Members of the families Pseudomonadaceae, Paenibacillaceae, and Brucellaceae were only found in untreated and washed crickets implying that these microorganisms were more sensitive to the steaming and drying process than the microorganisms of the other families. In contrast, *Micrococcus luteus* who is belonging to the family Micrococcaceae was only found in the dried crickets. Osimani et al. (2017) also found members of the family Micrococcaceae in whole dried crickets but their found species did not belong to the genus *Micrococcus*. *Micrococcus luteus* can be found on human skin and can be released into air (Kooken et al., 2012) and the presence of *Micrococcus luteus* in the dried crickets might be related to contamination during the drying process. However, in the cricket flour *Micrococcus luteus* was not found but it has to be taken into account that only grown microorganisms from the highest dilution were identified. Since the viable count was slightly higher after pulverization due to better accessibility to the microorganisms it might be possible that other microorganisms were more dominantly grown on the growth media and therefore the presence of *Micrococcus luteus* might be suppressed in the other samples. Pseudomonadaceae and Paenibacillaceae were detected in cricket powder by Garofalo et al. (2017) and Osimani et al. (2018), respectively, but in both studies culture-independent methods were used in comparison to the culture-dependent method used in our study. Using 16S rRNA gene amplicon sequencing, Vandeweyer et al. (2018) showed that heat-treated tropical house crickets had a similar microbial community composition as crickets during rearing even though the viable count decreased. They suspected that not all of the DNA was destroyed and was therefore detected by the cultivation-independent analysis method applied. However, it was shown that the microbial community composition of dried tropical house crickets is comparable to the microbial community composition of live tropical house crickets. In accordance, comparable microbial community composition of the crickets during processing to cricket flour was revealed in our study. Nevertheless, it has to be noted that the abundances of the different families within the microbial composition changed during processing indicating different resistances of the microorganisms toward the applied heat and drying processes.

During processing of crickets to flour a washing step was applied to remove the adherent dirt and feed substrate from the crickets. In consequence, the wash water may contain high amounts of microorganisms as well as organic matter (Gil et al., 2015). The high content of organic matter (e.g., proteins, carbohydrates, and lipids) lead to difficult disposal problems and potentially contribute to severe pollution problems (Hansen and Cheong, 2019). The microbial load of the cricket process water as well as its protein content was evaluated in this study and the results are presented in **Table 6**. The protein content of the cricket wash water from the first washing step was 65.6 μg/ml and in the wash water from the second and third washing step the protein contents were 27.7 and 27.8 μg/ml, respectively. The protein content of the water from the steaming process was not significant different from the protein content of the tap water. The cricket wash water had a TVC of $\sim$ 6$\log_{10}$ CFU/ml in all washing steps and since the used tap water had a TVC of $\sim$2 $\log_{10}$ CFU/ml the microbial load of the wash water is the result of the migration of microorganisms from the crickets into the water. The process water from the steaming step only had a TVC of 0.5 $\log_{10}$ CFU/ml. Wynants et al. (2017) also found an increase of the TVC in mealworm rinsing water and concluded a removal of microorganisms from the mealworm. Process water from meat, poultry, and seafood processing is characterized by a high content of proteins and fats and may contain significant amounts of pathogens (Hansen and Cheong, 2019). Since crickets are also of animal origin and microbial criteria of minced meat or seafood are suggested for edible insects, the occurrence of pathogens should also be taken into account in cricket wash water. Therefore, the grown microorganisms on the non-selective growth medium were also identified by MALDI-ToF MS to gain knowledge about the microbial community structure in the wash water (**Figure 4**). For the tap water 71 grown colonies were analyzed and thereof 20.8 $\pm$ 12.5% could not be identified. The identified culturable microbial community of the tap water consisted of the two families Comamonadaceae and Sphingomonadaceae whereby Comamonadaceae were composed only of *Acidovorax temperans* and Sphingomonadaceae included the genera *Sphingomonas* spp. and *Sphingobium*. In contrast, all four grown colonies from the steaming water could not be identified. The low number of microorganisms analyzed is due to the fact that the total bacterial count in the steaming water was very low. 28.3 $\pm$ 4.6% of the 88 analyzed colonies from the first wash water could not be identified and the identified microbial community consists of members of the families Staphylococcaceae, Enterococcaceae, Streptococcaceae, Alcaligenaceae, Moraxellaceae, and Metschnikowiaceae with abundances in descending order. In comparison to the first wash water, Enterobacteriaceae was additionally found in the second wash water. Altogether, 87 colonies were analyzed from the second wash water with 25.0 $\pm$ 4.4% unidentified microorganisms. Enterobacteriaceae could not be found among the 91 analyzed colonies from the third wash water, instead Flavobacteriaceae were detected. 30.3 $\pm$ 0.6 of the analyzed colonies in the third wash water remained unidentified. The results showed that the microbial community structure of the wash water is almost composed of the same microorganisms as that of the crickets whereby the relative abundances of the species were different. However, it should be noted that

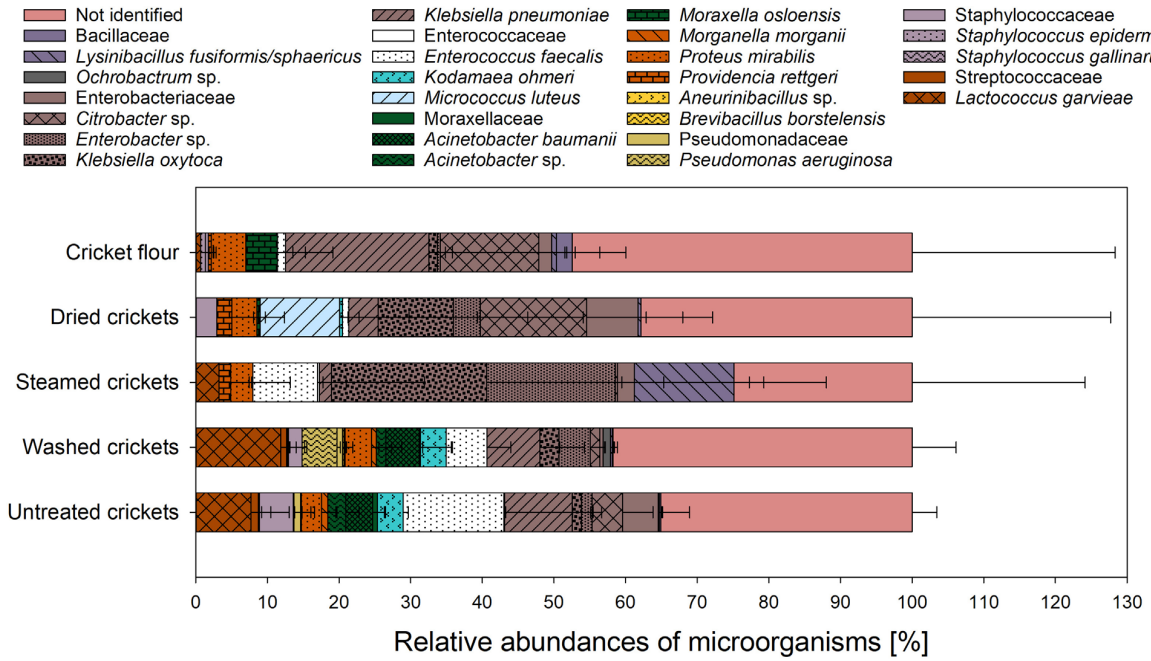

FIGURE 4 | Relative abundances of analyzed microorganisms from process water along the processing chain of cricket flour.

only the TVC was examined for the process water and that additional selective culture media were used for the crickets, leading to the possibility of a resulting shift in the relative abundances of the microorganisms. Nevertheless, Alcaligenaceae and Flavobacteriaceae were only found in the wash water and not on the crickets. This could be due to the fact that these microorganisms were found only on the surface of the insects and could therefore more easily migrate into the washing water. In consequence, these microorganisms were more common in the wash water than on the crickets and were therefore methodically found only in the wash water and not on crickets. The migration of microorganisms from the crickets into the wash water during processing has to be considered and monitored to avoid a transmission of potential pathogens from the wash water into the environment.

CONCLUSION

It is important to ensure microbial safety in edible insects and products thereof in order to avoid risks to human health. As there are currently no valid microbiological criteria for placing edible insects on the market, the microbiological criteria for minced meat are consulted. This can be viewed critically since insects are consumed with intestinal tract and therefore the microbiota of the intestinal tract strongly increases the microbial load. The higher microbial load due to the use of whole insects results in the need for appropriate inactivation procedures to reduce the microbial load in order to ensure microbial safety. Consumer's acceptance for the consumption of insects is higher if the insects are presented in unrecognizable form such as powder or pastes. The manufacture of such products requires suitable processes that produce a microbiologically safe product. When processing, it is important to implement and comply with hygiene regulations and to determine suitable process parameters as well as to introduce critical control points. In order to establish microbiological criteria for edible insects and the products thereof, detailed knowledge of the microbial community structure of the insects as well as the dynamic changes of the microbial community structure during processing is necessary.

The processing chain of cricket flour including steaming as inactivation step effectively reduced the TVC of the crickets which was maintained during drying and pulverization. The analysis of the culturable microbial community structure changes during processing using MALDI-ToF MS revealed that the microbial community of cricket flour is similar to the microbial community of untreated crickets but relative abundances of the microorganisms changed, indicating different sensitivities of the microorganisms against the different processing steps. While Enterobacteriaceae were the predominant microorganisms in all samples, the amount of Bacillaceae was higher in cricket flour than in the untreated crickets revealing a higher resistance of Bacillaceae against the applied inactivation treatments. However, an increased growth of potential human pathogenic microorganisms as a result of a treatment measure could not be derived from these results. Nevertheless, the influence of processing on both harmful microorganisms and endemic microflora (loss of the protective function of microbial colonization) must also be considered. The analysis of the process water revealed a migration of microorganisms into the water as

well as an increase of organic matter which has to be taken into account to avoid environmental pollution and possible release of potential pathogens. From the results it can be concluded that, in addition to the total bacterial count, the composition of the microbial community should also be examined in order to ensure the microbial safety of the products. The detection of unexpected pathogens is of importance to develop tailored concepts of hygienic procedures and proper decontamination techniques preventing risks to consumers' health.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

AF conceived and designed the experiments, performed the experiments, analyzed and interpreted the data, and wrote the manuscript. SB and JD contributed to the design of experiments, performed the experiments, and proofread the manuscript. OS contributed to the design of experiments, contributed reagents, material, analysis tools or data, and proofread the manuscript.

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Fermentation Versus Meat Preservatives to Extend the Shelf Life of Mealworm (*Tenebrio molitor*) Paste for Feed and Food Applications

An Borremans, Ruben Smets and Leen Van Campenhout*

Faculty of Engineering Technology, Department of Microbial and Molecular Systems (M2S), KU Leuven, Geel, Belgium

Freshly prepared pastes from blanched mealworms (*Tenebrio molitor*) are highly perishable and prone to microbial and chemical changes upon storage due to their high water activity, near–neutral pH, and their rich nutrient profile. Their shelf life is short unless preservation methods are used. In this study, the effects of preservatives (sodium nitrite and sodium lactate) and lactic acid fermentation (with the starter cultures Bactoferm® F-LC and *Lactobacillus farciminis*) on the microbiological and the chemical stability of mealworm pastes stored at 4°C were compared. During the storage experiment, the pH, water activity, color, microbial counts, and fat oxidation were monitored. In addition, the prevalence of the pathogens *Bacillus cereus*, *Salmonella* spp., and *Listeria monocytogenes* were studied. Microbial quality evaluation of the mealworm pastes showed that the addition of preservatives did not inhibit microbial growth during refrigerated storage, reaching the upper limits for consumption between seven and 14 days. By contrast, the acid medium (pH $<$ 4.50) created by fermentation stabilized all microbial populations investigated, indicating that these pastes could be consumed up to (at least) 8 weeks of refrigerated storage. *L. monocytogenes*, *Salmonella,* and *B. cereus* were not detected in any of the samples and lipid oxidation of the samples was minimal. Altogether, this study shows that lactic acid fermentation can be used successfully to inhibit microbial growth, to maintain chemical quality, and to extend the shelf life of mealworm pastes stored at 4°C.

Keywords: mealworms, microbial stability, chemical stability, preservatives, fermentation

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Gianluigi Mauriello,
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Reviewed by:
Luis Patarata,
University of Trás-os-Montes and Alto Douro, Portugal
Wei-Min Wu,
Stanford University, United States

****Correspondence:***
An Borremans
an.borremans@kuleuven.be

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INTRODUCTION

Edible insects such as the yellow mealworm [*Tenebrio molitor* Linnaeus (Coleoptera: Tenebrionidae)] are valuable alternatives to conventional meat, because their nutrient content is comparable, and the ecological impact of insect rearing is lower than that of traditional animal husbandry (Oonincx and de Boer, 2012; van Huis, 2015). In some EU member states, such as Belgium and The Netherlands, a variety of food products containing mealworms has been launched on the market since they were authorized for human consumption at national level. Currently, these products can stay on the market at least until their submitted Novel Food dossiers have been

evaluated [Regulation (EU) 2015/2283]. Mealworms are incorporated in these products either as whole insects in fresh or dried form, or processed into a powder or paste, or as an extract of protein, fat or chitin to increase their nutritional value or functionality. The use of mealworms as aquafeed and petfood ingredient is already allowed in Europe and they may be authorized in feed for other animal species in the future (International Platform of Insects for Food and Feed [IPIFF], 2019). Several European pet food companies incorporate dried mealworms in their feed formula as a mean to diversify their products' range or to replace soybean and fishmeal. However, fresh mealworms are perishable. After harvesting and killing, they have a short shelf-life because of microbial growth, lipid oxidation, Maillard reactions, and/or enzymatic reactions (Jeon et al., 2016; Lenaerts et al., 2018; Melgar-Lalanne et al., 2019). These phenomena can cause rancidity with off-flavors, color alterations, and/or loss of nutrients (Chan et al., 1993). In addition, food-borne pathogens may compromise microbial safety. Therefore, to maintain quality and safety of insects and derived products, fresh edible insects need to be stabilized.

Freezing and freeze-drying are the most commonly used methods to convert fresh insects to a storable form after harvesting (Efsa Scientific Committee, 2015). However, both conservation strategies impose drawbacks. Frozen storage implies frozen transport, which adds to the cost price. Moreover, when thawing is not performed correctly (too long and/or no refrigeration after thawing), food safety problems are likely to occur. Freeze-dried mealworms, on the other hand, are chemically and microbiologically stable, but freeze-drying is expensive and also preserves the microbiota, which may cause food safety problems upon processing of the larvae into food products (FASFC, 2018). Also, the brittle texture of freeze-dried larvae limits their applications. The feed and food industries are aware of these restrictions and therefore other preservation technologies have to be explored.

Refrigeration is the best method to retain the sensorial properties of fresh insects. However, this method can only be used to store (already to some extend processed) mealworms for a few days. According to Borremans et al. (2018), blanched mealworms can be stored refrigerated for ten to 12 days without exceeding the general food spoilage level for foods of 7.0 log cfu/g (Sperber and Doyle, 2009). De Smet et al. (2019) observed substantial microbial growth but limited fat oxidation in pastes prepared from steamed mealworms. These mealworm pastes were stored for three weeks at 4°C but were unacceptable for consumption within 14 days. The addition of meat preservatives (sodium nitrite and sodium lactate) did not improve the shelf life of these pastes.

Previous work has shown that a paste produced from blanched mealworms can be fermented using commercial meat starter cultures (Borremans et al., 2019). It was not yet investigated so far whether the shelf life of these pastes can be improved by fermentation and whether the pH can be reduced sufficiently and fast enough by fermentation to prevent spoilage organisms and pathogens from growing (Özogul and Hamed, 2018). Fermentation was demonstrated to extend the shelf life of shrimp paste for weeks or even months (Fagbenro, 1996). In this study, the impact of fermentation on the shelf-life of pastes produced from blanched mealworms was investigated. The shelf life was compared with that of unfermented pastes and unfermented pastes containing typical meat preservatives being sodium nitrite and sodium lactate.

MATERIALS AND METHODS

Preparation of Mealworm Samples

Living mealworms (Nusect, Ledegem, Belgium) were killed by freezing and stored at −18°C until further use. Fermented mealworm samples were prepared as described before (Borremans et al., 2018, 2019) without the addition of the curing agent sodium nitrite. Briefly, 7.0 kg of frozen larvae were blanched (40 s), and mixed into a paste using a kitchen mixer (Bosch CNHR 25). One half of the paste was inoculated with the commercial starter culture Bactoferm® F-LC (Chr. Hansen Holding A/S, Hoersholm, Denmark, 501091, consisting of a mixture of *Pediococcus acidilactici*, *Lactobacillus curvatus*, and *Staphylococcus xylosus*), according to the manufacturers' instruction (25 *g*/100 kg). The other half was inoculated with the pure culture *L. farciminis* (Chr. Hansen Holding A/S, Hoersholm, Denmark, 501167) to reach a level of ±6.5 log cfu/g paste. The ability of these two starter cultures to ferment a mealworm paste produced at laboratory scale was demonstrated in previous work (Borremans et al., 2018, 2019). Similar to raw meat fermentations, 2.8% NaCl (w/w), and 0.75% d(+)-glucose (w/w) were added to both pastes to provide flavor, to control the background microbiota, and to fuel the fermentation as the fermentable sugar content of mealworms is low (Morales-Ramos et al., 2016). After thorough mixing, the pastes were distributed over sterile 50 ml Falcon tubes (Sarstedt, Antwerp, Belgium) and incubated at 35°C for seven days. After seven days, non-fermented mealworm pastes were prepared (so that their storage period could start together with that of the fermented pastes) by blanching 5.0 kg frozen larvae (40 s) and mixing them into a paste using the same kitchen mixer. An aliquot of 1.5 kg of freshly prepared paste was supplemented with 150 mg $NaNO_2$/kg paste (EMSURE®, ACS, Reag. Ph. Eur. Analytical reagent, Merck Millipore, Overijse, Belgium), an equal amount was supplemented with 50 *g*/kg paste of a 60% sodium DL-lactate solution (Syrup, 60% w/w, synthetics, Sigma Aldrich, Overijse, Belgium), and the remaining paste remained without preservatives (control). After thorough mixing each type of paste, the pastes were also distributed over sterile 50 ml Falcon tubes (Sarstedt, Antwerp, Belgium) which were completely stuffed. The approach in this study differed from that used by De Smet et al. (2019) who also determined the shelf life of mealworm pastes: in our study, the mealworms were blanched instead of steamed, the mealworm pastes were stored in smaller volumes (50 mL versus 250 mL), the storage period for unfermented pastes was four (rather than three) weeks, and in particular fermented samples were included in the storage period [not in De Smet et al. (2019)].

Storage Conditions and Sampling Plan

All samples were stored at 4 ± 1°C in a home type refrigerator (Miele, Belgium). Immediately after processing as

well as during storage, pH, water activity, color, and microbial counts were monitored. Unfermented samples were analyzed weekly during four weeks after production, while the fermented samples had an additional sampling point after eight weeks of storage. Per sampling time point, three Falcon tubes per condition were withdrawn with each analysis performed once per Falcon tube. In addition, fat oxidation and a selection of food pathogens were monitored after processing and after four and eight (for the fermented samples) weeks of storage. For both pathogen detection and fat oxidation, the contents of six Falcon tubes were mixed and all analyses were performed in triplicate.

pH, Water Activity and Color Parameters

The pH was measured using a digital pH-meter (Portamess 911, Knick, Germany) equipped with a SI analytics electrode (Germany). The water activity (a_w) was determined using a LabMaster a_w, Novasina (Lachen, Switzerland) as described by Wynants et al. (2018) and a colorimeter (CR-5 Konica Minolta) was used to determine the color of the pastes in the CIELAB space. Browning indices, characterizing the intensity of the brown color, and total color differences, using the initial color of the pastes as reference, were determined using the methods described by De Smet et al. (2019).

Microbial Analyses and Pathogen Detection

The microbiological quality of the mealworm pastes during cold storage was monitored assessing total (an)aerobic bacteria, lactic acid bacteria (LAB), psychrotrophic bacteria (PSY), yeast and molds (Y&M), Enterobacteriaceae (ENT), sulphite reducing clostridia (SRC), and (an)aerobic bacterial spores as described by Borremans et al. (2019), Vandeweyer et al. (2017b), and Wynants et al. (2019).

The prevalence of *Bacillus cereus, Salmonella* spp., and *Listeria monocytogenes* was studied according to ISO 7932 (plate counts), ISO 6579 (absence in 25 *g*), and the ISO method AFNOR BRD 07/4-09/98 (absence in 25 *g*), respectively.

Fat Oxidation

To analyze fat oxidation, fat was extracted from the pastes following a modification of the procedure proposed by Folch et al. (1957). Briefly, 70 *g* of paste was mixed with 150 mL chloroform/methanol mixture (2:1 v/v, VWR) solution, shaken for 5 min, and then filtered. The solid phase was extracted two more times with 50 mL of the chloroform/methanol mixture. The obtained filtrates were pooled and washed twice with 50 mL of a 0.88% sodium chloride solution. After phase separation, the organic phase was dried using anhydrous magnesium sulfate (VWR) after which the solvent was removed using a rotary evaporator (Büchi). The fat extracts were stored at −18°C until further analysis.

The fat obtained by the Folch method was used to determine the peroxide and *p*-anisidine values. Peroxide values, expressed as units of meq. O_2/kg fat sample, were determined using the modified method of Wu and Mao (2008) as described by Lenaerts et al. (2018). *p*-anisidine values were determined using the method described by Tenyang et al. (2017) and Lenaerts et al. (2018).

Statistical Analysis

One-way ANOVA was performed to check whether there were any significant differences between the pH, a_w, color parameters, microbial numbers, and peroxide and *p*-anisidine values of the different samples during storage. When significant ($p < 0.05$), *post hoc* tests with the Tukey adjustment were performed. All analyses were performed using IBM SPSS Statistics 23.

RESULTS AND DISCUSSION

pH, Water Activity and Color During Storage

Changes in pH, water activity (a_w), and color parameters during the refrigerated storage of the pastes are shown in **Table 1**. Freshly prepared mealworm pastes were characterized by a near-neutral pH (6.54) and high a_w values (1.003), which were in line with the findings of Vandeweyer et al. (2017a,b), Lenaerts et al. (2018), and De Smet et al. (2019) for fresh and blanched mealworms and pastes prepared from steamed mealworms. As expected, the pH and a_w values of the fermented pastes were significantly ($p < 0.05$) lower than those of the non-fermented pastes at the start of the storage period (day zero), with values ranging from 4.35 to 4.51 and from 0.964 to 0.968, respectively. The decrease in pH is a desirable effect of the metabolic activity of the added starter cultures (Bintsis, 2018). The reduction in a_w can be attributed to the addition of D(+)-Glucose (0.75%, w/w) and sodium chloride (2.8%, w/w) to the pastes prior to fermentation. During the first week of storage, the pH of the control and the fermented pastes significantly ($p < 0.05$) decreased and increased, respectively, but stabilized thereafter until the end of storage. The addition of the preservatives sodium nitrite and sodium lactate to the mealworm pastes had no significant effect ($p < 0.05$) on the pH during the entire storage period. This last result is in contrast to the observations of De Smet et al. (2019), where neither of the two preservatives could prevent a reduction in pH during the refrigerated storage of pastes prepared from steamed mealworms. This difference can likely be explained by the fact that prior to the production of the pastes in the latter study, the mealworms were steamed and not blanched. The microbial reduction caused by steaming is lower than that obtained by blanching as demonstrated by De Smet et al. (2019). A higher microbial load remaining after heat treatment can cause the mealworms to spoil faster with a resulting decrease in pH due to metabolic activity, i.e., organic acid production.

As to the a_w of the pastes in the current study, the parameter did not vary significantly during storage, regardless of the preservative treatment and the storage time.

The color of the mealworm pastes was shown to be altered during storage as demonstrated by the browning index (BI) and the total color difference (ΔE). Browning can be a significant problem as it affects the visual quality of food products. As

TABLE 1 | Means ± standard deviation (n = 3) of pH, a_w, browning index (BI), and total color difference with the initial value (ΔE) during storage of pastes produced from blanched mealworms (*Tenebrio molitor*) without additive (control), with the additives sodium nitrite or sodium lactate, or fermented with the starter culture Bactoferm® F-LC or *Lactobacillus farciminis*.

Sample	Sampling day	pH	a_w	BI [-]	ΔE
Control	0	$6.54 \pm 0.02^{a,A}$	$1.00 \pm 0.00^{a,A}$	$36.35 \pm 0.37^{a,A}$	–
	7	$5.88 \pm 0.03^{b,A}$	$1.00 \pm 0.00^{a,A}$	$38.27 \pm 0.56^{a,b,A}$	$4.27 \pm 0.12^{a,b}$
	14	$6.09 \pm 0.25^{b,A}$	$1.00 \pm 0.01^{a,A,B}$	$38.11 \pm 0.88^{a,b,A}$	$3.63 \pm 0.40^{b,c}$
	21	$5.96 \pm 0.03^{b,A}$	$1.00 \pm 0.01^{a,A,B}$	$40.64 \pm 0.40^{b,A}$	5.19 ± 0.21^{a}
	28	$6.09 \pm 0.13^{b,A}$	$1.00 \pm 0.00^{a,A}$	$37.29 \pm 1.76^{a,A,B}$	2.73 ± 0.57^{c}
Sodium nitrite	0	$6.54 \pm 0.02^{a,A}$	$1.00 \pm 0.00^{a,A}$	$36.35 \pm 0.37^{a,A}$	–
	7	$6.65 \pm 0.01^{a,B}$	$1.01 \pm 0.01^{a,A}$	$32.46 \pm 0.24^{b,B}$	1.33 ± 0.04^{a}
	14	$6.59 \pm 0.08^{a,B}$	$1.00 \pm 0.00^{a,B}$	$32.26 \pm 0.09^{b,c,B}$	1.40 ± 0.09^{a}
	21	$6.62 \pm 0.04^{a,B}$	$1.01 \pm 0.00^{a,B}$	$31.75 \pm 0.09^{c,d,B}$	1.60 ± 0.08^{a}
	28	$6.38 \pm 0.29^{a,A,B}$	$1.00 \pm 0.00^{a,A}$	$31.47 \pm 0.30^{d,C}$	1.71 ± 0.04^{a}
Sodium lactate	0	$6.54 \pm 0.02^{a,A}$	$1.00 \pm 0.00^{a,A}$	$36.35 \pm 0.37^{a,A}$	–
	7	$6.64 \pm 0.01^{a,B}$	$0.99 \pm 0.01^{a,B}$	$32.87 \pm 0.38^{b,c,B}$	1.24 ± 0.19^{a}
	14	$6.49 \pm 0.23^{a,B}$	$0.99 \pm 0.01^{a,A}$	$32.59 \pm 0.33^{c,B}$	$1.51 \pm 0.09^{a,b}$
	21	$6.49 \pm 0.2^{a,B}$	$0.99 \pm 0.01^{a,A}$	$32.92 \pm 0.17^{b,c,C}$	1.31 ± 0.14^{a}
	28	$6.61 \pm 0.03^{a,B}$	$0.99 \pm 0.01^{a,A}$	$33.43 \pm 0.09^{b,C,D}$	1.75 ± 0.08^{b}
Bactoferm® F-LC	0	$4.50 \pm 0.01^{a,B}$	$0.97 \pm 0.00^{a,B}$	$37.50 \pm 0.47^{a,B}$	–
	7	$4.60 \pm 0.04^{b,C}$	$0.97 \pm 0.00^{a,C}$	$39.96 \pm 0.16^{b,C}$	0.89 ± 0.14^{a}
	14	$4.57 \pm 0.01^{b,C}$	$0.97 \pm 0.00^{a,C}$	$38.96 \pm 0.57^{b,c,C}$	0.53 ± 0.16^{a}
	21	$4.62 \pm 0.01^{b,C}$	$0.97 \pm 0.00^{a,C}$	$39.12 \pm 0.20^{b,c,D}$	0.69 ± 0.13^{a}
	28	$4.57 \pm 0.02^{b,C}$	$0.97 \pm 0.00^{a,B}$	$38.44 \pm 0.55^{a,b,A}$	0.57 ± 0.14^{a}
	56	$4,60 \pm 0.02^{b,A}$	$0.97 \pm 0.00^{a,A}$	$38.12 \pm 0.48^{a,b,A}$	0.52 ± 0.32^{a}
L. farciminis	0	$4.39 \pm 0.04^{a,C}$	$0.97 \pm 0.00^{a,B}$	$37.29 \pm 0.11^{a,B}$	–
	7	$4.47 \pm 0.03^{b,D}$	$0.97 \pm 0.01^{a,C}$	$36.78 \pm 0.49^{a,b,D}$	0.37 ± 0.21^{a}
	14	$4.46 \pm 0.02^{b,C}$	$0.97 \pm 0.00^{a,C}$	$36.60 \pm 0.50^{a,b,A}$	0.33 ± 0.14^{a}
	21	$4.50 \pm 0.04^{b,C}$	$0.97 \pm 0.00^{a,C}$	$35.58 \pm 0.74^{b,c,E}$	0.58 ± 0.26^{a}
	28	$4.46 \pm 0.03^{b,C}$	$0.97 \pm 0.00^{a,B}$	$35.39 \pm 0.40^{b,c,B}$	$0.66 \pm 0.17^{a,b}$
	56	$4.47 \pm 0.02^{b,B}$	$0.97 \pm 0.00^{a,A}$	$34.33 \pm 0.82^{c,B}$	1.16 ± 0.27^{b}

a, b, c, and d Mean values per treatment type with the same superscript are not statistically different ($P > 0.05$). A, B, C, D, and E Mean values per storage time with the same superscript are not statistically different ($P > 0.05$).

shown in **Table 1**, the browning index of the control pastes increased as storage time proceeded, which is not unexpected since mealworms are prone to browning upon processing (Van Campenhout et al., 2017). In the presence of sodium nitrite and sodium lactate, a significant ($p < 0.05$) reduction of the BI was observed during the first week of storage, but then it remained almost constant during the rest of the storage period. Using the same preservatives, De Smet et al. (2019) observed a similar evolution of the BI during storage, which might indicate that both preservatives have some antibrowning activity. Fermentation of the pastes did not present a definite trend in BI during storage but, as indicated by ΔE, the initial color of these pastes was preserved the best. In contrast, the most significant color changes were noticed in the control samples, where color differences up to 5.19 were recorded during storage. According to Wibowo et al. (2015), this differences can be visible. The total color difference of the pastes treated with additives remained below 1.75 during storage, which most likely is not visible.

Microbial Counts During Storage

Microbiological analysis of freshly prepared mealworm pastes revealed the presence of LAB at 2.6 ± 0.1 log cfu/g, ENT at 2.6 ± 0.6 log cfu/g, and PSY at 1.5 ± 0.8 log cfu/g. Total aerobic and anaerobic plate counts ranged between 2.7 and 3.9 log cfu/g, which were in line with the findings of De Smet et al. (2019) for pastes prepared from steamed mealworms. At the start of the storage experiment, the total counts and LAB counts of the fermented samples were significantly ($p < 0.05$) higher than those of the unfermented samples due to the inoculation of the starter strains. Pastes inoculated with the starter culture Bactoferm® F-LC had total counts and LAB counts of approximately 7.9 log cfu/g, compared to levels of 6.0 log cfu/g in samples inoculated with the starter culture *L. farciminis*. These levels, obtained after one week of fermentation, were similar to those reported in earlier studies on the fermentation of blanched mealworms (Borremans et al., 2018, 2019).

Microbial changes in the mealworm pastes during refrigerated storage at 4°C are shown in **Figure 1**. Throughout the storage period, total counts and LAB counts of the fermented pastes remained relatively constant. In contrast, in control pastes and pastes containing a preservative, total counts and LAB counts (except the LAB counts of pastes with SL) increased dramatically ($p < 0.05$) during the first two weeks of storage, reaching values between 2.3 and 6.2 log cfu/g at day seven and between

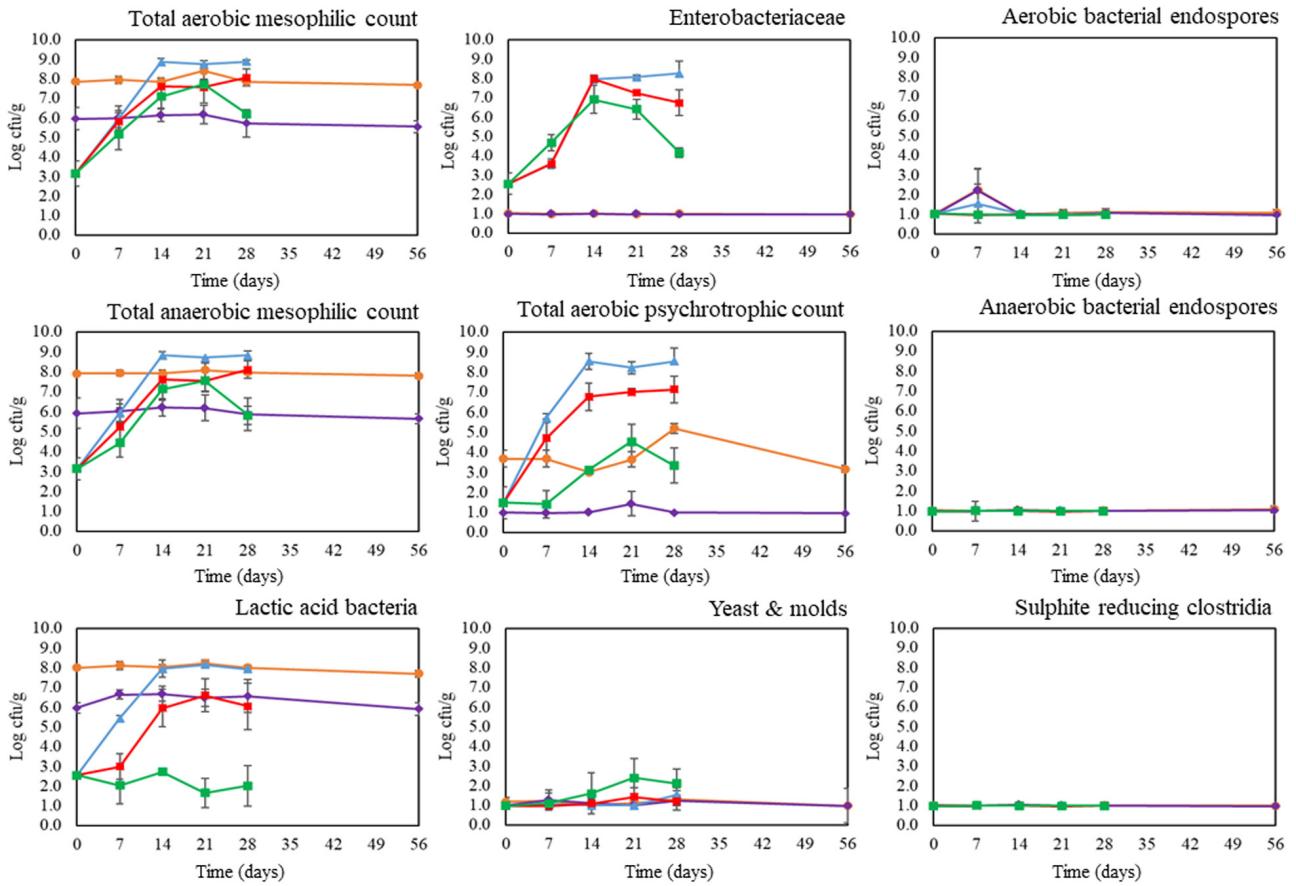

FIGURE 1 | Microbial counts of control pastes (blue), pastes with sodium nitrate (red), pastes with sodium lactate (green), pastes fermented with Bactoferm® F-LC (orange), and pastes fermented with *Lactobacillus farciminis* (purple) during storage at 4°C. Graphs show averages and standard deviations of three repetitions. The dotted horizontal line indicates the detection limit for the microbial counts.

6.7 and 9.1 log cfu/g at day 14. These counts are close to or above the level of 7.0 log cfu/g, which is considered as the microbiological acceptability limit for foods (Sperber and Doyle, 2009). Therefore, based on these counts, the microbiological shelf-lives of unfermented mealworm pastes would be between seven and 14 days, regardless of whether a preservative was added or not, and regardless of the type of preservative added. Using similar test conditions, De Smet et al. (2019) reached the same conclusions for pastes prepared from steamed mealworms. In this study, total counts of pastes with and without additives ranged between 2.8 and 5.6 log cfu/g at the start of the storage experiment, between 4.9 and 6.6 log cfu/g after one week of refrigerated storage and between 7.2 and 9.1 log cfu/g after two weeks of refrigerated storage.

As the total counts and the LAB counts of fermented samples were high due to the inoculation of the starter strains, other microbial counts like PSY, ENT, Y&M, SRC, and spore counts were chosen as factors determining the microbiological shelf-lives of fermented pastes. The presence of some psychrotrophs and Enterobacteriaceae in the mealworm pastes at the start of storage might involve a risk of spoilage in a chilled environment (Hwang and Tamplin, 2005; Martínez et al., 2007). Unfermented samples experienced a significant ($p < 0.05$) increase in both microbial counts during storage. Moreover, between one and two weeks of storage, the number of ENT exceeded their maximum limit of 5.0 log cfu/g for insects and insect-based products as proposed by the Federal Agency for Safety of the Food Chain (FASFC, 2018). In contrast, during storage of fermented samples, the acidified conditions completely inhibited the growth of ENT (<1.0 log cfu/g), whereas the levels of PSY remained (relatively) stable at acceptable levels (<5.2 log cfu/g). Note hereby that, both at the start as during storage, the PSY counts of the pastes fermented with the starter culture Bactoferm® F-LC were significant ($p < 0.05$) higher than those of the pastes fermented with the starter culture *L. farciminis*. This can be explained, and was also confirmed by microbiological analysis of the starter cultures, by the fact that one or more strains of the mixed culture Bactoferm® F-LC can grow at refrigerated temperatures. Edible insects are also prone to spoilage caused by yeast and molds. During storage, the highest numbers of yeast and molds, up to 3.0 log cfu/g, were found in the pastes with the preservative sodium lactate. These counts were still below the minimum limit of 3.7 log cfu/g for fungi (FASFC, 2018). Finally, no or limited growth in SRC and aerobic and anaerobic

bacterial endospores were observed in all samples throughout the storage period.

Bacillus cereus, *Salmonella* spp., and *Listeria monocytogenes* were not detected in any of the mealworm samples tested (<100 cfu/g, absence in 25 grams, and absence in 25 grams, respectively). The fact that microbial counts increased substantially in unfermented samples can be an indication that, if certain pathogens would be present in the starting material, they may grow as well and potentially cause safety problems. Whether fermentation can prevent this, still needs to be confirmed, for instance via challenge tests. Overall, the microbial counts of the fermented pastes in this study were below the permissible limits, which indicated that the product was safe for consumption, during the whole test period, i.e., eight weeks.

Lipid Oxidation During Storage

Mealworms are rich in fats (10–30% DM) and contain high amounts of unsaturated fatty acids (70–75%), which makes them vulnerable to lipid oxidation during storage (Lenaerts et al., 2018; Kröncke et al., 2019). The peroxide and *p*-anisidine values, used as an index for primary and secondary lipid oxidation, respectively, are shown in **Table 2**. Similar as in the mealworm pastes prepared from steamed larvae (De Smet et al., 2019), no primary oxidation occurred in the pastes investigated in this study. During storage, the peroxide values remained below the detection limit of 0.5 meq. O_2/kg fat. However, primary oxidation products are very unstable and decompose rapidly into secondary oxidation products that are responsible for sensory deterioration (Domínguez et al., 2019). Thus, when evaluating the fat quality, both parameters have to be considered. As shown in **Table 2**, *p*-anisidine values greater than zero were found for all samples. The *p*-anisidine values of the fermented pastes were higher than those of the unfermented pastes at the start of the storage period indicating that oxidation already occurred during fermentation. While their values increased toward the end of the experiment, *p*-anisidine values of the unfermented samples decreased. Despite these differences, all anisidine values were far below the EFSA criterion for fish oil (*p*-anisidine < 20), which is often used as a reference for edible insects. So it can be concluded that the oxidation status of all the pastes were acceptable until the end of the storage period, regardless of the preservative treatment applied.

TABLE 2 | Peroxide and *p*-anisidine values of pastes prepared from blanched mealworms without additive (Control), with the preservatives sodium nitrite or sodium lactate or fermented with the starter cultures Bactoferm® F-LC or *Lactobacillus farciminis* and stored at 4°C for 28 (unfermented pastes) or 56 days or 8 weeks (fermented pastes).

Sample	Sampling day	Peroxide value (meq.O_2/kg fat)	*p*-anisidine value (–)
Control	0	<LOD	$1.39 \pm 0.16^{a,A}$
	28	<LOD	$1.05 \pm 0.01^{b,A,B}$
Sodium nitrite	0	<LOD	$1.39 \pm 0.16^{a,A}$
	28	<LOD	$0.86 \pm 0.21^{b,A}$
Sodium lactate	0	<LOD	$1.39 \pm 0.16^{a,A}$
	28	<LOD	$0.72 \pm 0.20^{b,A}$
Bactoferm® F-LC	0	<LOD	$1.99 \pm 0.17^{a,B}$
	28	<LOD	$1.84 \pm 0.27^{a,C}$
	56	<LOD	$2.76 \pm 0.24^{b,A}$
L. farciminis	0	<LOD	$1.85 \pm 0.21^{a,B}$
	28	<LOD	$1.76 \pm 0.50^{a,B,C}$
	56	<LOD	$2.75 \pm 0.79^{a,A}$

LOD = Limit of detection (0.5 meq.O_2/kg fat). a, b Mean values per treatment with the same superscript are not statistically different (P > 0.05). A, B, C Mean values per storage time with the same superscript are not statistically different (P > 0.05). Values are means of three replicates ± standard deviation.

Cost-Effectiveness and Application Potential

While fermentation shows to be a promising conservation strategy for mealworm pastes, a number of aspects still need further research to assess the cost-effectiveness and application potential of fermented pastes. In the first place, future research on mealworm fermentation and storage of fermented pastes also needs to consider the impact on taste and consumer acceptance. Having a pH of around 4.5, the fermented product likely contains a large amount of lactic acid which causes a favorable or unfavorable taste for animals and humans. Whether fermentation can also be applied for other insect species that are reared at industrial scale, also remains to be investigated. If the taste is (at least) acceptable, then a range of applications in feed, petfood and food industry can be envisaged. Currently, when insects are used in a feed formulation for farm animals, mealworms (and other insects) are dried (or frozen and then dried), because it is the only conservation technology known so far and applied in industry to provide a shelf life long enough to set up a proper logistic chain. However, freezing, freeze drying, and oven drying are known to imply a substantial cost price, mainly due to energy consumption (Lenaerts et al., 2018). Feed producers are able to include up to 10% of liquid ingredients in their dry feed formulas, which makes them interested in insects as feed ingredients that are stabilized in other (and more cost-efficient) ways than drying (Heutink, ForFarmers, personal communication). If their taste is accepted by pets, fermented insect pastes may well find an application as palatant or digest to be sprayed on kibbles or included in wet foods. Also, petfood manufacturers are increasingly looking at opportunities to claim probiotic effects for their wet and dry formulas (Anonymous, 2016; Coman et al., 2019), which could be an asset of integrating fermented insects as well. In the same way as for animal feed, in the food industry fermented (and hence stabilized) insect pastes may likely be incorporated in foods as alternative to dried insects, or new functional ingredients may be developed from fermented insects.

CONCLUSION

The addition of a sodium nitrite and a sodium lactate preservative at their maximally allowed concentration to pastes prepared from blanched mealworms could not prevent microbial spoilage during refrigerated storage. In contrast, lactic acid fermentation of the pastes with Bactoferm® F-LC or *L. farciminis* avoided microbial spoilage during the whole test period of eight weeks

Typical food pathogens such as *Bacillus cereus*, *Salmonella* spp., and *Listeria monocytogenes* were not detected in any of the mealworm samples tested. Future research with mealworms artificially contaminated with food pathogens has to elucidate whether fermentation can also prevent outgrowth of those pathogens. Microbial growth occurred at a faster rate than lipid oxidation and was the major determinant of shelf life. This study demonstrates that lactic acid fermentation is a valuable preservation technique for mealworm pastes.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

AB designed and performed the experiments, processed the data, and drafted the manuscript with input from all authors. RS assisted with the fat oxidation measurements. LV supervised the project, responsible for the financing and edited and proofread the text. All authors contributed to the article and approved the submitted version.

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Reduction of Bacteria in Relation to Feeding Regimes When Treating Aquaculture Waste in Fly Larvae Composting

Ivã Guidini Lopes[1,2], Cecilia Lalander[2], Rose Meire Vidotti[1,3] and Björn Vinnerås[2]*

[1] Aquaculture Center of Unesp (Caunesp), São Paulo State University (UNESP), São Paulo, Brazil, [2] Department of Energy and Technology, Swedish University of Agricultural Sciences, Uppsala, Sweden, [3] São Paulo Agency of Agribusiness and Technology (APTA), Polo Regional Centro-Norte, Pindorama, Brazil

This study evaluated the impact of feeding regimes on process performance and inactivation of microorganisms during treatment of aquaculture waste with black soldier fly (BSF) larvae. In three treatments (T1–T3), a blend of reclaimed bread and aquaculture waste was used as substrate for BSF larvae. In T1, the substrate was inoculated with four subtypes of *Salmonella* spp. and *Escherichia coli* (both at 1% *w/w*), and offered only once, at the beginning of the 14-day trial. In T2 and T3, the substrate was supplied on three different days, with contaminated substrate provided only the first event in T2 and in all three events in T3. Provision of a lump sum feeding (T1) proved unfavorable for larval growth and process efficiency, but did not affect the microbial reduction effect. The total reduction in *Salmonella* spp. was approximately 6 $\log_{10}$ in T1 and T2, and 3.3 $\log_{10}$ in T3, while the total reduction in *E. coli* was approximately 4 $\log_{10}$ in T1 and T2, and 1.9 $\log_{10}$ in T3. After removing the larvae, the treatment residues were re-inoculated with *Salmonella* spp. and *E. coli*. It was found that the inactivation in both organisms continued in all treatments that originally contained BSF larvae (T1–T3), suggesting that antimicrobial substances may have been secreted by BSF larvae or by its associated microbiota.

Keywords: BSF, ecotechnology, hygienization, *Escherichia coli*, Salmonella Senftenberg, *S.* Typhimurium, *S.* Typhi, *S.* Dublin

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****Correspondence:***
Ivã Guidini Lopes
ivanguid@gmail.com

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INTRODUCTION

Global fisheries and aquaculture production exceeded 170 million tons in 2016. Of this, 47% was produced exclusively by aquaculture, a fast-growing industry providing high-quality animal protein worldwide (FAO, 2018). Generation of solid organic waste occurs throughout all production steps in aquaculture, from nurseries to fattening stages, at fish processing plants, research centers and up to the final consumer (Love et al., 2015; Lopes et al., 2019). Fish waste (e.g., whole carcasses and body parts) typically contains high nutrient loads, which can be detrimental to the environment if inadequately disposed, causing soil and water contamination (Erondu and Anyanwu, 2005). In addition, as this waste stream decomposes rapidly, its microbial communities multiply during decomposition, thus posing a risk of disease transmission, as these residues may contain pathogens (Ghaly et al., 2010; Sousa et al., 2014).

It is well established that fish carry a diverse community of microorganisms in their gut and skin (Leroi and Joffraud, 2011). These include autochthonous microbiota and pathogenic bacteria originating from the aquatic environment where the fish are reared and from inadequate processing (Mol and Tosun, 2011). Cross-contamination may also occur and compromise non-contaminated materials, e.g., when the same processing equipment is used for contaminated and non-contaminated fish (Ghaly et al., 2010). The different microorganisms present include Gram-positive bacteria such as *Enterococcus* spp. and *Clostridium* spp. (Leroi and Joffraud, 2011), and Gram-negative bacteria belonging to the Enterobacteriaceae family, such as *Escherichia coli* and *Salmonella* spp. (Morris et al., 1970; Marchaim et al., 2003). Under favorable conditions (e.g. moisture and temperature), fish spoilage proceeds from autolytic deterioration to microbial degradation within 2–5 days (Shawyer and Pizzali, 2003).

Sanitization of organic wastes can be achieved by various treatment methods, such as thermophilic composting (Soobhany et al., 2017) and anaerobic digestion (Grudziñsk et al., 2015). A novel treatment using larvae of the black soldier fly (BSF) (*Hermetia illucens* L.) (Diptera: Stratiomyidae) has also been shown to be promising in relation to the sanitization of different biodegradable waste streams. During treatment by BSF larvae, initial waste volumes can be reduced by up to 85% (wet basis), while generating two marketable products: a protein-rich larval biomass to be used in replacement of soybean and fishmeal in animal diets (Wang and Shelomi, 2017), and an organic treatment residue that can be used as a soil amendment (Setti et al., 2019), both representing more sustainable alternatives for the transition to a circular economy. Additionally, there is evidence that BSF larvae treatment of organic wastes have an impact on the concentration of selected microorganisms, thus improving its hygiene quality (Erickson et al., 2004; Choi et al., 2012; Lalander et al., 2013).

In a study examining the effect of BSF larvae treatment of chicken manure inoculated with *E. coli* and *S. enterica*, Erickson et al. (2004) observed that the presence of larvae accelerated inactivation of both bacteria. However, they also found that, while the observed reductions in microbial levels were significant, they were insufficient to ensure complete safety of the treated manure as a soil amendment. Similarly, Lalander et al. (2013) found significant reductions in *Salmonella* spp. when treating human feces with BSF larvae, but detected the pathogen in the gut of the larvae at the end of treatment. They recommended an additional processing step for the product to ensure its hygienic quality.

Although several studies have demonstrated the capacity of BSF larvae to inactivate microorganisms, the mechanism behind this inactivation remains poorly understood. Among several existing interactions between BSF larvae and the environment, two possible mechanisms for the inactivation have been suggested: (1) passage through the BSF larvae gut and associated exposure to low pH (Coluccio et al., 2008); and (2) secretion of antimicrobial substances, such as organic acids and peptides that bind to the bacterial cell wall, by BSF larvae (Choi et al., 2012; Park et al., 2014; Vogel et al., 2018). Other studies have demonstrated strong interactions between the medium and BSF larvae microbiota, with significant interferences related to biotic and abiotic factors that could lead to distinct microbiological responses in the treatment of wastes as a whole (Wynants et al., 2018; Jiang et al., 2019). The mechanism that contributes most to microorganism inactivation, and whether different feeding regimes generate different results in terms of inactivation, remain to be determined.

Different systems (batch or semi-batch feeding systems) for organic waste treatment using BSF larvae have produced promising results in terms of waste sanitization, in particular for two microorganisms: *E. coli*, an indicator microorganism of fecal contamination, and *Salmonella* spp., a zoonotic pathogen (Hasan et al., 2019). Erickson et al. (2004) observed the inactivation of these microorganisms in a batch-mode system using different manures as growth substrate, and observed significant reductions in the populations of both (1.5 – 5 log for *E. coli* and 0.5 – 4.0 log for *Salmonella* spp. at varying temperatures). In a continuous BSF larvae reactor to which substrate was added three times a week and small larvae twice a week, Lalander et al. (2015) obtained significant reductions in *Salmonella* spp., but observed small inactivation in thermotolerant coliforms, where *E. coli* is the dominant species (Hachich et al., 2012). These findings suggest that there could be an impact of treatment system, the time in the treatment at which contaminated waste is added and feeding regime, on microorganism inactivation in BSF larvae treatment.

The aims of this study were to investigate the impact of feeding regimes and time of bacterial contamination on inactivation of selected microorganisms, and assess whether the treatment residue had antimicrobial properties.

MATERIALS AND METHODS

Materials

Reclaimed bread of different brands and nutritional composition was donated by a local distribution company in Uppsala, Sweden (Fazer®). The selected model substrate for aquaculture waste (*Oncorhynchus mykiss* carcasses) was supplied by a commercial fish farm (Nordic Trout®) located in Mora (Sweden). Upon collection, the bread was manually shredded and placed inside plastic bags, while the aquaculture waste was milled in a meat grinder, homogenized, and placed in plastic bags. Both materials were kept at −18°C until use.

In a pre-trial performed before the start of the experiment, it was observed that the pH of the substrate (aquaculture waste and bread) declined significantly (<4.0) after approximately 72 h. This confounded the aim of the study, as pathogen survival may be jeopardized by low pH conditions. In order to avoid this drop in pH, the bread was moistened with a phosphate buffer solution (pH 7.2; SVA, Sweden) prior to the beginning of the experiment, to a moisture content of approximately 65%.

The larvae used in the experiments were obtained from a colony that has been running continuously since 2015, located at the Swedish University of Agricultural Sciences (SLU, Uppsala).

Newly hatched larvae were reared on a substrate containing chicken feed (Granngården Hönsfoder Start) 20% dry matter, DM) and larvae treatment residues (1:1 ratio), for approximately 7 days. Larvae (1.2 mg wet mean weight) were separated by sieving (1 mm mesh), and three batches of 100 larvae were counted and weighted for batch weight estimation.

The pathogens used in this experiment were four serotypes of the Gram-negative bacteria *Salmonella enterica* (*S.* Senftenberg, *S.* Typhimurium, *S.* Typhi, and *S.* Dublin), and *E. coli* ATCC 13706, a specific microorganism used as an indicator of fecal contamination. The Gram-positive bacteria *Enterococcus* spp. was also evaluated throughout the study. It was not inoculated into the substrate, but was already present in the fish carcasses, at an approximate concentration of 10^5 CFU g^{-1}. *E. coli* (unknown strain) was also present in the carcasses at low concentrations (10^3 – 10^4 CFU g^{-1}) – which would be further diluted by the addition of bread – thus we chose to inoculate this bacteria in the substrate in order to begin with higher concentrations and more stable populations.

Bacterial inoculate solutions were prepared according to the following procedure: the selected bacterial strains were grown at 37°C for 2 h, at 200 revolutions per minute (rpm), in 5 mL nutrient broth (Oxoid AB, Sweden). This concentrated solution was diluted in 45 mL of nutrient broth and kept in the same conditions for 24 h. Finally, the bacterial solution was centrifuged at 4500 rpm for 15 min at 15°C, and the pellet was collected and dissolved in 50 mL of Tween buffer (buffered NaCl peptone water with Tween 80 at pH 7; SVA, Sweden). The acquired concentrations of *Salmonella* spp. and *E. coli* in these concentrated solutions were approximately 10^7 and 10^6 CFU g^{-1}, respectively, being determined by plating different diluted solutions originated from the concentrated solutions. The inoculation was performed directly on the combined substrate, at a rate of 1% *w/w*, before it was added to the treatments.

Experimental Set-Up and Sampling

Two experiments were performed, aimed at: (1) determining the impact of feeding regime on BSF larvae treatment; and (2) investigating the inactivation potential of BSF larvae in the treatment of contaminated wastes, in order to evaluate the antimicrobial potential of the treatment residue, which serves as an indication of the presence of antimicrobial substances. The first experiment consisted of a control (CT) and three treatments (T1–T3), representing different feeding strategies. In T1, the feeding substrate was inoculated with four salmonella strains and *E. coli* and added once, on the first day of the experiment. In T2 and T3, the substrate was added three times, on day 1, day 4, and day 7, but was only inoculated on the first feeding (day 1) in T2, whereas the substrate in all three feedings was inoculated in T3. The control treatment followed the same protocol as in T1, but without addition of larvae, in order to determine the impact of these organisms (**Figure 1**).

The experimental units used in the first experiment were plastic containers (21 × 17 × 11 cm) covered with netting to prevent escaping of larvae. Three replicates per treatment were assembled. A total of 1000 larvae were placed inside each unit, giving an approximate density of 4 larvae cm^{-2}, and a feeding rate of 0.25 g volatile solids (VS) $larvae^{-1}$ was applied (80% aquaculture waste, 20% bread waste on wet basis). The pH of the treatment residues was measured on days 1–4, 7, 10, and 14, as was the total ammonium nitrogen (TAN) concentration. On day 14 of the experiment, the larvae were manually separated from treatment residues and one sample of approximately 30 g was collected per replicate (larvae and residue) for analysis of DM, VS, and total nitrogen (N_T) content (**Figure 1**).

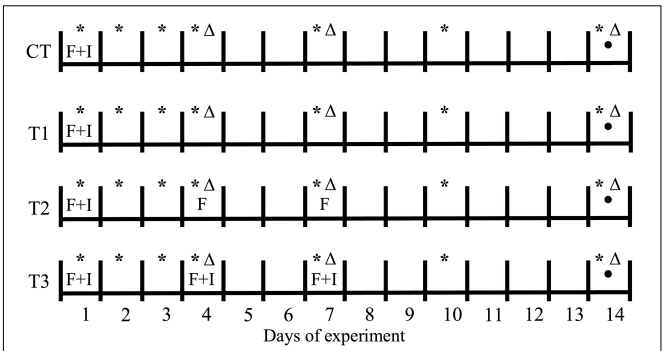

FIGURE 1 | Schematic representation of the feeding and pathogen inoculation protocols throughout the first 14-day experiment with BSF larvae. CT, treatment without BSF larvae; F, feeding event; I, inoculation of pathogens; (*) pH measurement; (Δ) evaluation of total ammonium nitrogen (TAN) in the treatment residues; (•) total nitrogen (N_T) measurement.

In the second experiment (called regrowth trial), all treatments considered in the first trial, including CT, were included. Triplicate, 5 g samples, of the treatment residues (materials remaining in the experimental units after the first experiment) from each replicate were collected using a sterile spoon, individually transferred to 50 mL Falcon tubes, and inoculated with the same concentrated solutions of *Salmonella* spp. and *E. coli* as were used in the first experiment, with an approximate concentration of 10^6 CFU g^{-1} and 10^5 CFU g^{-1}, respectively. After 24, 72, and 120 h, the concentrations of these bacteria were assessed, as described ahead. Both experiments were conducted in a Class II microbiological laboratory. The mean temperature inside the laboratory was 28.8 ± 1.8°C throughout the experiments.

Physico-Chemical Analysis

All samples of larvae and treatment residues (30 g) were dried at 105°C for 14 h for DM determination and combusted at 550°C for 6 h for total VS evaluation. For pH measurement, 5 g of treatment residues from each experimental unit were dissolved in 20 mL of deionized water and agitated in a vortex mixer for 2 min. After 1 h at room temperature, the pH was measured using a bench pH meter (InoLab pH level 1).

For N_T analysis, 0.5 g of sample was diluted in 15 mL of concentrated H_2SO_4 and brought to a "rolling boil" for approximately 20 min on a heating plate, and cooled to room temperature. A 1 mL sample from this mixture was diluted 50 times in deionized water, and the pH was neutralized to a range of 4–7, using a 10 M NaOH solution. A 10 mL sample of this solution was digested following the instructions of the analysis kit Spectroquant® Crack-Set 20 (1.14963.0001), and the

nitrate concentration was measured using a spectrophotometer at 340 nm, following the provider's instructions of the kit Spectroquant® Nitrate Test (1.09713.0002). For analysis of TAN concentrations in treatment residues, 1 g of sample was dissolved in 4 mL of deionized water, mixed in a vortex mixer for approximately 2 min, and the concentration was measured using a spectrophotometer at 640 nm, following the instructions of the provider of the analysis kit Spectroquant® Ammonium Test (1.00683.0001).

Microbiological Analysis

Microbial concentrations were assessed on a daily basis in the first experiment and on days 1, 3 and 5 in the second experiment, according to the following procedure: 5 g of treatment residues were dissolved in 45 mL of Tween buffer (this dilution was named 10^{-1}), and serial dilutions were prepared from this concentrated solution after a 15 min resting period. A 100 μL volume of the selected dilution was spread on xylose lysine desoxycholate agar (XLD) with novobiocin (Oxoid AB, Sweden) for *Salmonella* spp. enumeration, and on chromocult coliform agar for *E. coli* enumeration, both incubated at 37°C for 24 h. A 100 μL volume was also spread on Slanetz-Bartley agar (Oxoid AB, Sweden) and incubated at 44°C for 48 h for *Enterococcus* spp. enumeration (only the first experiment). Three plates were prepared daily for each of the pathogens evaluated per replicate in the first experiment and on the three evaluation days in the regrowth experiment. In order to reduce the detection limits of *Salmonella* spp. and *E. coli* concentrations, a 200 μL volume of the concentrated solution (10^{-1}) was spread on five plates for each sample and enumerated with a detection limit of 10 CFU mL^{-1}.

Calculations

DM losses and N volatilization were assessed by deducting the amount found in the residues and larvae at the end of the experimental period from the total amount added to the treatment. Survival rates were calculated in T1, T2, and T3, and process performance was evaluated based on material reduction, bioconversion ratio (percentage of substrate converted into insect biomass), and protein conversion ratio (proportion of added protein converted into larval protein), all presented in percentage.

The material reduction was calculated as:

$$\text{Material reduction} = 1 - \frac{m_{DMres}}{m_{DMsub}} \quad (1)$$

where m_{DMres} and m_{DMsub} are dry mass of final residues and initial substrate, respectively.

The bioconversion ratio (BCR) on dry matter basis was calculated as:

$$BCR = \frac{m_{DMlarvae}}{m_{DMsub}} \times 100 \quad (2)$$

where $m_{DMlarvae}$ is dry mass of larvae at the end of the experiment.

The protein conversion ratio (PCR) was calculated as:

$$\text{ProteinCR} = \frac{m_{DMlarvae} \times \%Pr_{larvae}}{m_{DMsub} \times \%Pr_{sub}} \times 100 \quad (3)$$

where $\%Pr_{larvae}$ and $\%Pr_{sub}$ are total percentage of crude protein (DM basis) in larvae and initial substrate, respectively.

A further three indices were used to assess pathogen inactivation: inactivation rate constant (k), which reveals the $\log_{10}$ reduction per time unit ($\log_{10}\ d^{-1}\ h^{-1}$); decimal reduction ($D_{90}$), representing the time (days) needed for a 1 $\log_{10}$ (90%) reduction in the microorganisms initially present in the contaminated material; and total logarithmic pathogen reduction (ΔLogRed), which represents the total pathogen inactivation from beginning to the end of the experimental period.

Rate constant k was calculated as:

$$k = \frac{(\log_{10} N_t - \log_{10} N_0)}{(N_t - N_0)} \quad (4)$$

where N_t and N_0 are bacterial concentration at time t and at the beginning, respectively.

D_{90} was then calculated as:

$$D_{90} = \left(\frac{-1}{k}\right) \quad (5)$$

$\Delta LogRed$ was calculated as:

$$\Delta\text{LogRed} = \log_{10}\left(\frac{CM_{at\ t=0}}{CM_{at.out\ t=i}}\right) \quad (6)$$

where $CMat_{t=0}$ is the estimated initial concentration in treatment residues, and $CMat.out_{t=i}$ is the final concentration at time i.

Statistical Analysis

Statistical analyses were performed using R software, version 3.5.3 (R Core Team, 2019), and GraphPad Prism, version 8.2.1. The assumptions of normality of error (Shapiro–Wilk's test) and homoscedasticity of variance (Levene's test) were verified for all process efficiency and pathogen inactivation data. One-way analysis of variance (ANOVA) with 95% confidence interval was performed to compare larval growth and process efficiency parameters, and the variables relating to pathogen inactivation. When significant differences were found, a Tukey *post hoc* test was performed at 5% significance level, to look for differences between treatments in the variables analyzed. Linear regressions were performed to assess the effect of time on pathogen inactivation.

RESULTS

Process Efficiency

The substrate biomass reduction exceeded 65% (DM basis) in all treatments containing larvae, which was significantly higher

than in the control treatment (CT) without larvae (**Table 1**). Larvae growth also differed significantly between treatments. After 14 days, larvae fed only once at the beginning of the trial (T1) were on average 35% lighter than larvae fed three times (T2 and T3). Hence, the bioconversion and protein conversion ratios were approximately 30 and 20% lower, respectively, in T1 than in T2 and T3. Larval survival and crude protein (%) did not vary significantly between treatments (**Table 1**). The difference in bacteria inoculation regime between T2 and T3 (where T3 received *Salmonella* spp. and *E. coli* at each feeding and T2 only at first feeding) did not affect the parameters evaluated. The larvae recovered at day 14 were all pre-pupae in all trials.

The pH of the feeding substrate at the beginning of the experiment was around 6.8, while at the end of the experiment it varied from 6.1 (CT) to 6.8 (T1), being significantly higher in T1 in comparison to other treatments. DM loss was higher in T1, where a single batch of substrate was supplied on the first day, than in T2 and T3. In all treatments, the remaining residues were dry after 14 days, with a DM content of 80–88%. The N_T concentrations in the treatment residues were not significantly different between treatments with larvae, but N volatilization was significantly higher (51%) in T1 than in T2 and T3 (38%) (**Table 2**).

The TAN concentrations increased with time in all treatments. Significant differences between treatments were found only at day 14, when treatments containing BSF larvae showed higher TAN concentrations than CT (**Figure 2**).

Pathogen Inactivation

The concentrations of *Salmonella* spp. and *E. coli* were significantly reduced in all treatments after 14 days, while *Enterococcus* spp. concentration was not reduced in any of the treatments. Additionally, the differences between feeding regimes resulted in differences in microbial reduction patterns (**Figure 3**). The initial concentration of *Salmonella* spp. and *E. coli* in the inoculated substrate was approximately 10^7 and 10^6 CFU g^{-1}, respectively, in all treatments. At the first day post-inoculation, increases of approximately 1 $\log_{10}$ were found for both bacteria in all treatments, while *Enterococcus* spp. populations increased by 1 $\log_{10}$ during the 14 days of the experiment (**Figure 3**).

The reduction in both *Salmonella* spp. and *E. coli* populations started within approximately 1 week of treatment and continued until the end of the experiment (**Figure 3**). In T3, where inoculated substrate was added three times, the concentrations of the evaluated microorganisms increased on subsequent days. However, the reduction in *Salmonella* spp. and *E.* coli in T3 at the end of the experiment was approximately 2.6 $\log_{10}$ and 2.2 $\log_{10}$ lower, respectively, than in the other treatments containing BSF larvae (**Figure 3**).

The reduction of *Salmonella* spp. was 5.8 $\log_{10}$ in T1, 6.2 $\log_{10}$ in T2, and 3.4 $\log_{10}$ in T3, while the reduction in the control (CT) with no larvae was 0.4 $\log_{10}$ (**Table 3**). The reduction rate (k; $\log_{10}$ CFU g^{-1} day^{-1}) was similar over time for both *Salmonella* spp. and *E. coli* in T1 and T2, but significantly faster than in the control (**Table 3**). Similarly, the decimal reduction (D_{90}) calculations

TABLE 1 | Efficiency of treatment of aquaculture waste and bread using black soldier fly (BSF) larvae.

	CT	T1	T2	T3
Bioconversion ratio (%)	–	16.7 ± 2.2^b	24.2 ± 1.5^a	24.3 ± 0.4^a
Protein conversion ratio (%)	–	14.3 ± 3.1^b	19.2 ± 1.8^a	18.1 ± 1.0^a
Material reduction (%)	35.9 ± 5.6^b	70.1 ± 2.6^a	65.6 ± 1.9^a	66.2 ± 2.1^a
Final weight (mg)	–	109.0 ± 13.0^b	165.0 ± 23.9^a	171.9 ± 12.7^a
Larval CP ($\%_{DM}$)	–	47.6 ± 4.9	44.1 ± 2.3	41.5 ± 3.1
Survival (%)	–	86.1 ± 2.7	93.6 ± 10.7	90.1 ± 5.7

CP, larval crude protein; CT, wastes inoculated with pathogens without larvae; T1, larvae fed once with inoculated wastes; T2, larvae fed three times, with wastes inoculated only the first time; T3, larvae fed three times, with wastes inoculated all three times. Values presented are mean ± SD. Different letters row-wise indicate significant differences ($p < 0.05$) between treatments.

TABLE 2 | Physico-chemical characteristics of the treatment residues obtained when black soldier fly (BSF) larvae were fed aquaculture waste and bread, and losses of dry matter and nitrogen after 14 days of treatment.

	CT	T1	T2	T3
Residue DM (%)	88.4 ± 1.3^a	83.2 ± 2.0^b	79.1 ± 2.3^b	82.2 ± 0.4^b
Residue VS (%)	94.5 ± 0.9^a	91.4 ± 1.2^{ab}	89.7 ± 1.7^b	89.6 ± 0.8^b
DM loss (%)	35.9 ± 5.6^b	53.4 ± 0.5^a	41.4 ± 2.4^b	41.9 ± 1.9^b
N_T (g kg^{-1})	ND	24.3 ± 1.3	24.4 ± 0.3	26.0 ± 1.7
N volatilization (%)	ND	51.3 ± 1.3^a	38.8 ± 1.6^b	37.9 ± 0.9^b
Final TAN (mM kg^1)	11.3 ± 2.5	12.9 ± 1.9	13.1 ± 6.8	12.3 ± 3.8
Initial pH	6.7 ± 0.1	6.8 ± 0.1	6.8 ± 0.0	6.9 ± 0.1
Final pH	6.1 ± 0.2^c	6.8 ± 0.1^a	6.5 ± 0.1^b	6.4 ± 0.1^b

DM, dry matter; VS, volatile solids; N_T, total nitrogen; TAN, total ammonium nitrogen; ND, not determined; CT, wastes inoculated with pathogens without larvae; T1, larvae fed once with inoculated wastes; T2, larvae fed three times, with wastes inoculated only the first time; T3, larvae fed three times, with wastes inoculated all three times. Values presented are mean ± SD. Different letters row-wise indicate significant differences ($p < 0.05$) between treatments.

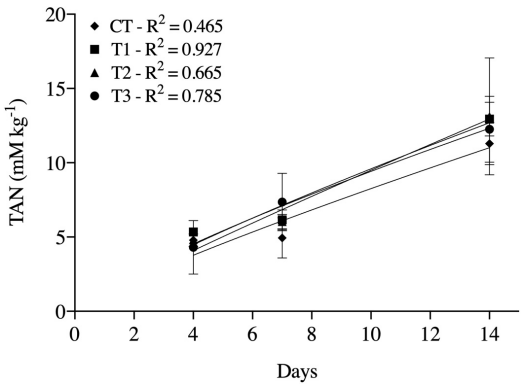

FIGURE 2 | Total ammonium nitrogen (TAN) generated at days 4, 7, and 14 in treatment of aquaculture waste and bread with black soldier fly (BSF) larvae. CT, wastes inoculated with pathogens without larvae; T1, larvae fed once with inoculated wastes; T2, larvae fed three times, with wastes inoculated only the first time; T3, larvae fed three times, with wastes inoculated all three times. R^2 values are coefficient of determination of the regression lines fitted for each treatment.

revealed that the required time to reduce *Salmonella* spp. and *E. coli* populations by 90% (1 $\log_{10}$) was 2.4 days and 3.5–4.5 days in T1 and T2, respectively.

Based on the D_{90} and k values obtained for T3, a 90% reduction in both *Salmonella* spp. and *E. coli* from the first to last day of experiment took twice as long as in T1 and T2. Since T3 received inoculated substrate again on days 4 and 7, additional calculations of D_{90} and k were performed to assess the inactivation potential of this particular feeding regime. The D_{90} value from the day post final feeding (day 8) to the final day (day 14) for *Salmonella* spp. and *E. coli* was found to be lower than the value based on the concentrations found on days 1 and 14 of the experiment. Similarly, the k was found to be lower, 0.291 and 0.198 $\log_{10}$ CFU g^{-1} day^{-1} for *Salmonella* spp. and *E. coli*, respectively (**Table 3**).

Regrowth Trial

The regrowth of microorganisms in the treatment residues (without larvae) in all treatments was evaluated for the four *Salmonella* strains and *E. coli*. The initial achieved concentration of *Salmonella* spp. and *E. coli* was approximately 10^6 and 10^5 CFU g^{-1}, respectively. After 3 days, no reduction was observed for neither of these microorganisms. However, after 5 days, a reduction of approximately 2.5 $\log_{10}$ was observed in the treatments that previously had larvae (T1, T2, and T3), while the concentration remained unchanged in the control that had no had BSF larvae (CT). The total reduction in *E. coli* was similar to that in *Salmonella* spp. on residues from T1 and T2, as revealed by the $\Delta\log_{10}$Red and k values after 5 days of the experiment. However, the total reduction on residues from T3 was significantly lower for *E. coli* (total reduction of 1.8 $\log_{10}$), while an increased concentration was found for the control treatment residue after 5 days (**Table 4**).

DISCUSSION

Efficiency of BSF Larvae Treatment in Different Feeding Regimes

The proportion between aquaculture and bread waste was adopted based on the results obtained by Lopes et al. (2020), whom observed that the addition of more than 25% of aquaculture waste to bread makes the treatment of this waste unfeasible. Supplying aquaculture-bread wastes as feeding substrates following different protocols (T1–T3) resulted in differences in growth and bioconversion ratios of BSF larvae, but did not affect survival of the larvae. Larvae in treatments T2 and T3 gained more weight than those in T1, which resulted in lower bioconversion ratio and protein conversion ratio (DM basis) in T3. However, the substrate biomass reduction was similar (∼65%) in all treatments containing larvae, indicating that more of the material was microbially degraded with only one substrate supply (T1), and that BSF larvae more efficiently converted a larger proportion of the substrate when three feedings were performed (T2 and T3).

Physico-chemical changes during fish waste microbial spoilage have been shown to result in lipid oxidation and protein degradation, through which N is lost by volatilization (Ghaly et al., 2010). Our results support this hypothesis, as N volatilization was higher in T1 than in T2 and T3 (**Table 2**).

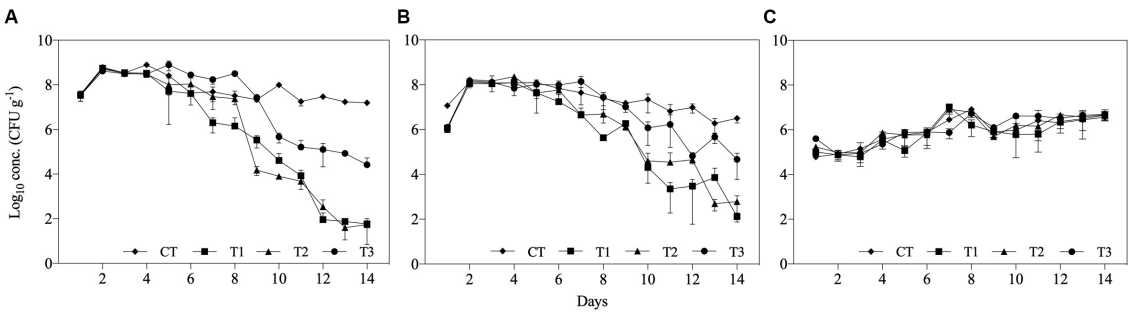

FIGURE 3 | $\log_{10}$ concentrations (CFU g^{-1}) of **(A)** *Salmonella* spp., **(B)** *Escherichia coli*, and **(C)** *Enterococcus* spp. over 14 days of treatment of aquaculture waste and bread treated with black soldier fly (BSF) larvae. CT, wastes inoculated with pathogens without larvae; T1, larvae fed once with inoculated wastes; T2, larvae fed three times, with wastes inoculated only the first time; T3, larvae fed three times, with waste inoculated all three times.

TABLE 3 | Values of the reduction constant (k, log_{10} $CFU\ g^{-1}\ day^{-1}$), decimal reduction (D_{90}, days), mean log_{10} reduction (Δlog_{10}Red), and p-value for different microorganisms in black soldier fly (BSF) larvae treatment of aquaculture waste and bread.

	Salmonella spp.				*E. coli*				*Enterococcus* spp.			
	k	D_{90}	Δlog_{10}Red	p for time effect	K	D_{90}	Δlog_{10}Red	p for time effect	k	D_{90}	Δlog_{10}Red	p for time effect
T1	-0.412^{a}	2.4^{c}	5.75^{a}	***	-0.281^{a}	3.5^{b}	3.97^{a}	***	0.117	–8.5	–1.63	*
T2	-0.413^{a}	2.4^{c}	6.22^{a}	***	-0.229^{a}	4.4^{b}	3.32^{ab}	***	0.102	–9.8	–1.41	**
T3	-0.222^{b}	4.5^{b}	3.28^{b}	***	-0.102^{b}	9.7^{c}	1.87^{bc}	*	0.077	–12.9	–1.07	**
CT	-0.027^{c}	36.4^{a}	0.34^{c}	**	-0.040^{c}	24.8^{a}	0.59^{c}	**	0.126	–7.9	–1.51	*
T3‡	–0.291	3.4	4.25	***	–0.198	4.4	3.14	**	–	–	–	–

*CT, wastes inoculated with pathogens without larvae; T1, larvae fed once with inoculated wastes; T2, larvae fed three times, with wastes inoculated only the first time; T3, larvae fed three times, with wastes inoculated all three times. Different letters column-wise indicate significant differences ($p < 0.05$) for k, D_{90} and Δlog_{10}Red values. ‡Recalculated indexes for T3, considering the period between day 8 (1 day post final feeding) and day 14 (end of experiment); *$p < 0.01$; **$p > 0.001$; ***$p < 0.0001$.*

Therefore, the slower BSF larvae growth in T1 could have been caused by nutrient imbalance in the degraded substrate, or by increased bacterial respiration in the substrate. In contrast, Banks et al. (2014) observed larger final body weight, but slower growth, of BSF larvae fed feces substrate in a lump sum rather than every two days. They concluded that aging feces had lower nutritional quality than fresh feces, and therefore the BSF larvae consumed larger amounts of the substrate in order to compensate for this deficiency. Based on the obtained results, the treatment of aquaculture waste is better performed when three batches of the substrate is supplied instead of only once.

Inactivation of Microorganisms

Escherichia coli is one of the most commonly used indicator organisms for fecal contamination (Carlos et al., 2010), and it occurs naturally in decaying carcasses of different fish species (Leroi and Joffraud, 2011), in accordance with the findings in this study. *Salmonella* spp., on the other hand, is a zoonotic bacteria that can infect most animals (Hasan et al., 2019) and several international standards demand absence of this microorganism in consumable products by animals and humans. It may also be found in decaying carcasses (Morris et al., 1970), however, this microorganism was not naturally observed in the carcasses used in this study. *Salmonella* spp. has been reported to be reduced in BSF larvae treatment, but the fate of *E. coli* is not consistent in previous studies. Therefore, they were chosen here as model microorganisms to evaluate the effect of treatment of aquaculture wastes.

Black soldier fly larvae treatment had strongest impact on *Salmonella* spp., with reductions of up to 6 log_{10} observed in T1 and T2. Based on larval growth and process efficiency parameters, *Salmonella* spp. inactivation was also affected by time of contamination: the treatment that received three contaminated feedings (T3) displayed a D_{90} of 4.5 days, in comparison with 2.4 days in T1 and T2, which resulted in lower total inactivation ($\sim$3.3 log_{10} reduction) after 14 days in T3. A pronounced lag phase was observed in the effect on *Salmonella* spp. growth in T1-3, as the concentration started to be reduced within approximately 5–7 days after the beginning of the experiment (**Figure 3A**). Therefore, the difference in reduction potential of T1 and T2 compared with T3 seemed to be a consequence of adding contaminated substrate more than once (**Figure 1**).

A few studies have demonstrated that different pathogens in the Enterobacteriaceae family can be inactivated in BSF larvae treatment, using different feeding systems. For example, using a continuous fly reactor, in which BSF larvae were fed with inoculated substrate three times a week and prepupae was continuously collected, Lalander et al. (2015) observed significant reductions in *Salmonella* Typhimurium (>7 log_{10}), beginning after 2 weeks of treatment and persisting for the remainder of their 9-week experiment, while the impact on thermotolerant coliforms was small. The authors hypothesized that a continuous system could improve the hygienic quality of the treated material compared with a batch-mode system, due to possible interactions of an established microbial and fungal community. As shown in the present study, it is not possible to adopt this BSF larvae treatment as a sole hygienization method, regardless of the system

TABLE 4 | Concentrations (log_{10}) of *Salmonella* spp. and *Escherichia coli* in treatment residues derived from black soldier fly (BSF) larvae treatment of aquaculture waste and bread, at the first (**In**) and fifth (**Out**) days of the regrowth trial, and values of the reduction constant (*k*, log_{10} *CFU* g^{-1} day^{-1}) and the mean log_{10} reduction (Δlog_{10}Red) achieved.

	***Salmonella* spp. (log_{10})**				***E. coli* (log_{10})**			
	In	**Out**	**Δlog_{10}Red**	***k***	**In**	**Out**	**Δlog_{10}Red**	***k***
T1	6.76	4.14^{b}	2.61^{a}	-0.186^{a}	5.80	3.44^{c}	2.37^{a}	-0.169^{a}
T2	6.69	4.09^{b}	2.60^{a}	-0.185^{a}	5.86	3.23^{c}	2.63^{a}	-0.187^{a}
T3	6.87	4.56^{b}	2.31^{a}	-0.165^{a}	6.05	4.22^{b}	1.84^{b}	-0.131^{a}
CT	7.04	7.11^{a}	-0.07^{b}	0.005^{b}	5.59	6.35^{a}	-0.76^{c}	0.054^{b}

CT, wastes inoculated with pathogens without larvae; T1, larvae fed once with inoculated wastes; T2, larvae fed three times, with wastes inoculated only the first time; T3, larvae fed three times, with wastes inoculated all three times different letters column-wise indicate significant differences ($p < 0.05$).

used, because, although some bacteria in the Enterobacteriaceae family are inactivated, the degree of inactivation is not sufficient to guarantee a completely safe end-product, regarding both larvae and treatment residues.

Gram-negative *E. coli* was inactivated over time up to 4 log_{10}, while in the study of Lalander et al. (2015), a small inactivation was verified for thermotolerant coliforms. Erickson et al. (2004) verified only a small reduction in *E. coli* at 27°C (1.5 log_{10}) and a more significant reduction (5 log_{10}) at 32°C in one of the three feeding substrates (chicken manure) they evaluated. As reported by the authors of that study, such a strong inactivation of *E. coli* at 32°C in comparison to lower temperatures, might be due to increased concentration of uncharged ammonia in the manure, which is known to increase with temperature (Nordin et al., 2009). Zdybicka-Barabas et al. (2017) studied the immune response of BSF larvae challenged with Gram-positive (*Micrococcus luteus*) and Gram-negative (*E. coli*) bacteria, and verified that the larvae produce specific sets of antimicrobial peptides to fight those bacteria, while other microorganisms remained unaffected. In addition, Vogel et al. (2018) suggested that the immune response of BSF larvae is at least partly diet-dependent, and that protein-rich diets might lead to stronger immune defenses. The authors found stronger antimicrobial activity against *E. coli* in larvae fed proteins, in comparison to larvae fed a plant oil-rich diet or a lignin-rich diet. In this sense, it is possible to assume that *E. coli* inactivation in the present study may have partly occurred due to excreted antimicrobial peptides induced by the influence of diet, which was protein-rich as fish carcasses were included.

No reduction in the Gram-positive bacteria *Enterococcus* spp. was found in this study (**Figure 3C**). Different types of antimicrobial peptides have been isolated from the BSF larvae that displayed significant effects against different bacteria strains. Choi et al. (2012) found antimicrobial peptides in methanol extracts of BSF larvae that showed antimicrobial properties against members of the Enterobacteriaceae family, but with no impact on Gram-positive bacteria, while Shin and Park (2019) successfully isolated attacin from BSF larvae, a type of antimicrobial peptide that has shown antimicrobial properties to selected Gram-negative and Gram-positive bacteria. However, in this last case, the antimicrobial substances were isolated from the larvae after an immunization process in which the production of these substances were induced in the larvae. Considering that Lalander et al. (2013) found high levels of *Enterococcus* spp. in BSF larval gut, suggesting that this is not a threat to the larvae, it may be that the BSF larvae does not produce any antimicrobial substance effective against this microorganism as an immune response. Alternative, the conditions under which the experiment was undertaken or similar to the case for *E. coli* the substrate, did not induce the production of these peptides in this case. Furthermore, other properties may have an impact. *Enterococcus* spp. has specific characteristics in their cell wall that assures higher endurance, such as greater thickness, composition with more peptidoglycans in comparison to Gram-negative bacteria, and higher resistance to ruptures (Hancock et al., 2014).

Another impacting factor could have been the TAN concentrations, as they were higher in treatments containing larvae (T1-3) than in the control (CT) without larvae (**Figure 2**). In a study that evaluated *Salmonella* spp. inactivation using urea and ammonia, Fidjeland et al. (2016) obtained significant inactivation of this pathogen at different temperatures (5–32°C) using high concentrations of ammonia (>50 mM). In the present study, TAN concentrations did not exceed 13 mM kg^{-1}, thus it is possible to assume that the effect of TAN on pathogen inactivation was possibly less relevant than the direct action of the BSF larvae. Similarly, although substrate temperature was not investigated directly in our study, it may have influenced microbial survival, as thoroughly demonstrated by Liu et al. (2008).

Bacteria Regrowth

It is difficult to ensure that microorganisms are entirely eliminated when treating organic wastes, regardless of the method used, as bacterial regrowth may occur under appropriate conditions (e.g. moisture, temperature, and pH), even after the end of treatment (Soobhany et al., 2017). The degree of maturity of compost has been demonstrated by Elving et al. (2010) to affect bacterial regrowth; they found negative correlations between the growth potential of *Salmonella* Typhimurium and the degree of maturity of an organic compost. The maturity of a material in a composting process (e.g. thermophilic composting and BSF larvae treatment) can be determined either by the self-heating capacity of the material (Brinton et al., 1994) or by assessing the CO_2 and NH_3 emissions

in the material, using simple commercially available tests (Changa et al., 2003). The treatment residue from fly larvae composting has been found to be that of raw compost (Lalander et al., 2018), so it is thus unlikely that the maturity of the residue was a main driver for continued inactivation in this study.

Although the impact of antimicrobial substances may play a key role in the inactivation of selected microorganism in a BSF larvae treatment, as discussed above, it is not clear whether the material has to pass through the larval gut to be in contact with these substances or whether they are excreted by the larvae. In the studies that have isolated antimicrobial substances, the substances were isolated from the larvae. Additionally, interactions between BSF larvae intrinsic bacteria and the medium bacteria may occur and further affect the dynamics of these microorganisms over time (Wynants et al., 2018; Jiang et al., 2019). The regrowth trial demonstrated that, after inoculating more microorganisms into the treatment residues, inactivation continued to occur even in the absence of BSF larvae, suggesting that antimicrobial substances were present in the treatment residue, or even that larval microbiota somehow affected microorganisms' survival. The same patterns in inactivation of studied microorganisms that were seen in the composting phase was also seen in the regrowth trial, further supporting the hypothesis that antimicrobial substances excreted into the material (in this case treatment residue) contributed to the observed reduction in *Salmonella* spp. and *E. coli.*

CONCLUSION

Feeding regimes were found to have an impact in BSF larvae composting of aquaculture waste. Larvae growth, bioconversion and protein conversion ratios was higher when the same amount of aquaculture waste was provided three times during the 14-days BSF larvae composting process as compared to when it was provided in a lump sum at the start of the treatment. Significant inactivation of *Salmonella* spp. (6 log) and *E. coli* (3.5 log) were achieved when the substrate was provided as a lump sum at the start, but when the substrate was inoculated three times these reductions were less pronounced. *Enterococcus* spp. was not affected by the treatment regardless of feeding regime. Even when larvae was removed from the treatment residues, both *Salmonella* spp. and *E. coli* continued to be inactivated in the material for 5 days, suggesting that there are antimicrobial substances present in the fly larvae composted material. Whether these substances originate from the larvae or from the larvae associated microbial community has to be investigated further. Regardless of the adopted feeding regimes, BSF larvae treatment cannot be considered a hygienization method, as it only improves the hygiene quality of the materials, thus it is recommended that both larval biomass and treatment residues undergo a post-treatment in order to ensure complete sanitization.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: Mendeley Data repository – http://dx.doi.org/10.17632/n52h7d4gk2.1.

AUTHOR CONTRIBUTIONS

IL: conceptualization, investigation, formal analysis, writing the original draft, and visualization. CL: conceptualization, investigation, formal analysis, resources, supervision, funding acquisition, and writing – review and editing. RV: conceptualization and supervision. BV: conceptualization, resources, supervision, funding acquisition, and writing – review and editing. All the authors contributed to the article and approved the submitted version.

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Salmonella Typhimurium Level in Mealworms (*Tenebrio molitor*) After Exposure to Contaminated Substrate

Annette Nygaard Jensen, Sussie Hjort Hansen and Dorte Lau Baggesen*

Division of Microbiology and Production, National Food Institute, Technical University of Denmark, Kongens Lyngby, Denmark

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***Correspondence:**
Annette Nygaard Jensen
anyj@food.dtu.dk

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Findings of viable *Salmonella* spp., which are important foodborne pathogens, are seemingly not reported in mealworms (*Tenebrio molitor*) for feed and food. Still, the bacterial load of mealworms is naturally high and includes members of the Enterobacteriaceae family to which *Salmonella* belong. This indicates that *Salmonella* may be able to thrive in mealworms if introduced into the production. Therefore, this study aimed to assess the quantitative level of *Salmonella enterica* serovar Typhimurium (ST) in mealworms over a 14-day course after exposure to substrate contaminated with ST levels from 1.7 to 7.4 log CFU/g at start (i.e., day 0). The level of ST found in larvae was below the quantitative detection level (1 or 2 log CFU/g) on day 1 in larvae exposed to contamination levels of 1.7, 3.4, and 3.6 log CFU/g opposed to contamination levels of 5.4, 5.6, and 7.4 log CFU/g, respectively. The maximum level of ST detected in individual 1-g larvae samples was 5.8 log CFU/g, but the level varied among the triplicate samples from each sampling, and the highest average value was 5.3 ± 0.3. Beyond day 7, only larvae exposed to the contamination level of 7.4 log CFU/g were >1.0 log CFU/g in the triplicate samples. However, qualitative testing (10 g) showed the presence of ST in larvae until the end of the experiment on day 14 except for the lowest contamination level of 1.7 log CFU/g. Parallel testing of surface disinfected larvae indicated that some larvae may be ST-positive due to *Salmonella* residing on the surface only. Still, any detection of *Salmonella* is of concern from a food safety perspective. In substrate with contamination levels below 3.6 log CFU/g, the level of ST was below the quantitative detection limit within a few days. Still, ST was detected until the end of experiment on day 14 except for the lowest contamination level of 1.7 log CFU/g. This study indicates the importance of avoiding introduction of *Salmonella* into the production, e.g., via contaminated substrate in order to avoid *Salmonella*-positive larvae as they remained positive for at least 14 days (except at the lowest contamination level).

Keywords: *Tenebrio molitor*, *Salmonella*, contamination level, persistence, food safety

INTRODUCTION

The interest in rearing of edible insects as a new valuable source of food and feed rich in proteins has increased significantly in the Western world following the publication 'Edible insects – Future prospects for food and feed security' from the Food and Agriculture Organization (FAO) in 2013 (van Huis et al., 2013). As for any other food and feed production chain, it is important to assess and control potential hazards associated to the production of insects and products derived thereof (Schlüter et al., 2016; van der Fels-Klerx et al., 2018; Raheem et al., 2019; Cappelli et al., 2020; Imathiu, 2020). Accordingly, The European Food Safety Authority (EFSA) prepared a risk profile related to production and consumption of insects as food and feed (EFSA Scientific Committee, 2015). Despite a scarcity of knowledge at that time about the specific risks associated to insects as a new form of mini-livestock, this risk profile emphasized the importance of hygienic conditions of the substrate and need for assessing the specific risks especially if other than food and feed-grade substrates are used (which is banned at present by EU regulation).

The EFSA risk profile also provided a list of insect species with high farming potential, which included the yellow mealworm (*Tenebrio molitor*) (EFSA Scientific Committee, 2015). As no appreciable consumption of mealworms took place in EU before May 15, 1997, mealworms are considered as Novel Foods by EU Regulation (EU) No 2015/2283 implying the requirement of authorization from EFSA before commercialization. Further, the general EU food law [Regulation (EC) No 178/2002] specifies that food and feed shall not be placed on market if unsafe. This also applies for insects even though the European Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs has set no specific criteria for mealworms (yet).

Due to a naturally high bacterial load of around 8 log CFU/g in *T. molitor* including opportunistic human pathogens, heat treatment or other processing is recommended to reduce the bacterial load before consumption (EFSA Scientific Committee, 2015; Schlüter et al., 2016; Stoops et al., 2016; Vandeweyer et al., 2017; Osimani et al., 2018; Wynants et al., 2018; Garofalo et al., 2019). The Enterobacteriaceae family is one of the highly represented bacterial groups in *T. molitor* larvae, and although *Salmonella* spp. belongs to this family, no findings of viable *Salmonella* has to our knowledge been reported yet (Grabowski and Klein, 2017; Vandeweyer et al., 2017; Osimani et al., 2018). This may be due to the use of feed-grade substrates as several insects as well as mealworms have vector potential for carrying *Salmonella* after exposure (Skov et al., 2004; Roche et al., 2009; Blazar et al., 2011; Nordentoft et al., 2017; Crippen et al., 2012, 2018; Wynants et al., 2019). Another explanation may be a potential capability of insects to fight incoming pathogens (Wu et al., 2018; Jo et al., 2019; Keshavarz et al., 2020).

As *Salmonella* spp. are important foodborne pathogens with >91,000 reported salmonellosis cases in EU annually, significant efforts are generally made to prevent their presence in feed and food production chains, e.g., stated by Regulation (EC) No 2160/2003 (European Food Safety Authority [EFSA], and European Centre for Disease Prevention, and Control [ECDC], 2019). A broad range of animals can carry *Salmonella* spp. and often without any symptoms, causing fecal excretion of *Salmonella* into the environment and potential transmission to other animals, crops and water reservoirs. Consequently, contaminated substrate, insufficient hygienic measures or lack of biosecurity for preventing entrance of infected wild insects, rodents and pets may introduce *Salmonella* spp. into insect production facilities.

For documenting the absence of *Salmonella* spp. in food, qualitative testing of the presence of *Salmonella* spp. by pre-enrichment of 10-g or 25-g samples is usually applied (e.g., ISO, 2017 6579-1). However, the level of *Salmonella* contamination is also of interest as dose–response modeling of *Salmonella* using outbreak data indicated that both the risk of infection and the risk of illness given infection increase with dose (Teunis et al., 2010). The dose response model found that the number of CFUs causing infection or illness in 50% (ID50) of exposed people were 7 and 36 CFU, respectively. Noteworthy, the bacterial cell count obtained from a sample, will depend on the ability to retrieve cells adequately from the sample matrix, which is affected by the nature of the matrix (e.g., fat content, physical structure) and the homogenization method applied (Rohde et al., 2015). Accordingly, proper sample treatment is important for enumerating the actual number of cells present and avoiding underestimation or false negative results. Still, there seem to be no suggestions for or evaluations of a proper and standardized treatment method for mealworm samples before enumeration or detection of the bacterial content.

One recent study assessed how the presence of *Salmonella* sp. during rearing of mealworms affected the survival in substrate and transmission to larvae (Wynants et al., 2019). The time course was here 7 days and it is of interest to see if longer time, e.g., will support clearance of *Salmonella*, which is relevant concerning mitigation options. In addition, more contamination levels, higher frequency of samplings and quantitative testing may help to a better understanding of the fate of *Salmonella* after introduction into a mealworm production site as well as the importance of the *Salmonella* contamination level.

In this study, we firstly aimed to test the ability to recover *Salmonella* Typhimurium from mealworm samples with the applied detection method. Secondly, we aimed to assess the quantitative level of *S.* Typhimurium in disinfected and non-disinfected mealworms over a 14-day course after exposure to substrate contaminated with different levels of *Salmonella* at start, as well as the fate of *Salmonella* in the substrate.

MATERIALS AND METHODS

Mealworms

Mealworms (*Tenebrio molitor*, L.) provided by the Danish Technological Institute (Aarhus, DK) were reared in-house at the National Food Institute, Technological University of Denmark (DTU) (Kgs. Lyngby, DK) on a flour-based dry substrate provided by Adival A/S (Billund, DK) and spent grain acquired from DTU Brewery (Kgs. Lyngby, DK) as wet feed. The rearing

room had a temperature of 26.5 ± 0.6°C and a relative humidity of 55.0 ± 3.6%.

Salmonella Typhimurium Contamination Strain

A Danish strain of *Salmonella enterica* serovar Typhimurium DT12 (*S.* Typhimurium or ST) previously made resistant to rifampicin (Jensen et al., 2006) was used to contaminate the substrate in an experimental laboratory study. A colony from an overnight (o.n.) plate-spread on tryptic soy agar with sheep blood (TSASB) (98763, SSI Diagnostica, Copenhagen, Denmark) was transferred into 10 mL Buffered Peptone Water (CM1049, Oxoid, DK) and incubated o.n. at 37°C. A 10-fold dilution series of the o.n. culture was prepared in 0.9% NaCl solution to reach expected contamination levels ranging from approximately 2 to 7 log CFU/g, and 0.1 mL of each dilution was plate-spread onto TSASB to determine the exact concentration of ST.

Quantitative Estimation of *S.* Typhimurium

To estimate the level of *S.* Typhimurium in larvae, 1 g of larvae was sampled and added 1 mL 0.9% NaCl solution before homogenization in-tube (12 mL round-bottomed tube) by grinding with a sterile Thomas pestle tissue grinder made of teflon and with at stainless steel shaft (Thomas Scientific, Swedesboro, NJ, United States). Additional 8 mL saline was added to the tube (i.e., 10^{-1} dilution) before vortexing and preparation of a 10-fold dilution series in 0.9% NaCl. Appropriate dilutions were spread onto Nutrient Agar (NA) plates with 50 μg/mL rifampicin (R3501, Sigma-Aldrich Chemie, DE) (NA^{rif}). For samples expected to have concentrations <100 CFU/g, 1 mL of the 10^{-1} dilution was distributed onto three agar plates to reach a detection limit of 10 CFU/g, otherwise 0.1 mL was plated.

Substrate samples of 1 g were handled similarly, except that the homogenization step was performed simply by vortexing 15 s.

The agar plates were incubated o.n. at 37°C before enumeration of ST presumptive colonies. Suspect colonies were verified by sub-culturing onto indicative plates as well as NA and NA^{rif} plates and agglutination serotyping as described in Section "Qualitative Detection of *S.* Typhimurium."

Qualitative Detection of *S.* Typhimurium

Qualitative detection of *S.* Typhimurium in homogenized larvae and substrate samples (10 g) was done by addition of 90 mL of buffered peptone water (BPW) (94515, SSI Diagnostica) before o.n. pre-enrichment at 37°C. The 10 g larvae was homogenized by grinding with a pestle tissue grinder as above, but in a 50 mL tube added 2 mL BPW, and with transfer of the larvae material to a 150 mL cup by flushing the tube with additional 88 mL BPW in total. The o.n. culture was spotted onto modified semi-solid Rappaport–Vassiliadis (MSRV) agar (CM0910 and SR0161, Oxoid, DK) in three droplets (approx. 0.1 mL). After o.n. incubation at 41.5°C, material from presumptive *Salmonella* swarming zones was sub-cultured onto indicative plates Brilliant Green Agar plates (PO5033A, Oxoid) and Xylose Lysine Deoxycholate (XLD) agar plates (PO5057A, Oxoid) and incubated at 37°C o.n.

The ST contamination strain was verified by slide agglutination of colony material from the indicative plates with O4 (23839, SSI Diagnostica, DK) and O5 (40272, SSI Diagnostica) antiserum according to the Kauffmann–White scheme (Grimont and Weill, 2007) as well as confirmation of the resistance marker by sub-culturing onto NA and NA^{rif}.

Detection Limit for *S.* Typhimurium in Mealworms

The ability to detect *Salmonella* in mealworms was assessed by testing larvae artificially contaminated with specified levels of *S.* Typhimurium. A ST suspension (in duplicate) was prepared as described in Section "*Salmonella* Typhimurium Contamination Strain" and diluted tenfold. Then 100 μl of each dilution was added to 1-g larvae samples to reach *Salmonella* concentrations from 1 to 10^5 CFU/g larvae. The added cells were allowed to settle on the larvae for 10 min, while kept at 5°C to avoid growth of the added cells and to calm the larvae before quantification of *Salmonella* as described in Section "Quantitative Estimation of *S.* Typhimurium."

Additional, qualitative detection of *Salmonella* was performed as described in Section "Qualitative Detection of *S.* Typhimurium." Larvae samples of 10 g were added ST in numbers from approximate <1 to 100 CFU from appropriate dilutions of the suspension (100 μl was added).

Experimental Exposure of Mealworms to *Salmonella*-Contaminated Substrate

In two successive experiments, 50–60 days old larvae close to the stage of pupation were exposed to substrate experimentally contaminated with different concentrations of *S.* Typhimurium at start of the experiment. Each experimental period lasted for 14 days. The experimental trays with larvae were placed in a separate incubator in the laboratory to avoid accidental contamination of the rearing room at temperatures of 25.5 ± 0.3°C and 25.6 ± 0.1°C and a RH of 55.9 ± 6.1% and 39.6 ± 4.6% in trials 1 and 2, respectively.

Experimental Trial 1

For the experimental set-up, larvae with an average weight of 126 ± 20 mg were sieved to remove the substrate provided during rearing. Two days prior to exposure on day zero, larvae, and fresh flour-based dry substrate (Adival A/S) were allocated in a 1:1 ratio of 150 g into each of 4 aluminum foil trays (19 cm × 12.5 cm × 4.5 cm, 1.1 L). One additional tray was added substrate only (150 g).

On day zero, 1.7 mL of appropriate dilutions of the *S.* Typhimurium suspension was added to 50 mL Greiner tubes each containing 20 g spent grain (DTU Brewery). After thorough vortexing, the contaminated spent grain was transferred to the aluminum foil trays with larvae for estimated final ST concentrations of approximately 2, 4, and 6 log CFU per g of substrate in each tray, respectively, see **Table 1**. Additionally, contaminated spent grain was added to the tray with substrate

TABLE 1 | Set-up for experimental exposure of mealworm to *Salmonella*-contaminated substrate.

	***Salmonella* contamination level (expected log CFU/g)[a]**			
Tray	**Description**	**1st trial**	**2nd trial**	**Tray content[b]**
1	Low	2	3	Larvae: substrate (1:1)
2	Medium	4	5	Larvae: substrate (1:1)
3	High	6	7	Larvae: substrate (1:1)
4	Neg. control	0	0	Larvae: substrate (1:1)
5	Control	4	5	Substrate

[a]Appropriate dilutions of the S. Typhimurium suspension were added via 20 g spent grain to reach an 'expected' final contamination level in the substrate at start of the experiment. [b]Larvae were mixed with fresh dry substrate in a 1:1 ratio of 150 g 2 days prior to contamination with Salmonella.

only (4 log CFU/g of substrate) referred to as control, while 20 g uncontaminated spent grain was added to a tray with larvae as a negative control for cross-contamination between trays. During the experimental period, the larvae were provided water by addition of 20 g spent grain after each sampling.

Experimental Trial 2

The set-up was similar to the first trial but with the following modifications. The average larvae weight was 131 ± 9 mg. The fixed volume the of *S.* Typhimurium suspension used to contaminate the spent grain was reduced to 1 mL and the contamination level was based on the results of the first trial increased to estimated final ST concentrations of approximately 3, 5, and 7 log CFU per g of substrate, respectively, see **Table 1**. The control tray without larvae was added 5 log CFU per g of substrate.

Sampling of Larvae and Substrate

The first sampling was performed approximately 10 min after addition of spent grain, referred to as day 0. During each experimental period, samplings were performed on day 0, 1, 2, 5, 7, 9, 12, and 14 or until a sample rendered test-negative by qualitative testing. The negative controls were sampled days 0, 7, and 14.

From each tray, 1 g of larvae was transferred to a round-bottomed 12 mL tube with a sterile tweezer (n = 6) for quantitative detection of *S.* Typhimurium (see section "Quantitative Estimation of *S.* Typhimurium"). Half of the larvae samples from each sampling was surface disinfected before testing (see section "Surface Disinfection of Larvae"). Following a negative test result by the quantitative testing approach, 10 g of larvae was transferred to a 50 mL Greiner tube for qualitative testing for the presence of ST (see section "Qualitative Detection of *S.* Typhimurium").

Substrate samples of 1 g mainly consisting of the flour-based substrate (leaving out spent grain residues) were collected from each tray with a plastic spoon and transferred to a 50 mL Greiner tube (n = 3) for quantitative detection of ST (see section "Quantitative Estimation of *S.* Typhimurium"). As for larvae, a 10-g sample was collected for qualitative testing when the preceding sample was test negative.

Surface Disinfection of Larvae

To remove *Salmonella* present on the surface of larvae, 1 g larvae samples were collected into a tube and added 3 mL of 70% w/w ethanol and vortexed for 10 s before removal of the ethanol. The larvae were left for 2 min before being rinsed twice with 5 mL Milli Q water.

This method had been tested prior to this study (data not shown). Shortly, individual larva was submerged in a suspension of the ST strain (approx. 10^7 CFU/mL), then 1 g larvae (n = 3) were pooled and disinfected by the method described above before individual larva was pour plated in 25 mL NA^{rif} and incubated overnight at 37°C.

Statistical Analyses

The quantitative detection of ST in larvae was compared with the concentration of ST added (**Table 2**) by pairwise t-test in GraphPad Prism version 8.3.1 for Windows, GraphPad Software, San Diego, CA, United States, www.graphpad.com (RRID:SCR_002798). For substrate, ST counts for each contamination level were compared between sampling moments (**Table 3**) using repeated measures ANOVA in GraphPad Prism. When counts were below the detection limit applied, i.e., 1 or 2 log CFU/g depending on the expected count, a value representing half of the detection limit, i.e., 0.5 or 1.0 log CFU/g, respectively, was chosen as a value to be included in the statistical analysis. Qualitative testing was applied following a sampling with a ST count below the detection limit, where values of 0.0 and −0.3 were assigned for positive and negative results, respectively.

RESULTS

Detection Limit for *S.* Typhimurium in Mealworms

Colonies of the *S.* Typhimurium contamination strain on NA^{rif} agar plates were counted based on morphology of colonies verified to be the contamination strain as described in Section "Quantitative Estimation of *S.* Typhimurium" and Section

TABLE 2 | Quantitative detection level for *S.* Typhimurium in mealworms.

ST in mealworm samples (log CFU/g)			
1st trial		**2nd trial**	
Cells added[a]	**Cells detected**	**Cells added[a]**	**Cells detected**
5.01	4.90	5.21	5.10
4.01	4.00	4.21	4.08
3.01	2.62	3.21	2.60
2.01	2.15[b]	2.21	2.20[b]
1.01	ND[c]	1.21	1.70[c]
0.01	ND[c]	0.21	ND[c]

[a]Addition of 100 μL ST cell suspension (10-fold dilution series) to 1 g mealworm. [b]Weighted average of CFU/g for plating of 100 μL (in duplicate) and 1 mL of the 10^{-1} dilution. [c]Plating of 1 mL of the 10^{-1} dilution ND, none detected.

TABLE 3 | Level of *Salmonella* Typhimurium found in substrate, larvae and disinfected larvae over a 14 days period after a single contamination event of substrate with ST at start.

Sample	*Salmonella* added[a] (log CFU/g)	*S.* Typhimurium log CFU/g[b]							
		Day after contamination of substrate							
		0	1	2	5	7	9	12	14
Larvae disinfected	1.7	0.5 ± 0.9	<1.0	<1.0	–	–	–	–	–
	3.4	1.4 ± 1.2	1.1 ± 1.9	Neg	Pos	–	Pos	Neg	–
	3.6	2.2 ± 0.4	<2.0	<2.0	–	–	Neg	–	Neg
	5.4	3.8 ± 0.5	<2.0	1.6 ± 2.7	1.0 ± 1.7	<1.0	<1.0	Pos	Neg
	5.6	4.1 ± 0.3	<2.0	<2.0	0.7 ± 1.2	<2.0	Pos	<2.0	Neg
	7.4	5.3 ± 0.3	2.0 ± 1.8	2.2 ± 2.0	<1.0	2.3 ± 2.4	<2.0	<1.0	Pos
Larvae	Control	Neg	–	–	–	Neg	–	–	Neg
	1.7	0.8 ± 1.4	<1.0	Neg	Neg	Neg	–	–	–
	3.4	1.5 ± 0.5	<1.0	Neg	Pos	–	Pos	Pos	Pos
	3.6	3.0 ± 0.4	<2.0	Pos	Pos	Pos	Pos	Pos	Pos
	5.4	3.8 ± 1.3	2.4 ± 2.1	<2.0	2.1 ± 0.5	1.2 ± 2.1	<1.0	Pos	Pos
	5.6	5.0 ± 0.1	2.4 ± 2.2	2.0 ± 3.5	Pos	Pos	Pos	Pos	Pos
	7.4	5.2 ± 0.5	2.1 ± 1.8	<2.0	3.1 ± 0.5	3.1 ± 1.2	2.3 ± 2.2	2.3 ± 0.3	1.9 ± 0.3
Substrate with larvae	Control	Neg	–	–	–	Neg	–	–	Neg
	1.7	0.9 ± 1.5	0.7 ± 1.2	<1.0	–	Neg	–	–	–
	3.4	1.6 ± 1.4	<1.0	Neg	Pos	–	Pos	Pos	Pos
	3.6	2.5 ± 2.2	0.7 ± 1.2	<2.0	–	Pos	Pos	–	Pos
	5.4	4.1 ± 0.7^{AC}	1.4 ± 1.2^{BC}	$<2.0^{AB}$	3.7 ± 0.4^{AC}	3.2 ± 0.3^{CD}	1.8 ± 0.5^{BD}	2.5 ± 0.4^{BCD}	2.3 ± 0.2^{AB}
	5.6	4.7 ± 0.8^{AB}	3.3 ± 0.5^{A}	2.5 ± 0.4^{B}	2.1 ± 1.8^{AB}	3.0 ± 1.2^{AB}	2.8 ± 0.8^{B}	2.0 ± 2.0^{AB}	2.0 ± 2.0^{AB}
	7.4	6.0 ± 0.4^{AC}	3.4 ± 0.2^{AB}	$<2.0^{C}$	3.8 ± 0.1^{AB}	4.6 ± 0.5^{AB}	3.7 ± 0.1^{B}	3.5 ± 0.2^{B}	3.7 ± 0.1^{B}
Substrate without larvae	3.4	1.7 ± 1.5	<2.0	<2.0	–	Pos	Pos	Pos	Pos
	5.4	4.5 ± 0.5	<2.0	<2.0	0.4 ± 0.8	0.3 ± 0.6	Pos	Pos	Pos

[a] *Estimated concentration of S. Typhimurium in substrate after the contamination event on day 0; Concentrations of 1.7, 3.6, and 5.6 log CFU/g were added in trial 1 and 3.4, 5.4, and 7.4 log CFU/g were added in trial 2; no ST was added to the controls.* [b] *Average values of triplicate samples ± standard deviation, with application of a value of zero for each individual sample negative in the quantitative testing. Triplicate samples all negative in the quantitative testing are indicated as below the applied detection limit, i.e., <1 or <2 log CFU/g. Detection of ST by qualitative testing (10-g samples) is shown as Pos while no detection of ST is shown as Neg; –, no testing; ST counts within each row that share a letter in superscript, did not significantly (p ≥ 0.05) increase of decrease between sampling days, as was shown from repeated measures ANOVA.*

"Qualitative Detection of *S.* Typhimurium." The quantitative detection of the ST contamination strain, showed that the detection level was close or similar to the level of cells added ($p = 0.45$), see **Table 2**.

The added cell levels of 1.01 and 1.21 log CFU/g in trials 1 and 2, respectively, are close to the theoretical detection level of 1 log CFU/g when 1 mL of the 10^{-1} dilution is plated, which may explain the negative test-result obtained for 1.01 log CFU/g in trial 1.

For the qualitative detection method, *S.* Typhimurium was detected in 1 out of 2 larvae samples at a contamination level of 0.6 CFU per 10 g sample, while both sample replicates were positive when the contamination level was 10-fold higher, i.e., 6 CFU or higher.

Experimental Exposure of Mealworms to *Salmonella*-Contaminated Substrate

Mealworms were at start of each of the two experimental periods exposed to substrate contaminated with *S.* Typhimurium in concentrations ranging from 1.7 to 7.4 log CFU/g. The quantitative level of ST found in larvae and substrate during the 14-day study period is shown as an average of triplicate samples (1 g each) in **Table 3**. In case individual samples tested negative, i.e., below the detection limit of 1 or 2 log CFU/g, a value of zero was applied for calculation of the average.

The level of ST found in larvae was below the quantitative detection level in all three samples (in **Table 3** shown as <1 or <2 log CFU/g) already within 1 day in larvae exposed to contamination levels of 1.7, 3.4, and 3.6 log CFU/g opposed to contamination levels of 5.4, 5.6, and 7.4 log CFU/g, respectively. The maximum level of ST detected in individual 1-g larvae samples (i.e., 8 larvae) was 5.8 log CFU/g detected on day 0. Beyond day 7, only larvae exposed to the highest contamination level, i.e., 7.4 log CFU/g were >1.0 log CFU/g, but often with variation of the ST level in individual larvae samples with findings of, e.g., <2.0, 2.4, and 4.4 log CFU/g on day 9. At end of the experiment on day 14, the ST level in these larvae was 1.9 ± 0.3 log CFU/g.

When testing of 1-g larvae samples (non-disinfected) from each experimental tray reached the detection limit, 10-g samples

were tested qualitatively for the presence of ST in the succeeding samplings. This showed the presence of ST in non-disinfected larvae at all contamination levels until the end of the experiment on day 14 as indicated by Pos for positive in **Table 3** (i.e., at least 1 CFU/g) except for the lowest contamination level of 1.7 log CFU/g. Here, the ST level was <1.0 log CFU/g on day 1 and no ST was found in three succeeding samplings on days 2, 5, and 7.

In the negative control tray where no ST was added, weekly testing of larvae and substrate showed absence of ST, i.e., no indication of cross-contamination between the experimental trays.

In this experiment as well as under natural rearing conditions, mealworms inhabits their substrate and therefore surface disinfection was applied in an attempt to disclose whether detected *S.* Typhimurium derived from ingested ST or merely from contamination on the surface of the larvae. The applied disinfection method with 70% ethanol and washing twice in MilliQ water had been tested prior to this study (see section "Surface Disinfection of Larvae"), where visual inspection of the larvae embedded in the agar plates showed no growth of ST on 20 out of the 21 larvae disinfected in total. ST was found on a single larva but with markedly lower growth of ST than on control larvae rinsed with water only, overall indicating efficacy of the applied disinfection method.

For surface disinfected larvae, ST was on day 0 detected in 15 out of 18 larvae samples and not in levels markedly different from non-disinfected larvae. However, at later samplings, the disinfected larvae generally rendered test-negative earlier than the untreated larvae, and only at the highest contamination level of 7.4 log CFU/g was ST found present in disinfected larvae at the end of experiment on day 14 (see **Table 3**). The qualitative testing of disinfected larvae was performed less frequently not to skew the 1:1 ratio between substrate and larvae too much when removing samples of 10 g, and further, it was considered superfluous if the non-disinfected counterpart tested negative already.

In the substrate, the level of *S* Typhimurium on day 0 shortly after contamination was up to 1.4 log lower than the expected level of ST added (see **Table 3**). At the three lowest contamination levels (1.7–3.6 log CFU/g), the ST level in the substrate was below the detection limit on day 2 and onwards. Still, ST was found until the end of experiment by qualitative testing except for the low contamination level, i.e., 1.7 log CFU/g. At the contamination levels of 5.4, 5.6, and 7.4 log CFU/g, the level of ST detected in the substrate on day 14 was 2.3 ± 0.2, 2.0 ± 2.0, and 3.7 ± 0.1 log CFU/g, respectively.

The level of *S.* Typhimurium in substrate from trays without larvae (control) was compared with ST levels in the trays containing larvae at contamination levels of 3.6 and 5.4 log CFU/g in trials 1 and 2, respectively (see **Table 3**). In the first trial, ST was generally below the quantitative detection level within the first days both in trays with and without larvae present although ST was detected until the end of the experiment. At the higher contamination level in the second trial, the ST level in the substrate remained quantifiable in trays with larvae, while the substrate without larvae after day 7 was ST-positive by pre-enrichment only.

DISCUSSION

In order to assess the level of *Salmonella* Typhimurium cells in mealworm (*T. molitor*) larvae after exposure to contaminated substrate, we made a preliminary test of the ability to recover *S.* Typhimurium from mealworm samples with the applied detection method. A standard approach for determination of bacterial cell numbers in a sample is spreading of a sample dilution series on agar plates. This approach though, has a theoretically limit of detection depending on the portion of sample plated. Further, the high bacterial load generally present in mealworms may challenge the specific detection of the *S.* Typhimurium target strain if outcompeted by the inherent bacteria (Vandeweyer et al., 2017; Wynants et al., 2018). Therefore, a *S.* Typhimurium strain with an antibiotic resistance marker (rifampicin) previously proven adequate for detection in pig fecal samples (Jensen et al., 2006) was chosen for the study to facilitate detection. Other strains of S. Typhimurium or other serovars may not have elicited the exact same outcome, however, *S.* Typhimurium is one of the most important serovars in human cases of salmonellosis and was considered a good candidate for the exposure study as a start.

Proper retrieval of bacterial cells from a sample is also important to obtain a good recovery of the bacteria of interest (Rohde et al., 2015). In this study, in-tube homogenization of mealworm samples by grinding with a sterile pestle tissue grinder was applied, as a small preliminary study indicated a good recovery of cells by this method as compared to stomaching and crushing by hands. This preliminary evaluation was based on total aerobic count of the inherent bacterial population (data not shown), as artificial spiking with the bacteria of interest is unlikely to reflect the natural binding or embedment of cells within a sample matrix. The in-tube homogenization approach appears convenient opposed to, e.g., blending or use of a mortar as there is no need for transferring the sample material after homogenization. Moreover, such transfer may lead to loss of material if the sample is weighed beforehand or failure in achieving a fully representative sample if weighing is performed on a sample not completely homogenous, which is likely for mealworms and other insects due to their exoskeleton parts.

The preliminary assessment of the detection limit for *S.* Typhimurium in mealworm samples indicated a good recovery of the cells added both for the quantitative estimation (**Table 2**) and the qualitative detection method applied in this study. The level of ST added in trial 1 was estimated to 1.01 log CFU/g, i.e., very close to the theoretical detection limit of 1 log CFU/g when 1 mL of the 1:9 (10^{-1}) dilution is plated. So the lack of detection at this level seemed to be within the expected precision of the plate spreading method rather than suppression of ST growth, generally implying a low risk of incorrect conclusions due to false-negative test results.

In the experimental exposure study, the lowest contamination level of 1.7 log CFU/g was the only one not resulting in ST positive mealworms at the end of the experiment, i.e., on day 14 for the non-disinfected larvae. In a study by

Wynants et al. (2019), a *Salmonella* contamination level of 2 log CFU/g also resulted in test-negative larvae on day 7 where the experiment ended. At a contamination level of 4 log CFU/g, those authors found 4 of 6 larvae replicates *Salmonella*-negative (qualitative testing only) on day 7 in one experiment, while *Salmonella* was <1.0 log CFU/g in another experiment. Similarly, in the current study, contamination levels slightly lower at 3.4 and 3.6 log CFU/g resulted in *Salmonella*<1.0 log CFU/g and <2.0 log CFU/g on day 1. A detection limit of 2 log CFU/g was applied for samples expected to reach this level, but the obtained ST counts proved to be lower in some cases, and here a detection limit of 1 log CFU/g would have been more informative. Nevertheless, the non-disinfected larvae remained ST positive throughout the experimental period of 14 days. Even though this may imply a *Salmonella* presence as low as 1 CFU per gram, the ID50 of 7 CFU for causing infection emphasizes the significance of these findings (Teunis et al., 2010). It also supports the recommendations concerning heat treatment or other processing to ensure the food safety of mealworms, although no specific food safety criteria [Regulation (EC) No 2073/2005] for edible insects have been established (yet).

The surface disinfected larvae generally turned ST-negative somewhat earlier than the corresponding non-disinfected larvae, indicating that some ST resided on the surface only. Surprisingly though on day 0, the ST levels were similar in the disinfected and non-disinfected larvae. Given efficacy of the disinfection method, this implies a rapid ingestion of substrate (*Salmonella*), as sample collection were started approximately 10 min after addition of the contaminated spent grain. To our knowledge, no other studies have made a parallel testing of disinfected and non-disinfected larvae in the same experiment to shed light on this. Inefficient disinfection could also explain the high ST counts on day 0, and despite our promising pre-evaluation of the method based on 70% ethanol, results by Crippen and Sheffield (2006) indicated that 70% ethanol alone was inefficient for surface disinfection of beetles of the lesser mealworm (*Alphitobius diaperinus*). However, for their assessment, disinfected beetles were submerged completely into the growth medium, and although shortly, excretion of internal bacteria into the medium during the submersion cannot be excluded. Regardless, any detection of *Salmonella* either internal or external is of concern from a food safety perspective, as the whole larvae will be processed.

At a contamination level of 7 log CFU/g, Wynants et al. (2019) observed no decrease in the *Salmonella* level in larvae on day 7 (4.1 ± 1.1 log CFU/g) and it was discussed whether it was simply a matter of longer time needed to exert a reduction or if numbers were too abundant for a reduction to happen. The only other contamination level tested in that experiment was 4 log CFU/g, which resulted in <1 log CFU/g in larvae at day 7, i.e., the fate of *Salmonella* at contaminations between 4 and 7 log CFU/g is uncertain. In our study, the ST counts for 7.4 log CFU/g were 5.2 ± 0.5, 3.1 ± 1.2, and 1.9 ± 0.3 log CFU/g on days 0, 7, and 14, respectively and at 5.4 log CFU/g, the ST counts were <1.0 log CFU/g on day 9 but ST positive until day 14. This indicates a decrease over time but also that the additional 7 days in the current study were insufficient for clearing the contamination in the larvae. Also Crippen et al. (2012) found that the lesser mealworm excreted *Salmonella* into their feces (frass) for 6–12 days after a 2 h exposure to 8 log CFU/mL, and where 33 and <10% of the larvae shed *Salmonella* on day 9 and day 12, respectively. In that study, however, the larvae were isolated from the source of contamination after 2 h, i.e., not reflecting real life rearing conditions where larvae inhabit their substrate, where contaminations may persist and constitute a continuous exposure. Although the ST counts found in our larvae depended on the initial contamination level, absence of ST within the 14-day course was not observed in non-disinfected larvae for contaminations levels ≥3.4 log CFU/g. So one can still pose the questions whether longer time (>14 days) was needed to clear the contamination in the larvae or if there is a certain threshold (>2 log CFU/g based on our study and Wynants et al., 2019) above which *Salmonella* will persist until harvest prior to pupation. Further, the answers may depend on several other factors like type of substrate, larval density or the larval stage at which the contamination event occurs, as younger larvae may elicit less colonization resistance to foreign microorganisms (Wynants et al., 2019). Moreover, it is uncertain how the design of the experimental set-up affects the results. For example, the volume of *Salmonella* suspension chosen for contaminating the substrate will affect the moisture content and hence growth potential of bacteria, while the method of distribution may influence the actual level of contamination experienced by individual larvae.

Concerning the current experimental design, the ST was added via the wet substrate (spent grain) to avoid clumping of the dry flour-based substrate. The spent grain constituted 12% of the substrate in total, and despite mixing efforts it may have caused an uneven distribution of ST and partly explain the discrepancy (up to 1.4 log) between the ST counts obtained on day 0 and the ST numbers added (**Table 3**). However, *Salmonella* contaminations occurring under natural rearing conditions will most likely result in a heterogeneous distribution of cells as well, and although larvae activity probably aid a more homogeneous distribution of contaminants over time, the triplicate substrate and larvae samples had variable ST counts as evident from the standard deviations of the averages. This possible variation between samples will also need consideration when sampling for monitoring purposes.

In substrate contaminated at levels ≤3.6 log CFU/g, ST counts were soon below the detection limit, although only ST-negative on day 14 for the lowest contamination level (1.7 log CFU/g) as the case for the larvae. At the 5.4 and 5.6 log CFU/g contamination levels, the ST counts in substrate were reduced to 2.3 ± 0.2 and 2.0 ± 2.0 on day 14, respectively, but counts were not significantly different from the initial ST level counted on day 0 (**Table 3**). Noticeable, although the ST contamination level found in substrate on day 14 in these trays was close to the initial contamination level of 1.7 log CFU/g where larvae samples rendered ST-negative within the first days, the larvae in these trays were still ST-positive on day 14. Similarly, the initial 7.4 log CFU/g contamination level in

substrate decreased significantly to 3.7 ± 0.1 log CFU/g on day 14, but this level was sufficient for ST counts reaching 1.9 ± 0.3 log CFU/g in the larvae opposed to trays with initial contamination levels of 3.4 and 3.6 log CFU/g. All together indicating the importance of the initial contamination level in the substrate for the resulting ST counts in the larvae. Still, it is uncertain for how long *Salmonella* will remain present at contamination levels >2 log CFU/g as only contamination levels ≤2 log CFU/g resulted in absence of ST at the end of experiments. Further, this study was based on a single contamination event while it is uncertain if a repetitious introduction of *Salmonella* might support longer persistence of *Salmonella* in the larvae even at low contaminations levels.

Wynants et al. (2019) found that *Salmonella* survived well in the wheat bran without larvae during the experimental period of 7 days. Further, the substrate without larvae had higher *Salmonella* counts or more *Salmonella*-positive replicates than substrate with larvae, which indicated that the larvae supported reduction of *Salmonella*. Contrary, our results for the contamination level of 5.4 log CFU/g indicated that ST counts in substrate were higher when larvae were present. Irrespective, at least no proliferation of *Salmonella* was observed in neither substrate nor larvae in both studies. For substrate, which is mainly dry, this may be explained by a required water activity a_w level of minimum 0.93 to facilitate growth of *Salmonella*, while *Salmonella* easily survive under dry conditions (Podolak et al., 2010). However, the humid rearing conditions for mealworms (often 50–70% RH) and addition of wet substrate as water source can be suspected to create 'wet spots' facilitating growth. No a_w measurements of the contaminated substrate were conducted in this study, but previous in-house measurements had indicated an a_w of 0.86 1 h after addition of spent grain in rearing boxes, i.e., well below the growth supportive level. So it is uncertain if the higher ST counts observed in substrate with larvae in our study were partly due to the continuous addition of spent grain increasing the moisture content, opposed to the single addition to trays without larvae to prevent molding. Further, as the gastrointestinal tract of the larvae provides humid conditions, it can be speculated if the passage of *Salmonella* through the larval gut is actually conducive for their survival after excretion considering the higher level of *Salmonella* found in substrate with larvae. The pH is 5.6 in the anterior and middle midgut of *T. molitor* larvae and 7.9 in the posterior midgut, i.e., there is no significant gastric acid barrier in the larvae acting on *Salmonella* that tolerate pH down to 4 (Moreira et al., 2017). This is in contrast to pH values as low as 2.0 and 3.1 in the middle midgut of larvae of black soldier flies (*Hermetica illucens*) and house flies (*Musca domestica*), respectively, assumingly reducing the change of *Salmonella* surviving passage through these fly larvae (Terra and Regel, 1995; Bonelli et al., 2019). Still, in *T. molitor* as in other insects, are antimicrobial peptides (AMP) reported to act in the defense against bacterial infections (Wu et al., 2018; Jo et al., 2019; Keshavarz et al., 2020). Nevertheless, Crippen et al. (2012) found that larvae of lesser mealworm excreted live *Salmonella* into their frass.

CONCLUSION

Considering the long-time efforts made for optimizing and standardizing bacteriological culturing protocols for monitoring and safety control of other food and feed production chains, similar standardization of methods for monitoring of insect production or products would help to ensure validity and comparability of test results within this completely new area. Not least if specific hygiene process criteria or food safety criteria for edible insects are to be established in future.

This study indicated the importance of avoiding introduction of *Salmonella* into the mealworm production site, e.g., via contaminated substrate in order to avoid *Salmonella*-positive larvae as they remained positive for at least 14 days when the initial contamination levels were ≥3.4 log CFU/g. However, this study was based on a single contamination event and the impact of repetitious introduction of *Salmonella* should be assessed to neglect contaminations <2 log CFU/g. The initial contamination level affected the resulting *Salmonella* count in both larvae and substrate, and although the *Salmonella* level generally decreased over time and no proliferation of *Salmonella* was observed, proper treatment before consumption would be needed to ensure the food safety of larvae.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

AJ and SH contributed design and conductance of study and data analysis. AJ wrote the first draft of the manuscript and performed statistical analysis. DB and AJ contributed conception and management of the project. All authors contributed to discussion of results, manuscript revision, and read and approved the submitted version.

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Identification of Bacteria in Two Food Waste Black Soldier Fly Larvae Rearing Residues

*Moritz Gold[1,2], Fabienne von Allmen[1,2], Christian Zurbrügg[2], Jibin Zhang[3] and Alexander Mathys[1]**

[1] Sustainable Food Processing Laboratory, Department of Health Science and Technology, Institute of Food, Nutrition and Health, ETH Zürich, Zurich, Switzerland, [2] Department Sanitation, Water and Solid Waste for Development (Sandec), Eawag: Swiss Federal Institute of Aquatic Science and Technology, Dübendorf, Switzerland, [3] State Key Laboratory of Agricultural Microbiology, National Engineering Research Center of Microbial Pesticides, College of Life Science and Technology, Huazhong Agricultural University, Wuhan, China

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***Correspondence:**
Alexander Mathys
alexander.mathys@hest.ethz.ch

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Significant economic, environmental, and social impacts are associated with the avoidable disposal of foods worldwide. Mass-rearing of black soldier fly (*Hermetia illucens*) larvae using organic wastes and food- and agro-industry side products is promising for recycling resources within the food system. One current challenge of this approach is ensuring a reliable and high conversion performance of larvae with inherently variable substrates. Research has been devoted to increasing rearing performance by optimizing substrate nutrient contents and ratios, while the potential of the substrate and larval gut microbiota to increase rearing performance remains untapped. Since previous research has focused on gut microbiota, here, we describe bacterial dynamics in the residue (i.e., the mixture of frass and substrate) of black soldier fly larvae reared on two food wastes (i.e., canteen and household waste). To identify members of the substrate and residue microbiota, potentially associated with rearing performance, bacterial dynamics were also studied in the canteen waste without larvae, and after inactivation by irradiation of the initial microbiota in canteen waste. The food waste substrates had similar microbiota; both were dominated by common lactic acid bacteria. Inactivation of the canteen waste microbiota, which was dominated by *Leuconostoc*, *Bacillus*, and *Staphylococcus*, decreased the levels of all rearing performance indicators by 31–46% relative to canteen waste with the native microbiota. In both food waste substrates, larval rearing decreased the bacterial richness and changed the physicochemical residue properties and composition over the rearing period of 12 days, and typical members of the larval intestinal microbiota (i.e., *Providencia*, *Dysgonomonas*, *Morganella,* and *Proteus)* became more abundant, suggesting their transfer into the residue through excretions. Future studies should isolate members of these taxa and elucidate their true potential to influence black soldier fly mass-rearing performance.

Keywords: *Hermetia illucens*, microbiota, insects, bioconversion, waste, frass, probiotics

INTRODUCTION

Significant economic, environmental, and social impacts are associated with the avoidable disposal of foods worldwide (Gustavsson et al., 2011; Papargyropoulou et al., 2014; Chen et al., 2020). Measures to reduce food loss and waste amount exceeding 1.3 billion tons per year (Gustavsson et al., 2011) include avoiding surplus food production, followed by redistribution and reuse of surplus foods (Papargyropoulou et al., 2014). Once produced, food loss and wastes should be recycled by using them as animal feed (Dou et al., 2018) or compost (Li et al., 2013), and extraction of energy should be the least preferred approach (Papargyropoulou et al., 2014). In recent years, mass rearing of the black soldier fly (*Hermetia illucens* L., Diptera: Stratiomyidae) larvae (BSFL) has emerged as an additional solution for food waste recycling (Gold et al., 2018; Zurbrügg et al., 2018; Berggren et al., 2019; Varelas, 2019). BSFL convert a range of organic wastes (e.g., food waste, animal manure) and food- and agro-industry side products (e.g., breweries, food processing industry) (Nyakeri et al., 2017; Barragán-Fonseca et al., 2018; Lalander et al., 2019; Gold et al., 2020a) into larval biomass and a compost-like residue (i.e., mixture of frass and substrate). The larval biomass is rich in proteins and lipids, and thus, serves as a raw material for various applications within the food system, such as proteins and lipids in feeds for pets (Bosch et al., 2014) and livestock (e.g., fish, poultry, swine) (Barragán-Fonseca et al., 2017; Wang and Shelomi, 2017), and processing of the larval exoskeleton into chitosan (Hahn et al., 2019). Next to this recycling of waste nutrients, according to circular economy principles (Cappellozza et al., 2019), waste treatment by BSFL (Ermolaev et al., 2019; Mertenat et al., 2019; Pang et al., 2020) and animal feed products (Smetana et al., 2016, 2019) with BSFL can have lower environmental impact than the *status quo* (i.e., composting and commercial feed ingredients such as fish meal).

One current challenge for BSFL rearing is to obtain reliable and high rearing performance (e.g., >200 mg for harvested BSFL) (Gold et al., 2018). Researchers have previously improved rearing performance by optimizing substrate nutrient contents and ratios (Nyakeri et al., 2017; Barragán-Fonseca et al., 2018; Gold et al., 2020a), however, few studies exist regarding the manifold roles in which BSFL-associated microbiota may influence rearing performance (De Smet et al., 2018). BSFL guts, rearing substrates and residues (i.e., the mixture of frass and substrate) all have rich and diverse microbiomes (Bruno et al., 2019; Klammsteiner et al., 2020) varying due to different biotic (e.g., initial rearing substrate microbiome) and abiotic (e.g., temperature) factors among rearing systems (Wynants et al., 2019; Raimondi et al., 2020). Similar to many insects (Douglas, 2009; Engel and Moran, 2013; Lee and Brey, 2013), Dipteran larvae such as those of *Drosophila melanogaster* (Diptera: Drosophilidae) and *Musca domestica* (Diptera: Muscidae) engage in complex interactions with their gut microbiota, as these influence larval immunity (Broderick and Lemaitre, 2012) and metabolism (Shin et al., 2016), growth signaling (Storelli et al., 2011), and nutrient provision (Zurek and Nayduch, 2016). Microbiota associated with BSFL may have similar functions (Ao et al., 2020) considering their similar ecological niche and phylogenetic order (Gold et al., 2018; Zhan et al., 2020). Identification and manipulation (e.g., by addition of bacterial mixtures) of microbiota in BSFL rearing is an additional promising approach to increase rearing performance, next to the optimization of substrate nutrient contents and ratios.

Members of the BSFL gut microbiota (e.g., *Bacillus natto*, *Bacillus subtilis*, *Lactobacillus buchneri*, and *Kocuria marina*) can increase performance when added to rearing substrates (Yu et al., 2011; Xiao et al., 2018; Rehman et al., 2019; Somroo et al., 2019; Mazza et al., 2020). Members of the substrate and residue microbiota are an additional yet unexplored pool of potentially beneficial bacteria. Fly larvae may support and sustain certain microbiota in the substrate and residue to favor decomposition and digestibility (Zhao et al., 2017) and thus larval development (Storelli et al., 2018) and also protect from other insects and microbes competing for the same resources (Bernard et al., 2020). Bacterial candidates associated with rearing performance could be identified by studying microbiota throughout the rearing time in the substrate and rearing residue. For BSFL, previous studies have typically only determined the bacterial community in the initial substrate as well as the final residue. Jiang et al. (2019) were the first to determine bacterial community dynamics throughout one rearing cycle and found that BSFL rearing affects and alters the substrate bacterial community over time to increase the capacity to decompose the substrate. Lalander et al. (2013) and Erickson et al. (2004) also reported a reduction in certain bacteria during BSFL rearing, and Cai et al. (2018) and Liu et al. (2020) reported a reduction in antibiotic resistance genes in bacteria. These results contradict the findings of Bruno et al. (2019), who did not identify a significant influence of BSFL on substrate microbiota during rearing. Changing the bacterial community in the rearing substrate may also be beneficial for rearing of insects for food and feed applications, when considering that the substrate microbial community may include human and animal pathogens (e.g., *Bacillus cereus* and *Enterococcus faecalis*) (Lalander et al., 2013; Van der Fels-Klerx et al., 2018; Wynants et al., 2019).

The aim of this study was to identify groups of bacteria potentially associated with BSFL residues and the rearing performance. We assessed the bacterial community dynamics when using two food waste substrates during BSFL rearing. We also studied the bacterial community dynamics in food waste after inactivation by irradiation of the initial microbiota in food waste with and without BSFL. We hypothesized that an inactivation of the initial microbial community in the substrate should decrease rearing performance, revealing that some important bacteria are associated with rearing performance. In addition, we hypothesized that certain groups of bacteria become more abundant during BSFL rearing in comparison to controls without larvae.

MATERIALS AND METHODS

Food Waste Substrates

Two types of food waste were collected in containers treated with 70% ethanol. Canteen waste included a mixture of discarded pasta, meat, fish, bread, and vegetables from the

Polyterrasse canteen at ETH Zürich in Switzerland. Household waste included discarded fruit peels, vegetables, eggs, bread, herbs, and food leftovers collected from a household organic waste bin in Zürich. Each collected substrate mixture was homogenized with a kitchen blender to mimic typical waste processing before BSFL rearing (Dortmans et al., 2017). Pictures of the fresh and homogenized rearing substrates are included in **Supplementary Table 1**. Following homogenization, the rearing substrates were stored at 4°C for 48 h. During this storage time, part of the canteen waste was sterilized with a high-energy electron beam. This substrate was fed to BSFL in parallel to the non-sterile food wastes to assess the influence of the loss of the initial substrate microbiota on rearing performance and bacterial dynamics. Irradiation was completed by a commercial provider (Leoni Studer AG, Däniken, Switzerland) with a 10 MeV electron beam (Rhodotron TT300, IBA Corp., Louvain-la-Neuve, Belgium) at a dose of 32 kGy in accordance with the ISO 11137-3:2017 standard [International Organization for Standardization (ISO), 2017]. These treatment conditions produced sterile substrates without microbial growth (Gold et al., 2020b).

Since substrate composition influences microbial communities and BSFL rearing performance, substrate gross nutrient composition, pH, and moisture content were determined using standard procedures described in detail in Gold et al. (2020a). The pH was measured with a portable meter and the pH probe HQ40d (Hach Lange GmbH, Rheineck, Switzerland). Moisture and organic matter were determined using an automatic thermogravimetric instrument (TGA-701, Leco, St. Joseph, MI, United States). Nitrogen content was determined using a C/N analyzer (Type TruMac CN, Leco). Glucose (D-Glucose GOPOD K-GLUC, Megazyme, Wicklow, Ireland) and starch (Total Starch Assay K-TSTA, Megazyme), and fructose (Available Carbohydrates K-ACHDF, Megazyme) were determined using commercial enzyme assays. For fructose determination, absorbance differences of >0.07 were used instead of >0.1, as recommended by the manufacturer. Neutral and acid detergent fibers were assessed using a fiber bag system (Fibretherm, Gerhardt Analytical Systems, Königswinter, Germany). Lipids were analyzed by Eurofins Scientific, Schönenwerd, Switzerland, according to Regulation (EC) No 152/2009 (European Commission, 2009). Protein was estimated by multiplying the nitrogen results with 5.4 (based on results for meat, fish, cereals, and vegetables) (Mariotti et al., 2008), and the caloric content was estimated by multiplying the mean lipid, non-fiber carbohydrate, and protein results with their gross caloric content of 9.4, 5.4, and 4.1 kcal/g, respectively (Merrill, 1973; Wu, 2016). Hemicellulose content was determined as the difference between the neutral and acid detergent fibers. The sum of glucose, fructose, and starch was assumed to reflect the total non-fiber carbohydrate content.

Fly Larva Rearing

BSFL were reared on homogenized food waste substrates using the following experimental setup. BSFL were obtained from a colony operated at Eawag (Dübendorf, Switzerland) since 2017, based on the protocol of Dortmans et al. (2017). The hatched larvae were fed *ad libitum* with poultry feed (UFA 625, UFA AG, Herzogenbuchsee, Switzerland) for 7–9 days to a weight of 0.5 mg dry mass (DM)/larva. Thereafter, larvae were manually separated from the poultry feed residue and 12 replicates with approximately 200 larvae per replicate were prepared for each treatment. To eliminate possible contaminations by airborne microbes, cross-contamination between substrates, and contamination with human microbes during rearing, larvae were reared in an one-time-feeding bench-scale batch experiment. Larvae were placed in sterile plastic containers (diameter: 100 mm, height: 80 mm) (O118/80, Eco2 NV, Ophasselt, Belgium) with substrates at a feeding rate of 22 mg DM/larva per day for 12 days, resulting in a larval density of 2.5 larvae/cm^2. Plastic containers were covered with lids (OD118 Filter XL, Eco2 NV, Ophasselt) permitting air flow, while being impermeable to microbes. Containers were placed in a random order in a climate chamber (HPP 260, Memmert GmbH, Büchenbach, Germany), providing a microclimate of 28°C and 70% relative humidity. Temperature was automatically recorded every 10 min in the substrate/residue of one replicate per treatment (DS1922L iButton, Maxim Integrated, San Jose, CA, United States).

For each treatment, every 3 days, for a total of 12 days, three containers were removed from the climate chamber. One residue sample was collected per removed container to determine the physicochemical (see section "Physicochemical Properties and Composition of the Residue") and microbial parameters in the residue (see section "Microbial Counts and Bacterial Communities"). Larvae were manually separated from the residue, cleaned with tap water, and dried with paper towels. Larvae were counted, weighed, freeze-dried and then stored at 4°C before larval protein content (see section "Microbial Counts and Bacterial Communities") measurement. Residue samples were analyzed for water activity and pH, thereafter freeze-dried and stored to later measure other physicochemical parameters. Weight loss in larvae and residue samples during freeze drying was used to correct all results for moisture content. All manipulations with rearing containers and collection of substrate/residue samples for microbial parameters were performed using sterile techniques under a laminar flow cabinet.

Controls With No Larvae

Sterile and non-sterile canteen waste without larvae underwent experimental and environmental conditions identical to those used for BSFL rearing. After 12 days in sterile containers in the climate chamber, samples were collected and processed in the same way as the rearing residue samples.

Physicochemical Properties and Composition of the Residue

Changes in the residue composition were measured through physicochemical parameters which are relevant for microbial growth. Portable meters were used to measure water activity (HygroPalm23-AW, Rotronic, Bassersdorf, Switzerland) and pH (HQ40d, Hach Lange GmbH) in the fresh residue samples. A thermogravimetric instrument (TGA-701, Leco) and a C/N analyzer (Type TruMac CN,

Leco) were used to measure moisture and organic matter, and carbon and nitrogen on the freeze-dried residue samples, respectively.

Rearing Performance

Residue and larval dry weights as well as larval protein content were used to calculate typical rearing performance indicators per replicate. Larval weight, bioconversion rate, and waste reduction were calculated according to Gold et al. (2020a). The total larval protein per biological replicate (which had 200 larvae) was calculated using equation 1.

$$\textit{Total larval protein}\left(\frac{\text{g DM protein}}{\text{replicate}}\right) = \text{larval weight}\left(\frac{\text{g DM}}{\text{larva}}\right)\text{x}$$
$$\textit{larval protein content}\left(\frac{\text{g DM protein}}{\text{100 g DM}}\right)\text{x}\frac{\text{200 larvae}}{\text{replicate}} \quad (1)$$

Each larval protein content was estimated as nitrogen content × 4.67, as proposed by Janssen et al. (2017). Nitrogen was measured on freeze-dried samples using a C/N analyzer (Type TruMac CN, Leco) and corrected for residual moisture with thermogravimetric determinator (TGA-701, Leco).

Microbial Counts and Bacterial Communities

Culturable microbial counts (i.e., CFU: colony forming units) in the substrate and residue were estimated using plate counts from a dilution series. Microbes were extracted from samples (10 g) by 2 min Stomacher treatment in a medium for recovery of organisms (Difco Maximum Recovery Diluent, BD Diagnostics, Le Pont-de-Claix, France). For each sample, 50 μL of the dilution series were spread in duplicate on Petri dishes (diameter: 90 mm) divided into four quadrants. Since we partially recorded colonies within the representative range of 20–250 for different dilutions and replicate plates, counts in the Stomacher-homogenate were calculated using equation 2 (Maturin and Peeler, 2001)

$$\frac{CFU}{mL} = \frac{\sum \text{c}}{\text{V} \times (1\text{n}_1 + 0.1\text{n}_2) \times \text{d}} \quad (2)$$

where Σc is the number of colonies on all plates, V is the volume added to each plate (0.05 mL), n_i is the number of quadrants counted of the i_{th} dilution, and d is the dilution. Total viable counts (TVC) were determined using standard agar (15 g/L Agar, VWR International, Leuven, Belgium; 30 g/L Tryptic Soy Broth No. 2, Sigma Aldrich GmbH, Buchs, Switzerland), lactic acid bacteria (LAB) on De Man, Rogosa, and Sharpe Agar (MRS, VWR International), and fungi on Dichloran Rose Bengal Chloramphenicol Agar (DRBC, Sigma Aldrich GmbH) after incubation at 30°C for 20 to 48 h. Media and incubation conditions were selected based on the manufacturer's directions and previous work by Wynants et al. (2019).

The bacterial community was determined by high-throughput 16S rRNA gene sequencing using the MiSeq Illumina platform. Total genomic DNA was extracted from 0.2 g of substrate (in single) or residue (single to quadruplicate per substrate and sampling day, see **Figures 1**, **2**) sample using the DNeasy PowerSoil Kit (QIAGEN, Hilden, Germany) with one modification. To enhance DNA extraction, three sterile metal beads (diameter: 3 mm; Uiker AG, Freienbach, Switzerland) were added to each PowerBead Tube with the lysis buffer and homogenized with a Bead Ruptor (Omni International, Kennesaw GA, United States; speed 5.5, 2 × 20 s with 30 s break between rounds). Purity (NanoDrop ND 1000 Spectrophotometer, Thermo Scientific, Wilmington MA, United States) and concentration Qubit dsDNA HR Assay Kit on a Spark 10 M microplate reader (Tecan, Männedorf, Switzerland) of the extracted DNA was determined. Library preparation followed a two-step protocol. Limited-cycle PCR was conducted in a 25 μL reaction volume using KAPA HiFi HotStart ReadyMix (12.5 μL) (Kapa Biosystems, Wilmington, MA, United States), template DNA (5 μL), forward and reverse primer (0.75 μL; 0.3 mM each), and molecular-grade water (6 μL). All primers included a hexanucleotide barcode and Illumina adapters (Illumina Inc., San Diego, CA, United States). The prokaryotic V3-V4 hypervariable region was amplified in triplicate using the primer pair 341F (5′- CCT ACG GGN GGC WGC AG 3′) and 806R (5′- GGA CTA CNV GGG TWT CTA AT - 3′). PCR conditions were an initial denaturation at 95°C for 300 s, 1 cycle at 98°C for 60 s, 26 cycles of 98°C for 20 s, 51°C for 20 s, and 72°C for 12 s, and a final extension at 72°C for 120 s (Hugerth et al., 2014). Negative controls were run by replacing template DNA with molecular grade water. A positive control was run by replacing template DNA with a known mixture of bacterial DNA (i.e., mock sample). Products from the first PCR were pooled, cleaned using solid-phase reversible immobilization beads (ETH Zürich Genetic Diversity Center, Zurich, Switzerland) and used as a template for the second PCR to attach dual indices using the Nextera XT Index Kit v2 (Illumina Inc.). Index PCR included the first PCR product (2 μL), KAPA HiFi HotStart ReadyMix (10 μL), molecular grade water (4 μL), and Nextera indexing primers (2 μL). PCR conditions were an initial denaturation at 95°C for 180 s, 10 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 300 s. Index PCR products were cleaned and DNA concentration was determined using the High Sensitivity D1000 Kit on a 2200 TapeStation (Agilent Technologies Inc., Santa Clara, CA, United States). Cleaned PCR products were pooled equimolar to a library concentration of 2 nM. The concentration and purity of the pooled library were controlled using a Qubit Fluorometer (Invitrogen Q32857, Carlsbad, CA, United States) and the TapeStation, respectively. Paired-end sequencing was performed using 19 pM of the prepared library in a single MiSeq 2 × 300 bp flow cell, using the MiSeq Reagent Kit v3 and a 10% PhiX concentration according to the manufacturer's directions (Illumina Inc.).

Bioinformatics

Initial quality control of the sequencing data was conducted using FastQC (version 0.11.2). Subsequent bioinformatics data preparation included trimming of read ends with USEARCH (version 11.0.667) and merging of pairs into amplicons with FLASH (Magoč and Salzberg, 2011). Following removal of primer

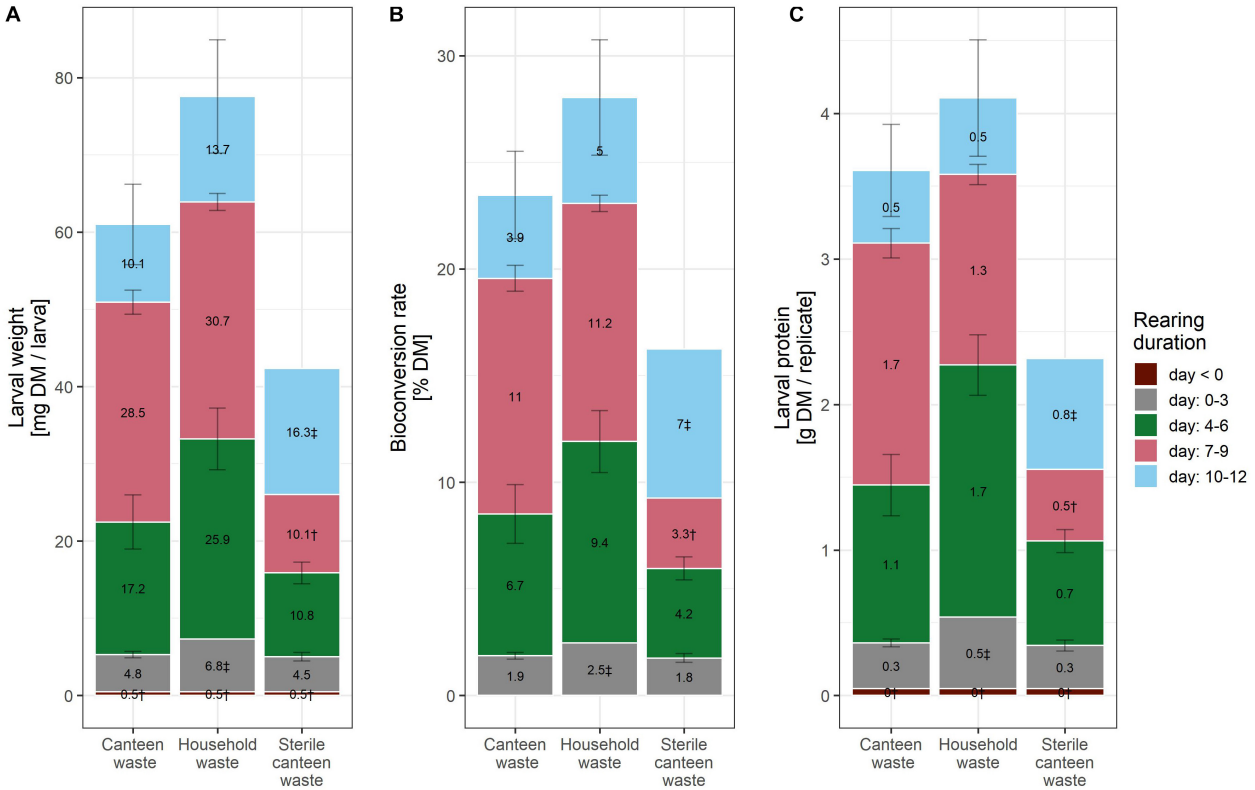

FIGURE 1 | Rearing performance of BSFL on canteen and household food waste. Bar plot of larval weight **(A)**, bioconversion rate **(B)**, and larval protein per replicate **(C)**. The bar plot label shows the mean increase in the levels of the performance indicator between the measurement days. † (n = 1) and ‡ (n = 2) indicate results with fewer than three biological replicates.

sites with USEARCH, quality-filtering was performed with the PRINSEQ-lite (version 0.20.4) (Schmieder and Edwards, 2011). The resulting high-quality reads were de-noised and clustered into zero-radius operational taxonomic units (ZOTUs) using the UNOISE3 algorithm (Edgar, 2016b). The taxonomic origin of each ZOTU was determined with the SINTAX algorithm (version 11.0.667) (Edgar, 2016a) in USEARCH using Silva 16S (V128) as the reference database. Taxonomic assignments were considered reliable when bootstrap confidence values exceeded 0.85.

Downstream Data Analyses

Data were analyzed using R version 312 3.6.2 (R Core Team, 2020). Rare ZOTUs with less than 10 total counts and samples with less than 2000 reads (i.e., the highest number of reads in the control samples) were removed before downstream analyses. ZOTUs belonging to the phylum *Cyanobacteria* and to the family *Mitochondria* were also removed, as they likely belonged to eukaryotic 16S rRNA (Michelou et al., 2013), given the plant-based nature of the rearing substrates. We abstained from statistical analyses among sampling days for all parameters due to the small number of replicates (n = 1–3). Instead, we compared the results visually or using the mean and standard deviation (when $n > 2$, difference between values for n = 2). The mean and standard deviation were calculated for rearing performance indicators, physicochemical residue parameters, and microbial counts. Differences in rearing performance indicators between measurement days were calculated by subtracting the mean values. Pearson correlation coefficients ($p < 0.01$) were calculated following visual assessment of normality (see **Supplementary Figures 2**, **3**) to identify linear dependencies between rearing performance indicators and the physicochemical residue composition.

Heat maps at the phylum and genus levels were produced in *ampvis2* (Andersen et al., 2018) after conversion of reads into percent abundance per sample. The same package was used to identify shared ZOTUs between samples with Venn diagrams (frequency cutoff >80% and abundance cutoff >0.01%). Alpha diversity (i.e., observed richness, Chao1, Shannon index, and Simpson Index) and beta diversity [i.e., non-metric multidimensional scaling (NMDS)] were calculated in *phyloseq* (McMurdie and Holmes, 2013). NMDS was used to illustrate the bacterial dynamics of ZOTUs that accounted for more than 1% of relative abundance in all samples using weighted UniFrac distance to account for phylogenic distances between ZOTUs. Distance-based redundancy analysis (dbRDA) was performed on the same data in *vegan* (Oksanen et al., 2019) with the capscale function using the Bray-Curtis Dissimilarity matrix to determine correlations between physicochemical residue properties and composition, rearing performance, and bacterial community

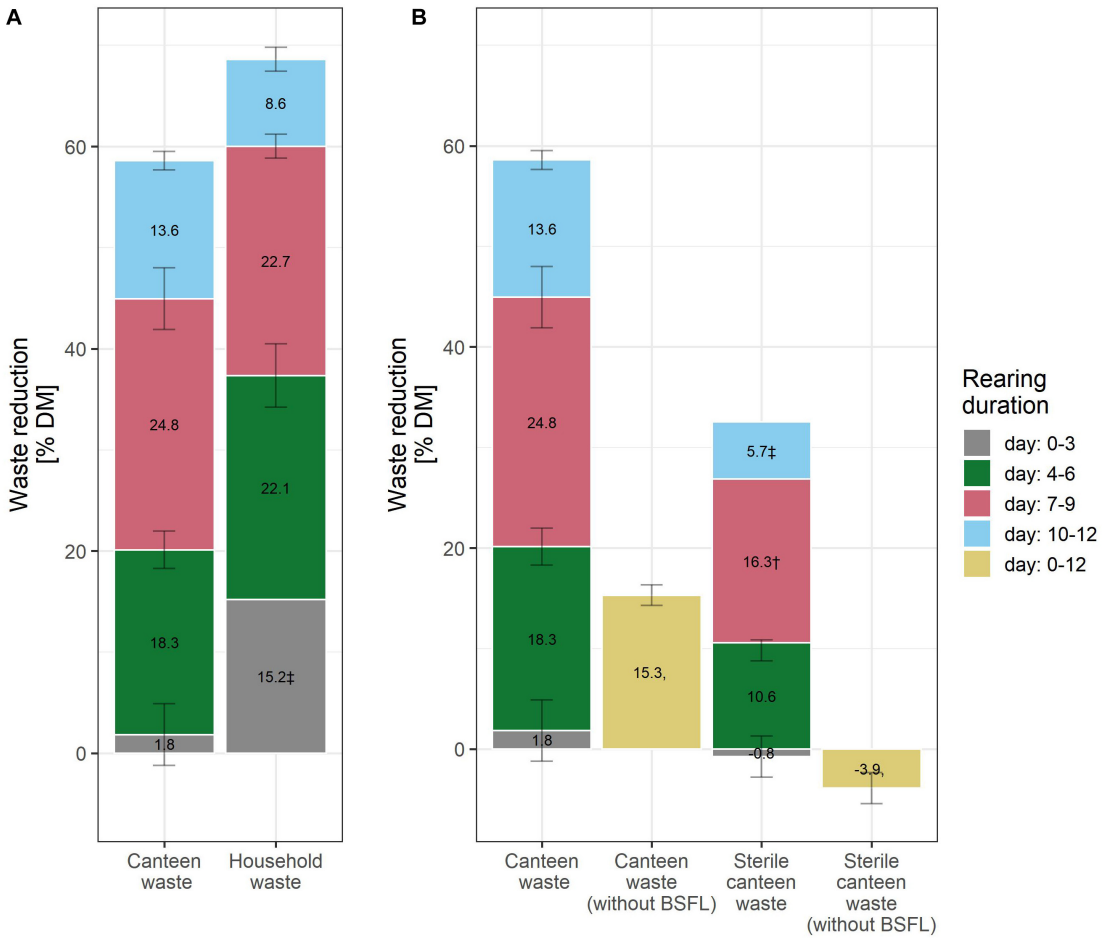

FIGURE 2 | Waste reduction during BSFL rearing with canteen and household food waste **(A)**, and on canteen waste without larvae, and sterile canteen waste with and without larvae **(B)**. The bar plot label shows the mean increase in the levels of the performance indicators between the measurement days. † (n = 1) and ‡ (n = 2) indicate results with fewer than three biological replicates.

dynamics. Prior to analysis, parameters with co-linearity were excluded from the data with a Pearson correlation matrix (see **Supplementary Figure 1**) and variance inflation factors (VIF) using the *usdm* function. A VIF value > 5 indicated multi-collinearity. The remaining parameters were scaled and centered. The significance of the model and the correlation of each parameter with the bacterial community was determined by the permutation test (1000 iterations) with a p-value < 0.05, denoting significance.

RESULTS

Waste Nutrient Composition

The household and canteen waste rearing substrates had similar protein contents, but canteen waste was richer in hemicellulose, lipids, and non-fiber carbohydrates (**Table 1**). Household waste had more cellulose and lignin, as well as glucose and fructose, that was almost absent in the canteen waste.

Rearing Performance

Considering the mean and standard deviation, the bioconversion rate and larval protein were similar between the two rearing substrates (**Figures 1B,C**), but larval weight (**Figure 1A**) and waste reduction (**Figure 2A**) were higher for household than for canteen waste. Following 12 days of rearing, mean (standard deviation) bioconversion rate and larval protein were 28.0 (2.7)% DM and 4.1 (0.4) g protein/replicate for household waste, and 23.5 (2.1)% DM and 3.6 (0.3) g protein/replicate for canteen waste. Larval weight and waste reduction were higher for household than for canteen waste: 77.6 (7.3) mg DM and 68.6 (1.2)% DM for household waste, and 61.0 (5.2) mg DM and 58.6 (1.0)% DM for canteen waste.

Inactivation of the initial canteen waste microbiota by irradiation reduced rearing performance (**Figures 1**, **2**). In rearing with sterile canteen waste, larval weight, bioconversion rate, larval protein levels, and waste reduction were reduced by 18.7 mg DM, 7.3% DM, 1.3 g protein/replicate, and 26.8% DM, respectively. Without larvae, 15.3 (1.0)% DM was lost from

TABLE 1 | Mean nutrient composition of canteen and household waste used for rearing.

	Non-fiber carbohydrates					*Fiber*					
	Protein	**Total**	**Glucose**	**Fructose**	**Starch**	**Total**	**Hemicellulose**	**Cellulose and lignin**	**Lipids**	**P:NFC[1] ratio**	**Caloric[2] content**
Canteen waste	15.3 (0.4)	38.6 (0.1)‡	0.3 (0.1)	0 (0.0)‡	37.2 (2.0)	36.1 (2.3)‡	26.9 (1.2)‡	9.1 (1.1)‡	24.5†	1:2	471
Household waste	16.3 (1.1)	29.5 (4.1)‡	5.9 (0.7)	7.5 (1.5)‡	16.5 (2.4)	22.9 (1.5)‡	8.0 (1.1)‡	14.9 (0.4)‡	18.1†	1:2	412

Nutrients are in percent dry mass and caloric content in kcal per 100 g dry waste. In parenthesis: standard deviation for samples where n ≥ 3 and differences between analyses where n = 2. [1] *P:NFC = ratio of protein to non-fiber carbohydrates (NFC).* [2] *gross caloric content of protein, NFC, and lipids.* † *n = 1,* ‡ *n = 2.*

canteen waste, and −3.9 (1.6)% DM was lost from sterile canteen waste (**Figure 2B**).

Physicochemical Residue Composition

Rearing performance indicators were positively correlated with residue moisture content (r = 0.75–0.86, $p < 0.01$) and pH (r = 0.76–0.81, $p < 0.01$) (**Table 2**). Moisture and nitrogen content (**Table 2**) increased throughout the rearing experiment in the canteen and household waste residue in comparison to the substrates. The residue pH decreased from the substrate value in the first half of rearing, and then increased above the initial substrate value in the second half of rearing. Residue organic matter had different trends for the two rearing substrates. It decreased from the value in the household waste, but not in the canteen waste substrate. Values for carbon (range: 49.2–56.0% DM), water activity (range: 0.95–0.99), and temperature (range: 27.5–29.9°C) showed low variability throughout the rearing experiment from the initial value ($\leq\pm$ 5%, see **Supplementary Table 2**). The residue composition changed much less when the initial canteen waste microbiota was inactivated. Except for the residue moisture content and the carbon to nitrogen ratio, the residue composition was $\leq\pm$ 5% relative to the initial value in the substrate.

Microbial Dynamics

Canteen and Household Waste Rearing Substrates

Canteen and household waste had similar counts of TVC, LAB, and fungi (**Table 2**). Throughout the rearing experiment, residue microbial counts deviated by ±1–3 $\log_{10}$ CFU/g from the counts in the substrate. At the end of the rearing experiment, TVC in the canteen waste residue was 1 $\log_{10}$ CFU/g higher and in the household waste residue 1 $\log_{10}$ CFU/g lower than that in the substrate. LAB counts decreased by 1–2 $\log_{10}$ CFU/g and fungal counts increased by 2–3 $\log_{10}$ CFU/g in the residue in comparison to the counts in the substrate.

Considering all 45 samples, gene sequencing using DNA produced a total of 1,783,860 reads, with an average of 44,597 reads/sample, and a total of 275 ZOTUs. Rarefaction curves (see **Supplementary Figure 4**) demonstrate that samples were sequenced to an extent sufficient to approximate true diversity. The canteen waste and household waste bacterial community consisted of 119 and 125 ZOTUs, respectively (**Table 2**). Canteen waste was dominated by a few genera of *Firmicutes*, and household waste by *Firmicutes* and *Proteobacteria* (**Figure 3**). Dominant genera were *Leuconostoc*, *Bacillus*, *Staphylococcus* in the canteen waste and *Leuconostoc*, *Weissella*, *Pseudomonas*, *Oxalobacteraceae*, and *Lactococcus* in the household waste. Sixty two percent of the relative abundance of the two wastes was due to 28 shared ZOTUs, while 49 ZOTUs were unique to canteen waste and 34 ZOTUs were unique to household waste (see Venn diagram in **Supplementary Figure 5**). Unique high-abundance (>10%) species were from *Pseudomonas* and *Oxalobacteraceae* in household waste and *Staphylococcus* and *Bacillus* in canteen waste.

The results for the bacterial community alpha and beta diversity demonstrated that the addition of BSFL to the substrates

TABLE 2 | Physicochemical properties, composition, microbial counts, and bacterial community alpha diversity (i.e., richness and diversity) in the substrates and residues.

Substrate	Day	Moisture content	pH	Nitrogen	C/N	Organic matter	TVC	LAB	Fungi	Observed richness	Chao 1	Shannon Index	Simpson's Index
		%	–	%DM	–	%DM	log_{10}/g	log_{10}/g	log_{10}/g	–	–	–	–
BSFL rearing													
Canteen waste	0	69.5 (0.2) ‡	4.4 (0.0) ‡	2.9†	19.3†	95.9 (0.0)‡	9.2 (0.2)	9.2†	5.2 (0.5)	119†	140†	2.4†	0.8†
	3	69.0 (1.0)	3.8 (0.0)	3.5†	15.2†	95.8 (0.1)	8.4 (0.2)	8.4 (0.2)	6.8 (0.0)	113 (14)	148 (45)	2.4 (0.2)	0.8 (0.0)
	6	71.8 (0.3)	3.7 (0.1)	3.4 (0.3)	16.2 (1.4)	95.2 (0.2)	7.7 (0.0)	7.7 (0.1)	6.7 (0.1)	92 (5)	103 (10)	2.3 (0.1)	0.8 (0.0)
	9	77.4 (1.0)	4.6 (0.1)	3.5 (0.1)	16.0 (0.4)	94.1 (0.2)	8.2 (0.0)	8.2 (0.1) ‡	7.1 (0.1)	73 (6)	95 (14)	2.5 (0.1)	0.9 (0.0)
	12	81.1 (0.7)	5.6 (0.2)	3.9 (0.1)	14.3 (0.4)	95.0 (0.2)	8.6 (0.5)	8.1 (0.1)	8.7 (0.1)	57 (5)	76 (11)	2.5 (0.1)	0.9 (0.0)
Sterile canteen waste	0	69.5 (0.2)‡	4.4 (0.0) ‡	2.9†	19.3†	95.9 (0.0)‡	n.a.	n.a	n.a	125†	143†	2.4†	0.8†
	3	68.2 (0.5)	3.9 (0.1)	3.1 (0.2)	17.6 (1.4)	95.9 (0.4)	8.6 (0.0)	8.6 (0.1)	6.4 (0.1)	78 (12)	101 (10)	1.5 (0.2)	0.6 (0.1)
	6	69.5 (0.2)	3.8 (0.0)	3.0 (0.1)	18.2 (0.7)	95.4 (0.1)	8.5 (0.1)	8.5 (0.1)	6.9 (0.2)	62 (5)	84 (20)	1.4 (0.2)	0.6 (0.1)
	9	72.0†	3.9†	3.0†	18.0†	95.3†	8.3†	8.0	7.2†	53†	66†	2.0†	0.8†
	12	73.6 (1.2)‡	4.2 (0.0)	3.1 (0.1)	17.9 (0.8)‡	95.8 (0.1)‡	9.3 (0.1)‡	8.2 (0.1)‡	9.0 (0.1)‡	63 (14)‡	89 (30)‡	2.4 (0.1)‡	0.9 (0.0)‡
Household waste	0	76.7 (0.1)‡	4.8 (0.0) ‡	3.1†	16.7†	94.3 (0.1)‡	9.2 (0.1)	9.0 (0.3)‡	5.4 (0.2)	122†	147†	2.6†	0.9†
	3	79.4 (0.2)‡	3.9 (0.0) ‡	3.3†	15.8†	93.7 (0.0)‡	8.6 (0.1)‡	8.7 (0.1)‡	6.6 (0.1)‡	122 (52)‡	142 (61)‡	2.0 (0.6) ‡	0.7 (0.2)‡
	6	83.0 (0.5)	3.9 (0.0)	3.2 (0.1)	16.5 (0.4)	92.1 (0.4)	8.0 (0.1)	7.9 (0.1)	6.3 (0.2)	99 (7)	122 (13)	2.3 (0.1)	0.8 (0.0)
	9	88.2 (0.4)	4.5 (0.1)	3.2 (0.1)	15.7 (0.5)	88.8 (0.4)	7.6 (0.1)	7.6 (0.1)	5.0 (0.1)	67 (9)	89 (14)	2.1 (0.0)	0.8 (0.0)
	12	90.2 (0.3)	6.5 (0.6)	3.6 (0.1)	13.8 (0.5)	85.5 (0.6)	10.0 (0.5)	7.5 (0.0)	7.5 (1.3)‡	62 (5)	88 (9)	2.2 (0.1)	0.8 (0.0)
Without BSFL													
Canteen waste	12	72.2 (0.3)	4.8 (0.3)	3.8 (0.0)‡	14.8 (0.2 ‡	95.2 (0.1)‡	n.a	n.a	n.a	106 (4)‡	122 (10)‡	2.7 (0.0)‡	0.9 (0.0)‡
Sterile canteen waste	12	66.9 (0.4)	4.0 (0.1)	3.1 (0.1)	17.2 (0.5)	95.7 (0.0)‡	n.a	n.a	n.a	97 (9)	133 (11)	1.6 (0.3)	0.6 (0.1)

*See **Supplementary Table 2** for the carbon, water activity, and temperature results. In parenthesis: standard deviation for samples where n ≥ 3, differences between analyses where n = 2.* † *n = 1,* ‡ *n = 2, n.a., not analyzed. C/N: carbon to nitrogen ratio. Richness: The number of ZOTUs determined with 16S rRNA gene sequencing based on DNA extracted from samples. Chao 1: the total number of ZOTUs estimated by infinite sampling. A higher number indicates a higher richness (Chao, 1984). Shannon Index: Measure richness and evenness. Increases with community richness and evenness (Shannon and Weaver, 1949; Lemos et al., 2011). Simpson's Index: Measures community evenness. Index increases as diversity decreases (Simpson, 1949; Lemos et al., 2011).*

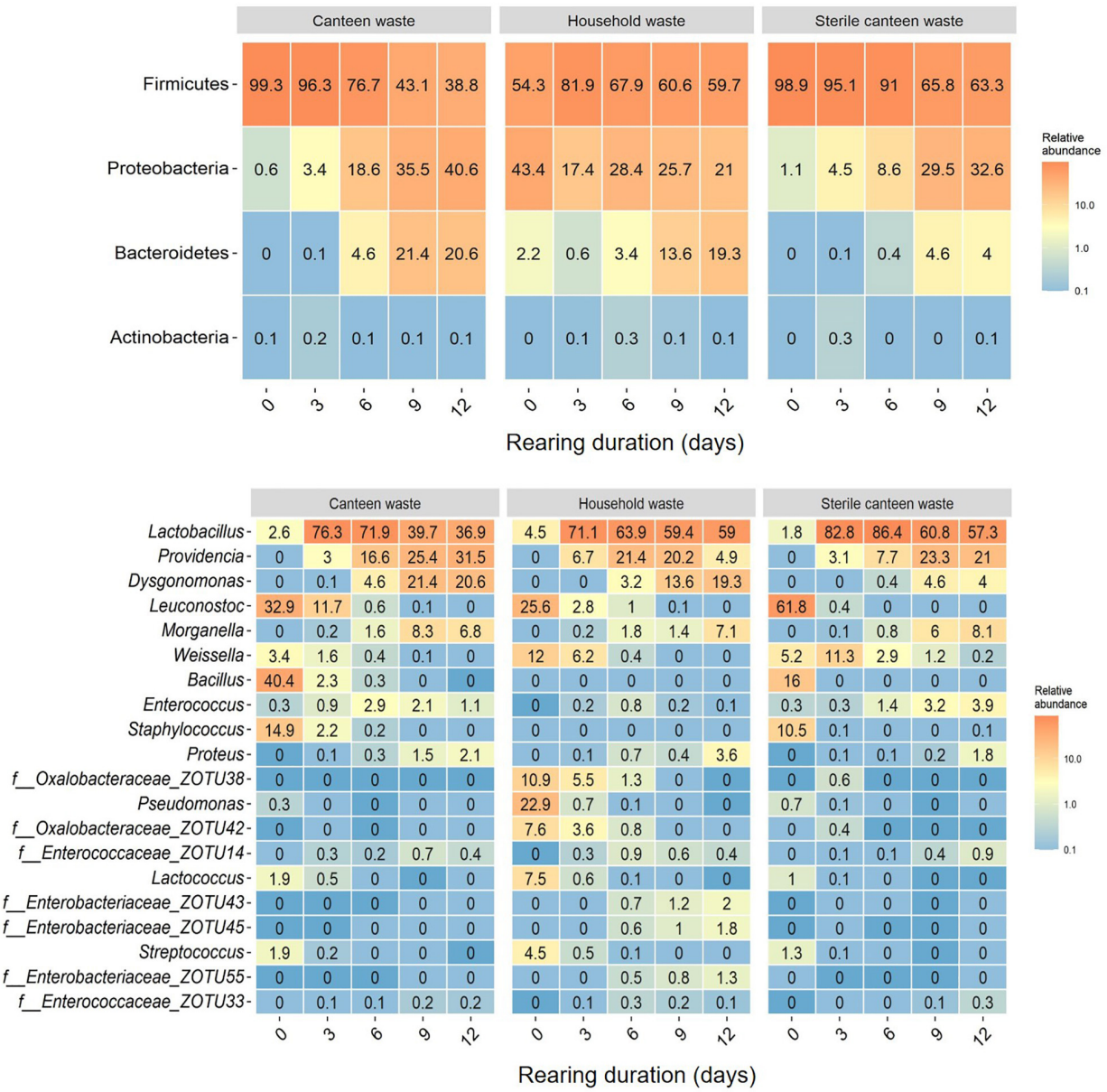

FIGURE 3 | Substrate and residue bacterial community at different days (0–12 days) of BSFL rearing. Heatmaps of the top phyla **(top)** and top 20 genera **(bottom)** based on the relative abundance of ZOTUs in all samples. Relative abundances are the mean of replicate samples rounded to one digit. If no clear assignment to a genus was possible, the family assignment is shown together with the ZOTU.

dramatically changed the bacterial richness (**Table 2**) and community (**Figures 3**, **4**). Community richness decreased on both wastes throughout rearing, and the bacterial community between the two substrates became more similar. Replicate samples clustered well based on UniFrac distances according to rearing day and substrate. After 3 days of rearing, the bacterial communities were more similar to each other than the initial wastes (**Figure 4A**). Changes in the bacterial community were the largest within the first 6 days. The similarity between bacterial communities decreased again following 9 days. In both residues, most bacteria belonged to the genus *Lactobacillus*. Throughout the rearing period, the phyla *Proteobacteria* and *Bacteroidetes*, and the genera *Providencia*, *Dysgonomonas*, *Morganella*, and *Proteus* became more abundant than in the substrate.

Sterile Canteen Waste

Despite complete microbial inactivation by irradiation, the sterile canteen waste substrate had a similar bacterial community as the non-sterile canteen waste (**Table 2** and **Figures 3**, **4B**). Sterile canteen waste and canteen waste shared ZOTUs that accounted for 99.5% of the relative abundance (see Venn diagram in **Supplementary Figure 6**). Bacterial dynamics were also similar

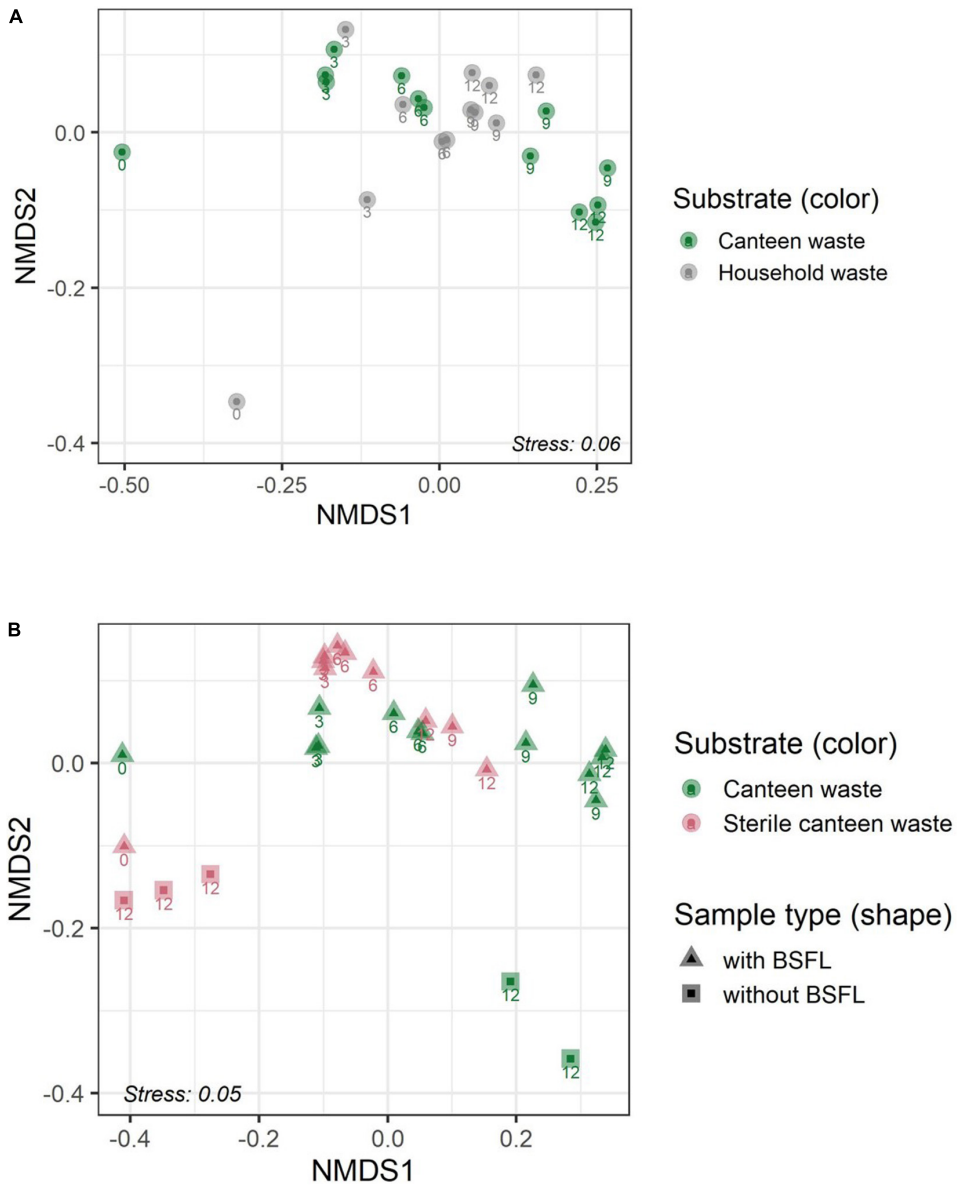

FIGURE 4 | Residue bacterial community dynamics in the two food wastes **(A)** and in canteen waste (sterile, non-sterile) **(B)** during rearing illustrated by non-metric multidimensional scaling (NMDS) of bacterial communities in the substrate and residue based on weighed UniFrac dissimilarity. Numbers adjacent to the symbols indicate the sampling day during BSFL rearing (0–12 days).

between sterile and non-sterile canteen waste (**Figure 4B**). The addition of BSFL to the sterile canteen waste led to a repopulation of the substrate to microbial counts similar to those determined in the non-sterile canteen waste (**Table 2**). *Lactobacillus* was also highly abundant in the sterile canteen waste residues, decreased in abundance during rearing, and *Providencia*, *Dysgonomonas*, *Morganella*, and *Proteus* became more abundant, but to a smaller extent compared to non-sterile canteen waste.

Substrates Without Larvae

The bacterial community of sterile canteen waste was similar to the initial bacterial community of the canteen waste substrates, after 12 days of storage (**Figure 4B**). However, for both sterile and non-sterile canteen waste, it differed noticeably from the bacterial communities in the rearing residues (**Figure 5**). The substrates stored without larvae had a higher bacterial richness (**Table 2**) and *Leuconostoc*, *Stenotrophomonas*, *Hafnia-Obesumbacterium, Lactococcus,* and *Enterobacteriaceae* were highly abundant but absent in the rearing residues (**Figure 5**). Genera that became more abundant in the rearing residues (see section "Substrates Without Larvae," i.e., *Providencia*, *Dysgonomonas*, *Morganella,* and *Proteus)* were absent from the substrates stored without larvae.

TABLE 3 | Literature summary of bacterial communities in BSFL rearing residues.

References	Substrate	Major families*	Major genera**	Feeding rate	Feeding interval	Larval density
				mg DM/day	–	BSFL/cm^2
This study	Canteen waste Household waste	***Lactobacillaceae***, ***Enterobacteriaceae***, *Porphyromonadaceae*	***Lactobacillus***, ***Providencia,*** *Dysgonomonas*, ***Morganella***	22	One time	2.5
Jiang et al. (2019)	Food waste	*Corynebacteriaceae*, ***Bacillaceae***	*Corynebacterium*, ***Bacillus***, ***Lactobacillus***, *Enterococcus*	125	Every 2 days	2.9
Bruno et al. (2019)	Substrate for fly rearing	***Flavobacteriaceae***, *Comamonadaceae, Sphingobacteriaceae, Stenotrophomonas*	*Flavobacteriaceae, Comamonas,* ***Sphingobacterium***, *Stenotrophomonas*	*ad libitum*	Every 1–2 days	1.0
	Vegetable waste	***Lactobacillaceae***, *Methylocystaceae*	***Lactobacillus***, *Methylocystaceae*			
	Fish waste	***Planococcaceae***, *Clostridiales, Bacilli,* ***Peptostreptococcaceae***, *Clostridiaceae*	*Planococcaceae, Clostridiales, Bacilli, Tissierella*, ***Clostridium***			
Wynants et al. (2018)	Fruit and vegetable waste	***Enterobacteriaceae***, ***Bacillaceae***, *Planococcaceae*	*Cosenzaea*, ***Bacillus***, ***Morganella***, ***Providencia***, ***Sporosarcina***	*ad libitum*	Every 3–4 days	–
	Supermarket/ restaurant waste	*Lactobacillaceae, Enterobacteriaceae*	***Lactobacillus***, ***Morganella***, ***Providencia***			
	Poultry blood	***Lactobacillaceae***, ***Enterobacteriaceae***, *Clostridiaceae,* ***Peptostreptococcaceae***	***Lactobacillus***, *Buttiauxella*, ***Clostridium***, *Pediococcus, Peptostreptococcus*			
	Poultry manure	***Bacillaceae***, *Carnobacteriaceae*	***Gracilibacillus***, *Virgibacillus, Lentibacillus,* ***Atopostipes***, ***Amphibacillus***, ***Bacillus***, ***Oceanobacillus***			
	Brewery waste, fruit waste	*Pseudomonadaceae, Sphingobacteriaceae,* ***Flavobacteriaceae***, ***Bacillaceae***	*Pseudomonas*, ***Sphingobacterium***, ***Flavobacterium***, *Myroides*, ***Bacillus***, ***Oceanobacillus***, *Mucilaginibacter*			
	Vegetable waste, mill by-products, yeast	***Bacillaceae***, ***Planococcaceae***	***Gracilibacillus***, ***Oceanobacillus***, ***Sporosarcina***			
	Fruit and vegetable waste, brewery waste, former food products, food waste	***Bacillaceae***, *Pseudomonadaceae, Carnobacteriaceae,* ***Flavobacteriaceae***, ***Bacillaceae***	***Bacillus***, ***Amphibacillus***, *Thiopseudomonas,* ***Atopostipes***, ***Flavobacterium***, *Gracilibacillus*			
Shelomi et al. (2020)	Soy pulp, Canteen waste	*Cellvibrionaceae, Cytophagaceae, Caulobacteraceae*	*Cellvibrio, Leadbetterella, Brevundimonas*	*ad libitum*	Daily	–

*Families and genera identified in at least two studies are highlighted in bold. The bacterial community was identified in all studies by 16S rRNA gene sequencing. * relative abundance >10%, in order of descending relative abundance. ** relative abundance >5%, in order of descending relative abundance; whenever the genera could not be identified, the lowest available taxonomic rank (i.e., family or class) is reported.*

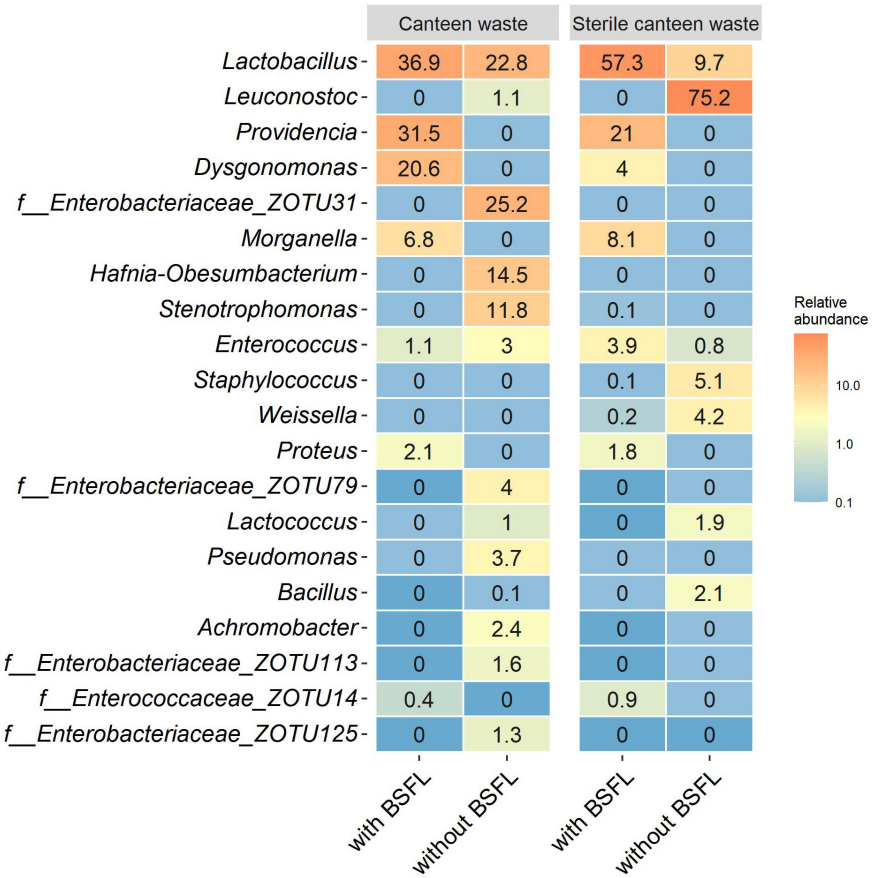

FIGURE 5 | Bacterial community in canteen waste (sterile, non-sterile) with and without larvae following 12 days of rearing or storage. Heatmaps of the top 20 genera based on the relative abundance of ZOTUs in all samples. Relative abundances are the mean of replicate samples rounded to one digit. If no clear assignment to a genus was possible, the family assignment is shown together with the ZOTU.

Correlation Among Bacterial Community, Rearing Performance, and Residue Composition

Bacterial community dynamics correlated with rearing performance and physicochemical properties and composition of the residue (**Figure 6**). Of all parameters, larval weight, and residue pH, carbon, nitrogen, and water activity had the lowest co-linearity and were used in distance-based redundancy analysis (dbRDA). The global dbRDA model and the first two axes were statistically significant and explained 83.5% of the variability in the bacterial community.

DISCUSSION

Substrate Microbiota Contributes to Rearing Performance

We compared results for substrate and residue bacterial communities and BSFL rearing performance indicators between sterile and non-sterile canteen waste. We hypothesized that inactivation of the initial bacterial community in the rearing substrate will decrease rearing performance and reveal important members of the microbiota influencing improved rearing performance.

Our hypothesis was confirmed as results show that inactivation of microbes in the substrate reduced rearing performance (**Figures 1**, **2**), suggesting that the initial substrate microbial community contributes to substrate decomposition and/or larval growth. The initial canteen waste microbial community was dominated by LAB (**Figure 3**), which is typical for fermented foods (Wu et al., 2018) and grain-based substrates (Wynants et al., 2019) with high contents of digestible carbohydrates (i.e., starch) (**Table 1**) and low pH (**Table 2**). LAB that have been shown to promote the growth of *Drosophila melanogaster* (Shin et al., 2016; Storelli et al., 2018), are routinely used as feed additives to promote the growth of farmed animals (e.g., poultry, pigs, and cattle) (Vieco-Saiz et al., 2019), and the addition of LAB (e.g., *Lactobacillus buchneri*) to the substrate has been shown to increase BSFL rearing performance (Somroo et al., 2019; Mazza et al., 2020). The mechanisms by which LAB promote growth are debated and still part of ongoing research, but suggestions include an increase of the metabolic capacity by fermentation of substrate carbohydrates into short chain

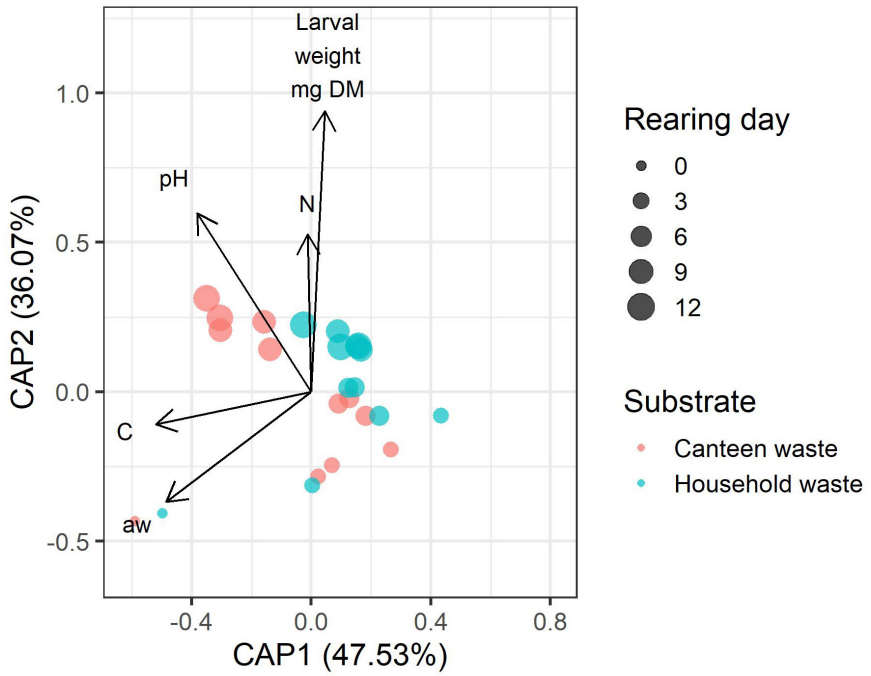

FIGURE 6 | Distance-based redundancy analysis (dbRDA) biplot of canteen waste and household waste samples showing the correlation between the physicochemical properties and composition of the residue (pH, N, C, a_w), larval weight, and the bacterial community. The length of the vectors indicates the relative importance of the parameter. The vector angle between variables indicates a correlation. Variables with smaller angles between vectors have a closer positive correlation. Perpendicular vectors indicate that there is no correlation. Vectors pointing in opposite directions indicate a negative correlation. Shorter distance between points indicates similarity between bacterial communities.

fatty acids (as indicated by the low pH in the residue, **Table 2**), growth signaling, immunity, and protection and maintenance of stable gut microbiomes (Holzapfel et al., 2001). Interestingly, bacteria and fungi in the digestive tract or on the surface of larvae repopulated the high-energy electron beam treated canteen waste during rearing (**Table 2**) but rearing performance indicators remained nevertheless lower given the loss of the initial microbial community (**Figures 1**, **2**).

Intact DNA after high-energy beam treatment interfered with further interpretation of bacterial dynamics in the sterile canteen residue. Despite lethal irradiation doses (Gold et al., 2020b), similar bacterial community between sterile and non-sterile canteen waste (**Figure 3**) after 12 days indicates that some bacterial DNA remained intact and was considered in the bacterial community based on DNA sequencing. As the bacterial communities identified in the sterile canteen waste residue may also include members without any major metabolic functions, these results should be interpreted with caution and are not further discussed. This finding agrees with recent research on the effect of irradiation on bacteria. Hieke and Pillai (2018) reported that *Escherichia coli* maintain their cell integrity post-irradiation, which protects DNA from denaturation. Since larvae and the associated microbiota digest bacteria (Gold et al., 2018), it is possible that the determined bacterial community becomes more representative of the viable bacterial community with increasing rearing duration.

Rearing Performance Between the Two Food Wastes

While it has been recognized that both the rearing substrate nutrient composition and the microbial community (De Smet et al., 2018) composition influence rearing efficiency and reliability, previous studies typically emphasized only one aspect (Bruno et al., 2019; Klammsteiner et al., 2020) or considered both aspects but in isolation (Wynants et al., 2019; Gold et al., 2020a). We determined both substrate nutrient contents and bacterial communities and this over the rearing duration. We purposely used two rearing substrates with similar nutrient contents. Thereby we expected that the differences in rearing performance could be more easily attributed to the different substrate bacterial communities and could reveal members associated with rearing performance rather than showing the effect of nutritional differences.

Rearing performance was high with both food wastes (**Figures 1**, **2**). We expected this based on the high nutrient contents (**Table 1**). Previous studies have reported lower bioconversion rates for food waste of 13.9–22.7% DM (Nyakeri et al., 2017; Lalander et al., 2019; Gold et al., 2020a) as compared to 23.5–28.0% DM in this study. Waste streams with low nutrient contents, such as digested waste water sludge, or cow and poultry manure, typically have even much lower bioconversion rates of 2.2–3.8% DM (Lalander et al., 2019; Gold et al., 2020a). Since the bioconversion rate is a key

indicator determining the economics of insect rearing and the environmental sustainability of insect-derived products (Smetana et al., 2019), the results show that food waste could be an especially viable substrate for BSFL rearing.

Rearing performance was similar between the two rearing substrates with regard to conversion efficiency (**Figure 1**). However, larval biomass production (**Figure 1**) and waste reduction (**Figure 2A**) were higher for household than for canteen waste substrates. As demonstrated by the reduction in rearing performance due to the loss of the initial microbial community, differences in the initial substrate bacterial community could have contributed to the differences in this rearing performance. Notably, between the two substrates, there was a considerably higher waste reduction within the first 3 days of rearing (**Figure 2A**). However, as the two substrates shared most taxa, no conclusions can be drawn on bacteria that may explain the differences in rearing performance between substrates. It appears to be more likely that the disparity in rearing performance between substrates is due to different content and digestibility of nutrients. For example, non-fiber carbohydrates in household waste are likely more digestible for BSFL, considering that they were mostly comprised of glucose and fructose (**Table 1**). Pimentel et al. (2018) demonstrated that *Musca domestica* larvae can directly absorb glucose in the anterior and posterior midgut. Starch, however, requires catalysis before absorption and comprises all non-fiber carbohydrates in the canteen waste (**Table 1**) therefore being less directly digestible. In addition, household waste digestibility may have been increased by the onset of microbial substrate decomposition during storage (i.e., in the order of hours to days) at the household level (Albuquerque and Zurek, 2014). In contrast, canteen waste was collected on the same day of waste generation and stored at 4°C.

In summary, these findings demonstrate the challenge of unambiguously identifying the causes for differences in rearing performance despite comprehensive analysis of nutrient contents and bacterial communities. Differences in rearing performance could be due to differences in the low-abundance taxon or due to differences on the species level for the same genus. In addition, although Bruno et al. (2019) and Klammsteiner et al. (2020) concluded that bacterial gut communities are rather similar between substrates with broadly similar nutrient contents (**Table 1**), differences in bacterial community structure and function among substrates, as demonstrated by Zhan et al. (2020), may also have contributed to rearing performance differences.

Common Fly Associated Bacteria Dominate the Rearing Residue

Microbial dynamics in the rearing residues of BSFL are still poorly understood. We studied bacterial community dynamics to identify taxa that were more abundant during BSFL rearing and that were absent in the controls (i.e., substrates stored under the same environmental conditions but without larvae). As suggested by previous researchers (Zhao et al., 2017; Ao et al., 2020), we hypothesized that bacteria enriched in the rearing residue contribute to substrate decomposition. This could imply that pure-culture bacteria and/or defined bacterial mixtures comprised of these bacteria from the residue could potentially increase large-scale BSFL rearing.

Previous studies have reported inconsistent results on the significance of altered substrate bacterial community during BSFL rearing (Bruno et al., 2019; Jiang et al., 2019; Cifuentes et al., 2020). Our study confirmed that BSFL rearing dramatically changes the substrate bacterial community and physicochemical properties and composition. For example, consistent with previous research, the pH increased in the residue during BSFL rearing (**Table 2**; Erickson et al., 2004; Lalander et al., 2014; Ma et al., 2018; Jiang et al., 2019; Wynants et al., 2019; Klammsteiner et al., 2020). Characteristic for food waste decomposition, the residue pH initially decreased due to the hydrolysis of proteins (Wu et al., 2018). Following 6 days of rearing, the pH increased beyond the initially value in the substrate, presumably due to the excretion of nitrogenous compounds by BSFL (e.g., uric acid) (Klammsteiner et al., 2020). Similar to the results of Jiang et al. (2019), ordination plots (**Figure 4**) revealed an obvious succession of the bacterial community throughout BSFL rearing. Consistent with previous research by Jiang et al. (2019) and Wynants et al. (2018), this study observed a reduction in bacterial richness (**Table 2**) in the residue in comparison to the substrate over the rearing duration. In contrast to Wynants et al. (2018) and Jiang et al. (2019), Bruno et al. (2019) did not observe significant differences in the bacterial community between substrates and residues, and Cifuentes et al. (2020) observed an increase in bacterial richness. Different abiotic (e.g., temperature, substrate nutrient content, and pH) and biotic (e.g., initial substrate bacterial communities) factors known to influence microbial ecology and the presence and stability of antimicrobial proteins by BSFL (De Smet et al., 2018; Vogel et al., 2018) may provide some explanation for discrepancies in findings among studies. Wynants et al. (2018) studied BSFL rearing with a variety of mostly grain-based substrates in several laboratory and industry-scale settings, Jiang et al. (2019) with food waste in an industry-scale setting, and Bruno et al. (2019) with a standard substrate for fly rearing, vegetables and fish waste in a laboratory setting (**Table 3**). Considering that substrate digestion by BSFL (Cai et al., 2018; Bruno et al., 2019) and other fly larvae (e.g., *Lucilia sericata*, Diptera: Calliphoridae) (Mumcuoglu et al., 2001; Lerch et al., 2003) decreases bacterial richness along the digestive tract (Gold et al., 2018; Vogel et al., 2018), different feeding rates, intervals, and larval densities among studies could be especially relevant (**Table 3**). One could expect that the substrate and residue bacterial community is altered to a greater extent by BSFL when less feed is provided per larvae (i.e., lower feeding rate and/or higher larval density). Our study and that of Jiang et al. (2019) had a 2–3-fold higher larval density and feed was provided less frequently when compared to Bruno et al. (2019), thus allowing more time for larval digestion (**Table 3**). Cifuentes et al. (2020) provided an insufficient rearing protocol to allow comparison with the other studies. A further study focusing more on microbial dynamics in BSFL under different rearing parameters is therefore recommended. However, considering the results of

this and previous studies, and that large-scale rearing facilities may have higher larval densities [e.g., 4 larvae per cm^2 by Dortmans et al. (2017)], a lower number of bacterial species can be expected in well-digested residues in comparison to the initial substrates.

Genera in the residues at the time of larval harvest are ubiquitous in the environment (e.g., soil, water, digestive tracts of humans, and farmed animals) and have been previously identified in different life stages of the BSF (**Table 3**; Bruno et al., 2019; Cifuentes et al., 2020; Klammsteiner et al., 2020; Raimondi et al., 2020; Zhan et al., 2020), *Musca domestica* (Zurek et al., 2000; Su et al., 2010; Gupta et al., 2012), and *Lucilia sericata* (Singh et al., 2014). Bacteria commonly associated with BSFL residues (**Table 3**) are from the *Lactobacillaceae*, *Bacillaceae*, *Enterobacteriaceae*, *Planococcaceae,* and *Flavobacteriaceae* families. One possible explanation for the recurrence of these taxa in fly larvae residues is through the transfer of intestinal commensal bacteria with larval secretions and excretions into the residue (Zhao et al., 2017; Storelli et al., 2018). The results of this study support this hypothesis. Recurring taxa in the residues belong to the phyla *Firmicutes*, *Proteobacteria*, and *Bacteroidetes*, which are the main intestinal bacteria in fly larvae (Zurek et al., 2000; Jeon et al., 2011; Boccazzi et al., 2017; Scully et al., 2017; Cifuentes et al., 2020; Liu et al., 2020). *Providencia* spp., *Dysgonomonas* spp., *Morganella* spp., and *Proteus* spp. present in the BSFL residues, and absent in controls without larvae, are highly abundant in the BSFL guts (Ao et al., 2020; Cifuentes et al., 2020; Klammsteiner et al., 2020; Raimondi et al., 2020). Identification of *Dysgonomonas*, *Providencia*, *Morganella,* and *Proteus* in the posterior midgut of BSFL suggest that members of these genera may survive gut passage (Bruno et al., 2019). The one-time feeding regime, low feeding rate, and high larval density in our experiments could have contributed to the more pronounced appearance of these genera than in previous studies (Bruno et al., 2019; Wynants et al., 2019), as the residue presumably passed the digestive tract more often than that at higher feeding rates and frequency and lower larval densities. However, it is important to bear in mind that these genera are typically present at low abundance (<0.01% in this study) in BSFL substrates (see **Figure 3**; Bruno et al., 2019; Shelomi et al., 2020). Consequently, the proliferation of these genera during BSFL rearing could also have been in part due to the observed changes in the residue physicochemical properties and composition, and not only the secretions/excretions of BSFL. For example, members of the genera *Proteus*, *Providencia*, and *Morganella* are involved in urea hydrolysis and thus may benefit from the nitrogenous compounds excreted by BSFL (Manos and Belas, 2006; Klammsteiner et al., 2020).

Dysgonomonas, *Providencia*, *Proteus,* and *Morganella* have important functions in the life cycle of fly species. Despite their prominence in the digestive tracts and residues, the ways in which they influence larvae and substrate decomposition are still poorly understood. Studies focusing on the role of these bacteria in BSFL rearing do not exist. *Morganella* spp. and *Providencia* spp. are typically transferred between generations in several fly species (Su et al., 2010), and *Proteus spp.* have been isolated from the egg surface of the BSF (Mazza et al., 2020) and the digestive tract of *Musca domestica* (Su et al., 2010; Gupta et al., 2012). One function of these bacteria appears to be stimulation of the fly ovipositor by the release of volatile compounds. Different species of these genera, such as *Proteus mirabilis,* have been shown to control fly oviposition in *Lucilia sericata* (Ma et al., 2012; Tomberlin et al., 2012; Uriel et al., 2020) and *Cochliomyia hominivorax* (Diptera: Calliphoridae) (DeVaney et al., 1973; Eddy et al., 1975). In addition, *P. mirabilis* may repel bacteria that are detrimental to larval development. *P. mirabilis* is associated with *Lucilia sericata* and exerts bactericidal effects (Erdmann and Khalil, 1986), but the antimicrobial excretion/secretion from *Lucilia sericata* is not active against *P. mirabilis,* suggesting a symbiotic host-microbe relationship (Barnes et al., 2010). In addition, bacteria may support the decomposition of substrate constituents (Zhao et al., 2017). Bruno et al. (2019) identified *Dysgonomonas* and Ao et al. (2020) *Providencia* as major genera in the BSFL digestive tracts and proposed that their members could be involved in the digestion of hemicellulose and proteins and lipids, respectively. Similarly, *Dysgonomonas* and Providencia could contribute to nutrient decomposition in the residue. However, it should be noted that there is considerable uncertainty with these claims as they are based on findings for phylogenetically different well-studied insects (e.g., honeybees and termites), correlations between bacterial communities and environmental parameters (e.g., substrate nutrients), or functional predictions based on DNA sequencing, which may not provide direct evidence for bacterial community functional capacities.

We demonstrated that there is considerable overlap between bacterial communities in BSFL residues and digestive tracts. We hypothesize that some members of these genera may influence substrate decomposition and larval development, and therefore have the potential to increase the performance of large-scale BSFL rearing. A natural progression of this work is to isolate members of these genera from residues or larval digestive tracts, and assess their potential to increase rearing performance by adding them to the rearing substrates *in vivo* (Yu et al., 2011; Xiao et al., 2018; Rehman et al., 2019; Somroo et al., 2019; Mazza et al., 2020) and *in vitro* (Gold et al., 2020c). Further research is needed to better understand the variable effectiveness among bacterial species (Mazza et al., 2020), and among strains of the same species (Yu et al., 2011), in influencing rearing performance under variable biotic and abiotic conditions typical in practice. These studies should use or imitate large-scale rearing conditions (e.g., larval densities) to ensure maximum transfer of results into practice. Even though we used realistic rearing conditions (i.e., feeding rates and larval densities), residue temperatures (27–30°C) influencing bacterial communities in BSFL (Raimondi et al., 2020) were below those found in large-scale rearing (e.g., 33–45°C) (Bloukounon-Goubalan et al., 2019) due to the bench-scale nature of our study.

Implications for Product Safety

Despite not being the main focus of this research, our results present relevant findings regarding BSFL rearing product

safety. BSFL substrates may have pathogenic microbes (Erickson et al., 2004; Lalander et al., 2013) and since BSFL live within their rearing substrate and pass it through their digestive tract, pathogenic microbes inside or on the harvested larvae are a hazard for product safety. Such pathogenic microbes in the harvested larval biomass can be eliminated by thermal or non-thermal inactivation technologies. An alternative approach could be the inactivation of pathogenic microbes in the substrate before BSFL rearing, for instance by irradiation. Our results suggest that such an approach may greatly decrease rearing performance. Future research should be undertaken to mimic more realistically rearing facility substrate inactivation technologies (e.g., pasteurization) and conditions (e.g., time, temperature). Some technologies may only lead to partial microbial inactivation and at the same time reduce particle size and increase nutrient digestibility, impacting positively on rearing performance. However, considering that fly larvae may live in close association with pathogens such as *Providencia rettgeri*, *P. mirabilis*, and *Morganella morganii*, post-harvest treatment of the residue (e.g., composting) and larval biomass (e.g., heat treatment such as pasteurization) may still be the most efficient and reliable approach.

CONCLUSION

Sustainable mass rearing of BSFL for feed and food applications requires efficient and reliable process performance. Complementing previous work on the larval microbiota, this study set out to identify bacterial taxa in two food waste rearing substrates and residues that are potentially associated with rearing performance. As expected, considering their high nutrient content, rearing performance was high with canteen and household food waste substrates, underlining their potential for efficient insect production. A loss of the initial food waste microbiota, dominated by lactic acid bacteria, decreased rearing performance, indicating that initial substrate microbiota influence the complex bioconversion process. Furthermore, the rearing performance could also be influenced by bacteria in the rearing residue. Rearing duration decreased the bacterial richness and changed the physico-chemical properties and composition of the residue, and typical members of the larval intestinal microbiota (that is, *Providencia, Dysgonomonas, Morganella*) became more abundant, suggesting their transfer into the residue through excretions. The present study provides a scientific basis for future studies that should isolate these bacteria and assess their true role in influencing rearing performance.

DATA AVAILABILITY STATEMENT

All original data presented in the study is publicly available. This sequencing data can be found at: https://www.ncbi.nlm.nih.gov/PRJNA646490. All other data and analyses can be found at: https://github.com/MoritzGold/BSFL_residue_microbiota.

AUTHOR CONTRIBUTIONS

MG: conceptualization, methodology, investigation, formal analysis, visualization, writing – original draft, and funding acquisition. FA: conceptualization, methodology, investigation, formal analysis, visualization, and writing – review and editing. CZ: conceptualization, supervision, project administration, funding acquisition, and review and editing. JZ: writing – review and editing. AM: conceptualization, supervision, project administration, writing – review and editing, and funding acquisition. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.582867/full#supplementary-material

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Effects of Bacterial Supplementation on Black Soldier Fly Growth and Development at Benchtop and Industrial Scale

*Emilia M. Kooienga[1], Courtney Baugher[1], Morgan Currin[1], Jeffery K. Tomberlin[2] and Heather R. Jordan[1]**

[1] Department of Biology, Mississippi State University, Starkville, MS, United States, [2] Texas A&M AgriLife Research, Department of Entomology, Texas A&M University, College Station, TX, United States

Historically, research examining the use of microbes as a means to optimize black soldier fly (BSF) growth has explored few taxa. Furthermore, previous research has been done at the benchtop scale, and extrapolating these numbers to industrial scale is questionable. The objectives of this study were to explore the impact of microbes as supplements in larval diets on growth and production of the BSF. Three experiments were conducted to measure the impact of the following on BSF life-history traits on (1) *Arthrobacter* AK19 supplementation at benchtop scale, (2) *Bifidobacterium breve* supplementation at benchtop scale, and (3) *Arthrobacter* AK19 and *Rhodococcus rhodochrous 21198* as separate supplements at an industrial scale. Maximum weight, time to maximum weight, growth rate, conversion level of diet to insect biomass, and associated microbial community structure and function were assessed for treatments in comparison to a control. Supplementation with *Arthrobacter* AK19 at benchtop scale enhanced growth rate by double at select time points and waste conversion by approximately 25–30% with no impact on the microbial community. Predicted gene expression in microbes from *Arthrobacter* AK19 treatment was enriched for functions involved in protein digestion and absorption. *Bifidobacterium breve*, on the other hand, had the inverse effect with larvae being 50% less in final weight, experiencing 20% less conversion, and experienced suppression of microbial community diversity. For those tested at the industrial scale, *Arthrobacter* AK19 and *R. rhodochrous 21198* did not impact larval growth differently as both resulted in approximately 22% or more greater growth than those in the control. Waste conversion with the bacteria was similar to that recorded for the control. Diets treated with the supplemental bacteria showed increased percent difference in predicted genes compared to control samples for functions involved in nutritional assimilation (e.g., protein digestion and absorption, energy metabolism, lipid metabolism). Through these studies, it was demonstrated that benchtop and industrial scale results can differ. Furthermore, select microbes can be used at an industrial scale for optimizing BSF larval production and waste conversion, while others cannot. Thus, targeted microbes for such practices should be evaluated prior to implementation.

Keywords: black soldier flies, microbiome, bacterial supplementation, benchtop scale, industrial scale

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****Correspondence:***
Heather R. Jordan
jordan@biology.msstate.edu

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INTRODUCTION

Global demand for food produced for human consumption is predicted to increase by 100% over the span of the next 40 years (Tubiello et al., 2014). Despite efforts to keep up, agricultural production is not predicted to meet the demand (Ray et al., 2013). Increased need for cattle and other animal proteins requires increased feed production with limited available land. The space and water needs of both livestock and production of their feed account for nearly 70% of all the land used in agricultural production (FAO, 2013). Furthermore, as both human and animal populations grow, there will be an increase in waste production. Manure, food, and agricultural waste all produce greenhouse gases and noxious odors as well as serve as potential incubators for pathogenic microbes (Mawdsley et al., 1995; Sahlstrom, 2003; Tyrrel and Quinton, 2003). Therefore, safe and effective waste management solutions must be developed.

The black soldier fly (BSF), *Hermetia illucens* (L.) (Diptera: Stratiomyidae) is one of about 2000 species of insects that is used as food or feed (Mitsuhashi, 2016). The adult BSF is not a pest when properly managed (Tomberlin and Huis, 2020). Additionally, adult BSF do not need to feed to survive and reproduce (Sheppard et al., 2002). BSF larvae (BSFL) are known as voracious feeders that consume and degrade most organic materials 55 up to 70% (Sheppard, 1983; Newton et al., 1992; Diener et al., 2011). They can degrade everything from fruit and vegetables (Nguyen et al., 2015), to animal remains (Tomberlin et al., 2005; Harnden and Tomberlin, 2016) and manure (Sheppard et al., 1994; Myers et al., 2008; Miranda et al., 2019). These wastes can then be converted into insect biomass that is rich in both proteins and fats (Liu et al., 2017).

Substitution or partial replacement of traditional diets with BSFL has had positive results. Weaned pigs fed a diet consisting of 50% BSFL showed a 9% improvement in feed efficiency (Newton et al., 1977; Sheppard et al., 1994). Similarly, a study conducted on rainbow trout showed that replacing up to 40% of the fish's diet with BSFL showed no negative effects on both the fish's physiology and the quality of meat, but unfortunately there were lower levels of healthy polyunsaturated fatty acids (Renna et al., 2017). BSFL have also been fed to poultry, usually because they are natural colonizers of poultry manure and have been used by farmers to help with waste management and prevent manure from becoming a pollution issue (Bradley and Sheppard, 1984; Bradley et al., 1984). In many studies, BSFL were deemed a fit substitute for soybean or corn meal feed. When used to feed broiler quail, there was no difference in yield between quail whose diet had been partially replaced with BSFL and those who ate their usual diet (Cullere et al., 2016); but they did have improved amino acid levels and increased saturated and monounsaturated fatty acids that pushed the meat toward more nutritious (Cullere et al., 2016). Another poultry study conducted with broiler chickens also found that while feeding BSFL to chickens did increase the levels of undesired fatty acids, defatting the BSFL decreased this effect (Renna et al., 2017).

BSF larvae are poised for mass production for proteins and oils as we know more about this species than any other insects that hold the same potential. Numerous companies both in the United States and abroad are attempting to rear BSFL for mass production as food, as feed, and as a waste management and conversion solution. However, the system has not been optimized for maximum production of proteins and lipids or for maximum waste degradation. The first step to their optimization is performing experiments on the benchtop, in order to determine variability and efficiency in methodology. It is important, however, to recognize differences may be found when results at a small scale are compared to those obtained at the industrial scale. Reasons for this may include the sheer number of larvae in an industrial scale, as nutrient availability and access to food for each individual will differ from the small scale. In small scale studies, larvae have less competition and easier access to their food substrate, as well as less surface area. On the industrial scale, they must compete with thousands, not hundreds, of other larvae for resources. This dynamic in turn will influence waste conversion and feeding efficiency. Similarly, moisture content and the heat of the entire system will not be the same as on the benchtop because of increased number of larvae seeking out food. Studies conducted on a small scale are important for initial results, determination of important variables, and fine-tuning methodology, but must be conducted on an industrial level before these methods can be considered for application to "real world" or to a commercial setting.

BSF larvae have been shown to decrease the amount of pathogens, such as *Salmonella enterica* (Erickson et al., 2004) and *Escherichia coli*, in their substrate (Liu et al., 2008; Lalander et al., 2013, 2015) and can become contaminated with the bacteria they encounter (Erickson et al., 2004). Furthermore, studies have shown beneficial effects through bacterial supplementation. For instance, inoculating poultry manure used to raise BSFL with a bacterium, *Bacillus subtilis*, increased larval growth (Yu et al., 2011). These studies show that BSFL are able to be influenced by microbes.

By definition, probiotics are "viable microorganisms that, when ingested, have a beneficial effect" (Havenaar and Huis In't, 1992). In human intestinal health, probiotics are able to inhibit adherence of pathogens, compete for nutrients, and stimulate immunity (Rolfe, 2000). In insects, probiotics have been found to have beneficial effects. One study showed that *Enterococcus kuehniella* isolated from larval moth feces and orally administered to red flour beetle larvae increased infection survival rates of the beetle larvae due to the probiotic's antimicrobial activity (Grau et al., 2017). However, in another study, bees fed sugar syrup supplemented with *Lactobacillus rhamnosus* (a commercially available probiotic) were more susceptible to disease, and had a shorter lifespan (Ptaszynska et al., 2016). The latter study underscores the importance of probiotic selection in measuring health and functional outcomes.

Bacteria also provide nutrition in the form of triglycerides and lipids that are essential for insect growth and reproduction and provide energy needed during extended non-feeding periods. This is particularly true during the larval stage where energy reserves are accumulated within the fat body to be utilized during metamorphosis. There is great diversity in the concentration

of lipids present in bacterial species. For instance, oleaginous microbes have a high lipid content, which composes about 20% or more of their biomass (Meng et al., 2009). Oleaginous microbes are excellent candidate organisms for the bioprocessing of chitinous waste, such as the exoskeletons of dead adult BSF, as many possess the enzymatic machinery to break down chitin and protein. Additionally, they can synthesize and accumulate triacylglycerides, similar in composition to vegetable oils, a primary material for biodiesel production (Castro et al., 2016). In a large scale, nearly zero waste system, rearing facilities could use adult flies allowed to emerge for breeding as a portion of the media used to grow the oleaginous microbes, eliminating waste output from the system and further cementing BSFL-rearing for protein as an environmentally conscious effort.

For these reasons, we selected two oleaginous microbial species to supplement into the BSFL diet mixture: *Arthrobacter AK19* and *Rhodococcus rhodochrous 21198*. We previously conducted a benchtop scale study with *R. rhodochrous* 21198, and demonstrated that *R. rhodochrous* supplementation increased larval mass (manuscript submitted elsewhere). Both *Arthrobacter* and *Rhodococcus* species have also been investigated as a means of bacterial hydrocarbon synthesis ultimately to be used in making biofuels (Srinophakun et al., 2017). Our hypothesis for studies outlined here was that the addition of fat-rich bacteria to the feeding substrate of BSFL would increase body mass, development rate, feed-to-body mass conversion, and nutrient density. We present methodology and results of both small scale and industrial scale research where BSFL were supplemented with oleaginous microbes. Additionally, we conducted a small scale study where we supplemented BSFL diet with *Bifidobacterium breve*, a well-characterized human probiotic (Shi et al., 2016). In all studies, our objectives were to measure growth, waste conversion, and gut microbiome composition in an effort to determine the utility of bacterial supplementation in BSFL rearing and industrialization, and the role gut microbes play in BSFL diet metabolism. Results of our work demonstrate the effectiveness of bacterial supplementation to BSFL food to BSFL growth and waste conversion, and the importance of scale and probiotic choice in feeding experiments.

MATERIALS AND METHODS

Fly Colony

Black soldier fly eggs were collected from a colony at the Forensic Laboratory for Investigative Entomological Sciences (FLIES) Facility at Texas A&M University, College Station, TX, United States. Eggs were collected in three layers of 2 × 3cm corrugated cardboard blocks placed above approximately 500 g of spent grain diet saturated with water. The cardboard was replaced daily, and cardboard containing eggs was placed in a 1 L deli cup and held in an incubator at 70% relative humidity, 27°C, and 12:12 L:D until the eggs have hatched. Larvae were shipped to Mississippi State University (MSU) Department of Biological Sciences when the larvae were 11-days-old for each of the experiments conducted at MSU, Starkville, MS, United States.

Bacterial Growth and Collection

Both *R. rhodochrous* 21198 and *Arthrobacter AK19* were grown on Luria nutrient agar and broth at pH 6.8 and 26°C for 3 days and then collected by either scraping the plates or centrifuging the broth and collecting the pellet. All of the collected bacteria were washed in a saline solution to remove residual nutrient media. *B. breve* was grown anaerobically at 37°C on plates and collected by scraping plates.

Arthrobacter AK19 Supplementation-Benchtop

Larvae were divided into replicates of 300, with each replicate placed into control or treatment containers in triplicate. A perforated plastic wrap was secured on the top of the containers to prevent escape. Larvae in control containers were fed daily with 18.00 g Gainesville diet (a standard plant based diet composed of 30% alfalfa meal, 20% corn meal, and 50% wheat bran with water; Hogsette, 1992), while treatment containers were fed 16.65 g Gainesville diet supplemented with 1.35 g (approximately 1×10^5 CFU, 7.5% of diet) of *Arthrobacter* AK19. Control diets received additional water in place of a supplement to make up for the moisture difference. Initial larval weights were recorded by randomly selecting 25 larvae from the containers and weighing them, as well as initial weight of the diets. Every 24 h the larvae were separated from their feeding substrate, and the larvae and waste in the container were weighed. Containers were kept in a controlled and constant environment at room temperature. After 10 days, the experiment was stopped and the larvae and waste were immediately weighed. Waste and larvae from each replicate were collected, weighed, and dried at constant temperature (55°C) for 5 days, and for 24 h, respectively, in a MyTemp Mini Digital Incubator (Benchmark Scientific) and then weighed again. Remaining larvae and waste from immediate collection were frozen in −20°C until further analysis.

Bifidobacterium breve Supplementation Experiment-Benchtop

A similar treatment plan was followed for *B. breve* as was described above, except instead of 300 larvae per cup only 100 larvae were placed into each treatment container. An identical feeding plan and percent bacteria were used. Instead of placing the bacterial supplement into each day's diet and then feeding, *B. breve* was grown on plates anaerobically until 1% (approximately 1×10^6 CFU) by weight of the total diet could be replaced with the supplement. The entire volume of diet required for a 10-days-experiment was weighed out and prepared with the appropriate volume of water in advance. The diet was placed in an anaerobic chamber, maintained by anaerobic packs that were changed out daily. The inoculum amount was added into the diet and allowed to colonize and the diet required for the entire experiment was kept at growing temperature. BSFL were left on the benchtop at room temperature in their treatment containers with a perforated plastic wrap on top of each cup to prevent escape. In order to make sure temperature

was not significantly different between the control and treatment groups, the diet and water for the control groups was also kept in the same incubator and was the same temperature during feedings.

Arthrobacter AK19 and *R. rhodochrous* 21198 Supplementation-Industrial Scale

The industrial scale experiments were conducted using *Arthrobacter* AK19 and *R. rhodochrous* 21198. Gainesville diet was used as a diet base where either 8 g (approximately 6×10^5 CFU/g) of *R. rhodochrous* 21198 or *Arthrobacter AK19* was added to 6 kg of diet per pan (four pans per treatment or control, $N = 16$ total), stirring with gloves for 30 s to homogenize the supplement. Following this, approximately 10,000 larvae were added to each pan of either non-supplemented or supplemented. Each treatment condition (*Arthrobacter*-supplemented, *Rhodococcus*-supplemented, and control) had four replicates. The larvae were allowed to feed constantly on the initially placed food substrate. The pans were mixed daily to ensure that temperature spikes due to composting did not occur. Every 3 days, a subset of 500 larvae was removed from the pans, weighed, and frozen for later analysis. A sample of the waste was also collected and frozen for later analysis. The experiment was carried out for 10 days. On day 10, 500 larvae from each replicate were removed, weighed, and frozen. The rest of the larvae were shifted from the waste and the total mass of the larvae, as well as the total mass of waste, was weighed. One liter of waste and 500 larvae were saved and dried for 5 days and 24 h, respectively, in a MyTemp Mini Digital Incubator (Benchmark Scientific) set at 55°C to determine moisture content.

DNA Extraction

Subsets of larvae were surface sterilized by submerging larvae in 10% bleach solution for 2 min, followed by subsequent submersion in two separate containers of molecular grade water for 2 min. Following this, larvae were cut lengthwise using a sterile scalpel, and the gut was removed. DNA from larval guts and waste were isolated using a modified protocol of that discussed in Williamson et al. (2014), quantified by a Qubit 2.0, and purified using a Qiagen DNA clean-up kit. Genomic Larval gut DNA was extracted from all replicates and the extracts were subsequently pooled. DNA was amplified with V4 primers and suggested protocols by the Earth Microbiome Project (Thompson et al., 2017) and visualized by gel electrophoresis. Verified amplifying DNA from larval guts was sent to Michigan State University Sequencing Facility, East Lansing, MI, United States, for paired-end 16S metagenome sequencing.

DNA Sequencing

Microbial DNA samples from larval guts were sequenced using Illumina MiSeq of 2 × 250 bp paired-end reads following 16S library construction, both performed by the Michigan State University Genomics Core Facility. The V4 hypervariable region of the 16S rRNA gene was amplified using dual indexed Illumina compatible primers 515f/806r as described by Kozich et al. (2013). PCR products were normalized using Invitrogen SequalPrep DNA Normalization plates and the products recovered from the plates pooled. This pool was cleaned with AMPureXP magnetic SPRI beads and quantified using a combination of Qubit dsDNA HS, Advanced Analytical Fragment Analyzer High Sensitivity NGS DNA and Kapa Illumina Library Quantification qPCR assays. Sequencing of the pooled amplicons was on an Illumina MiSeq v2 standard flow cell using a 500 cycle v2 reagent cartridge. Custom Sequencing and index primers were added to appropriate wells of the reagent cartridge as described in Kozich et al. (2013). Base calling was done by Illumina Real Time Analysis (RTA) v1.18.54 and output of RTA was demultiplexed and converted to FastQ format with Illumina Bcl2fastq v2.19.1.

Raw fastq files barcoded Illumina 16S rRNA paired-end reads were assembled, quality-filtered, demultiplexed, and analyzed in QIIME version 1.8.0 (Caporaso et al., 2010b). Reads were discarded if they have a quality score < Q20, contained ambiguous base calls or barcode/primer errors, and/or were reads with < 75% (of total read length) consecutive high-quality base calls. Chimeric reads were removed using the default settings in QIIME (Haas et al., 2011). After quality control, the remaining sequences were binned into OTUs at a 97% sequence similarity cutoff using UCLUST (Edgar, 2010). Assembled sequence reads were classified into operational taxonomic units (OTUs) on the basis of sequence similarity. The highest-quality sequences from each OTU cluster were taxonomically assigned using the RDP classifier (Wang et al., 2007) and identified using BLAST against reference sequences from the most current Greengenes 97% reference dataset[1] (DeSantis et al., 2006; McDonald et al., 2012; Werner et al., 2012). Representative sequences of all OTUs were aligned to the Greengenes reference alignment using PyNAST (Caporaso et al., 2010a), and low abundance OTUs (<0.0005% of reads in the total dataset) were removed (Bokulich et al., 2013). Samples were rarefied to achieve equal coverage per sample and those samples with fewer sequences were not used in subsequent analyses.

Sequence Archiving

Sequences were archived within the NCBI Sequence Read Archive[2] under Accession Number: PRJNA663337.

Quantitative PCR for Detection of *Arthrobacter* and *Rhodococcus* Over Time

Primers were designed for targeting the respective 16s regions of *R. rhodochrous* and *Arthrobacter* AK-19. Primers and Taqman probe targeting *R. rhodochrous* 21198 16s included forward primer: 5′ACGACGTCAAGTCATCATGC; reverse primer: 5′ GTATCGCAGCCCTCTGTACC; probe (VIC fluorophore): VICTATGTCCAGGGCTTCACACAMGBNFQ. Primers and Taqman probe targeting Arthrobacter AK-19 16s included forward primer: 5′ GTGGGTACGGGCAGACAGA; reverse primer: 5′

[1] http://greengenes.secondgenome.com

[2] https://submit.ncbi.nlm.nih.gov/subs/sra/SUB8135510/overview

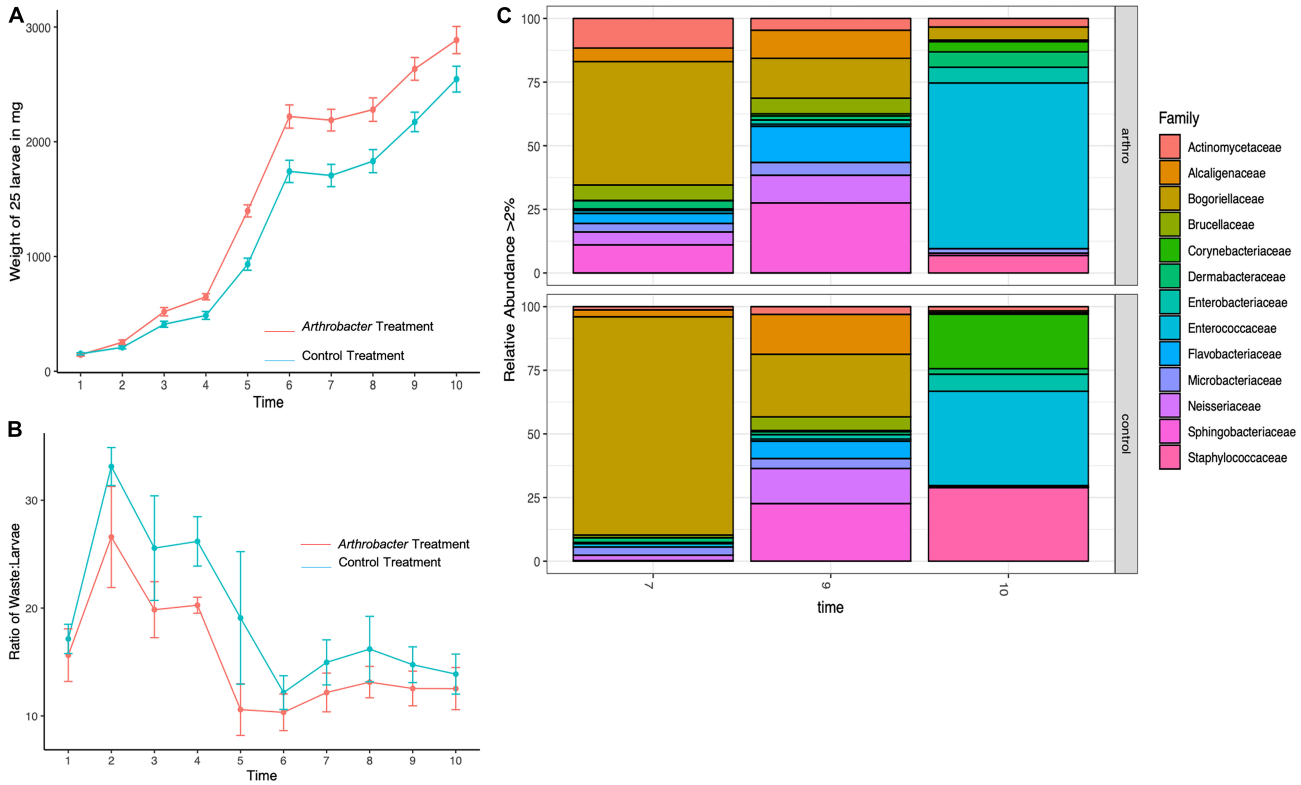

FIGURE 1 | Mean daily black soldier fly larval **(A)** weights, **(B)** waste:larvae ratio, and **(C)** microbial relative abundance for *Arthrobacter* supplemented larvae compared to control at benchtop scale.

CTACGCATTTCACCGCTACA; probe (FAM fluorophore): 6FAMGTGCAGTAGGGGAGACTGGAMGBNFQ. Ten-fold dilutions were created for standards using known concentrations of *R. rhodochrous* 21198 and *Arthrobacter* AK-19. Standards were analyzed in triplicate, samples were analyzed in duplicate, and qPCR reactions were multiplexed. Conditions for qPCR included 3 μL of template, 1 μL each of forward and reverse primers (2.5 μM), 2.5 μL each of probe (0.125 nM), and 12.5 μL Environmental MasterMix (ThermoFisher). Cycling conditions included an initial melting temperature of 95°C for 3 min, following by 40 cycles of 95°C for 1 min, 55°C for 30 s, and 72°C for 45 s.

Analyses of Microbial Diversity

Bacterial diversity of larval gut microbiomes was assessed through the Chao1 estimator and the Shannon index, calculating both indexes after subsampling with QIIME and data against the Greengenes Database, to avoid sequencing bias. Mann–Whitney or Kruskal–Wallis tests were used to test statistical significance of alpha diversity.

Relative abundance was also assessed and plotted at family level using the R vegan and phyloseq packages. Family level abundance less than 2% were not shown. Statistical significance of microbial variance between groups was analyzed using the adonis function in the vegan R package given a Bray–Curtis dissimilarity matrix of the taxonomic profiles and metadata. Adonis is a permutational multivariable analysis of variance (PERMANOVA) using distance matrices. Statistical significance of the fit was assessed using 99,999 permutation tests. Bonferroni correction was used when necessary.

Determination of Functional Capacity Using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt)

In order to predict genes from the metagenome, closed-reference OTUs were obtained from the filtered reads using QIIME version 1.8.0 (Caporaso et al., 2010b). The biom-formatted OTU table was then loaded to PICRUSt on the online Galaxy version in the Langille Lab (v1.1.1), alongside the Greengenes database (last updated June 2017). PICRUSt software estimates functional potential from the community metagenome using copy normalized 16S rRNA sequencing data whose gene contents are contributing to Kyoto Encyclopedia of Genes and Genomes (KEGG) identified pathways. Functionally annotated genes that were identified were compressed into 12 general gene families. Comparisons were made between differences in annotated gene abundance from control and treatment groups to determine the percent change of treatment as compared to control groups. Only those with gene abundance at or above 25% change were considered for analysis.

RESULTS

Arthrobacter AK19 Supplementation-Benchtop

The mean daily weights for the small scale *Arthrobacter AK19* supplementation study show the mean daily weight (**Figure 1A**). The mean daily weight of supplemented larvae was greater than that of the control-diet larvae, particularly at early timepoints. On the third day, treatment groups were 94% larger than control in mass and had increased 107% from day 2 to day 3, whereas control larvae only increased mass by 28% from day 2 to day 3. A similar but steadily diminishing trend was seen in later timepoints: On day 5, treatment larvae were 58% larger than control larvae and increased their mass by 113% from day 4. Despite the fact that control larvae had an 85% increase in mass at the day 4 timepoint, their overall mass was still less than treatment larvae. There was a significant difference in mean daily weight between treatment and control larvae at day 3 ($p = 0.007$), day 4 ($p = 0.0003$), day 5 ($p = 0.005$), day 6 ($p = 0.001$), day 7 ($p = 0.0006$), day 8 ($p = 0.007$), day 9 ($p = 0.002$), and day 10 ($p = 0.015$).

The waste:larvae ratio was calculated for timepoints 2–10 and shows the ability of the larvae to convert their feeding substrate (Gainesville Diet) into body mass (**Figure 1B**). An overall lower waste:larvae ratio was observed in *Arthrobacter* supplemented groups, revealing that the bacterial supplemented larvae had an increased capacity to digest the feeding substrate and convert the substrate to biomass. The waste:larvae ratio increased during the first few days, and peaked at day 2. As the larvae continued feeding on their food and received daily feedings, the ratio, while not statistically significant ($p = 0.793$) decreased until the larvae neared the prepupal stage from T6 to T10.

Arthrobacter was detected by qPCR in the treatment group larval guts over time (**Figure 2A**), where there was a slight increase from the initial inoculum within the first 7 days (from 1×10^5 to 6.85×10^5 CFU). *Arthrobacter* was also detected in larval guts on day 9 (3.78×10^5 CFU) and on day 10, though had decreased by two logs on day 10 of the experiment (7.36×10^3).

Family level microbial richness and evenness was higher in *Arthrobacter* treated larval gut microbiomes than controls on days 7 and 9 but this was reversed at day 10, at which time control samples showed an increased abundance. The difference in microbial richness and evenness between treatments at days 7, 9, and 10 were not statistically significant ($p = 0.4$).

Figure 1C shows the relative abundance of BSFL associated bacterial families. On day 7, the treatment group showed greater diversity and had increased abundance from every family represented, with the exception that the control group was composed of 39% more Bogoriellaceae and 9% more Enterococcaceae. Of those families that were increased in treatments at day 7, all showed over 100% increase except for an 11% increase in Microbacteriaceae and a 23% increase in *Staphylococcaceae*. At day 9, the differences were not as apparent. Treatment larvae saw a 21–36% decrease in Alcaligenaceae, Bogoriellaceae, and Neisseriaceae. Treatment larvae had 50% more Actinomycetaceae, 62% more Corynebacteriaceae, 44% more Dermabacteraceae, 26% more Microbacteriaceae, and over 100% increases in Flavobacteriaceae and Staphylococcaceae as compared to control larval associated microbial families. At day 10, another shift in abundance was identified. Bogoriellaceae, which was decreased from the previous timepoint, was over 300% more abundant in treatment groups. Families that saw a decrease in treatments from controls at day 10 were: Alcaligenaceae (66% decrease), Brucellaceae (21% decrease), Corynebacteriaceae (87% decrease), Enterobacteriaceae (40% decrease), Flavobacteriaceae (74% decrease), Neisseriaceae (70% decrease), and Staphylococcaceae (84% decrease). PERMANOVA of Bray–Curtis beta diversities indicated that timepoint differences explained microbial taxonomic variation (permutation test, $p = 0.02$, 99,999 permutations), where control and treatment samples were similar at days 9 and 10, but with notable differences on day 7. Treatment also explained microbial taxonomic variation as all treatments showed statistically significant microbial variation from each other ($p < 0.001$, 99,999 permutations).

PICRUSt was used to explore relationships between predicted functional gene annotations and identified metagenomes from *Arthrobacter* supplemented samples compared to controls. From this, 263 annotated genes were identified, compressed into 12 general gene families and used to compare gene abundance between treatment and control groups. Only families with gene abundance within *Arthrobacter* group at or above 25% change from control are shown in **Supplementary Figure 2**. Percent difference in *Arthrobacter* group gene abundance compared to control samples at 7 days revealed predicted genes enriched for functions involved in protein digestion and absorption (96.05%), Bile acid biosynthesis (82.21%), pollutant/contaminant digestion (55.82%), nucleic acid repair/replication/general metabolism (50.61%), antimicrobial metabolism/resistance (50.26%), motility and signaling (42.73%), some genes involved in lipid metabolism (42.39%), energy metabolism (44.47%), fatty acid metabolism (36.17%), amino acid metabolism (37.59%), and membrane transport (33.00%). However, compared to controls at 7 days, samples showed a decrease in functions for bile secretion (−69.69%) as well as decrease in some genes for lipid metabolism (−30.21%). At 9 days, fewer differences in gene abundance were found in the *Arthrobacter* group compared to control. Genes increased, as compared to control, included those for pollutant/contaminant degradation (43.98%), protein digestion and absorption (25.84%), and lipid metabolism (25.51%), whereas genes for functions involved in bile secretion (−54.21%) and motility and signaling (−48.15%) were decreased compared to control. At day 10, only two functional categories from the *Arthrobacter* supplemented group were increased compared to control. Those included genes involved in functions for nucleic acid repair/replication/general metabolism (−46.18%) and for bile secretion (39.05%). However, many functional categories within the *Arthrobacter* supplementation group showed a decrease in percent abundance compared to control (**Supplementary Figure 2**). These included genes involved in lipid metabolism (−91.59%), pollutant/contaminant degradation (−74.29%), amino acid metabolism (−59.07%), nucleic acid

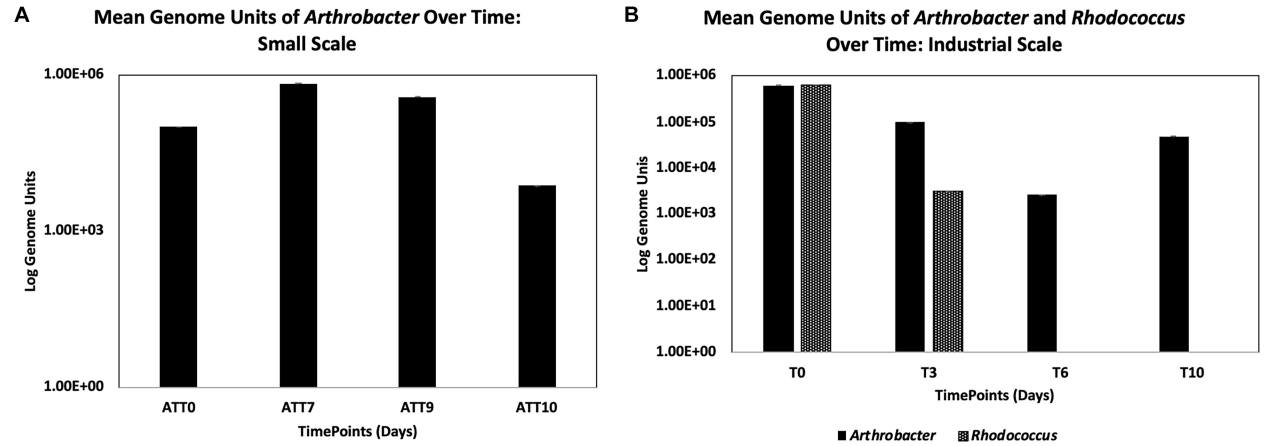

FIGURE 2 | Detection of *Arthrobacter* at small scale **(A)** and *Arthrobacter* and *Rhodococcus* at industrial scale **(B)** using qPCR targeting the respective 16s genes (with standard error bars).

repair/replication/general metabolism (−52.97%), antimicrobial metabolism and resistance (−47.17%), membrane transport (−46.63%), motility and signaling (−43.15%), protein digestion and absorption (−40.59%), energy metabolism (−38.82%), and fatty acid metabolism (−31.09%).

Bifidobacterium breve Supplementation Experiment-Benchtop

Supplementing with *B. breve* yielded lower weights over time compared to control BSFL (**Figure 3A**). Additionally, supplemented larvae appeared weak, slow, and discolored (data not shown). Also, the treatment BSFL waste:larvae ratio was much lower than control across all timepoints (**Figure 3B**).

Relative abundance of larval gut microbiomes from control groups showed an increased amount of Actinomycetaceae (97% increase), Bogoriellaceae (99% increase), Brucellaceae (85% increase), Cellulomonadaceae (89% increase) Enterobacteriaceae (96% increase), Enterococcaceae (96% increase), Moraxellaceae (99% increase), Sphingobacteriaceae (98% increase), and Xanthomonadaceae (72% increase) compared to treatment groups (**Figure 3C**). Treatment *B. breve* supplemented BSFL gut microbiomes showed an increase in Clostridiaceae (107.9% increase) and Promicromonosporaceae (510% increase) compared to controls at day 9.

PICRUSt identified 168 genes that were above 25% change from controls on day 9 of the experiment. These were compressed into 12 general gene families, as shown in **Supplementary Figure 3**. Samples with *B. breve* supplementation showed decrease in predicted functions for all gene families, compared to control. These included percent decrease in bile secretion (−13,601%), transport (−1,333%), protein digestion and absorption (−1,946%), pollutant/contaminant degradation (−1,958%), motility/signaling (−1,231%), lipid metabolism (−2,023%), fatty acid metabolism (−1,696%), energy metabolism (−1,491%), bile biosynthesis (−2,503%), antimicrobial metabolism/resistance (−1,491%), amino acid metabolism (−1,482%), and nucleic acid replication and repair, and general metabolism (1,488%).

Arthrobacter AK19 and *R. rhodochrous 21198* Supplementation-Industrial

Arthrobacter and *Rhodococcus* supplemented larvae were not statistically different from each other for the duration of the study (**Figure 4A**). However, both treatment groups weighed statistically significantly more than control larvae at day 3 ($p = 0.02$) where *Arthrobacter* treated larvae were 21.6% larger than controls, and *Rhodococcus* treated larvae were 22.2% larger than controls. At day 6, treatment groups were not statistically different from controls, likely due to large variation in control larvae ($p = 0.06$), though *Arthrobacter* treated larvae were 29% larger than controls, and *Rhodococcus* treated larvae were 25% larger than controls. At day 10, control larvae weighed 6.3% more than *Arthrobacter* treatments, and 12.0% more than *Rhodococcus* treated larvae. The waste:larvae ratio from day 10 was not significantly different between treatments ($p = 0.793$, **Figure 4B**).

Arthrobacter and *Rhodococcus* were detected by qPCR through time to determine growth of the supplemented bacteria within the larval guts (**Figure 2B**). We found an initial decrease of one log (from 6.0×10^5 CFU initial inoculum to 9.76×10^4 CFU on day 3) from the initial *Arthrobacter* inoculum within the first 3 days of the experiment. There was continued decrease by another log by day 6 of the experiment (2.56×10^3 CFU). However, 4.72×10^4 *Arthrobacter* genome units were detected at day 10 (**Figure 2B**).

Richness was lower for larval gut microbiomes from the *Arthrobacter* treatment on day 3 than for larval gut microbiomes from the control or *Rhodococcus* treatments (**Figure 5A**), but was similar to *Rhodococcus* on days 6 and 10 where control richness was higher than both bacterial treatments (**Figures 5B,C**). The *Arthrobacter* treatment also showed lower evenness than the control and *Rhodococcus* treatments on day 3 (**Figure 5A**). This shifted on days 6 and 10 where both *Arthrobacter* and *Rhodococcus* treatments showed

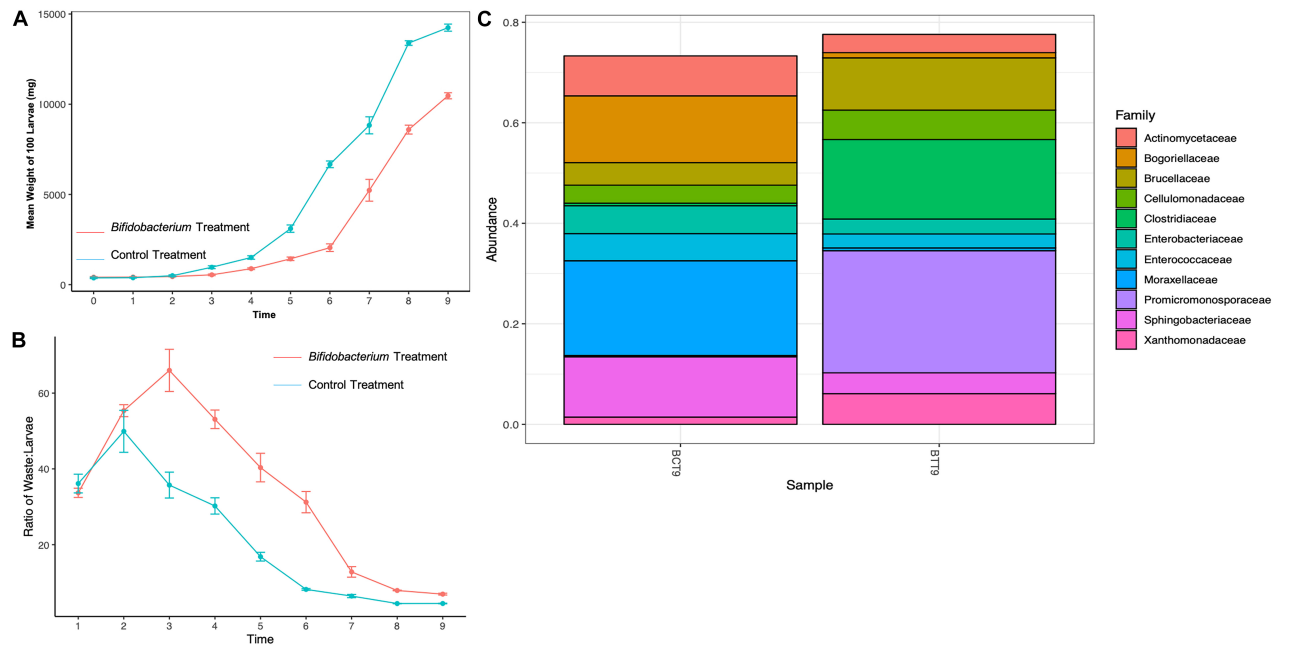

FIGURE 3 | Mean daily black soldier fly larval **(A)** weights, **(B)** waste:larvae ratio, and **(C)** microbial relative abundance for *Bifidobacterium breve* supplemented larvae compared to control on day 9 of the experiment.

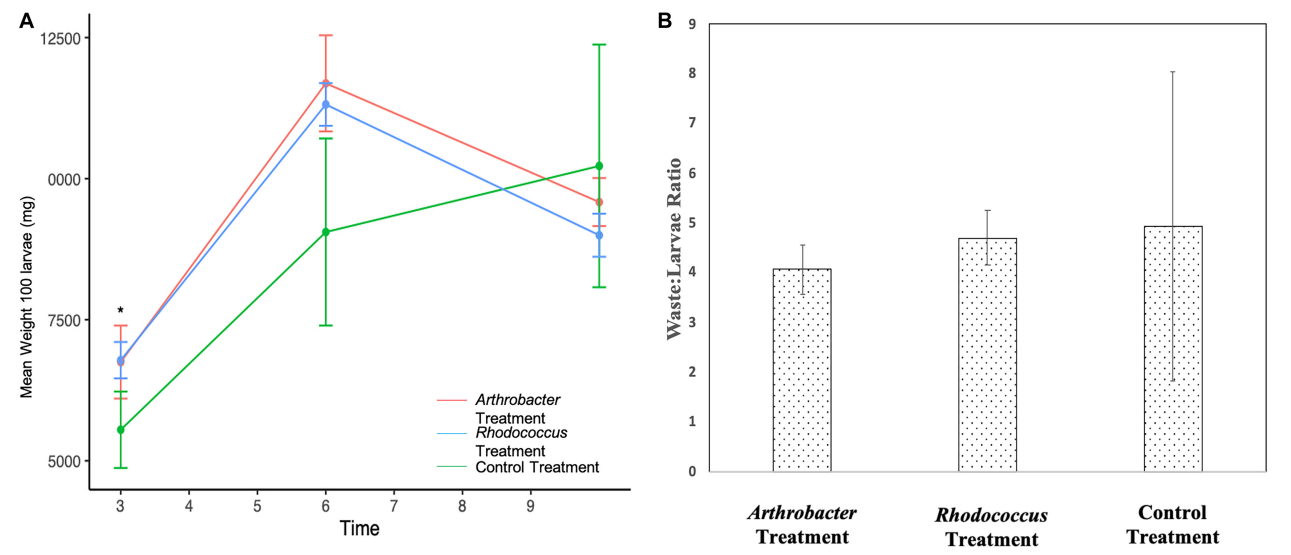

FIGURE 4 | (A) Mean larval weight of 100 *Arthrobacter* and *Rhodococcus* supplemented black soldier fly larvae compared to control at industrial scale. Standard error bars are included. **(B)** waste:larvae ratio on day 10 across all treatments.

higher evenness than control (**Figures 5B,C**). There was no statistical significance with any of the alpha diversity metrics ($p = 0.4$).

At day 3, *Arthrobacter* and *Rhodococcus* larval gut microbiomes had similar relative abundance compared to control (**Figure 5D**). At day 6, compared to control, *Arthrobacter* treated larval microbiomes had 894% more Enterobacteriaceae, 12% more Enterococcaceae, 766% more Flavobacteriaceae, 157% more Neisseriaceae, 2295% more Pseudomonadaceae, 564% more Sphingobacteriaceae, 4794% more Xanthomonadaceae, 38% less Bacillaceae, 27% less Comamonadaceae, 41% less Paenibacillaceae, and 65% less Planococcaceae. Day 6 *Rhodococcus* treated larval gut microbiomes, when compared to controls had 97% more Pseudomonadaceae, 89% more Sphingobacteriaceae, 95% more Flavobacteriaceae, 24% more Enterbacteriaceae, 10%

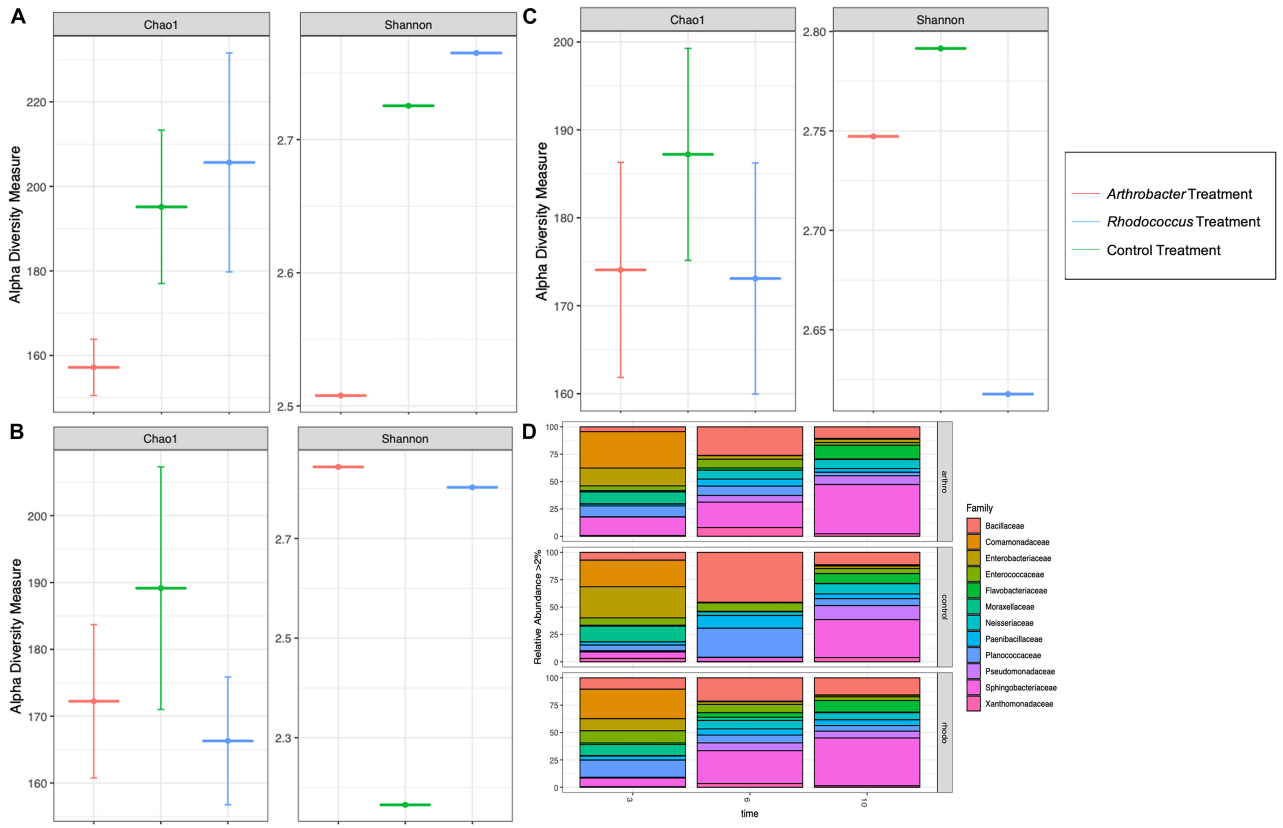

FIGURE 5 | Alpha diversity measures of numbers of observed families (Chao1) and abundance and evenness (Shannon) in *Arthrobacter* and *Rhodococcus* supplemented black soldier fly larvae compared to control at industrial scale at **(A)** day 3, **(B)** day 6, and **(C)** day 10. **(D)** The microbial relative abundance of *Arthrobacter* or *Rhodococcus* supplemented black soldier fly larvae compared to control at industrial scale.

more Planococcaceae, 95% more Xanthomonadaceae, 45% Bacillaceae, 87% less Enterobacteriaceae, and 93% less Moraxellaceae. At day 10, species richness was similar in all groups. However, differences in relative abundance were noted from day 6 to day 10. For instance, the *Arthrobacter* group had notable differences at day 10 with 52% less abundance in Bacilliaceae, 72% less Planococcaceae, 72% less Entercoccaceae, 60% less Paenibacillaceae, 60% less Comamonadaceae, 34% less Enterbacteriaceae, but 35% more Moraxellaceae in controls than in *Arthrobacter* supplemented larvae (**Figure 5D**). Permutational analysis of variance (ANOVA) of Bray–Curtis beta diversities indicated that timepoint differences explained microbial taxonomic variation (permutation test, $p = 0.004$, 99,999 permutations), and by treatment where each sample showed statistical variance from the other ($p < 0.001$, 99,999 permutations).

Arthrobacter supplemented BSF larval gut microbiomes showed increased percent difference in predicted genes compared to control samples for functions involved in protein digestion and absorption (58.93%), energy metabolism (42.77%), lipid metabolism (39.28%), pollutant/contaminant digestion (35.62%), motility and signaling (34.22%), nucleic acid replication/repair/general metabolism (27.19%), and antimicrobial metabolism/resistance (25.97%, **Figure 6**). Additionally, other genes for energy metabolism (−60.87%), nucleic acid replication/repair/general metabolism (−108.80%), and bile secretion (−113.19) were decreased compared to control (**Figure 6A**). At day 6, *Arthrobacter* treatments showed enrichment in all general gene families, with the highest percent change from control being bile secretion (82.26%), followed by lipid metabolism (53.91%), antimicrobial metabolism and resistance (51.47%), pollutant/contaminant degradation (45.01%), motility and signaling (42.50%), fatty acid metabolism (41.29%), protein digestion and absorption (39.83%), energy metabolism (39.33%), amino acid metabolism (38.12%), bile acid synthesis (35.73%), and transport (34.42%). Only two gene families were decreased from control at day 6 including motility and signaling (−88.48%) and energy metabolism (−51.53%, **Figure 6A**). At day 10, *Arthrobacter* treatments only had increases in genes functionally predicted for lipid metabolism (40.41%). However, genes associated with all gene families were decreased compared to control. Those included genes for bile secretion (−201.94%), motility and signaling (−88.06%), lipid metabolism (−82.63%), transport (−81.46%), nucleic acid replication/repair/general metabolism (−76.07%), antimicrobial metabolism and resistance (−75.27%), bile acid biosynthesis (−74.67%), fatty acid metabolism (−74.36%),

pollutant/contaminant degradation (−72.51%), energy metabolism (−68.67%), amino acid metabolism (−60.46%), and protein digestion and absorption (−57.99%)(**Figure 6A**).

At day 3, *Rhodococcus* treated BSFL microbiomes showed percent increase compared to control in genes involved in protein digestion and absorption (62.69%), nucleic acid replication/repair/general metabolism (38.38%), and lipid metabolism (39.33%, **Figure 6B**). Nine of the 12 gene families were decreased from control including bile secretion (−113.19%), nucleic acid replication/repair/general metabolism (−49.81%), protein digestion and absorption (−47.71%), energy metabolism (−39.69%), antimicrobial metabolism and resistance (−31.75%), transport (−31.15%), pollutant/contaminant degradation (−29.49%), motility and signaling (−25.82%), and bile acid biosynthesis (−25.60%). All gene families were increased compared to control samples, with lipid metabolism being the most increased (71.03%). Following this, bile acid biosynthesis (69.28%), antimicrobial metabolism/resistance (60.74%), protein digestion and absorption (59.38%), energy metabolism (57.88%), fatty acid metabolism (54.96%), pollutant/contaminant degradation (54.12%), nucleic acid replication/repair/general metabolism (52.21%), transport (50.32%), amino acid metabolism (48.63%), motility and signaling (48.20%), and bile secretion (33.29%). Two gene families showed percent decrease compared to control at day 6, including motility and signaling (−95%) and energy metabolism (−71.46%). On day 10, *Rhodococcus* samples showed no increased genes compared to control. Functional genes for bile secretion (−130.46%), motility and signaling (−70.40%), pollutant/contaminant degradation (−66.75%), transport (−64.09%), antimicrobial metabolism and resistance (−61.48%), amino acid metabolism (−60.46%), fatty acid metabolism (−59.57%), nucleic acid replication/repair/general metabolism (−59.06%), energy metabolism (−51.33%), lipid metabolism (−49.69%), bile acid biosynthesis (−45.87%), and protein digestion and absorption (−41.80%).

DISCUSSION

We hypothesized that the addition of oleaginous microbes such as *Arthrobacter* AK19 and *Rhodococcus* would increase BSFL mass. A previous benchtop scale study conducted with *R. rhodochrous* 21198 demonstrated this effect (manuscript submitted elsewhere). *Arthrobacter* AK19 was chosen for additional studies at small scale because the bacterium possesses a high concentration of lipids, usually accumulating greater than 40% lipid in dry biomass (Meng et al., 2009). With this high lipid concentration, we expected to see an amplified result in larval weight; data confirmed this expectation.

Arthrobacter is a well-characterized microbe commonly found in soil and in decomposition environments (Jones and Keddie, 2006). *Arthrobacter* can reduce a variety of aromatic compounds, herbicides and pesticides, hexavalent chromium and 4-chlorophenol in contaminated soil (Nordin et al., 2005), increasing interest in their use in bioremediation (see http://eawag-bbd.ethz.ch/servlets/pageservlet?ptype=allmicros for a database list of 22 *Arthrobacter* species involved in biodegrading a wide variety of compounds; Eschbach et al., 2003). *Rhodococcus* is a related genera to *Arthrobacter* and has similar biodegradation capabilities (visit http://eawag-bbd.ethz.ch/servlets/pageservlet?ptype=allmicros for a list of 30 *Rhodococcus* species involved in degrading a number of compounds). Furthermore, *Arthrobacter* and *Rhodococcus* species can degrade lignocellulosic biomass for lipid biosynthesis (Brink et al., 2019; Zhaoxian et al., 2019), and could thus be "pre-digesting" the food for the larvae, allowing an increase in nutrient availability (Jones and Keddie, 2006).

Another potential explanation is that *Arthrobacter* and *Rhodococcus* are colonizing the gut of the larvae and, like human probiotics, assisting with the digestive process. Our data indicate that *Arthrobacter* may be colonizing the gut, as we detected *Arthrobacter* by qPCR throughout the course of both studies. However, *Rhodococcus* was only detected by qPCR on day 3 of the experiment, and was below detectable limits at the remaining timepoints, suggesting only transient *Rhodococcus* passage through the gut. Another possible explanation is that *Arthrobacter* and *Rhodococcus* change the initial environmental conditions allowing other bacteria to proliferate, and may be maintained in the BSFL waste, particularly as the waste becomes more alkaline. Day 7 of the small scale study yielded percent increases in all functional groups as compared to control. This is reflective of both the taxa present as well as the relative abundance of those taxa. Unfortunately, we did not measure the microbial community within the waste in this study, nor for *B. breve*, though this is a logical next step and currently underway in subsequent studies.

Our 16s sequencing data included families where *Arthrobacter* and *Rhodococcus* reside, but detected only 184 Micrococcaceae (Family for *Arthrobacter*, data not shown) combined abundance from all treatment samples, and at all timepoints for the small scale study. We also detected Micrococcaceae from sequencing industrial scale larval guts, and found 635 combined abundance with larval gut treatments, with detected abundance at day 6 and day 10 of the study. Nocardiaceae (Family for *Rhodococcus*, data not shown) were also detected at every timepoint, with a combined abundance of 957, with decreased detection as timepoints increased. Differences in sensitivity and specificity of the two methods, along with relative abundance associated with 16s sequencing, where increase of one taxon leads to the equivalent decrease of remaining taxa, likely account for these differences (Yang et al., 2015; Jian et al., 2020).

The industrial scale experiment showed that mean daily weights of *Rhodococcus* and *Arthrobacter* supplemented larvae were consistently larger than control larvae throughout the study (**Figure 3**), except at the final day of the experiment. A potential explanation for the decrease in mean weights on the last day could be attributed to pupation. As the BSFL prepares for pupation, it moves into the prepupal stage. In this stage, larvae stop feeding and their integument begins to harden and darken (Lalander et al., 2019; Nyakeri et al., 2019). Their digestive system empties and they exhibit a crawl-off behavior as they seek out a safe place away from the feeding substrate where they can pupate (Dortmans et al., 2017). If the *Arthrobacter* and *Rhodococcus* supplementation was able to accelerate development, then it

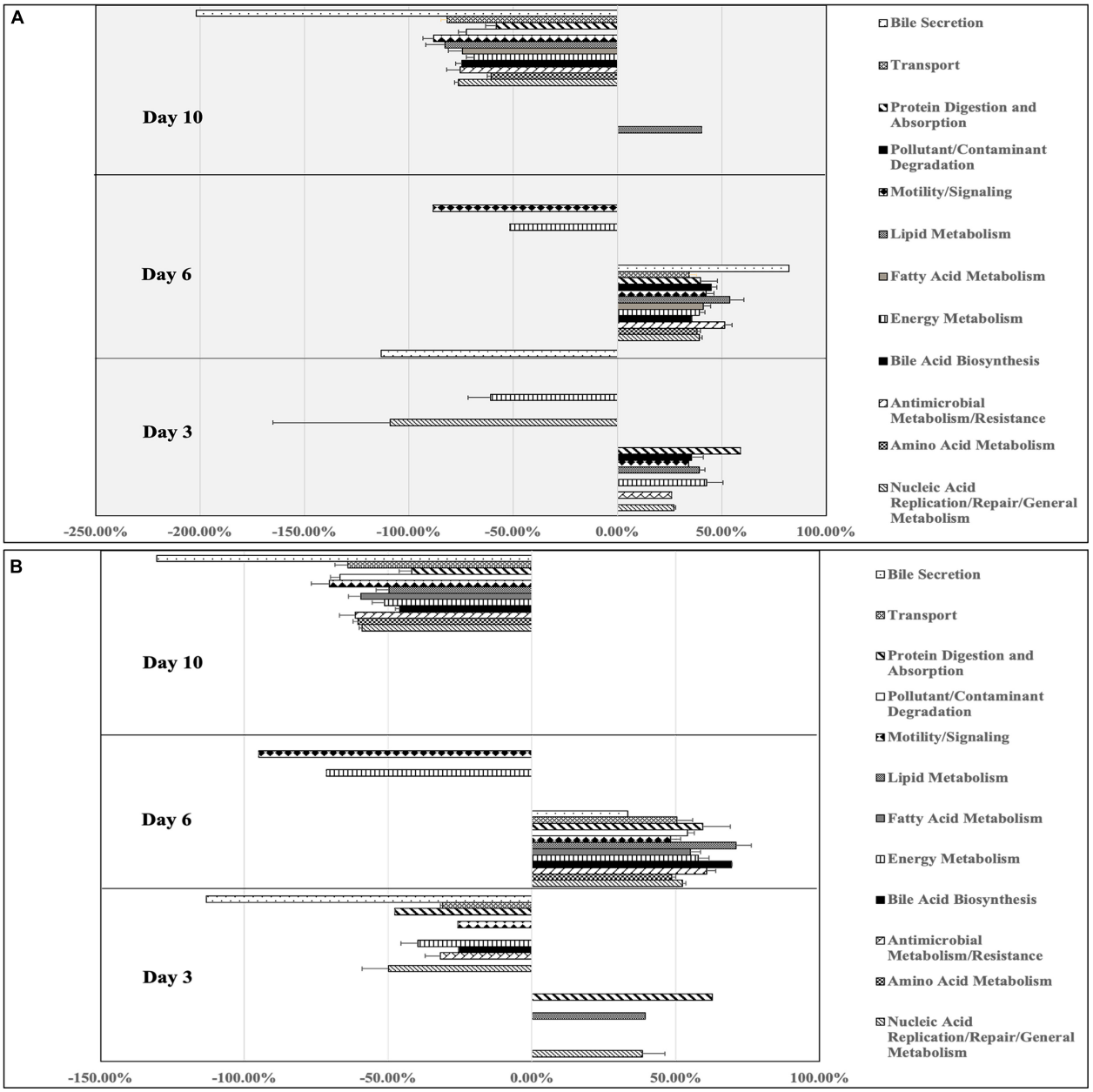

FIGURE 6 | Percent differences in predicted gene functions from microbial metagenomes of black soldier fly larvae supplemented with **(A)** *Arthrobacter* or **(B)** *Rhodococcus* compared to control black soldier fly larvae at industrial scale on days 3, 6, and 10 of the experiment.

would potentially undergo this process sooner. In this case, when we collected samples to weigh, BSFL would have been in an advanced stage and likely weighing lighter. Additionally, there was no statistical differences in the waste:larvae ratios between the groups at industrial scale. This was not surprising since the waste:larvae ratio was only measured on day 10, where there were no differences in the larval weights for that timepoint (**Figure 3A**). However, because the *Arthrobacter* supplemented group still showed a better conversion ratio even at that timepoint, statistically significant differences in waste:larval ratios might have been found if measured at earlier timepoints where there was less variation between replicates.

We also note that bacterial supplementation yielded somewhat comparable results at small and large scales, depending on the timepoints. For instance, on day 3, *Arthrobacter* supplemented BSFL weighed 21% versus 22% more than control BSFL, and on day 10, 11% versus 6.7% more than control BSFL at bench and industrial scale, respectively (**Figures 1A**, **3A**). And on day 6, *Arthrobacter* supplemented BSFL weighed 35 versus 29% more than control BSFL at benchtop versus industrial scale,

respectively. Bacterial supplementation at industrial scale also yielded changes in gut microbiome species presence and relative abundance, with greatest differences found from day 6 samples, whereas large differences in gut microbiome relative abundance were also observed at day 7 or the benchtop experiment. Predicted genes involved in all functional groups were also present, similar to benchtop scale (**Supplementary Figure 2** and **Figure 6**). But, both control and treatment larvae during both benchtop scale experiments had not reached peak weight and were still growing at the final timepoint (**Figures 1A**, **3A**). In contrast, treatment larvae at industrial scale had reached peak weight at day 6 of the experiment with an increased number of pupated larvae (data not shown), whereas control larvae at the industrial scale were also still growing (**Figure 4A**). Differences in scale, numbers of larvae, amount of substrate, and differing inoculum for benchtop and industrial scale experiments likely account for this. Also, as previously mentioned, scale is important—please see the Miranda et al. (2020) reference for a discussion on this topic.

Changes in the environmental substrate during larval feeding and through supplementation likely led to changes in species composition and relative abundance. BSFL responses to stimuli including changes in water availability (Cheng et al., 2017), pH (Ma et al., 2018; Meneguz et al., 2018), temperature (Tomberlin et al., 2009), toxicity (Purschke et al., 2017), and nutrient availability (Cammack and Tomberlin, 2017) would be important in this system as these changes are observed throughout the course of larval feeding. Organisms present in high abundance in the bacterial supplemented groups appeared to have broad systems for responding to these changes including those for bacterial motility and signaling, antimicrobial resistance and biosynthesis, and pollutant/contaminant degradation. An in-depth look at predicted genes within the functional groups showed enrichment for two component systems, and higher abundance of bacterial motility and flagella proteins and bacterial secretion systems compared to control, which play important roles in bacterial attachment, colonization, and chemotaxis. Many of the identified microbial families have been found to be involved in gut digestion in mammals and other animals, as well as degradation of organic aromatic compounds and other organic pollutants (Zhang et al., 2015; Jing et al., 2020; Lavelle and Sokol, 2020). Additionally, many of these are known to produce antimicrobial and other secondary compounds.

Taken together, these results with *Arthrobacter* and *Rhodococcus* are promising and, along with previous small scale data, point toward a potential for industrialization of this process. Larvae that reach a harvestable size sooner save industrial BSF production companies money and increase their yield. If the larvae are being used for waste management, their organic material degradation ability can be increased with the aid of these probiotics, helping them to process more waste in a shorter amount of time, with even the possibility of degradation of intractable materials through bacterial supplementation directly, or indirectly through a change in microbial populations toward those with these traits.

The effect that *B. breve* supplementation had on the BSFL was unexpected. We hypothesized that addition of this bacterial species would have a positive effect on the larvae in some way, just like this probiotic can aid digestion in humans. However, this was not the case. Supplemented larvae appeared discolored, slow, covered in a sticky exudate and overall unhealthy (data not shown). They stuck to each other, to the feeding substrate, and to the container. Healthy control larvae were tan-colored, active and moved through their feeding substrate without issue. The daily mean weight of supplemented larvae was lower than the control larvae, and their waste:larvae ratio was high. *B. breve* did not aid the larvae in converting their food to body mass. Additionally, *Bifidobacterium* treatments showed an increase in Clostridiaceae, and closer inspection revealed an increase in *Clostridium* genera. Despite the increase in Promicromonosporaceae and Cellumonadaceae which contain species with high concentrations of cellulases and xylanases, digestion and frass excretion also appeared stalled. Furthermore, there was a decrease in all predicted microbial functional categories compared to controls. This result is not conducive for overall insect health if the goal is to increase growth and waste conversion. Supplementation with *B. breve* may be useful if the goal is to slow growth and development. Additionally, data from this work suggest a new mechanism for *B. breve*'s role in decreasing obesity. However, more work should be conducted to confirm this, including sampling at earlier timepoints, using differing strains and also at industrial scale.

Overall, our results show that bacterial supplementation is beneficial to BSFL larval growth and waste conversion, though care should be taken toward the appropriate bacterial supplement. We showed that bacterial supplementation yielded somewhat comparable results at small and large scales, depending on the timepoints. There was also a difference in some bacterial taxa identified among microbiomes from the two experiments. This may be due to differences in feed batches or larvae initial microbiomes, as the experiments were not conducted at the same time. It will be important to repeat the industrial scale experiments in order to determine consistency in results, particularly if bacteria are to be targeted from the results for further experimentation.

Additional studies important to the field will include the inclusion of a wide variety of food substrates including those such as spent brewer's grain, manure, or food waste, and the inclusion of other potential probiotics. Furthermore, the effectiveness of the combination of *Arthrobacter* and *Rhodococcus* within the same treatment would be interesting. Other important data include transcriptomic data. While useful for our study, PICRUSt is limited in that genes may not be transcribed or translated, limiting the impact of their annotated function. Therefore, our conclusions about microbiome function derived from PICRUSt analyses of our metagenomes have been treated as hypotheses that require further in-depth validation through functional assays. Nevertheless, it was quite interesting that changes in functional predictions in our datasets could be related to relative abundance differences across time and treatment, based on gene annotations for a given taxa, giving insight into microbially mediated mechanisms of BSFL feeding and waste conversion. Another interesting finding was the number of taxa with functional potential for pollutant/contaminant degradation.

This was an exciting finding in that there is further potential of specific bacterial supplementation, particularly many of those enriched within our studies, and manipulation in the BSFL system to allow BSFL to degrade intractable materials and also have potential utility in bioremediation, while also increasing proteins and lipids of value.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, PRJNA663337.

AUTHOR CONTRIBUTIONS

EK, JT, and HJ participated in study design, conducted the study, analyzed the data, and wrote the manuscript. CB and MC aided in the setting up the study, collecting samples, and preparing samples for sequencing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.587979/full#supplementary-material

Supplementary Figure 1 | Alpha diversity measures of number of observed species (Chao1) and abundance and evenness (Shannon) in *Arthrobacter* supplemented black soldier fly larvae compared to control at **(A)** day 7, **(B)** day 9, and **(C)** day 10.

Supplementary Figure 2 | Percent differences in predicted functions from microbial metagenomes of *Arthrobacter* supplemented BSFL compared to control black soldier fly larvae at small scale on days 7, 9, and 10 of the experiment.

Supplementary Figure 3 | Percent differences in predicted functions from microbial metagenomes of *B. breve* supplemented and control black soldier fly larvae at benchtop scale on day 9 of the experiment.

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Impact of Processed Food (Canteen and Oil Wastes) on the Development of Black Soldier Fly (*Hermetia illucens*) Larvae and Their Gut Microbiome Functions

Thomas Klammsteiner[1], Andreas Walter[2], Tajda Bogataj[1], Carina D. Heussler[1,3], Blaž Stres[4,5,6], Florian M. Steiner[3], Birgit C. Schlick-Steiner[3] and Heribert Insam[1]*

[1] Department of Microbiology, University of Innsbruck, Innsbruck, Austria, [2] Department of Biotechnology and Food Engineering, MCI – The Entrepreneurial School, Innsbruck, Austria, [3] Department of Ecology, University of Innsbruck, Innsbruck, Austria, [4] Department of Animal Science, University of Ljubljana, Ljubljana, Slovenia, [5] Institute of Sanitary Engineering, University of Ljubljana, Ljubljana, Slovenia, [6] Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia

Canteens represent an essential food supply hub for educational institutions, companies, and business parks. Many people in these locations rely on a guaranteed service with consistent quality. It is an ongoing challenge to satisfy the demand for sufficient serving numbers, portion sizes, and menu variations to cover food intolerances and different palates of customers. However, overestimating this demand or fluctuating quality of dishes leads to an inevitable loss of unconsumed food due to leftovers. In this study, the food waste fraction of canteen leftovers was identified as an optimal diet for black soldier fly (*Hermetia illucens*) larvae based on 50% higher consumption and 15% higher waste reduction indices compared with control chicken feed diet. Although the digestibility of food waste was nearly twice as high, the conversion efficiency of ingested and digested chicken feed remains unparalleled (17.9 ± 0.6 and 37.5 ± 0.9 in CFD and 7.9 ± 0.9 and 9.6 ± 1.0 in FWD, respectively). The oil separator waste fraction, however, inhibited biomass gain by at least 85% and ultimately led to a larval mortality of up to 96%. In addition to monitoring larval development, we characterized physicochemical properties of pre- and post-process food waste substrates. High-throughput amplicon sequencing identified Firmicutes, Proteobacteria, and Bacteroidota as the most abundant phyla, and *Morganella*, *Acinetobacter*, and certain Lactobacillales species were identified as indicator species. By using metagenome imputation, we additionally gained insights into the functional spectrum of gut microbial communities. We anticipate that the results will contribute to the development of decentralized waste-management sites that make use of larvae to process food waste as it has become common practice for biogas plants.

Keywords: animal feedstuff, waste valorization, circular economy, metabolism, microbial communities, 16S amplicon sequencing, oil waste, growth parameters

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****Correspondence:***
Thomas Klammsteiner
thomas.klammsteiner@uibk.ac.at

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Abbreviations: ANOVA, analysis of variance; BSF, black soldier fly; BSFL, black soldier fly larvae; CFD, chicken feed diet; COD, chemical oxygen demand; DM, dry matter; FOG, fats, oil, and grease; FWD, food waste diet; NMDS, non-metric multidimensional scaling; OTU, operational taxonomic unit; OWD, oil waste diet; VFA, volatile fatty acids; VS, volatile solids.

INTRODUCTION

With a more fast-moving society in industrialized countries, amounts of food waste in these countries as large as the total net food production of sub-Saharan Africa come along (FAO, 2011). In addition, a recent study by Verma et al. (2020) indicates that the worldwide food waste might be even twice as high than previous statistics published by the Food and Agriculture Organization of the United Nations FAO (2011). In contrast to food loss that occurs early in the food supply chain due to a decrease in quality and improper handling, the extent of food waste originates in the consumers' values, behavior, and attitude (Principato, 2018). This mindset is also reflected by food waste accumulating in food service outlets such as canteens, cafeterias, and buffets. Canteens represent an important source of food waste in hubs of human productivity and many parameters influencing the extent of leftovers have been identified in the past: a lack of flexibility in adapting to consumer preferences, excessive portion sizes, displeasing consumption settings, and time constraints for lunch breaks can be factors for leaving behind food (Boschini et al., 2020).

As a generalist species, the black soldier fly (BSF; *Hermetia illucens*, Diptera: Stratiomyidae) is frequently used as decomposer of organic wastes, ranging from kitchen waste (Nguyen et al., 2015) and manure (Sheppard et al., 1994) to human excreta (Banks et al., 2014). The application of BSF larvae (BSFL) can range from small-scale lab populations (Nakamura et al., 2016) and household rearing systems (Klammsteiner et al., 2020c) to large-scale industrial rearing operations (Wynants et al., 2018). Food waste is heterogenous in its composition. Elevated salt (NaCl) content in meals is often discussed because of its adverse effect on human health (e.g., high blood pressure) and can account for up to 1.2% in hot meals and sandwiches handed out in canteens (Rasmussen et al., 2010). Cho et al. (2020) concluded that BSFL are suitable to treat food waste with even higher salt concentrations, since a significant inhibition of biomass gain and pupation was only observed at concentrations >3%.

The larval gut microbiome is considered essential for efficient food conversion by BSFL. In general, results from microbiome studies on BSFL can be challenging to compare due to variations in biotic and abiotic factors during the execution of experiments (De Smet et al., 2018; Bosch et al., 2020). Recent studies suggest that, although the larval gut microbiome does not remain unaffected when exposed to various biogenic wastes, a versatile core community of bacteria may contribute to the digestion of these substrates (Wynants et al., 2018; Zhan et al., 2019; Klammsteiner et al., 2020b). Although the BSFL gut microbiome has been shown to adapt to changing diets, the overlap between the gut residing communities and communities found in the respective diets is often low (Wynants et al., 2018). Little is known about the microbial metabolism taking place in the larval guts, since past studies have mostly focused on clarifying phylogenetic dynamics in relation to larval diet. Detailed investigations of metabolic processes taking place in larvae and attempts to exploit them for biotechnological applications are sparse (Lee et al., 2018; Song et al., 2018; Zhan et al., 2019). Tools imputing metagenomes to 16S rRNA gene-based amplicon data such as PICRUSt (Langille et al., 2013) or Tax4Fun (Aßhauer et al., 2015) have been used to provide insights into functional genes. Although originally developed for a human microbiome context, the reference databases are continuously growing also for other animals and environments (Breitwieser et al., 2017; Kostanjšek et al., 2019). To comply with the widespread use of amplicon sequencing, both platforms have recently been updated to follow-up versions (Douglas et al., 2020; Wemheuer et al., 2020). Imputation methods have also become a valuable tool to gain insights in metabolic processes of insects (Chen et al., 2016) and to assess the effect of insect-based diets on livestock such as poultry (Borrelli et al., 2017). In addition, they could provide an efficient approach to screen prokaryotic communities for novel bioactive compounds and find new means for the degradation of xenobiotics as has recently been investigated in marine sponges (Steinert et al., 2019).

In this study, we determined the effect of two major organic-waste fractions occurring in canteens (food waste from leftovers and oil waste from oil separators) on BSFL growth, the bacterial gut biota, and its functional makeup. The larval diet was changed in the experiments from the chicken feed diet (CFD) to either the food (FWD) or oil waste diet (OWD) 6 days after larvae had hatched. Guts were extracted at multiple time points to determine changes in microbial-community composition and function during their development. Finally, larval growth, waste degradation, and developmental time were documented, and meaningful process indices were derived from these data. We hypothesized that, due to its high nutritional value, canteen food waste represents a convenient substrate for BSFL rearing but presumed that it would induce shifts in gut microbiome composition due to varying nutrient patterns. Moreover, we considered that diet-induced shifts in the larval gut microbiota could provide an opportunity to identify diet-specific microbial biomarkers.

MATERIALS AND METHODS

Source of Black Soldier Fly Larvae and Colony Maintenance

Six-day old larvae were obtained from a bench-scale BSF colony at the Department of Microbiology (University of Innsbruck, Austria). Adequate and stable environmental conditions of 27°C, 60% relative humidity, and a light:dark photoperiod of 16:8 h using LED panels as described in Heussler et al. (2018) were created in a Fitotron SGC 120 (Weiss Technik, United Kingdom) climate chamber. Larvae, pupae, and adults were held in separate containers, and the density of individuals was manually controlled. An *ad libitum* amount of ground chicken feed diet (CFD; Grünes LegeKorn Premium, Unser Lagerhaus, Austria) mixed with water (40:60 w/v) was used for colony maintenance.

Experiment Preparation and Diets

Six days after hatching, 200 manually counted larvae were each transferred to four replicate boxes per diet, adding up to a total of 2,400 larvae equally distributed over twelve separate boxes with a density of 1.2 larvae cm^{-2}. The BSFL were kept under the

same conditions as described for the general colony maintenance. Sterilized and dried pine humus (20 g) was added to each box as litter for humidity regulation. The diets were defined as OWD, FWD, and CFD analogous to colony maintenance as control diet. OWD and FWD were obtained from a local canteen on a single day (DMS: 47°15′50.9″ N, 11°20′37.4″ E). For OWD, the upper layers from the content of an oil separator collection container were collected and manually homogenized. Fresh canteen waste for the FWD was shredded with a Vitamix TNC® electric blender (Vitamix, Olmsted Falls, OH, United States), thereby reducing the size of food residues and homogenizing its components. The diets were stored at −20°C until the start of the experiment. Feeding took place every second day for a maximum of 18 days (d_{0-18}), resulting in a maximum of ten feeding events per diet. After 20 days (d_{20}), the experiment was terminated. The amount of substrate to administer per larva and day was calculated using the content of organics in 100 mg CFD larva^{-1} as reference. OWD and FWD were thereby added in amounts of 70 and 170 mg larva^{-1} day^{-1}, respectively. Water and organic content were determined gravimetrically by measuring the loss of mass after drying the samples in a drying oven (UF110, Memmert, Schwabach, Germany) at 105°C for 24 h and subsequent incineration in a muffle furnace (CWF 1000, Carbolite, Neuhausen, Germany) at 550°C for 5 h. Substrate-water content was adapted to the moistest substrate (FWD) by adding 70 and 100 mg of water to CFD and OWD, respectively. Thereby, all three diets contained the same amount of water and organics.

Sample Collection and Preparation and Processing

Sampling followed the scheme illustrated in **Figure 1** and samples were stored at −20°C if not immediately processed. A climate room operating at 4°C was used to slowly thaw frozen samples. One larval sample consisted of ten randomly

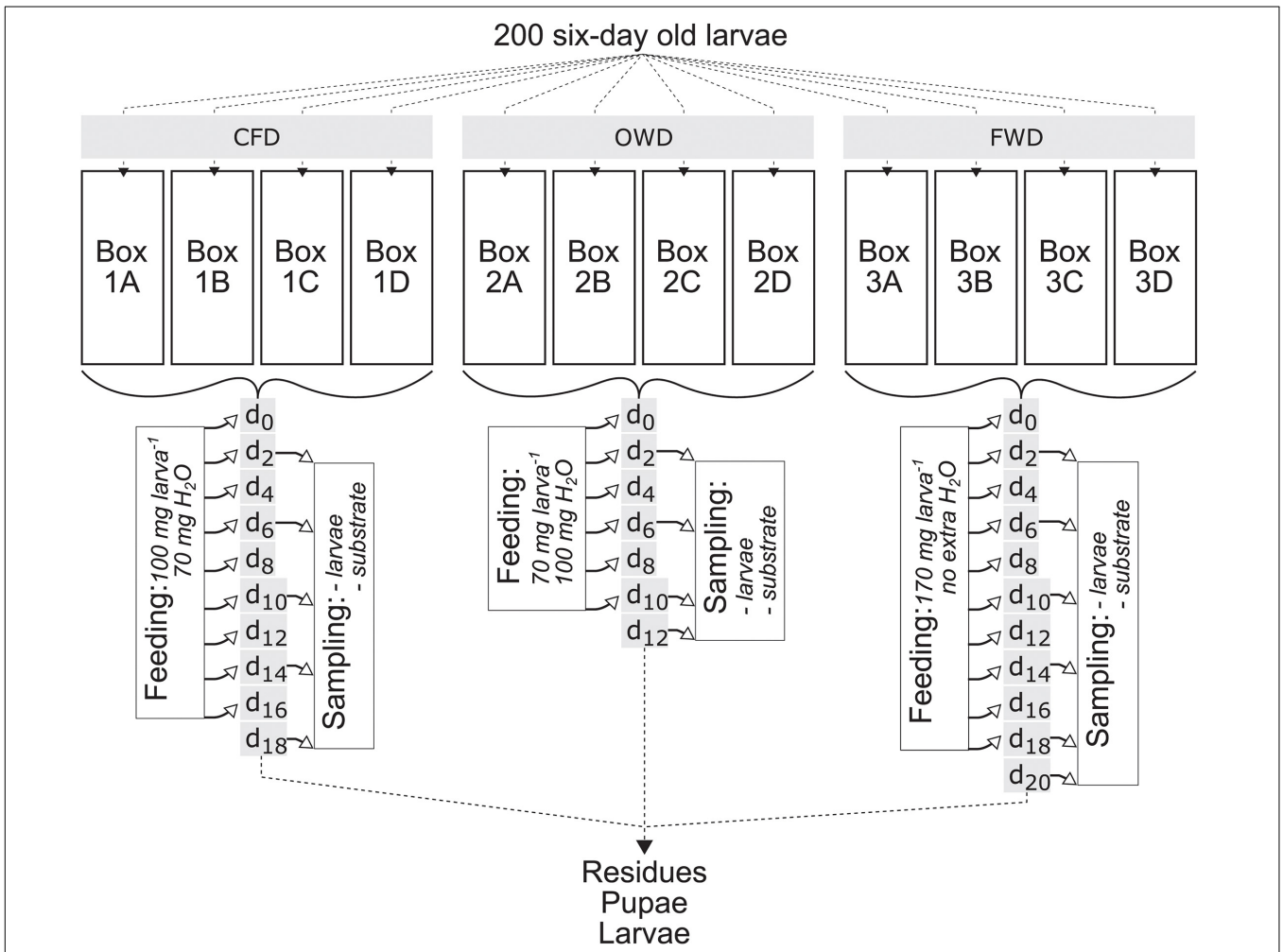

FIGURE 1 | Black soldier fly (*Hermetia illucens*) larvae feeding experiment scheme displaying sampling and feeding events after the introduction of two new diets (OWD, oil waste diet; FWD, food waste diet). The 6-day old larvae were obtained from a laboratory population kept on a chicken feed diet (CFD), with the control group continuing on the same diet as before the start of the experiment. Time points $d_{0\text{-}20}$ count the days of the experiment starting at 6 days post hatching of the larvae. Feeding events took place every other day for a maximum of 18 days.

collected larvae, of which five were used for determination of dry matter (DM) and volatile solids (VS), and another five were subjected to gut extraction. Fresh substrates and substrate residues were both physicochemically characterized prior and after the experiment, respectively. To prepare samples for the quantification of oxidizable organics [chemical oxygen demand (COD)], ammonium, total protein and reducing sugars, 10 g of sample was mixed in 25 ml diH_2O by briefly vortexing. After incubation at room temperature for 30 min, the mixtures were centrifuged at 12,000 × *g* for 30 min and subsequently filtered (MN 615 1/4 150 mm, Macherey-Nagel, Düren, Germany). The filtrate was used for further analyses.

Physicochemical Analyses

Total Solids, C/N Ratio, Volatile Solids, and Fatty Acids

Total solids were determined gravimetrically after drying the samples at 105°C for 24 h. The difference in fresh and dry mass was determined as water content. Volatile solids were inferred from the weight difference before and after incinerating the dry samples in a muffle furnace at 550°C for 5 h. Samples dried at 105°C for 24 h were finely ground using a pestle and mortar to quantify the C/N content using an elemental analyzer (TruSpec CHN Elemental Determinator, Leco, St. Joseph, MI, United States) by following the manufacturer's protocol. For the determination of volatile fatty acids, one g per sample was mixed into 1 ml sterile water and centrifuged for 15 min at 15,000 × *g*. Vials for high performance liquid chromatography were filled with the liquid supernatant and used for analysis following Wagner et al. (2017).

Chemical Oxygen Demand, Ammonium, Total Protein Content, and pH

After filtration (section "Sample Collection and Preparation and Processing"), the samples were diluted in diH_2O following the protocol enclosed in the quick test kits Nanocolor COD 1500 and Nanocolor Ammonium 50 (Macherey-Nagel, Düren, Germany), respectively, and transferred in the cuvettes. The cuvettes were incubated at 160°C for 30 min (Nanocolor Vario HC, Macherey-Nagel, Düren, Germany) and photometrically measured (Nanocolor UV/VIS, Macherey-Nagel, Düren, Germany). The Lowry protein assay following Noble and Bailey (2009) was used to determine the total protein content in sample filtrates. For the pH measurement, 10 g of substrate were mixed in 25 ml diH_2O and briefly vortexed. After 60 min incubation at room temperature the pH was determined with a 774 pH Meter (Metrohm, Herisau, Switzerland).

Statistical Analysis of Larval Development and Physicochemical Measurements

Growth rate, consumption index, and approximate digestibility were calculated based on Waldbauer (1968). Substrate reduction and the waste reduction index were determined as described by Jucker et al. (2020) and efficiency of conversion of the ingested and digested food was calculated as in Medrano and Gall (1976). Applied formulae are listed in **Table 1**. Statistical analyses were conducted in R (R Core Team, 2018). Processing of principal component analysis results and hierarchical clustering were conducted using the factoextra package (Kassambara and Mundt, 2020). All figures were created using ggplot2 (Wickham, 2016).

Harvesting of Larval Guts

After thawing, larvae were surface sterilized by briefly washing them in a Petri dish containing 70% EtOH. After the EtOH evaporated, a few mm were cut off from the anterior part of the larvae using a sterile scalpel and the gut was pulled out from this incision. From each time point × diet, 0.05 g guts were pooled from five larvae and transferred to a bead tube using sterile tweezers (Klammsteiner et al., 2020b).

DNA Extraction and 16S rRNA Gene Amplicon Sequencing

For DNA extraction from fresh substrates, 10 g of sampling material was mixed with 25 ml deionized water. After vortexing and subsequent incubation for 30 min at room temperature, the samples were shaken for 30 min at 120 rpm (Controlled Environment Incubator Shaker, New Brunswick Scientific,

TABLE 1 | Larval growth and degradation parameters based on data collected during the feeding trials (n = 4).

	CFD	FWD	OWD	
	mean ± SD	mean ± SD	mean ± SD	Formulae
Growth rate [mg d^{-1}]	9.0 ± 0.4^a	8.2 ± 0.8^a	1.1 ± 1.1^b	$GR = \frac{L_{end} - L_{initial}}{T}$
Substrate reduction [%]	65.6 ± 0.2^a	85.3 ± 0.7^b	2.4 ± 3.7^c	$SR = \frac{I-R}{I} \times 100$
Consumption index	96.9 ± 1.8^a	156.2 ± 3.9^b	17.5 ± 27.8^c	$CI = \frac{E}{T \times A}$
Waste reduction index	3.7 ± 0.0^a	4.3 ± 0.0^b	0.2 ± 0.3^c	$D = \frac{I-R}{I}$ $WRI = \frac{D}{T} \times 100$
Approximate digestibility [%]	47.7 ± 0.5^a	82.7 ± 0.9^b	–	$AD = \frac{E-R}{E} \times 100$
Efficiency of conversion of ingested food [%]	17.9 ± 0.6^a	7.9 ± 0.9^b	–	$ECI = \frac{B}{E} \times 100$
Efficiency of conversion of digested food [%]	37.5 ± 0.9^a	9.6 ± 1.0^b	–	$ECD = \frac{B}{E-R} \times 100$

CFD, chicken feed diet, FWD, food waste diet, OWD, oil waste diet; SD, standard deviation. Different superscript lower-case letters indicate differences between treatments ($p < 0.05$) according to the Tukey's HSD test. $L_{initial}$, initial mean weight of larvae; L_{end}, final mean weight of larvae; I, total feed input; R, residues; T, days; B, total larval biomass gained; A, mean fresh weight during feeding period; E, total feed ingested.

United States) before filtering them through folded filters (MN 615 1/4 150 mm, Macherey-Nagel, Düren, Germany). The DNA of substrate filtrates and guts was extracted following the manual of the used kit (NucleoSpin Soil, Macherey-Nagel, Düren, Germany). Quality and quantity of extracts were assessed via agarose gel electrophoresis and spectrophotometry (NanoDrop 2000c, Thermo Fisher Scientific, Waltham, MA, United States), respectively. Biological replicates of gut samples from three different boxes per treatment were collected and prepared at each sampling time point. Amplicon sequencing including a two-step PCR library preparation using a Nextera Index Kit, purification, pooling, and demultiplexing was performed by Microsynth (Balgach, Switzerland) on a Illumina MiSeq following the 2 × 250 bp paired-end approach. The universal bacterial and archaeal primer set 515f/806r (GTGCCAGCMGCCGCGGTAA/GGACTACHVGGGTWTCTAAT) was used to amplify the V4 region on the 16S rRNA gene (Caporaso et al., 2011). The provided reads were trimmed from adaptors and primers by the sequencing provider.

Data Analysis of Sequencing Data

Trimmed raw sequences were analyzed using mothur v.1.44.1 (Schloss et al., 2009). In the initial screening, ambiguous bases, homopolymers longer than eight and sequences >275 bases were removed. The SILVA database v.138 (Quast et al., 2013) was used as reference for alignment and classification steps. Potentially chimeric sequences were identified and subsequently removed using the vsearch algorithm. Sequences assigned to chloroplast, mitochondrial, archaeal, eukaryotic, and unknown lineages were filtered from the data. Downstream analyses were carried out in two comparative approaches: (I) binning at 97% sequence similarity and (II) identification of unique amplicon sequence variants (ASV). For data normalization, samples from both approaches were subsampled to (a) smallest sample size, (b) 15,000 sequences, and (c) 80,000 sequences. Distance matrices based on Bray-Curtis dissimilarity were calculated from the six abundance tables and pairwise comparison of matrices via Mantel test (Pearson correlation, 1000 permutations) was carried out to ensure stability of subsequent analyses (**Supplementary Figure 1**). The sampling effort was evaluated based on rarefaction curves (**Supplementary Figure 2**). Pairwise comparison of α-diversity (Shannon index; H') in samples grouped by diet and time was conducted using analysis of variance (ANOVA), pairwise *t*-test (including Bonferroni correction), and Tukey's honestly significant difference (HSD) test (**Supplementary Table 3**). Bartlett's test was applied to test for homogeneity of variances. Non-metric multidimensional scaling (NMDS) based on Bray-Curtis dissimilarity was used as β-diversity measure on filtered gut microbiome data (minimum prevalence of four in at least 10% of samples to remove overly sparse OTUs). Overall impact of the variables diet and time on microbial communities was computed by PERMANOVA (Bray-Curtis dissimilarity, 1000 permutations). All statistical tests were conducted using vegan v.2.5.6 (Oksanen et al., 2018). Pielou's evenness (J) was calculated as H'/log(species number). Congruent OTUs from linear discriminant analysis (LDA) effect size (Segata et al., 2011) implemented in mothur and multi-level pattern analysis as part of the R indicspecies package (De Cáceres and Legendre, 2009) were considered as indicator species.

Imputation of bacterial metabolic pathways was conducted using version 1.1.5 of the Tax4Fun2 R package and the KEGG database (Kanehisa and Goto, 2000) following the standard SOP (Wemheuer et al., 2020). Reference sequences were preclustered at 100% identity. All figures were created in R using the ggplot2 package (Wickham, 2016).

RESULTS

Maturation of Larvae and Dynamics of Waste Degradation

The feeding experiment was observed for a maximum timespan of 20 days (d_{20}) for FWD as the most prolific diet, resulting in 26-day-old larvae at the end of the experiment (**Figure 1**). The dietary treatments were terminated when all larvae had either pupated or died (CFD at d_{18}, OWD at d_{12}). Larvae on CFD and FWD both exhibited similar growth progress, with larvae offered FWD achieving a slightly higher biomass throughout the experiment (**Figure 2A**). However, fresh weights in the last sample were 5% lower in FWD, and biomass peaks were reached 2–6 days before the transition to pupal stage in CFD and FWD, respectively. After reaching the biomass peak at 206 ± 9 mg larva^{-1} on d_{14} and continuing their development toward pupae, biomass of FWD-fed larvae significantly decreased by 20 ± 3% until d_{20} ($p < 0.05$, ANOVA; decrease of 7 ± 5% in CFD). Drying and subsequent incineration of larvae additionally revealed similar dry matter (35–37%; of which 32–36% were volatile solids) and water contents (63–65%) in relation to fresh weight (**Table 2**). However, BSFL fed with OWD were strongly inhibited in their growth and further restricted by a mortality of 96 ± 2%, which ultimately led to the termination of this dietary treatment at d_{12}. The lethal effect became evident after d_5 and surviving larvae exponentially decreased until d_{12}. No larva exposed to OWD transitioned to the pupal stage. The endpoint biomass per larva reared on this diet added up to 21.5 ± 1.1 mg and was thus 87% below the weight of larvae reared on CFD and FWD.

With an average developmental time of 22 ± 3 days, BSFL raised on FWD developed slightly slower and with higher variability than larvae in the control group (21 ± 2 days). On CFD and FWD, first pupae appeared at d_{12} with a significantly higher initial biomass per pupa ($p < 0.001$) in FWD (**Figure 2B**). While pupae from FWD showed constant fresh weights of 169 ± 2 mg pupa^{-1} irrespective of the sampling event, pupae raised on CFD gradually increased from 116 ± 14 mg pupa^{-1} on d_{12} to 152 ± 2 mg pupa^{-1} in the experiment's last sampling event on d_{18}. In FWD, the last pupae were harvested on d_{20} and exhibited the largest variation in weight. Pupation in CFD reached its peak on d_{16} when biomass was highest (154 ± 5 mg pupa^{-1}), resulting in around 40% of larva transitioning to pupal stage in this time point.

In both CFD and FWD, larvae showed comparable growth rates (**Table 1**) with approx. 8.5 mg biomass gain per day. Although substrate reduction, consumption and waste reduction

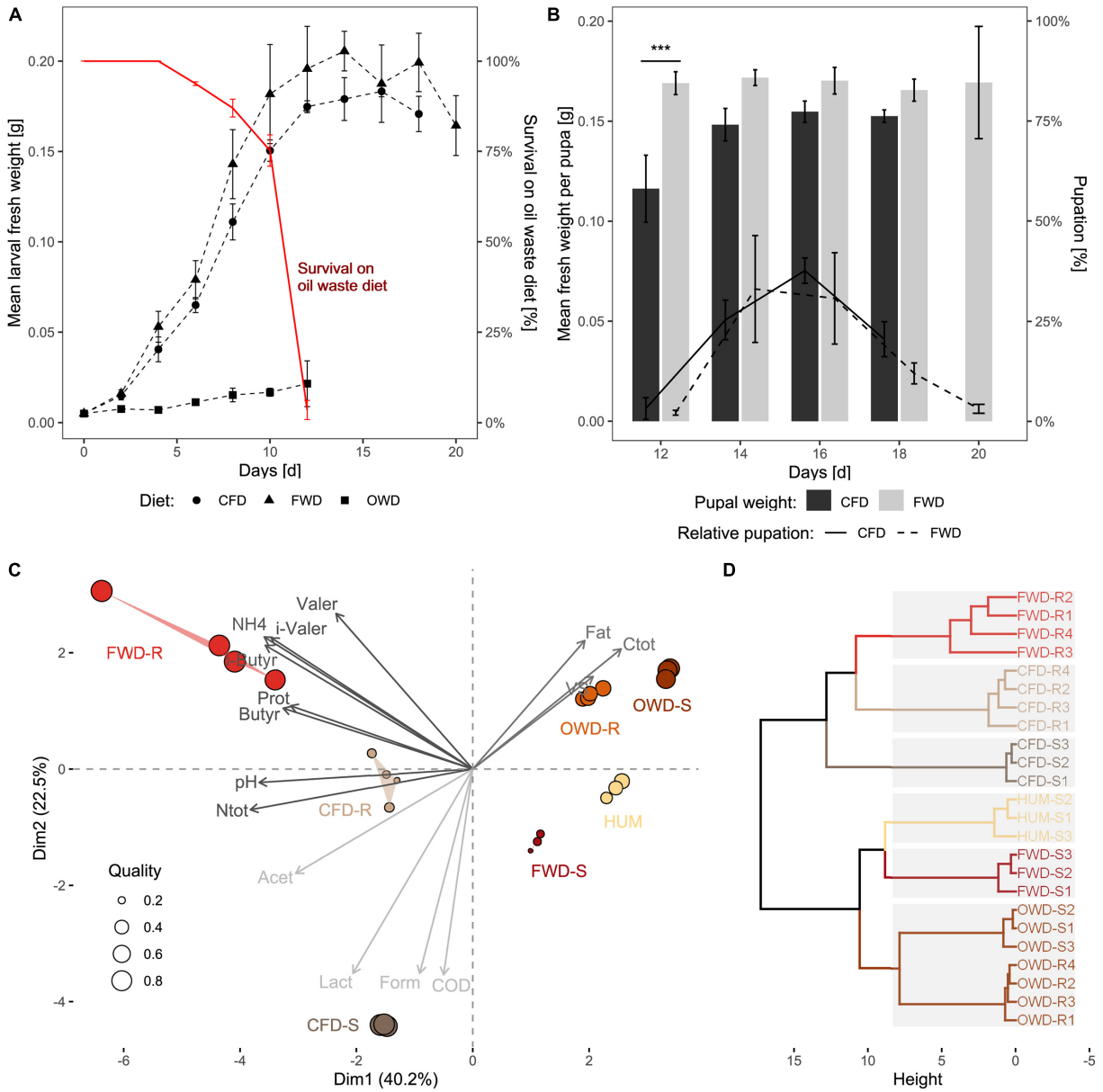

FIGURE 2 | Performance of black soldier fly larvae, pupae, and their three diets. **(A)** Dotted lines represent the impact of diet on the increase of larval fresh weight (n = 4). The red line highlights the survival of larvae on OWD. **(B)** Bars indicate the fresh weight of pupae in relation to their time of harvest. The lines show the fraction of larvae that transitioned to pupal stage in each sampling time point. Larvae fed with OWD did not reach pupal stage and are not shown in the graph (n = 4). **(C)** Principal component analysis based on physicochemical properties of fresh substrates (-S) and substrate residues (-R). The three clusters of variables represented by the arrows were determined by k-means clustering and are distinguished by different shades of gray (fresh: n = 3; residues: n = 4). **(D)** Hierarchical clustering of sample replicates. Gray backgrounds represent sample clusters determined by the gap statistics method. CFD, chicken feed diet; FWD, food waste diet; OWD, oil waste diet; HUM = pine humus; Prot, proteins; Ntot, total nitrogen; Ctot, total carbon; VS, volatile solids; COD, chemical oxygen demand; (i-)Valer, (i-)valerate; (i-)Butyr, i-butyrate; Form, formate; Lact, lactate; Acet, acetate. ***$p < 0.001$.

indices as well as approximate digestibility were significantly higher in FWD, the efficiency of larvae to convert ingested and digested food were more than two and four times higher in the CFD control diet, respectively. Larvae raised on OWD exhibited negligible substrate reduction. The consumption and waste reduction indices indicated insignificant degradation of the diet fed.

Physicochemical Characterization of Substrates

Principal component analysis was conducted on physicochemical data from pre-process fresh substrates used as diet (CFD-S, FWD-S, OWD-S) and in post-process substrate residues (CFD-R, FWD-R, OWD-R). The two principal components in **Figure 2C** explain a combined variance of 62.7% in the data. The

TABLE 2 | Endpoint characterization of larval and pupal biomass (n = 4).

		CFD	FWD	OWD
		mean ± SD	mean ± SD	mean ± SD
Larvae	Fresh weight* [g]	0.171 ± 0.008^{a}	0.164 ± 0.014^{a}	0.022 ± 0.011^{b}
	Water content* [g]	0.108 ± 0.006^{a}	0.107 ± 0.012^{a}	0.014 ± 0.006^{b}
	Dry weight* [g]	0.063 ± 0.003^{a}	0.057 ± 0.001^{a}	0.008 ± 0.005^{b}
	Volatile solids* [g]	0.055 ± 0.003^{a}	0.054 ± 0.001^{a}	0.008 ± 0.005^{b}
	Mortality [%]	0^{b}	0^{b}	96 ± 2^{a}
	Developmental time [d]	21 ± 2	22 ± 3	0
Pupae	Pupation rate [%]	87 ± 7^{a}	80 ± 9^{a}	0
	Fresh weight^{+} [g]	0.153 ± 0.003^{a}	0.169 ± 0.023^{a}	–
	Water content^{+} [g]	0.096 ± 0.001^{a}	0.107 ± 0.014^{a}	–
	Dry weight^{+} [g]	0.057 ± 0.001^{a}	0.062 ± 0.009^{a}	–
	Volatile solids^{+} [g]	0.050 ± 0.001^{a}	0.060 ± 0.009^{a}	–

CFD, chicken feed diet; FWD, food waste diet; OWD, oil waste diet; SD, standard deviation. Different superscript lower-case letters indicate differences between treatments ($p < 0.05$) according to the Tukey's HSD test.
**at last sampling time point (d_{20}).*
$^{+}$after harvesting of pupae.

15 most influential parameters represented by the arrows were aggregated in three groups by k-means clustering to clarify the coordination of samples. Increased NH_4 (20- and 30-fold in CFD-R and FWD-R, respectively) and protein (10-fold in all residues) contents strongly contributed in distinguishing pre- from post-process substrates (**Supplementary Table 1**). Differences between fresh and residue samples from OWD were less evident due to similar physicochemical characteristics featuring a high fat, C_{tot}, and volatile solid content, therefore, both groups sharing a similar coordination. The fresh control diet CFD-S stands out from the other diets due to its elevated lactate and formate content and higher chemical oxygen demand (COD). After processing by BSFL, the residues of the control diet (CFD-R) deviated from their initial properties and shared more similarities with FWD-R, being better represented by a higher pH and N_{total} content. While FWD-S featured a comparatively scarce VFA profile (**Supplementary Table 2**) and a weaker fit by the principal components, the residues from this diet (FWD-R) were characterized by high protein, NH_4, (i-)valerate, and (i-)butyrate concentrations. The VFAs together with nitrogen compounds (protein, NH_4, N_{total}) were most influential for the variation in the data. The sterilized and dried pine humus (HUM) used as neutral medium for moisture regulation during the feeding experiment contained negligible concentrations of VFAs, fats and nitrogen compounds. Relationships among groups of pre- and post-process substrates were further determined by hierarchical clustering (**Figure 2D**). Gap-statistics analysis (cut-off at $k = 0.61$) identified an optimal number of six clusters (from seven sample groups) represented by gray rectangles underlaying the dendrogram, summarizing the groups of fresh and residual OWD samples in one group.

Analysis of the Substrate Microbiota

After passing through quality control and filtering steps, a total of 7.01×10^5 reads from nine fresh substrate samples (three biological replicates per substrate) were used for downstream analysis. ANOVA and PERMANOVA of fresh substrate-derived microbial communities indicated significant differences between substrates ($p < 0.001$) on α-diversity level (defined by H'; **Figure 3A**) and in general community composition represented by β-diversity ($p < 0.05$), respectively. Significant pairwise differences ($p < 0.001$) were further confirmed via pairwise t-test and Tukey's HSD *post hoc* test (**Supplementary Table 3**). Firmicutes were found to be the most dominant phyla in both FWD and OWD with 92% and 57% relative abundance. CFD was dominated by Proteobacteria (44%) and contained overall lower abundances of Firmicutes (23%). Bacteroidota were most common in OWD (38%) but less abundant in FWD and CFD (28% and 14%, respectively).

With a share of 34.7 ± 3.5%, sparse OTUs with a relative abundance smaller than 1% contributed to a large part to the community in CFD compared with 8.9 ± 0.4% in OWD and 5.2 ± 0.8% in FWD. H' further indicated a heterogeneous distribution of abundances among few OTUs in FWD (H' = 1.14 ± 0.14) described by a low evenness (J = 0.33 ± 0.03). CFD was defined by a more even community (J = 0.79 ± 0.03) consisting of many less abundant OTUs (H' = 4.15 ± 0.21). For matters of clarity, only genera with abundances of >5% were included in **Figure 3B**. On genus level, FWD was largely dominated by *Leuconostoc* (77%) of the phylum Firmicutes. In OWD (H' = 2.67 ± 0.02; J = 0.66 ± 0.02), Firmicutes were mainly represented by unclassified Selenomonadaceae and shared their predominance (31%) with a similarly abundant *Prevotella* (36%).

Taxonomic Exploration of the Gut Microbiota

Sequencing of the 16S rRNA V4 genetic region yielded a total of 3.50×10^6 reads from 36 BSFL gut samples (three biological replicates per time × treatment) after passing through quality control and filtering steps. Mantel tests on distance matrices

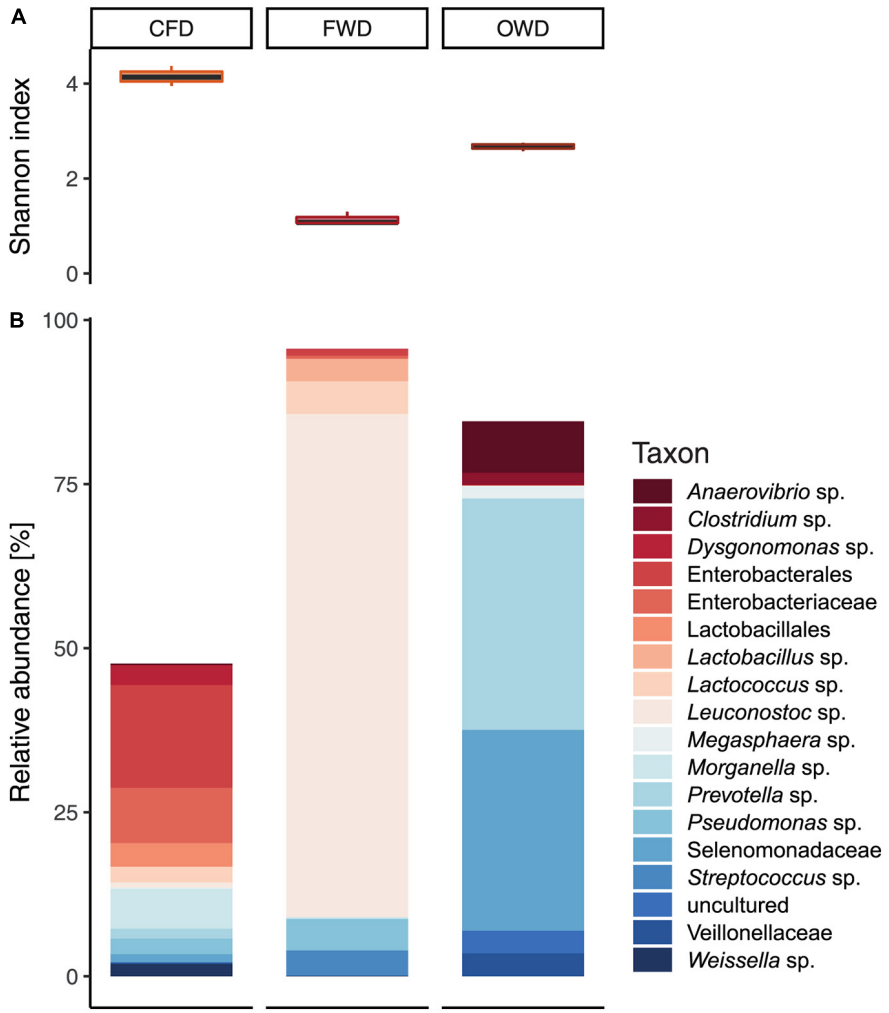

FIGURE 3 | Bacterial community characteristics in chicken feed (CFD), food waste (FWD), and oil waste (OWD) administered as diet in feeding experiments with black soldier fly larvae (n = 3). **(A)** Shannon diversity index observed in the three diets. **(B)** Bacterial community in substrates representing taxa with a relative abundance of at least 5%.

produced by the different clustering and subsampling approaches described in section "Data Analysis of Sequencing Data" resulted in no significant differences among the compared methods. This confirmed the overall congruency of the results irrespective of clustering method (97% similarity OTUs or unique ASVs) and subsampling size. The presented results are based on 97% similarity data subsampled to 36,878 reads (smallest sample size). The reads were clustered into 1,583 OTUs representing 491 genera within 39 phyla. Most abundant phyla across all guts were Firmicutes (30%), Proteobacteria (26%), Bacteroidota (15%), and Actinobacteriota (7%). The H' as well as J observed in the original population of 6-day-old larvae (INI; $H' = 1.89 \pm 0.05$, $J = 0.37 \pm 0.01$) remained stable throughout the CFD treatment ($H' = 1.65 \pm 0.04$, $J = 0.35 \pm 0.01$) but significantly decreased in later samples obtained from the FWD treatment ($H' = 0.59 \pm 0.18$, $J = 0.13 \pm 0.04$), $p < 0.05$) (**Figure 4A**). A similar decrease was observed in later samples from OWD-fed larvae ($H' = 1.25 \pm 0.07$, $J = 0.25 \pm 0.02$).

PERMANOVA based on Bray-Curtis dissimilarity of gut microbiome data confirmed the observed differences in larval gut microbiomes ($p < 0.001$) between sampling time points and dietary treatments. Pairwise PERMANOVA with samples summarized on diet level specified significant compositional divergence from INI in the gut microbiome of FWD and OWD-fed larvae but showed stability in the CFD control group (**Figure 4B** and **Supplementary Table 3**). While *Dysgonomonas* (38%) and *Providencia* (30%) were initially highly prevalent in FWD, over time they were displaced by *Morganella* (from 1% in d_2 to 88% in d_{20}; **Figure 4B**). A comparable development was observed in OWD, which contributed to an increasing similarity to FWD-fed larvae. NMDS analysis of the larval gut microbiome data (stress < 0.2) further explained spatial relationships between diets and highlighted the compositional overlap found in FWD and OWD-fed larvae (**Figure 5A**). Linear discriminant analysis of effect size found 30 OTUs to be explanatory for differences between groups, while 49 OTUs were identified to be strongly

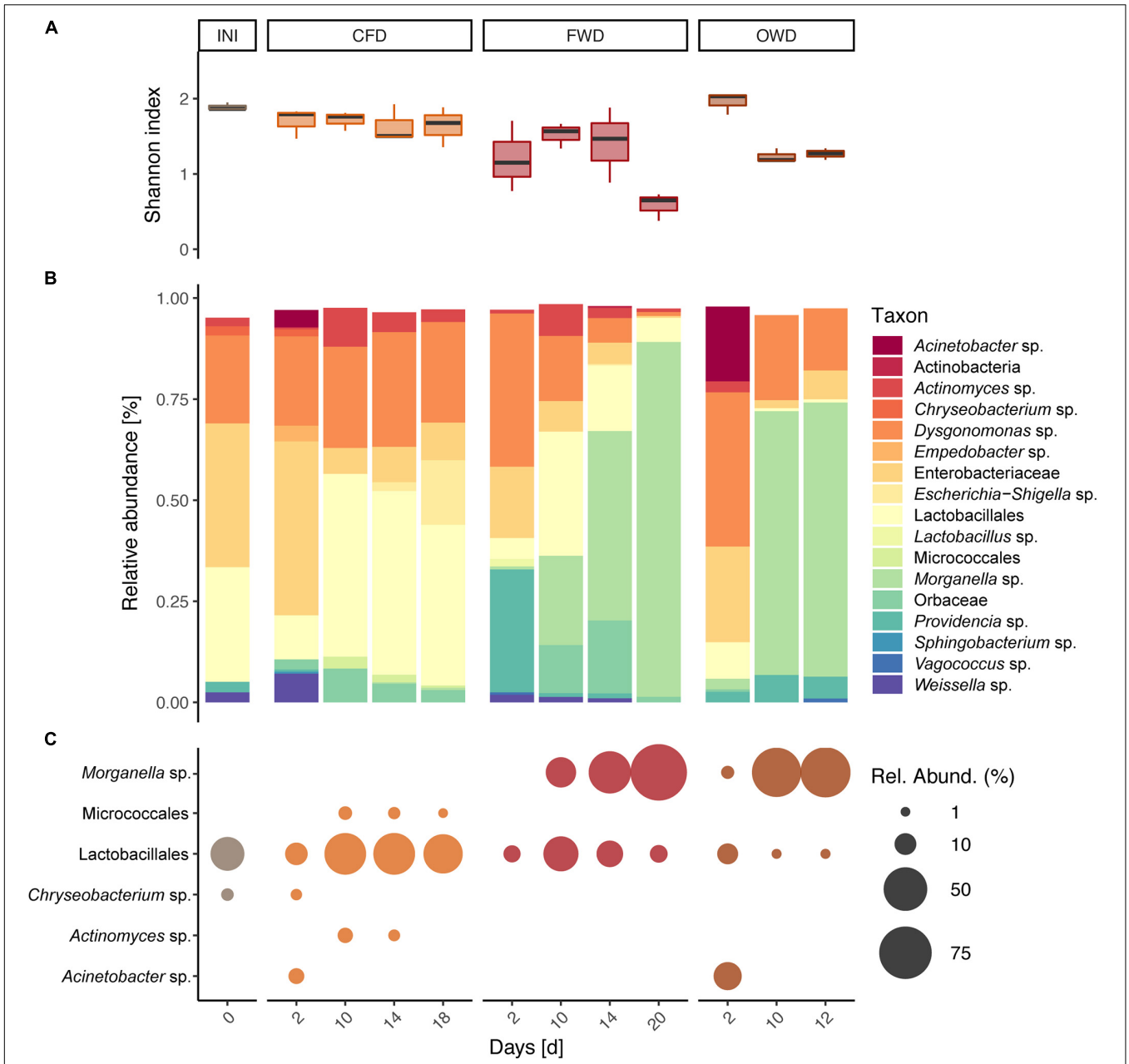

FIGURE 4 | Bacterial community characteristics in guts of black soldier fly larvae reared on chicken feed (CFD), food waste (FWD), and oil waste diet (OWD). Before introducing new diets, neonate larvae were reared on CFD for 6 days (INI). Data from each time point is presented as summary of three replicates (n = 3). **(A)** Shannon diversity index observed in guts of BSFL. **(B)** Bacterial community composition in guts of BSFL representing taxa with a relative abundance of at least 1%. **(C)** Indicator species identified by both linear discriminant analysis of effect size and multi-level pattern analysis with a relative abundance of at least 1%.

associated with the respective dietary treatment by multi-level pattern analysis. A congruent set of 17 OTUs recognized by both methods was defined as indicator species and assigned to 15 distinct genera (genera with relative abundance >1% in **Figure 4C**). Representatives of the order of Lactobacillales were found to be characteristic in guts of all treatments, but could not be assigned to a described genus. *Morganella* contributed with high abundances to the differentiation of mid to late stages of FWD- and OWD-fed larvae from the control group. However, CFD-fed larvae contained specific differentially abundant bacterial groups depending on their stage of growth. *Chryseobacterium* was found to be distinctive for early stages, while *Actinomyces* and Micrococcales were mostly found in mid and late stages. Moreover, the later samples spread further away from the initial community (INI) that consisted of predominantly Enterobacteriaceae (36%), Lactobacillales (29%), and *Dysgonomonas* (21%). Although similar in larval development time and growth rate, CFD and FWD-fed larvae

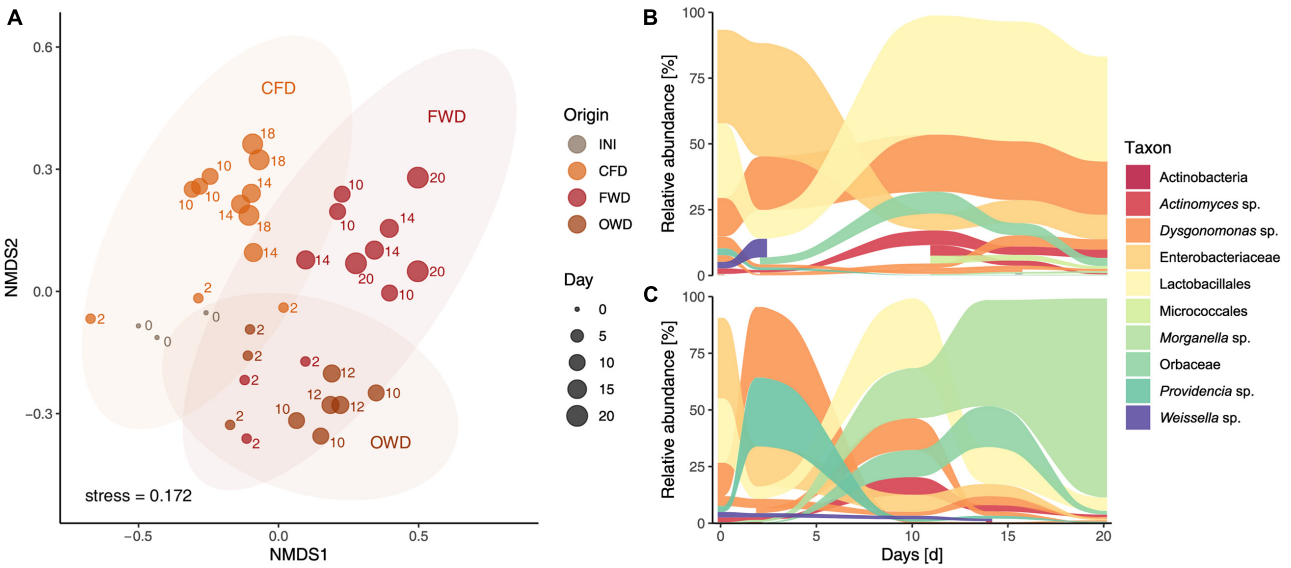

FIGURE 5 | Compositional relationships and dynamics in black soldier fly guts (n = 3). **(A)** Non-metric multidimensional scaling of larval gut microbiome data based on Bray-Curtis dissimilarity with ellipses representing a 95% confidence interval. Data was filtered to contain OTUs with a prevalence of four in at least 10% of the samples. INI, initial population of 6-day old larvae; CFD, chicken feed diet; FWD, food waste diet; OWD, oil waste diet. Dynamics of taxa with a relative abundance >1% present in at least three sampling events in black soldier fly larvae reared on chicken feed **(B)** and food waste **(C)**.

exhibited different dynamics of the most abundant taxa in their guts. Persistent genera with a relative abundance >1% and present in at least three sampling events are shown in **Figures 5B,C** for CFD and FWD-fed larvae, respectively.

Imputation of Main Metabolic Pathways of the Gut Microbiota

Approaches making use of metagenomic imputation as introduced by the R packages Tax4Fun2 were used to infer metabolic pathways from 16S rRNA gene-based amplicon data. On average, 70 ± 18% of the obtained sequences could be assigned to the reference database and used for the prediction of pathways. PERMANOVA analysis indicated significant time- and diet-dependent differences ($p < 0.001$) in metabolic profiles (**Supplementary Table 3**). Pairwise comparison of data grouped on diet-level via pairwise PERMANOVA revealed that both OWD and FWD differ from the CFD control group, but only OWD diverged from the metabolic pathways found in larval gut communities of INI. In contrast to the deviating development of FWD from INI on a phylogenetic level, this difference was not observed in a metabolic context. In general, the distinction between treatments on the level of metabolic pathways was much blurrier than on a taxonomic level (**Figures 4B**, **6**).

DISCUSSION

Two major fractions of canteen waste – FWD and OWD – were compared with a commonly used CFD. Impact on the growth, the gut microbiome profiles, and the divergence from the initial 6-day old population of BSFL were determined.

Canteen Food Waste as an Easily Degradable and Growth-Promoting Substrate for BSFL

Food waste from canteens is highly heterogenous and mainly consist of staples (potatoes, rice, pasta), vegetables/fruits, and grain products with smaller amounts of meat and fish wastes (Silvennoinen et al., 2015). Our FWD derived from one batch of mixed organic wastes that accumulated on a single day in a local canteen. We found that larvae fed FWD took a highly similar course in biomass gain and development as the CFD-fed control group (**Figures 2A,B**). However, endpoint biomass of FWD-fed larvae were 5% below the average measured in the control. These results are congruent with previously published records of larvae raised on chicken feed (on average, 158 ± 20 mg larva^{-1}; Barragán-Fonseca et al., 2017). While CFD-fed larvae did not experience a considerable decrease in biomass once they had reached their peak weight, FWD-fed larvae approaching (pre-)pupal stage consistently lost mass. This is crucial in calculating cost efficiency and designing processes in the large-scale application of BSFL (Salomone et al., 2017). A biomass peak significantly higher a few days prior to the transition to pupal stage suggests not to use the self-harvesting ability of larvae in an industrial setting. Instead, a batch system with scheduled mechanical or manual harvesting cycles taking place during the fifth instar before onset of migration could represent a more efficient option. Although greenhouse gas emission and overall environmental footprint of treating organic wastes with BSFL were already low compared with traditional composting (Mertenat et al., 2019), timing the harvest of larvae could boost the efficiency of operating resources. Improved biomass output and more stable frequency of rearing cycles would be the

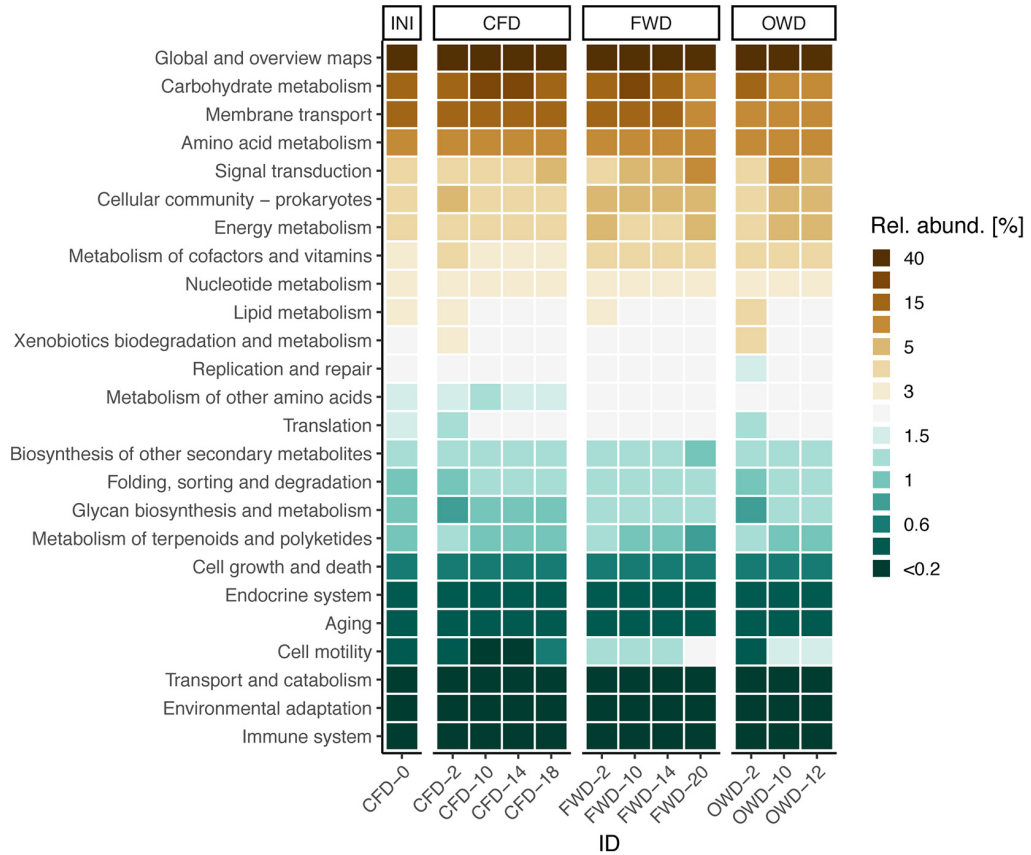

FIGURE 6 | Most abundant functional pathways in the gut microbiota of black soldier fly larvae before introduction of dietary treatments (INI) and after introduction of food waste (FWD) and oil waste (OWD) (n = 3). The control group was further fed with chicken feed (CFD). Pathways were obtained from Tax4Fun2 analysis and summarized on level 2 of the BRITE hierarchy based on KEGG orthology.

consequences. The advantages of harvest before the last instar became also evident when larvae were used for the production of biodiesel, since they contained a higher fraction of lipids and less chitin (Wong et al., 2019).

During the degradation process, FWD was highly modified by larval and microbial activity. This is indicated by high concentrations of VFAs such as valerate and butyrate, but also higher NH_4 contents (**Figure 2C**). The accumulation of nitrogen compounds is of interest for the commercialization of residues as soil amendment (Klammsteiner et al., 2020a). Residues from BSFL-processed household organic wastes showed similar C_{tot} and N_{tot} ratios to the FWD-R produced in our study and have been identified as an effective nitrogen source for plant growth (Kawasaki et al., 2020). Moreover, the generally high chitin content in residues produced by insects can improve the defense against phytopathogens by triggering plant-based immune responses (Sharp, 2013).

When food waste remains untreated, microbial spoilage dominated by lactic acid bacteria can cause the pH to rapidly drop to approx. 4.3 within 7 h (Aichinger et al., 2015; Wu et al., 2018). A similarly low initial pH of 4.5 was observed in the food waste fed to the larvae in our study (**Supplementary Table 1**), before shifting to a more neutral value in post-process residues (**Figure 2C**). This is particularly important from a hygiene perspective, as these acidic conditions can inhibit bacterial pathogens (Wu et al., 2018). In addition, BSFL have been shown to exhibit antimicrobial activity against multiple potentially human pathogenic bacteria by excreting antimicrobial compounds into their environment (Erickson et al., 2004; Liu et al., 2008; Choi et al., 2012).

Larvae fed with FWD showed significantly enhanced substrate reduction, better approximate digestibility of the substrate, and a higher consumption index compared with CFD (**Table 1**). However, a presumably lower nutrient availability in this diet required the ingestion of larger amounts to reach the same larval biomass as was observed in CFD (**Table 2**). This is emphasized by the comparably low efficiency of FWD-fed larvae to convert ingested and digested food to their own biomass. The chicken feed commonly used to maintain BSF lab populations was developed as a balanced, energy-rich diet for laying hens and optimized amounts for BSFL rearing have been investigated by Diener et al. (2009). The overall benefit created by using waste-derived substrates, however, can make up for the lower biomass yield and slower development. It is still not known whether the larvae adapt to a specific diet over time and how many generations are necessary to establish metabolic

stability. Nutrition-induced epigenetic inheritance from previous generations has been found to influence offspring phenotypes in other Dipterans such as *Drosophila melanogaster* (Wang et al., 2017). The potential distortion and its extent during impact assessment of newly introduced diets on BSFL growth and microbe-host relationships have yet to be quantified.

Oil-Separator Waste Is Strongly Reducing BSFL Development and Survival

The OWD treatment mainly derived from fats, oils, and grease (FOG) trapped as supernatant in the collection container of an oil separator. In addition to the lipid fraction, it also contained a smaller and heterogenous fraction of food residues from the cooking process. The fatty components of the OWD represent a more problematic fraction of the canteen waste since FOGs generally require more refined treatment methods (Husain et al., 2014). Supplying them to anaerobic digestion is a frequently chosen biotreatment (Long et al., 2012), but also composting of FOG compounds provided promising results (Lemus and Lau, 2002).

In our trial, the OWD changed its consistency within a few days and became more viscous, making it harder for the larvae to move. However, physicochemical properties and volatile fatty acid profiles remained largely unchanged (**Figures 2C,D** and **Supplementary Tables 1**, **2**). Solidification and deposition of FOGs are a widespread problem in sewer systems transporting wastewater from food service establishments (He et al., 2013). These processes are most likely driven by high concentrations of saturated fats and calcium, while moisture content is secondary (Keener et al., 2009). Due to the slow growth rate and negligible substrate reduction, the degradation parameters did not provide any meaningful information.

A combination of inhibited mobility and lack of easily degradable nutrients may have led to the starvation of the larvae. Moreover, the physical blocking of the larvae's spiracles by the oil could have led to their suffocation (Taverner, 2002). After approximately 7 days, the extent of this effect became visible by an exponentially increasing mortality (**Figure 2A**). No notable ingestion and digestion of the substrate could be assessed by the consumption and waste reduction index (**Table 1**). There have not been in-depth investigations on BSF lipid metabolism yet, but a recent study showed that BSFL do not only bioaccumulate fatty acids from their diet but are also able to synthesize fatty acids themselves (Hoc et al., 2020). Future studies with diets supplemented with defined combinations of FOGs could determine limits of BSFL fat tolerance in organic wastes to enable FOG utilization in BSFL feedstuff.

Food Waste Diet Shapes the BSFL Gut Microbiome

Chicken feed diet-fed larvae retained a stable bacterial diversity throughout the experiment and did not diverge microbiome-wise from the initial population of 6-day old larvae raised on the same diet (**Figures 4A,B**). These observations are in line with Cifuentes et al. (2020), who found that a chicken-feed-based diet contributes to an overall stable gut microbiome during larval development. The introduction of FWD and OWD treatments had a decisive impact on the development of the BSFL gut microbiome. Over time, the initially abundant Lactobacillales, Enterobacteriaceae, and *Dysgonomonas* were displaced by mostly *Morganella* in both diets. This compositional change further led to a decrease in α-diversity by crowding-out other groups and thereby. Even though larval growth (**Table 2**) and substrate degradation parameters (**Table 1**) reported negligible conversion of OWD, the gut microbiomes of these larvae developed similarly to FWD-fed larvae (**Figure 4B**). However, in contrast to Raimondi et al. (2020), we could not observe a meaningful increase of gut microbiome complexity over time.

As recently stated by Raimondi et al. (2020), Proteobacteria, especially *Providencia*, dominated guts of vegetable-fed larvae irrespective of the rearing temperature used in their study. In our trial, however, *Providencia* was mostly found in early larval stages and in low abundances throughout the development of OWD-fed larvae. This discrepancy is likely caused by the nutritional differences between their standard vegetable diet and the FWD and OWD used in our study. Larval ingestion of corn flour, wheat bran, and alfalfa flour contained in the vegetable diet might favor the prevalence of *Providencia* (Raimondi et al., 2020).

Furthermore, the Gram-negative and motile genus of *Morganella*, predominant in FWD and OWD-fed larvae, is known to share several biochemical aptitudes such as the (oxidative) deamination of certain amino acids with *Providencia* (Manos and Belas, 2006). It has previously been found to play a significant role in the BSFL gut microbiome due to its prevalence across various feeding schemes and larval developmental stages (Wynants et al., 2018; Cifuentes et al., 2020; Klammsteiner et al., 2020b; Liu et al., 2020; Raimondi et al., 2020). In our study, *Morganella* strongly contributed as indicator species to the distinction of FWD- and OWD-shaped gut microbiomes from the control group (**Figure 4C**). It is an environmentally widespread genus of the Enterobacterales so far only consisting of the species *Morganella morganii* and is often found as commensal in human guts (Manos and Belas, 2006). Due to its potential to cause severe infections, it has been cited as a relevant pathogenic microorganism associated with insects grown for feed and food (Schlüter et al., 2017; Raimondi et al., 2020). As opposed to rearing BSFL raised on CFD, food waste favored the enrichment of *Morganella* in larval guts. In fresh FWD and OWD substrates, *Morganella* was only present in comparatively low relative abundances of 0.1–0.3% while fresh CFD contained 6% but did not promote its accumulation in larvae. Additionally, extensive gut colonization by other bacteria was inhibited as has previously been observed in carrion degrading burying beetles: while endogenous *M. morganii* not only contributed to the stabilization of the beetle gut microbiome, it also helped to outcompete rival bacterial communities and prevent colonization by potentially entomopathogenic bacteria (Duarte et al., 2018; Wang and Rozen, 2018). High abundances of this species were also reported for other Diptera such as wild populations of the Mediterranean fruit fly *Ceratitis capitata* (De Cock et al., 2019). On the contrary, for mass-reared Mexican fruit flies (*Anastrepha ludens*), inoculating the substrate with only 105 CFUs ml^{-1}

of *M. morganii* led to 100% mortality in larvae while lower concentrations reduced emergence and flight ability of adults (Salas et al., 2017). Although *Morganella* did not negatively alter BSFL performance in our trial, the role of other entomopathogens as a risk factor in industrial *H. illucens* production remains to be investigated. Immunological responses of larvae in form of antimicrobial peptides have been addressed (Vogel et al., 2018). However, microorganisms (bacteria, fungi, and viruses) potentially endangering the uprising BSF industry – this includes fly populations as well as workplace safety – are due to be characterized (Joosten et al., 2020).

Since previously reported studies rarely match in rearing conditions, protocols, and locations, detailed comparisons are strongly limited (De Smet et al., 2018). Shifts, and thereby adaptations, of gut microbial communities to new diets might be primarily driven by the functional needs involved in degrading the available substrate. By clarifying whether in BSFL metabolic competence is preferred over enrichment of ingested exogenous microbes, the accumulation dynamics of specific phylogenetic groups in the larval gut could further be explained. In this context, investigating also the modification of the inherent digestive enzymatic toolkit and gut cell morphology in relation to diet uptake constitutes a crucial approach to gain a comprehensive picture of larval metabolism (Bruno et al., 2019; Bonelli et al., 2020). Nevertheless, we think that especially the pursuit to convert organic wastes known to carry a high bioburden into larval biomass should provide further motivation to find appropriate pre-treatment methods for substrates.

Indicator Species as Traits for Dietary Adaptation of Microbial Communities in the Gut

Besides *Morganella*, Lactobacillales pervaded guts from all treatments (**Figure 4C**). They have been identified to be a common driving factor for the divergence of dietary treatment groups. Although the OTUs representative of this group could not be classified at the genus level, a selection of Lactobacillales taking place based on the ingested diet is probable. This order also includes lactic acid bacteria such as *Lactobacillus*, *Leuconostoc*, and *Lactococcus*. In extensively studied honeybees and fruit flies, probiotic lactic acid bacteria were found to improve pesticide resistance and gastrointestinal pathogen control (Trinder et al., 2015; Daisley et al., 2017). Moreover, inoculation of organic wastes with Lactobacilli was shown to yield a higher biomass output and a better nutritional spectrum in BSFL compared with artificial feed-amended wastes (Somroo et al., 2019). Depending on their distribution at a higher phylogenetic resolution (e.g., species level), Lactobacillales identified as indicator species could act as candidate biomarker for larva-substrate interactions. Wu et al. (2018) found that lactic acid bacteria, and in particular Lactobacillales, strongly prevail during the microbial degradation of food waste and shape its microbiome by changing physicochemical parameters.

In the CFD-fed control group, a temporal succession of low-abundant indicator species was observed. *Chryseobacterium* defined the guts of early CFD-fed larvae and was previously linked to the digestion of high-fiber diets in guts of American cockroaches (Dugas et al., 2001). Two Actinobacteria represented by *Actinomyces* and Micrococcales, were found to be specific for mid to late-stage larvae. These findings are consistent with observations made by Raimondi et al. (2020), who identified Micrococcales (in particular *Brevibacterium*) as biomarker in prepupae. In a previous study, we identified *Actinomyces* as a main member of the gut core microbiome in larvae raised on low-bioburden diets such as commercial chicken feed (Klammsteiner et al., 2020b).

Main Metabolic Features Are Not Primarily Defined by Community Composition

Deducing metabolic pathways from short reads and microbial abundances comes with limitations but can maximize the informative value of marker-gene data. Especially environmental samples have more shortcomings compared with human-derived samples. Poorer availability of closely related reference genomes and the reliance on comparatively short sequence fragments that are often highly similar among distinct bacterial families have to be kept in mind (Knight et al., 2018). However, deducing links between taxonomy and function from 16S rRNA gene-based amplicon data can also be a preferable method to shotgun metagenomic sequencing, when, e.g., the host genome DNA strongly interferes with the microbiome signal at sequencing step (Langille, 2018). This could pose a problem when using metagenomic sequencing to investigate gut microbial communities in BSFL, as samples still contain host derived tissues.

Because of the known limitations, we decided to conduct our comparative analysis on the second level of the KEGG hierarchy instead of focusing on distinct pathways and/or metabolic functions. Although the administered diets induced clear shifts in the composition of microbial communities, the differences in the main metabolic pathways between dietary treatments were not as clear (**Figure 6**). A similar conclusion was reached by Zhan et al. (2019) on the larval transcriptome level: to help with the digestion of food waste as well as poultry, swine, and dairy manure, larvae use a common genetic toolkit instead of expressing diet-specific genes. These basic findings are directly in line with findings of The Human Microbiome Project Consortium (2012). A comprehensive study of the human microbiome led to the conclusion that metabolic pathways are mostly stable despite larger variations in microbial community structure. Pathways taking care of general and specific metabolic functions (carbohydrate, amino acid, energy, cofactor, and vitamin metabolism), membrane transport, and cell communication were amongst the highest abundant pathways in BSFL gut microbiomes.

So far, the investigation of metabolic functions of the BSFL gut microbiome has received little attention. Integrating metagenomic and metatranscriptomic analyses in future studies as expansion of imputed metagenomics could help to find a consensus in describing the functional framework in which BSFL convert organic wastes to biomass.

CONCLUSION

Decentralized collection and processing centers in hubs of social activity could represent a feasible way to valorize food waste. By premixing the wastes collected from various sources, a more average and stable larval feedstuff composition could be achieved. The high content of fat, oil, and grease in the oil waste fraction of canteen wastes was found to be lethal for BSFL, but the conversion of the food waste fraction proceeded efficiently. Diet-induced shifts in gut microbiota were observed throughout the larval development. In contrast to the diet adaption on a phylogenetic level, general functional competences described as metabolic pathways resulted in similar patterns irrespective of the diet. This indicates that metabolic competence is a strong filter in the selection of larval gut colonizers, meaning that different communities can do the same job. Yet, many aspects of BSFL metabolism, especially fatty-acid digestion and accumulation as well as substrate-larva-microbiome interdependencies are still largely undescribed. Special attention should also be given to the assessment of risk factors such as entomopathogens and enrichment of potentially human-pathogenic microorganisms introduced by contaminated wastes.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ebi.ac.uk/ena, PRJEB39545.

AUTHOR CONTRIBUTIONS

TK performed statistical and bioinformatical data analysis, created the figures, and wrote the manuscript. AW designed and supervised the experiment. TB carried out sample collection and preparation, physicochemical analyses, and extraction of DNA. CH maintained the BSF colony, provided larvae for the experiment, and assisted during the experimental analyses. BS assisted with bioinformatical analysis and writing. FS, BS-S, and HI contributed equally to the study by supervising the planning, execution, and analysis of the study and provided scientific input during writing. All authors contributed important intellectual content and approved the final version of manuscript for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.619112/full#supplementary-material

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Organic Waste Substrates Induce Important Shifts in Gut Microbiota of Black Soldier Fly (*Hermetia illucens* L.): Coexistence of Conserved, Variable, and Potential Pathogenic Microbes

*Chrysantus M. Tanga[1†], Jacqueline Wahura Waweru[1†], Yosef Hamba Tola[1,2], Abel Anyega Onyoni[1], Fathiya M. Khamis[1], Sunday Ekesi[1] and Juan C. Paredes[1]**

[1] International Centre of Insect Physiology and Ecology (ICIPE), Nairobi, Kenya, [2] Department of Biochemistry, Genetics, and Microbiology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa

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***Correspondence:**
Juan C. Paredes
jparedes@icipe.org

[†] These authors have contributed equally to this work

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The sustainable utilization of black soldier fly (BSF) for recycling organic waste into nutrient-rich biomass, such as high-quality protein additive, is gaining momentum, and its microbiota is thought to play important roles in these processes. Several studies have characterized the BSF gut microbiota in different substrates and locations; nonetheless, in-depth knowledge on community stability, consistency of member associations, pathogenic associations, and microbe–microbe and host–microbe interactions remains largely elusive. In this study, we characterized the bacterial and fungal communities of BSF larval gut across four untreated substrates (brewers' spent grain, kitchen food waste, poultry manure, and rabbit manure) using 16S and ITS2 amplicon sequencing. Results demonstrated that substrate impacted larval weight gain from 30 to 100% gain differences among diets and induced an important microbial shift in the gut of BSF larvae: fungal communities were highly substrate dependent with *Pichia* being the only prevalent genus across 96% of the samples; bacterial communities also varied across diets; nonetheless, we observed six conserved bacterial members in 99.9% of our samples, namely, *Dysgonomonas*, *Morganella*, *Enterococcus*, *Pseudomonas*, *Actinomyces*, and *Providencia*. Among these, *Enterococcus* was highly correlated with other genera including *Morganella* and *Providencia*. Additionally, we showed that diets such as rabbit manure induced a dysbiosis with higher loads of the pathogenic bacteria *Campylobacter*. Together, this study provides the first comprehensive analysis of bacterial and fungal communities of BSF gut across untreated substrates and highlights conserved members, potential pathogens, and their interactions. This information will contribute to the establishment of safety measures for future processing of BSF larval meals and the creation of legislation to regulate their use in animal feeds.

Keywords: organic waste valorization, microbiota, safety, dysbiosis, feed industries, foodborne diseases

INTRODUCTION

The animal feed industry is currently facing considerable shortages in protein sources, and the situation is expected to aggravate further with human population growth and the increasing demand for meat in their diets (National Research Council, 2015; van Huis, 2013). Therefore, the urgent need for alternative protein sources to substitute the conventional expensive sources such as fishmeal and soya bean has become crucial. Insects are a promising and sustainable alternative protein source, and their applications as protein additives in animal feeds have been the subject of recent research (Rumpold and Schlüter, 2013). The black soldier fly (BSF; *Hermetia illucens* L.) larvae is an example of a high-quality edible insect whose adoption as an alternative animal feed presents several advantages. First, BSF larvae are rich in crude protein and has a well-balanced amino acid profile (particularly lysine, threonine, and methionine, which are cereal-limiting amino acids), fats, and micronutrients [iron and zinc (Zheng et al., 2013; De Smet et al., 2018; Shumo et al., 2019; Giannetto et al., 2020)]. Secondly, the use of BSF larvae as animal feed for poultry, fish, and swine has been deemed feasible due to the ease and economical production systems that depend largely on recycling organic waste into nutrient-rich biomass (Shumo et al., 2019). The utilization of organic wastes by the BSF has been considered as an excellent mitigation measure against environmental pollutants due to the high larval waste bioconversion efficiency (Müller et al., 2017; Wang and Shelomi, 2017). Thirdly, the insect is neither a pest nor a disease vector, making BSF larval production environmentally friendly, cost-effective, and safe (Diener et al., 2009; Shumo et al., 2019).

An efficient BSF larval production to satisfy market demands for alternative protein is nonetheless dependent on several factors such as the composition of the feeding substrates, conversion efficiency, and environmental conditions [e.g., temperature and humidity (Tomberlin et al., 2009; Wang and Shelomi, 2017)]. As such, many research efforts have focused on characterizing the nutrients and micronutrient assimilation by BSF larvae in response to different rearing conditions and substrates, as a step to optimize their yield and quality (Gold et al., 2018; Lalander et al., 2019; Shumo et al., 2019; Chia et al., 2020). Remarkably, very little research has been done on their gut microbiota and its role in BSF bioconversion and insect physiology and health.

In many insect species, symbionts—beneficial associated microbes—play crucial roles in host physiology, including nutrition, digestion, and immunity (Dillon and Dillon, 2004; Engel and Moran, 2013). Understanding the role of microbial communities in BSF has proven to be difficult since gut microbiota shifts importantly across diets and locations. Nonetheless, some evidence suggests that the gut microbiota plays important roles in larval biomass digestion (Jeon et al., 2011; Boccazzi et al., 2017; Bruno et al., 2019; Wynants et al., 2019; Klammsteiner et al., 2020). For instance, *Dysgonomonas* is thought to be involved in complex polysaccharide degradation, *Bacteroides* and *Parabacteroides* promote glycan metabolism, and *Actinomyces* facilitates the degradation of lignin and chitin (Yu et al., 2011; Zheng et al., 2013; Bruno et al., 2019; Jiang et al., 2019; Klammsteiner et al., 2020). Additionally, BSF diet supplementation with strains of gut bacteria such as *Bacillus subtilis* has been shown to promote larval growth. Studies have also reported antimicrobial properties of BSF gut microbes. These are of great interest since they might be able to reduce the load of harmful and undesired pathogens for humans in microbe-rich BSF substrates, such as manure (Erickson et al., 2004; Liu et al., 2008). For instance, it has been shown that *Trichosporon asahii* inhibits the growth of *Candida* species, most likely by the production of fungicidal molecules (Yu et al., 2011; Boccazzi et al., 2017). Other studies have shown that BSF larvae can effectively reduce *Escherichia coli* O157:H7, *Salmonella enterica* serovar enteritidis, and viruses in organic waste (Erickson et al., 2004; Lalander et al., 2014). It is therefore anticipated that pathogen waste reduction might be achieved by microbe ingestion and lysis in the gut and/or secretion of antimicrobial compounds directly into the substrate. In both processes, BSF gut microbiota might play crucial roles (Erickson et al., 2004).

Whereas the antimicrobial properties of BSF and its gut microbiota could lead to alternative valorization of the BSF system, several studies have reported as well that BSF can be a reservoirs of many significant foodborne pathogens that are detrimental to humans and animals (Bruno et al., 2019; Khamis et al., 2020). This is especially complex, when potential animal/human pathogenic bacteria associated with BSF are beneficial to the insect physiology. For example, *Providencia* in BSF is a vertically transmitted bacterium that enhances oviposition, while in humans, it causes gastroenteritis, urinary tract infections, and other nosocomial infections in immunocompromised patients (Galac and Lazzaro, 2011; De Smet et al., 2018). Other bacteria with important biosafety considerations include *Wohlfahrtiimonas*, which has been shown to cause sepsis upon myiasis infestation in two patients (Tóth et al., 2008; Almuzara et al., 2011; Kõljalg et al., 2015).

Therefore, in order to strategize ways to improve insect production, health, and safety, it is important to further characterize (i) BSF gut microbiota when reared on different substrates; (ii) identify potential conserved beneficial microbes; (iii) list and catalog potential animal and human pathogens harbored by larvae; and (iv) understand the nature of the interactions between beneficial microbes and BSF and between beneficial microbes and their pathogenic counterpart.

In this study, we characterized the gut microbiota (bacterial and fungal communities) of BSF larvae fed on four different substrates, brewers' spent grains, kitchen food waste, poultry manure, and rabbit manure, to evaluate the impact of the different substrates on microbiota shifts as well as the promotion or absence of potential pathogenic microbes. Further, we provide insights on microbial diversity, their consistency in the gut of BSF across substrates, and microbe–microbe interactions as a step toward understanding the role of the gut microbiota in insect physiology and health (Klammsteiner et al., 2020).

MATERIALS AND METHODS

BSF Larval Sample Collection and Preparation

Fifth-instar BSF larvae fed on four different substrates, brewers' spent grains, kitchen food waste, poultry manure, and rabbit manure, were collected from the Animal Rearing and Containment Unit (ARCU) at the International Centre for Insect Physiology and Ecology (*icipe*, Nairobi, Kenya). The brewers' spent grains used were sourced from the Kenya Breweries Limited, Nairobi, Kenya. Brewers' spent grains are the common substrate used at *icipe* to rear BSF larvae, and they were considered as the "control" substrate. On brewers' spent grains, the BSF larvae successfully complete their development in 16–21 days (Chia et al., 2018). The substrate has also been shown to promote high nutritional quality of the larvae (Meneguz et al., 2018; Chia et al., 2020). Samples of BSF larvae from the various rearing substrates were collected after 14 days and washed in 40% bleach, followed by 70% ethanol and finally 1 × PBS for 2 min to eliminate any external microorganism or contaminant DNA attached to the cuticle. Twenty entire guts per substrate were then dissected aseptically using forceps, and each gut was placed in a 2-ml microcentrifuge tube containing 750 μl CTAB solution (20 g CTAB in 100 ml CTAB base: 100 ml 1 M Tris-HCL pH 8, 280 ml 5 M NaCl, 40 ml 0.5 M EDTA pH 8, complete with Milli-Q H_2O to a liter), 2 μl beta-mercaptoethanol, and 100–200 μl beads (3 mm diameter). Samples were then stored at −80°C until DNA extraction.

DNA Extraction

The CTAB–phenol–chloroform DNA extraction method was used in this study adapted from Ausubel et al. (1988). Briefly, freeze-thawed gut samples (see described above) were homogenized using the QIAGEN TissueLyser II, and 1 ml of phenol was added, mixed vigorously for 10 s, and incubated at 64°C for 6 min. The CTAB/phenol homogenate was then put in a fresh 2-ml tube containing 400 μl of chloroform and mixed by inverting several times. Centrifugation at 13,000 rpm for 10 min at room temperature followed, and the aqueous layer was transferred into a new microcentrifuge tube, to which 500 μl of phenol–chloroform–isoamyl alcohol in the ratio of 25:24:1, respectively, was added. Contents in the tubes were then mixed and centrifuged at 13,000 rpm for 3 min at room temperature, and the aqueous layer in each tube was again transferred into a new microcentrifuge tube. Five hundred microliters of chloroform was then added to each tube, and then centrifugation at 13,000 rpm for 3 min at room temperature followed. Next, the resulting aqueous layer was transferred into a new microcentrifuge tube, and the DNA was precipitated by adding 900 μl of absolute ethanol and incubated overnight at −80°C. Precipitated DNA was then chilled on ice, followed by centrifugation at 13,000 rpm for 30 min at 4°C, resulting in a DNA pellet. The DNA pellet was washed twice with 1 ml of 70% ethanol and then dried at room temperature for 10 min. Lastly, the DNA was resuspended in 200 μl sterile water, and DNA concentration per sample was confirmed using NanoDrop (Thermo Scientific).

16S rRNA and ITS Gene Amplification and Sequencing

The DNA from 20 individual guts per substrate was shipped to Macrogen Europe BV (Meibergdreef, Amsterdam, Netherlands) for 16S rRNA (10 samples from 10 individual guts) and ITS (10 samples from 10 individual guts) region amplification and sequencing using the Illumina MiSeq platform. The V3–V4 region of the 16S rRNA was amplified using the 314F-CCTACGGGNGGCWGCAG and 805R-GACTACHVGGGTATCTAATCC primers while F5-GCATCGATGAAGAACGCAGC and R5-TCCTCCGCTTATTGATATGC were used to amplify the ITS2 region. Whereas poultry-fed larval samples followed the same treatment procedures during their DNA extraction and had comparable DNA starting material, ITS2 amplicons from these samples did not meet the DNA quantity and quality required for library preparation, three times. This was potentially due to low loads of fungi in poultry-fed larval samples.

Data Analysis

Paired-end sequences spanning the V3–V4 and ITS2 regions of the bacterial 16S rRNA and fungi, respectively, were analyzed using QIIME 2 (version 2020.2). Briefly, the reads were imported into QIIME 2, followed by quality checking, and all the present adapters and primers trimmed using Cutadapt (version 1.8) embedded in QIIME 2. Subsequently, low-quality bases were trimmed, the forward and reverse reads merged, and chimeras removed using the DADA2 pipeline in QIIME 2. Taxonomic classification was done against the SILVA 132 database using a pre-trained naïve Bayes classifier in the case of bacteria and the UNITE (version 7-99) database in fungi.

The taxonomy file and sequences were then imported into R for downstream analysis. We first filtered unwanted sequences, taxonomies, and features including those of chloroplast, Eukaryota, and Archaea and those that were unassigned to any taxonomic group by the SILVA database after reading the three files into R. Sequences not classified up to the genus level were extracted, and a BLAST search was performed to classify them. The taxonomizr package in R was used to complete the different taxonomic classification levels. The BLAST taxonomic classification results were combined with those from SILVA 132, the taxonomy table was merged to the abundance table, and a relative abundance barplot was plotted in R (**Supplementary Table S1**).

Next, we rarefied the data (**Supplementary Figure S1**) to even sampling depths and performed alpha diversity estimation to determine richness using chao1 and Faith's phylogenetic diversity, and diversity was determined using the Shannon diversity index with all the samples grouped according to the sample metadata. The statistical significance differences of gut microbiota richness and diversity across the different substrates were tested using the Kruskal–Wallis

H-test (Vargha et al., 1998). Beta diversity estimations on the other hand were done using Venn diagrams and weighted and unweighted UniFrac distance metrics at the genus level (Lozupone and Knight, 2005). PERMANOVA was done to compare microbial communities across different substrates. Lastly, the microbial abundance interaction among the most abundant microbiota was established using an abundance correlation-based network generated in R using the Psych package, to establish how the most abundant bacteria in the gut of BSF larvae interacted. This was also done to elucidate whether there are any bacteria in the gut of BSF larvae that influence the presence or abundance of others, especially potential pathogens.

RESULTS

BSF Larval Weight Gain in Four Untreated Substrates

We started by measuring the weight gain of BSF larvae in different substrates. We observed that the substrate that promoted the highest BSF neonate (5 days old, fifth-instar larvae) growth was kitchen waste with a larval weight of 0.76 g, followed by rabbit manure (0.56 g weight per larva), brewers' spent grains (0.44 g per larva), and chicken manure (0.32 g per larva) (**Figure 1**).

Bacterial Communities Associated With BSF Guts

In order to evaluate the role of the gut microbiota in BSF growth and uncover potential shifts in BSF gut microbiota induced by the diet, we characterized bacterial and fungal communities of the BSF gut. Out of the 39 BSF larval gut samples profiled in this study from four different substrates [brewers' spent grains, kitchen waste (food waste), poultry manure, and rabbit manure], we recovered a total of 4,665,023 paired-end sequences spanning the V3–V4 region of the bacterial 16S rRNA. By analyzing all the sequenced reads, we identified 3,282 amplicon sequence variants (ASVs). From our analysis, we characterized 21 genera, with an overall relative abundance higher than 0.5%, as BSF representative gut bacterial communities (**Figure 2A**, **Supplementary Figure S2A** and **Supplementary Table S2**). From our dataset, the dominant phylum was Bacteroidetes with the most abundant genera being *Dysgonomonas*, *Parabacteroides*, *Bacteroides*, and *Flavobacterium*. *Dysgonomonas* alone accounted for about 32% of total reads. The second was Proteobacteria, which included *Campylobacter*, *Desulfovibrio*, and *Morganella*, with *Campylobacter* alone accounting for about 27% of total reads. Firmicutes was the third phylum including *Lachnoclostridium*, *Erysipelothrix*, and *Enterococcus*. We also uncovered *Actinomyces*, *Providencia*, and *Wohlfahrtiimonas*, but with low relative abundance (>1%, **Figure 2A**). Among all the identified genera in this study, *Dysgonomonas*, *Campylobacter*, *Desulfovibrio*, *Erysipelothrix*, *Morganella*, *Enterococcus*, *Pseudomonas*, *Actinomyces*, and *Providencia* were among the most prevalent across samples with 99.9% of prevalence (**Supplementary Table S1**).

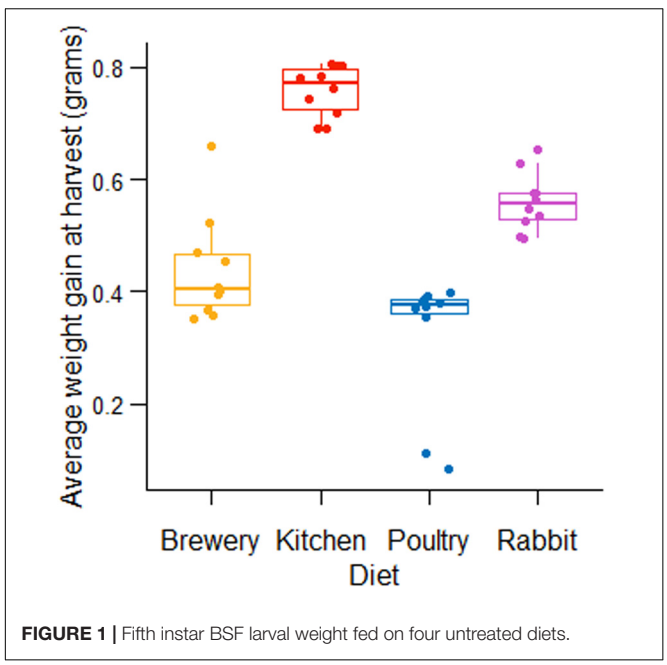

FIGURE 1 | Fifth instar BSF larval weight fed on four untreated diets.

Substrate Effect on Bacterial Communities

Forty-six bacterial genera were common across all substrate types. Rabbit manure harbored 126 unique bacterial genera, followed by chicken manure with 72 unique genera (**Figure 3A**). We observed that the alpha diversity in BSF guts varies considerably when richness (*chao1*: $p = 0.0001$, **Figure 4C**), phylogenetic distance (*Faith's phylogenetic diversity*, $p = 0.000073$, **Figure 4E**), and evenness (*evenness*: $p = 0.00007$, **Figure 4G**) were tested. We found that in these three metrics, the rabbit manure substrate was clearly set apart from the other three substrates. These results are consistent with the principal coordinate analysis (PCoA) using both unweighted and weighted UniFrac (**Figures 5A,C**). Larvae fed on kitchen waste and poultry manure showed bacterial communities that clustered together in both weighted and unweighted UniFrac, whereas brewers' spent grain clustered close to those fed on poultry manure using weighted but not unweighted UniFrac analysis (**Figures 5A,C**). Furthermore, the substrate effect was further confirmed by pairwise PERMANOVA, where substrate had a significant impact on gut bacterial communities with ($R^2 = 0.47485$, $p = 0.01$, **Supplementary Table S3A**). Jointly, the four substrates in this study had a significant impact on the gut bacterial communities of BSF larvae with rabbit waste having the most severe effect.

Fungal Communities Associated With BSF Guts

From 26 samples, we characterized the BSF gut fungal communities from larvae reared on three different substrates: brewers' spent grains, kitchen waste, and rabbit manure. A total of 3,840,437 paired-end sequences spanning the fungal ITS2 region

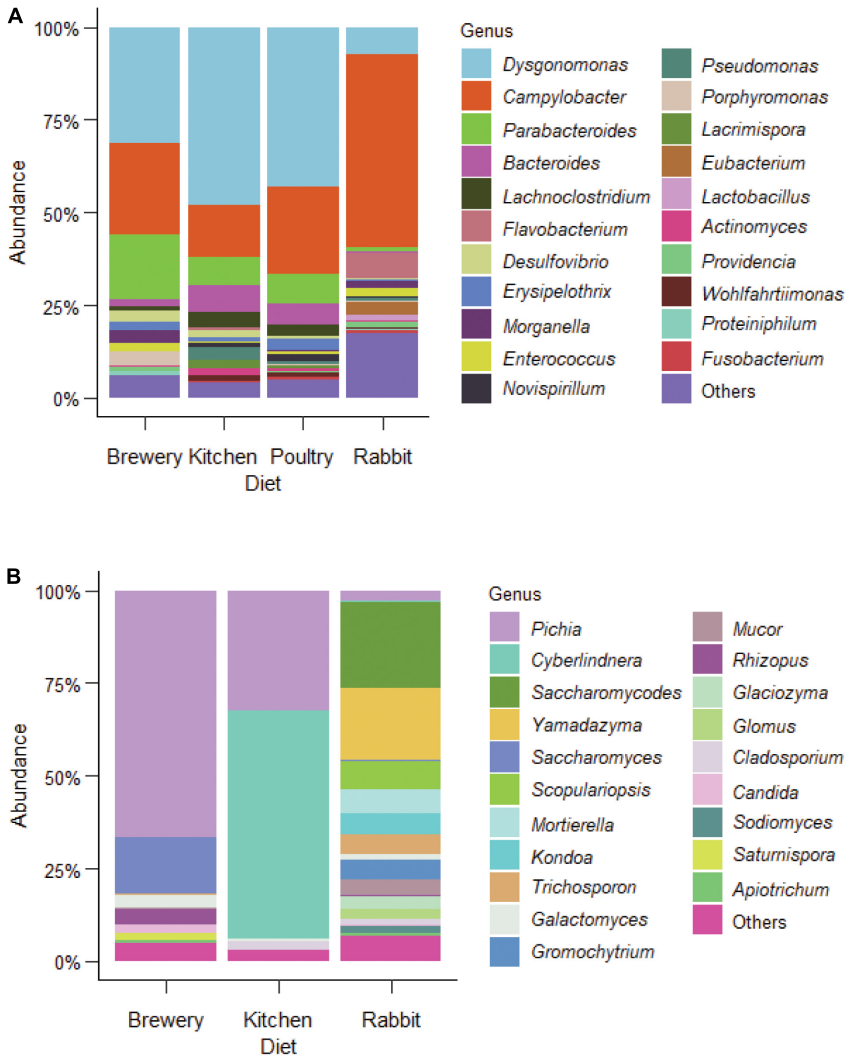

FIGURE 2 | Bacterial and fungal microbiota profiles in BSF larvae fed on different diets. **(A)** bacterial and **(B)**, fungal microbial profile from BSF larvae guts fed on brewers' spent grain, kitchen food waste, poultry manure, and rabbit manure.

were analyzed, and 654 fungal ASVs were identified, 277 of which were classified as yeast representing 69.2% of total reads. A total of 20 fungal genera with a relative abundance greater than 0.5% were presented in **Figure 2B**. The dominant genera were *Pichia*, *Cyberlindnera*, *Saccharomycodes*, *Yamadazyma*, *Saccharomyces*, and *Scopulariopsis*. At the species level, *Pichia kudriavzevii* dominated in the brewers' spent grain and rabbit manure (25.8%) with the highest prevalence (96%), while *Cyberlindnera jadinii* was dominant in BSF larvae fed on kitchen waste (15.3%, **Supplementary Figure S2B**). A total of 19 genera were common across substrates with their cumulative relative abundance representing 60.8% of total reads (**Figure 3B** and **Supplementary Table S1**).

Substrate Effect on Fungal Communities

We found a significant impact of diets on fungal microbiota richness, diversity, and evenness (Shannon, $p = 0.00003$; *chao1*, $p = 0.0013$; *Faith's phylogenetic diversity*, $p = 0.023$; *evenness*, $p = 0.00015$; **Figures 4B,D,F,H**) across substrates, especially when larvae were fed on rabbit manure. The dichotomy among substrates observed in **Figures 2–4** was further confirmed by beta diversity analysis. Unweighted and weighted UniFrac analysis (**Figures 5B,D**) and PERMANOVA pairwise analysis (**Supplementary Table S3B**, $R^2 = 0.81352$, $p = 0.001$) showed that the fungal communities in BSF guts clustered by substrate. Together, the substrate had a severe impact on the fungal community's composition and abundance in BSF larval gut.

Gut Microbiota Member Interactions

In our analysis, we found several pathogenic opportunistic bacteria including *Campylobacter*, *Morganella*, *Wohlfahrtiimonas*, and *Providencia* (**Figure 2A** and **Supplementary Figure S2A**) and opportunistic fungus teleomorph (the sexual reproduction stage) *Candida* species

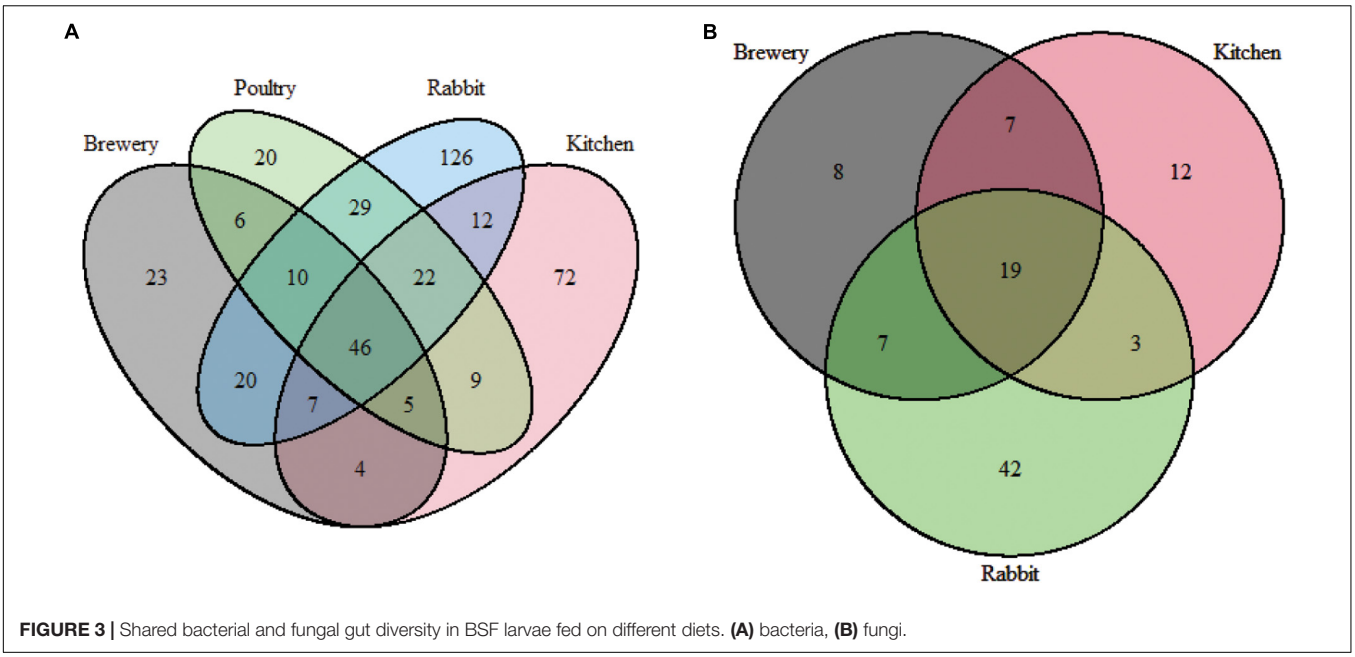

FIGURE 3 | Shared bacterial and fungal gut diversity in BSF larvae fed on different diets. **(A)** bacteria, **(B)** fungi.

(e.g., *Cyberlindnera* sp. and *Trichosporon* sp.) (**Figure 2B** and **Supplementary Figure S2B**). The relationship between potential bacterial pathogens and the rest of the gut bacterial microbiota is presented in an interaction network based on the correlation of the most abundant bacterial microbiota of BSF larvae (**Figure 6**). Among bacterial communities, *Enterococcus*, *Bacteroides*, and *Campylobacter* had numerous interactions with other bacterial species. *Enterococcus* was positively correlated with *Morganella*, which in turn was positively correlated to *Providencia*. Surprisingly, *Dysgonomonas*, the most dominant genus, did not interact with the other postulated core microbiota genera and only negatively correlated with *Eubacterium*. A similar pattern was found for *Actinomyces*, which only positively correlated with *Lachnoclostridium* (**Figure 6**).

DISCUSSION

In this study, we characterized the bacterial and fungal communities of the BSF larval guts raised in four different substrates. We observed that the substrate affected both the bacterial and fungal communities in BSF, but the effect was more severe in fungal microbiota. A close examination of the bacterial microbiota in individual samples revealed that some genera, reported in other studies, were present in almost all our samples (99.9%) despite the substrate, pointing toward a potential stable resident core gut microbiota in BSF. Additionally, we uncovered that the substrate can induce an important bacterial shift leading sometimes to dysbiosis, where one pathogenic bacterium dominated the gut microbiota without detrimental effect on the insect's growth performance and health. Finally, we established a microbiota network in an attempt to better understand microbe–microbe interactions and guide microbe-based strategies to improve insect production, health, and safety.

We uncovered previously reported genera in great abundance such as *Dysgonomonas*, *Morganella*, *Enterococcus*, *Pseudomonas*, *Actinomyces*, and *Providencia*, genera that have been postulated as the BSF core gut microbiota members by Klammsteiner et al. (2020). In their study, Klammsteiner et al. (2020) reported that *Dysgonomonas*, *Actinomyces*, and *Enterococcus* accounted for 44% of the total number of reads with 80% prevalence across all the samples. In this study, the three accounted for a similar abundance, 34.1% of total reads, while their prevalence was much higher, 99.9% across all the samples. *Dysgonomonas* has been reported to be always among the top three most abundant members (Bruno et al., 2019; Khamis et al., 2020; Klammsteiner et al., 2020), and in our study, it accounted for 32% of the reads. Whereas the prevalence of *Dysgonomonas*, *Morganella*, *Enterococcus*, *Pseudomonas*, *Actinomyces*, and *Providencia* across studies, substrates, and locations indicates that they are conserved members of the BSF larval gut microbiota, the evidence that they play positive interactions among them and with their host, and whether they have been associated with their host over evolutionary timescales, has not been studied. This remains crucial in order to determine if the BSF larva has a core gut microbiota and if these genera could be cataloged as such.

While *Campylobacter* have been found in other studies, they have never been reported as abundant across samples as in this study [27% of total reads (Wynants et al., 2019; Klammsteiner et al., 2020)]. Their dominance is even greater in rabbit manure where they account for more than 50% of total reads. *Campylobacter* are gastrointestinal pathogens that cause diarrhea in humans and are considered as opportunistic bacteria that are usually self-limiting. They can infect a range of animals including most food production animals (Blaser, 1997). Since the most common source of *Campylobacter* infection for humans is food and water, it is particularly important to evaluate their survival along the BSF food chain (BSF larval processing, storage,

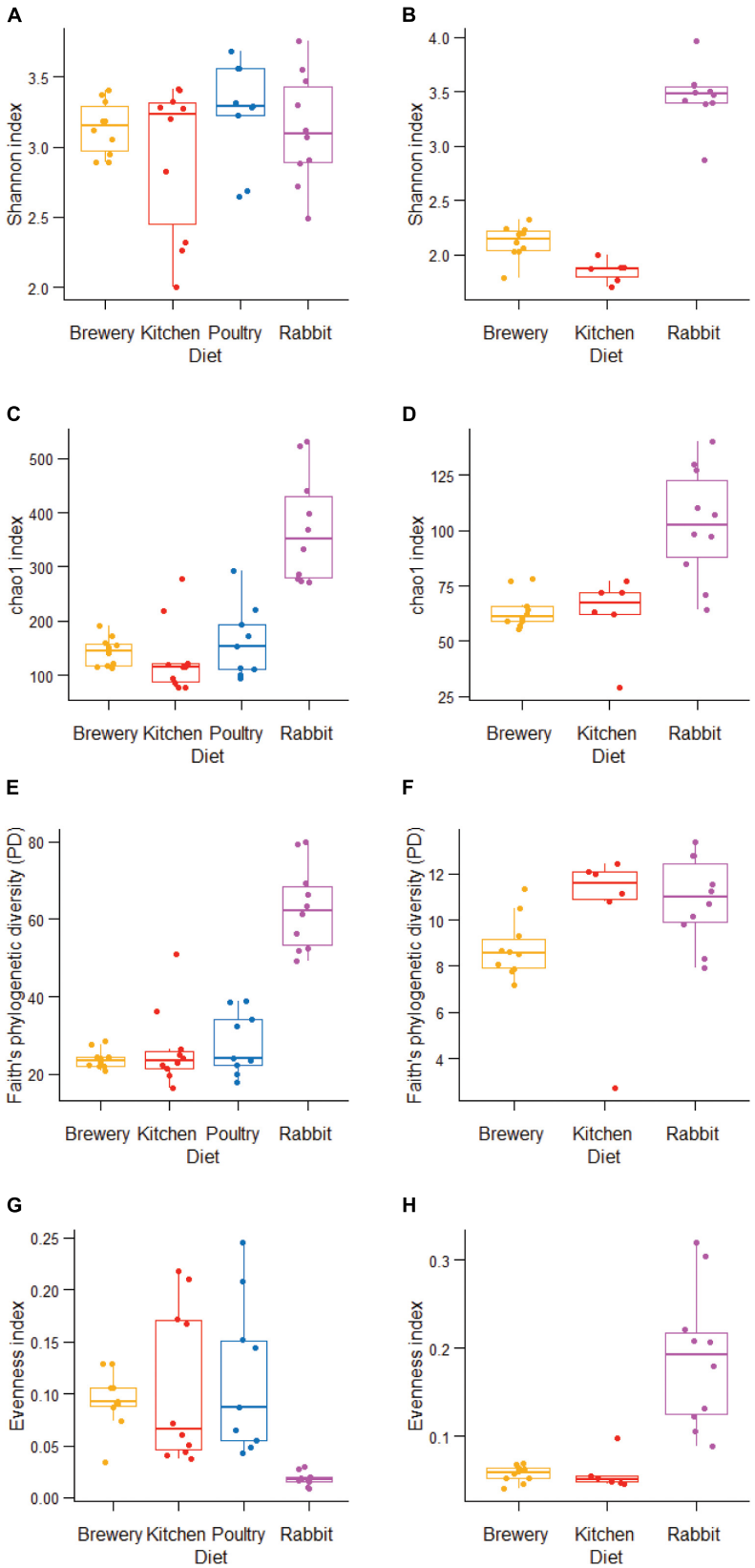

FIGURE 4 | Alpha diversity in bacterial and fungal gut communities in BSF larvae fed on different diets. **(A,B)** *Shannon* diversity index estimates, **(C,D)** *chao1* richness index estimates. **(E,F)** *Faith's Diversity* estimates. **(G,H)** *Evenness* estimates, from bacterial **(A,C,E,G)** and fungal **(B,D,F,H)** communities.

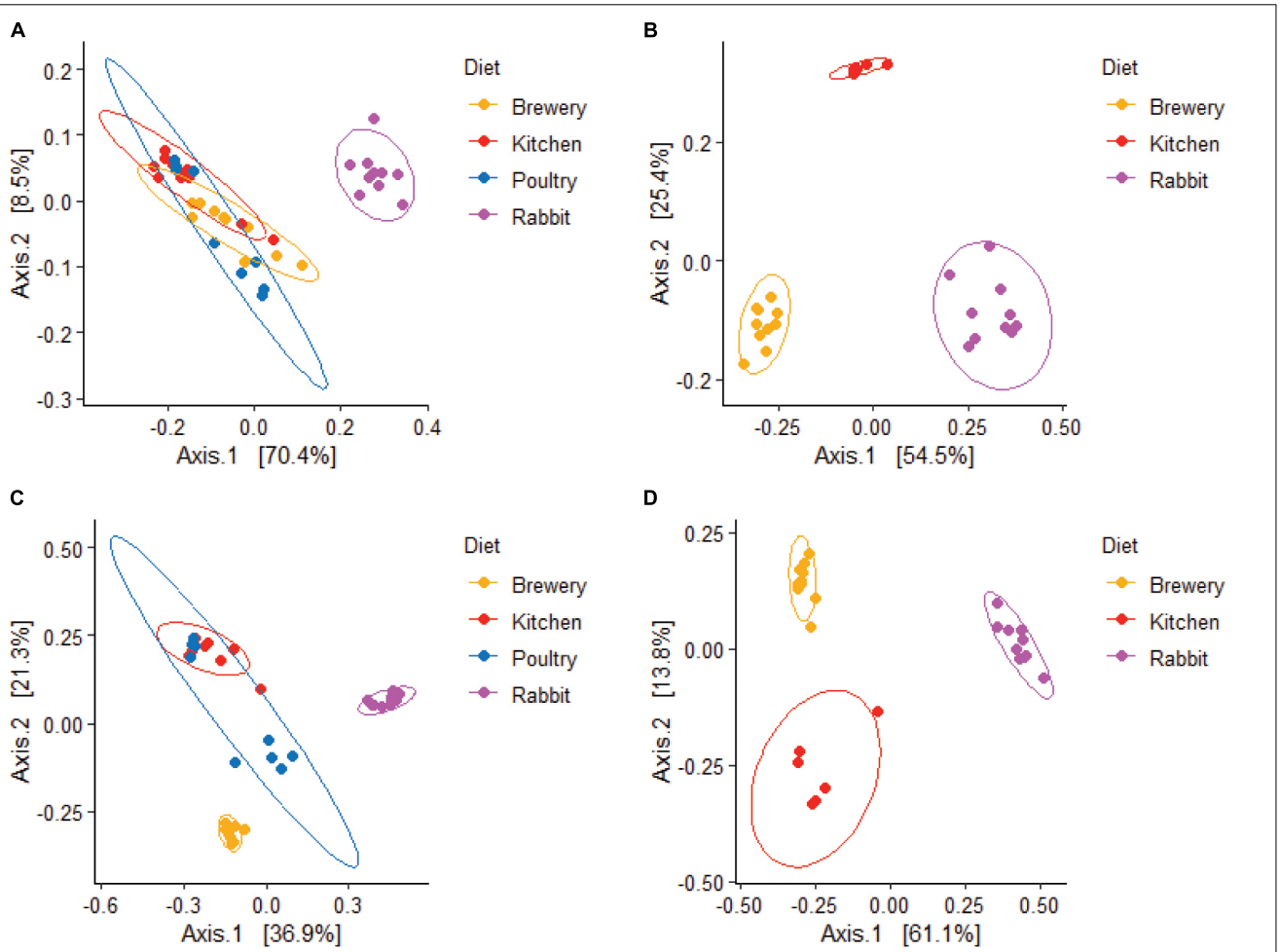

FIGURE 5 | Weighted and Unweighted unifrac distance estimates from gut communities in BSF larvae fed on different diets. Weighted **(A,B)**, and Unweighted **(C,D)**, unifrac distance estimates from bacterial **(A,C)**, and fungal **(B,D)**, communities.

BSF fed animal gut tract, and BSF fed animal meat) in order to evaluate their real potential risk and safety. It is noteworthy that *Campylobacter* might be seasonal or breeding place dependent since Khamis et al. (2020) did not report *Campylobacter* among the most abundant genera found in wild samples across the world but more importantly in Kenya. Additionally, rabbit manure-fed larvae presented the highest loads of *Campylobacter*; thus, we speculate that rabbit manure might be the source of contamination since rabbit manure is used in our rearing facility as an attractant for ovipositing flies in all substrates. However, further analysis of rabbit manure and egg chorion-associated bacteria should be performed to confirm its potential as source of contamination.

In addition to already reported genera, we found specific bacteria never described before in BSF gut such as *Lachnoclostridium*, *Flavobacteria*, *Desulfovibrio*, *Eubacteria*, and *Proteiniphilum* (Jeon et al., 2011; Zheng et al., 2013; Bruno et al., 2019; Wynants et al., 2019; Khamis et al., 2020; Klammsteiner et al., 2020). We speculate that these specific genera proliferated in BSF substrates as they are not found consistently in every study, suggesting that they might be acquired during feeding and transit through the gut without particular selection or retention in the host (Kolton et al., 2016; Tomazetto et al., 2018; Saha et al., 2020). This aspect together with the fact that no consistent subset of OTUs, besides the postulated core microbiota, are found among studies highlights that most of the bacteria found in the BSF gut are transient and are affected by the local environment.

While the substrate affects the diversity and abundance of bacterial and fungal communities found in BSF larval gut, it could lead to extreme cases of dysbiosis. Bruno et al. (2019) previously reported that an unbalanced substrate (fish based substrate) promotes Proteobacteria, mainly *Providencia*, microbiota dominance. In this study, rabbit manure also promotes Proteobacteria dominance, although in our case, it is *Campylobacter* (and in a minor extent, *Flavobacterium* from the Bacteroidetes phylum) but not *Providencia*. Whereas in Bruno et al. (2019), an unbalanced substrate reduced BSF performance, in our case, overproliferation of *Campylobacter* did not compromise larval growth and survival. This draws special attention to the fact that even if BSF is omnivorous,

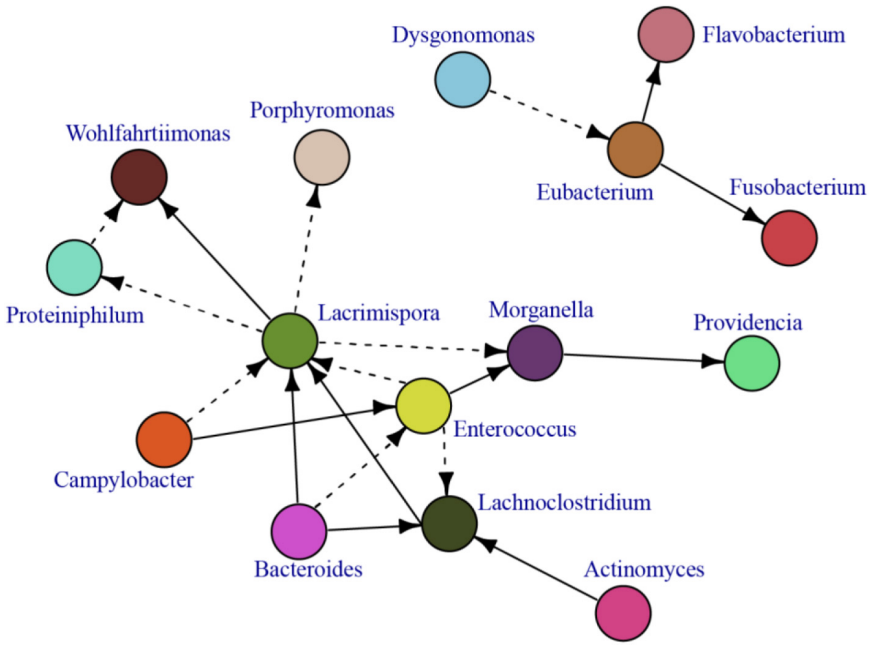

FIGURE 6 | A correlation network demonstrating the BSF gut bacterial interactions. A cut of 0.65 and above was used to distinguish the most significant correlation.

specific consideration has to be taken, particularly with the safety quality of the substrates, especially when BSF larvae performed well in such substrates. Although specific substrates might not directly or indirectly affect the performance and/or safety of BSF larvae, the economic value and safety of BSF larval meal (BSFLM) for use in animal feeds remains a major concern to be addressed.

Bacterial interaction analysis showed limited interactions (correlations in abundance) among all bacteria, but particularly few among the postulated core gut microbiota genera. *Enterococcus*, one of the most interconnected, positively correlated with *Morganella* and negatively correlated with *Lacrimospora* and *Lachnoclostridium*, and *Morganella* positively correlated with *Providencia*. Surprisingly, *Dysgonomonas* and *Actinomyces* showed poor interactions with others; *Dysgonomonas* negatively correlated only with *Eubacterium* and *Actinomyces* and positively correlated only with *Lachnoclostridium*. *Campylobacter*, the most dominant opportunistic clinical pathogen in this study, positively correlated with *Enterococcus* and *Lacrimospora*. Whereas analysis of more samples are needed to get a robust correlation network, these results give hints about the central role of *Enterococcus* modulating *Morganella* and *Providencia* abundance.

Mycobiome analysis in this study revealed that the fungal community in BSF guts was entirely substrate dependent as was also observed by Boccazzi et al. (2017). We found that only *Pichia* presented a high relative abundance and prevailed across all the substrates; the rest of the identified fungal communities were highly substrate specific. The high prevalence and dominance of *Pichia* in this study as well as in that of Boccazzi et al. (2017) points toward a stable association with BSF larval gut. *P. kudriavzevii*, the most prevalent species found in our samples, has been reported to encode the antibacterial toxin RY55 that is active against several human pathogens such as *E. coli*, *Enterococcus faecalis*, *Klebsiella* sp., *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Pseudomonas alcaligenes* (Boccazzi et al., 2017). Despite the fact that the mycobiome of BSF larvae depends on the substrate, their antimicrobial activities and ability to colonize the BSF gut open an important potential for substrate supplementation and, thus, increase BSFLM safety for its use for food and feed.

We conclude that whereas bacterial and fungal communities in the BSF larval gut varied greatly across substrates, few members remained constantly associated across substrates, pointing toward a potential ecologically distinctive core gut microbiota in BSF larvae. The presence of potentially opportunistic pathogens in the gut of freshly harvested BSF larvae from large-scale production systems underpins the need of pretreatment of substrates to minimize risk of pathogen contamination along the insect-based feed value chain. The hurdles that need to be overcome in order to introduce BSFLM as a viable protein additive option in animal feed include safety concerns and product applications. Results indicate that postharvest processes such as steaming, blanching, and drying would be important and effective tools to increase microbial safety. Further research into the kinds of substrates as well as the processing and storage parameters is required to ensure that BSFLM fulfills global food safety criteria. This information will feed directly into creating legislation to regulate the use of BSFLM for animal consumption, which will in turn make its utilization at industrial scale more attractive.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

JP, CT, FK, and SE: conceptualization. JW, YT, and AO: investigation. JW and YT: data curation. JW and JP: formal analysis, visualization and writing—original draft preparation. CT, FK, and SE: funding acquisition. JW, YT, and JP: methodology. JP, CT, and FK: writing—review and editing. CT: project administration. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.635881/full#supplementary-material

Supplementary Figure 1 | Bacterial rarefaction curve **(A)**, and fungal rarefaction curve **(B)**. Bacterial reads were rarefied to an even depth of 34750 while fungal reads were rarefied to an even depth of 78365.

Supplementary Figure 2 | (A) Bacteria and, **(B)** fungal gut communities at the species level in BSF larvae fed on different substrates.

Supplementary Table 1 | 16S rRNA and ITS sequencing data analysis.

Supplementary Table 2 | Relative abundance of the most prevalent bacteria **(A)**, and fungi **(B)**.

Supplementary Table 3 | PERMANOVA pairwise analysis from the BSF guts. Bacterial community's pairwise analyses are presented in **(A)**, while **(B)**, represents the analysis from the fungal communities.

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frontiers in Microbiology

ORIGINAL RESEARCH
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Starvation Alters Gut Microbiome in Black Soldier Fly (Diptera: Stratiomyidae) Larvae

Fengchun Yang[1], Jeffery K. Tomberlin[1] and Heather R. Jordan[2]**

[1] Department of Entomology, Texas A&M University, College Station, TX, United States, [2] Department of Biological Sciences, Mississippi State University, Starkville, MS, United States

Unlike for vertebrates, the impact of starvation on the gut microbiome of invertebrates is poorly studied. Deciphering shifts in metabolically active associated bacterial communities in vertebrates has led to determining the role of the associated microbiome in the sensation of hunger and discoveries of associated regulatory mechanisms. From an invertebrate perspective, such as the black soldier fly, such information could lead to enhanced processes for optimized biomass production and waste conversion. Bacteria associated with food substrates of black soldier fly are known to impact corresponding larval life-history traits (e.g., larval development); however, whether black soldier fly larval host state (i.e., starved) impacts the gut microbiome is not known. In this study, we measured microbial community structural and functional shifts due to black soldier fly larvae starvation. Data generated demonstrate such a physiological state (i.e., starvation) does in fact impact both aspects of the microbiome. At the phylum level, community diversity decreased significantly during black soldier fly larval starvation ($p = 0.0025$). Genus level DESeq2 analysis identified five genera with significantly different relative abundance ($q < 0.05$) across the 24 and 48 H post initiation of starvation: *Actinomyces*, *Microbacterium*, *Enterococcus*, *Sphingobacterium*, and *Leucobacter*. Finally, we inferred potential gene function and significantly predicted functional KEGG Orthology (KO) abundance. We demonstrated the metabolically active microbial community structure and function could be influenced by host-feeding status. Such perturbations, even when short in duration (e.g., 24 H) could stunt larval growth and waste conversion due to lacking a full complement of bacteria and associated functions.

Keywords: starvation, functional prediction, total RNA, viable microbiomes, black soldier fly

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Dorothee Tegtmeier, Fraunhofer Society (FHG), Germany
Juan Paredes, International Centre of Insect Physiology and Ecology (ICIPE), Kenya
Chrysantus Mbi Tanga, International Centre of Insect Physiology and Ecology (ICIPE), Kenya

****Correspondence:***
Heather R. Jordan
jordan@biology.msstate.edu
Fengchun Yang
springyang@tamu.edu

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INTRODUCTION

Sensations of hunger are caused by an imbalance between energy intake and expenditure (Friedman et al., 1999). One immediate response to hunger is assumed to be the drive to increase food consumption. Malnutrition, on the other hand, is not as simple as hunger, and is defined as either inadequate or excessive consumption of dietary substances ultimately leading to the development of undernutrition or obesity, respectively, and their corresponding health sequelae (Elia, 2017).

Though malnutrition is not the same as hunger, they may be connected (Friedman et al., 1999; Elia, 2017).

Recent studies have shown evidence of commensal microorganisms playing significant roles in influencing nutritional decisions, digestion, and metabolism (Read and Holmes, 2017). Many of these organisms have coevolved within their host to perform a number of functions the host would otherwise be unable to accomplish on its own. For instance, host-associated bacteria rapidly adapt to changes in host diet through changes in population and induction of signaling compounds and degradation enzymes that facilitate digestion through absorption and metabolism of complex molecules (Read and Holmes, 2017). Characterization (e.g., structure and function) of bacteria is therefore important for understanding the comprehensive physiology of the gastrointestinal tract microbiota and its relationship to both hunger and malnutrition.

Given their ease in rearing and manipulation, insects offer great benefits as model hosts for studying these processes. They represent the most diverse group of known organisms, and account for approximately 80% of Earth's animal species (IISE, 2011). Insects also occupy most habitats, and provide natural ecosystem services often taken for granted, such as herbivory, food for animals, and pollination. Additionally, many insects drive ecosystem processes such as nutrient recycling and decomposition.

Studies have investigated the impact of commensals, as well as gut microbiomes on nutrient absorption, brain function, behavior, overall health, and the consequences of diet on insect associated microbiomes (Feldhaar, 2011). However, very few have analyzed data from nutrient deprivation or starvation, and a literature review at the time this manuscript was prepared revealed only one study investigating the effects of prolonged nutrient deprivation on insect gut microbiota (Tinker and Ottesen, 2016). These studies will be important for our comprehensive understanding of how change in nutrient status impacts insect and other hosts' gut microbiomes, and subsequent changes in insect-microbe functional processes.

The black soldier fly, *Hermetia illucens* (L.) (Diptera: Stratiomyidae) is a well-known, non-pest fly species that, along with associated microbes, converts decomposing matter into proteins and lipids (Diener et al., 2009; Yu et al., 2011). Black soldier fly larvae undergo a fast-growing period and have a huge appetite. Individual black soldier fly eggs can develop into prepupae in about 3 weeks (Tomberlin et al., 2002 and unpublished data). Black soldier fly larvae consume a wide range of organic matter, such as animal manure (Sheppard et al., 1994; Newton et al., 2005; Zhou et al., 2013) and restaurant waste (Nguyen et al., 2015), and transform them into high quality protein approved for use in the aquaculture industry as feed (Bondari and Sheppard, 1987). Furthermore, data demonstrate black soldier fly larval digestion reduces pathogens from within the waste (Erickson et al., 2004; Liu et al., 2008; Lalander et al., 2013, 2015). To date, researchers have shown that black soldier fly larvae can reduce *Escherichia coli* O157:H7 and *Salmonella* Enteritidis in chicken manure (Erickson et al., 2004; Liu et al., 2008; Lalander et al., 2013, 2015), *E. coli* in dairy manure (Liu et al., 2008), and *Salmonella* spp. in human feces as well as in mixed organic wastes (Lalander et al., 2013, 2015). Consequently, industrialization of black soldier flies has been proposed as a means to sustainably convert organic waste (i.e., animal manure or food waste) to protein.

Although the industry is evolving and growing rapidly, many aspects of the basic biology of this species are still unclear. Some studies determined black soldier fly larval gut microbial community structure was significantly influenced by the type of the food consumed (Jeon et al., 2011; Zheng et al., 2013). Other studies demonstrated inoculation of either gut bacteria (Yu et al., 2011) or probiotics (Zheng et al., 2012) into waste could improve larval growth or waste conversion. However, the mechanism of how microbes influence the nutrient uptake ability of the larval host is unclear. Also, the system has not been optimized for converting waste to protein, and reliance on multiple or single batch feedings as a means to produce black soldier flies can result in different levels of production and waste conversion (Miranda et al., 2020). With both practices, windows for black soldier fly larval starvation could occur due to competition. Such delays in feeding could potentially impact black soldier fly larval microbial communities thus resulting in varied larval life history traits and subsequent production. Therefore, we took advantage of our knowledge of black soldier fly biology and feeding behavior, as well as its importance in nutrient recycling, to investigate the total associated *metabolically active* prokaryotic shifts due to host starvation and subsequent nutrient deprivation as a first step for understanding microbe-host interactive metabolism, and in contribution to the long-term goal of optimizing black soldier fly sustainable agricultural systems.

MATERIALS AND METHODS

Colony Maintenance

Black soldier fly eggs were collected from a colony maintained in the Forensic Laboratory for Investigative Entomological Sciences (FLIES) Facility at Texas A&M University. The eggs were collected in three layers of 2 (w) by 2 (h) × 3 (l) cm corrugated cardboard blocks taped to the inside a 2 L plastic bucket 3 cm above approximately 500 g of the Gainesville diet (30% alfalfa meal, 20% corn meal, and 50% wheat bran) saturated with water (Hogsette, 1992). Cardboard was replaced daily. Cardboard containing eggs was placed in a ∼1 L deli cup and maintained in an incubator at approximately 70% RH, 27°C, and 12:12 L:D until hatch.

Black Soldier Fly Feeding Conditions

Black soldier fly eggs, representing 66 clutches (approximately 40K eggs), were collected within an 8-h window using cardboard egg traps. Upon hatching, larvae were maintained using methods described in Sheppard et al. (2002). Larvae were fed 50 g 70% saturated Gainesville diet on the day of hatching (Sheppard et al., 2002). An additional 50 g of feed was given daily from day four to ten. On the eleventh day, 4,000 larvae were collected from the pool and partitioned equally into four containers (∼1L) (ChoiceHD brand from

webstaurantstore.com), representing four technical replicates denoted as A, B, C, and D.

For each replicate, larvae were fed 50 g Gainesville diet at 70% moisture daily for an additional 5 days in order to allow the larvae to acclimate to the new containers. On day 16, each replicate was split into two treatments The first treatment group represented the Control group (aka Fed group) and was fed 50 g Gainesville diet daily until the end of the experiment. The second group, representing the Starved treatment, was not fed for the remainder of the experiment. Each of the 4 replicates within the Fed group was given 20 g feed 2 h prior to larval sampling, and an additional 30 g feed after samples were taken (**Figure 1**).

Five sub-samples (80–100 mg each) of whole larvae (free of substrate) were taken from each replicate of the Fed and Starved groups at three time points (T0 H, T24 H, and T48 H) and placed in individual 2 ml sterile centrifuge tubes with 0.5 g glass beads and 1 mL Trizol®, immediately followed by manual tissue homogenization for individual RNA isolation. Whole larvae were collected in order to analyze the impact of starvation on external and internal larval microbiomes.

To have a basic understanding of the initial microbial structure in the starting feed, one subsample of the saturated 70% Gainesville diet as described above (100 mg) was taken at the beginning of the experiment. The feed sample was collected during the same time as initial larval sampling (i.e., approximately 2–3 h after being first saturated). The RNA of the feed samples was extracted using the same procedures abovementioned as all other larvae samples. Due to the single sample collection, this feed sample data was only included as a point for discussion.

Microbial Total RNA Isolation and Purification

Manually homogenized samples were further processed with a bead beater (BioSpec) for 2 min. After the first minute of homogenization, samples were placed on ice for a 2-min incubation period to avoid overheating and subsequent RNA degradation, and then processed to the second minute of bead beating. After homogenization, samples were incubated at room temperature for 5 min, followed by centrifugation at 12,000 × *g* for 10 min at 4°C to pellet excess particulate. Following this, the supernatant in each sample was transferred to a new tube containing 0.2 mL chloroform, followed by vortexing and incubation at room temperature for 3 min. Samples were centrifuged at 12,000 × *g* for 15 min at 4°C, and resulting product was distinguished into three phases: upper, aqueous RNA phase, the middle, DNA interphase, and lower, protein and lipid organic phase.

The upper, aqueous RNA phases were transferred to a clean tube containing 0.5 mL isopropanol and incubated at room temperature for 10 min followed by a centrifugation at 12,000 × *g* for 10 min at 4°C. Supernatants were removed, and RNA pellets were washed twice using 1 mL 75% ethanol and subsequent centrifugation at 7,500 × *g* for 5 min at 4°C. The RNA pellets were air-dried, then resuspended in RNase free

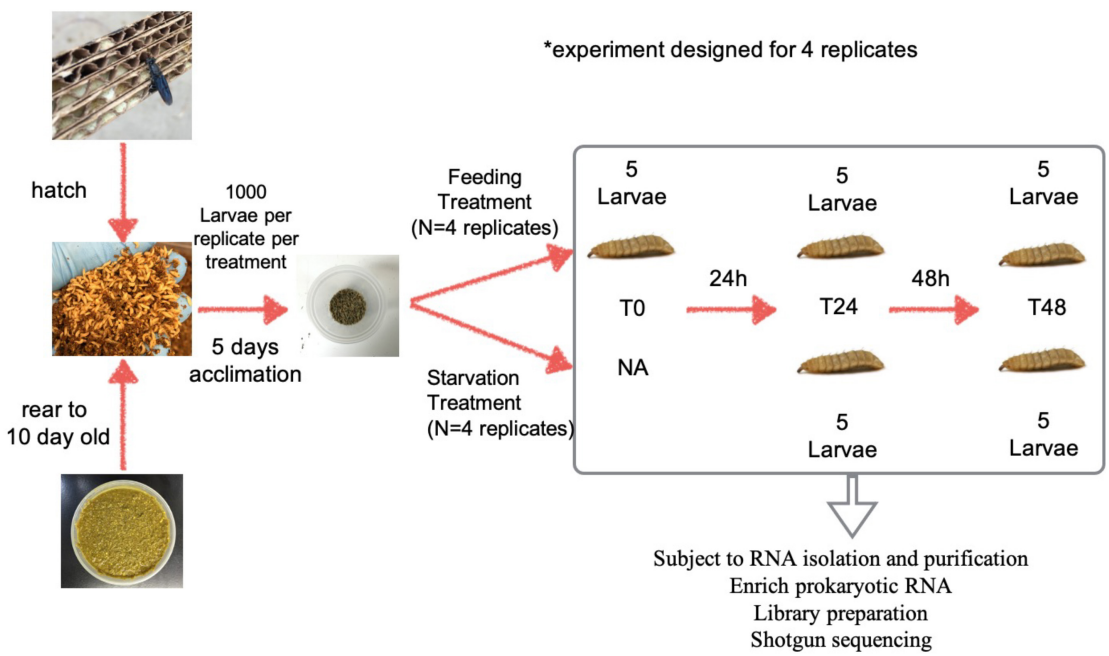

FIGURE 1 | The workflow of the experiment design. Neonates hatched from the eggs were fed 70% moisture Gainesville diet until 10 days old then partitioned into 1,000 larvae per lot for an additional 5-day feeding for acclimation. On the 15th day larvae and frass were further split into two treatments of 500 larvae with equal amount of the frass each. One cup represented the control (Fed) group, in which the larvae were being fed daily (20 g, 2 h prior to the sampling, and 30 g after the sampling), until the end of the experiment. The other cup represented the Starved treatment group, in which the larvae were no longer receiving any food for the rest of the experiment. Five subsamples were taken from each replicate and subjected to RNA isolation and library preparation for shotgun sequencing.

water. Resuspended RNA samples were pooled for each replicate, resulting in 20 RNA samples from larval samples and one RNA sample isolated from feed samples, that were stored at −20°C.

RNA was purified using the Direct-zol™ RNA MiniPrep kit, following the manufacturer's protocol. Resulting prokaryotic RNA was enriched using the MICROBEnrich™ Kit, following the MICROBEnrich™ Kit Protocol that captures and removes up to 90% of eukaryotic RNA, resulting prokaryotic RNA enrichment. If severe eukaryotic RNA contamination was detected, the respective samples were purified with the MICROBEnrich™ Kit for a second time. The enriched RNA products were then quantified with a Qubit® 2.0 Fluorometer, and ran on a gel to determine RNA quality.

The NEB Ultra RNA Library Kit (New England Biolabs) was used to convert the microbial RNA to cDNA, avoiding steps to remove rRNA, and preserving total RNA concentrations. Established cDNA libraries were multiplexed using NEBNext Oligos for Illumina to create five libraries. Resulting multiplexed libraries were submitted for shotgun whole microbial metatranscriptome sequencing using an Illumina® HiSeq 2000 instrument for 2 × 101 basepair reads.

Microbiome 16S Analyses and Functional Prediction

Raw paired-end reads from whole metatranscriptome sequences were demultiplexed, and trimmed, and adapters removed using the FasQC tool kit. Low-quality reads less than 36 nucleotides long, and low-quality nucleotide groups with a Phred + 33 score less than 30 were removed using the FasQC tool kit (Version 1.0.0). Sequences using Illumina® HiSeq, followed by quality filtering, yielded 10.4 GB of sequence data (862,168 sequences) from the total 20 samples, with 101 base pair sequence length for each sample. All reads passed FastQC quality check with a mean quality score over 30.

Parallel-Meta 3.3.1 was used for bacterial identification to multiple taxonomic levels using the unassembled metagenomic shotgun sequences. Taxonomic classification and KO functional prediction were assessed by setting sequence type as "Shotgun", and functional analysis as "Enabled". Additionally, "format check" was disabled as instructed through personal communication with the software developer, leaving other settings as default (Su et al., 2014). Parallel-Meta utilizes high performance data mining algorithms and curated comparator databases that elucidates hundreds of thousands to millions of short reads of a metagenomic sequence into discrete microbial genomes and genes. Parallel-Meta first constructed Hidden Markov Models using all bacterial 16S rRNA sequences of SILVA (version 123), and predicted the 16S rRNA gene fragments in our shotgun samples from both the forward sequences and reversed complementary sequences by HMMER, then extracted the 16S rRNA fragments from the shotgun sequences for profiling (Eddy, 2011; Jing et al., 2017). All 16S rRNA gene sequences were aligned to the Parallel-Meta reference database by Bowtie2 for OTU picking, taxonomical annotation and phylogeny construction. The Parallel-Meta customized database integrated GreenGenes (sequence similarity on 97% level) with SILVA consensus taxonomy annotation (assigned by BLASTN with e-value < 1e-30 and similarity > 97%) (Pruesse et al., 2007), RDP (Cole et al., 2009), and Oral Core (Griffen et al., 2011) to determine microbial identification to multiple taxonomic levels. Phylogenies for reference sequences were built by FastTree (Price et al., 2009, 2010). The Parallel-Meta algorithm also rarified sequences and calculated the precise relative abundance of each organism by 16S rRNA copy number calibration using the IMG database (Markowitz et al., 2012).

Extraction of 16S rRNA yielded an average of 22,455 16S rRNA sequences per sample for taxonomic analyses, including 10 phyla, 24 classes, 40 orders, 66 families, 117 genera, 26 species, and 10691 OTUs, with the genera level providing the most informative classification. Taxa representing less than 5% in mean relative abundance across all timepoints were classified as "other". Shannon diversity was used to measure alpha diversity, and two-tailed T-tests were performed to determine significance. Principal coordinate analysis ordination of a Bray-Curtis dissimilarity matrix was used to measure beta diversity distances, and PERMANOVA based on the Bray-Curtis dissimilarities with Bonferroni correction was used to measure significance across treatments and timepoints. To illustrate how microflora changed from the feed to larvae, we included a datum point from microbial sequencing Gainesville diet, which was collected and sequenced as a pilot study. Because we only sequenced one sample of the feed, and it cannot provide any statistical power, we only used this feed datum point for discussion to provide inferences on what microbes were originally in the feed.

Parallel Meta also implemented the PICRUSt algorithm using the KEGG database to estimate all functional gene potential within the microbiome 16S rRNA gene OTUs (Kanehisa and Goto, 2000; Langille et al., 2013). Predicted functional genes were annotated by KO (KEGG Ontology), KEGG pathways, and BRITE hierarchies (Kanehisa and Goto, 2000; Kanehisa, 2019). We determined cut-off values for a log2 fold change equaling a fold change of at least 1 in order to further rank the top 10 most significant predicted genes.

Statistical Analyses

Additional statistical analysis was performed using RStudio (Version 0.99.903), which was built on R software (Version 3.3.1) (R Core Team, 2013). The DESeq2 package was used to detect genera with differential abundance between treatments (Love et al., 2014). The Wilcoxon-Mann-Whitney test with Benjamini-Hochberg p-value correction was used to determine statistical differences in the distributions of microbial taxa in the Fed and Starved groups over time. Timepoints T24 H and T48 H were treated as separate events, where raw count data from Control T24 H were tested against Starved T24 H, and Control T48 H were tested against Starved T48 H. Significance was determined with FDR adjusted p value (q-value) less than 0.05.

Random Forest Feature Selection

The random forest machine learning algorithm returns an array of feature importance values of length equal to the array of input features (Liaw and Wiener, 2002). Feature importance was determined as part of the boot-strap method used for

assembling random decision trees, where feature importance is greater for variables with greater predive performance. Feature ranking was determined using a bootstrap method that randomly sampled 80% of the training data from BRITE hierarchies over a default of 1000 iterations. The highest ranking values associated with BRITE hierarchies were determined over all iterations. The Wilcoxon-Mann-Whitney test with Benjamini-Hochberg *p*-value correction was used to determine statistical differences in the predicted BRITE hierarchy abundances.

RESULTS

Phylum-Level Differences Between Treatments and Timepoints

The Shannon Diversity Index revealed a significantly higher diversity and a more even community overall in the Fed than in the Starved group ($p = 0.0025$) at the phylum level. The Fed group at T24 H had significantly higher Shannon diversity than the T0 H group ($p = 0.06$), as well as the group of Starved T24 H ($p = 0.031$), Fed at T48 H ($p = 0.038$), and Starved at T48 H ($p = 0.033$, **Figure 2A**). Shannon diversity of the Starved T24 H group was also significantly lower than the T0 H group ($p = 0.031$). Among the 11 identified phyla, six were significantly different between the Fed and Starved cohort relative abundance ($q < 0.05$). Actinobacteria was the most dominant phylum shared by samples from both Fed and Starved, followed by Proteobacteria, Firmicutes, Euryarchaeota, and Bacteroidetes (**Figure 2B**). While Actinobacteria was the most abundant phylum among all treatments, this phylum was found in significantly higher abundance in the Starved cohort ($q < 0.001$). Besides Actinobacteria, Bacteroidetes and Verrucomicrobia were also found with significantly higher abundance in the Starved cohort ($q < 0.01$ and $q < 0.001$, respectively; **Table 1**). On

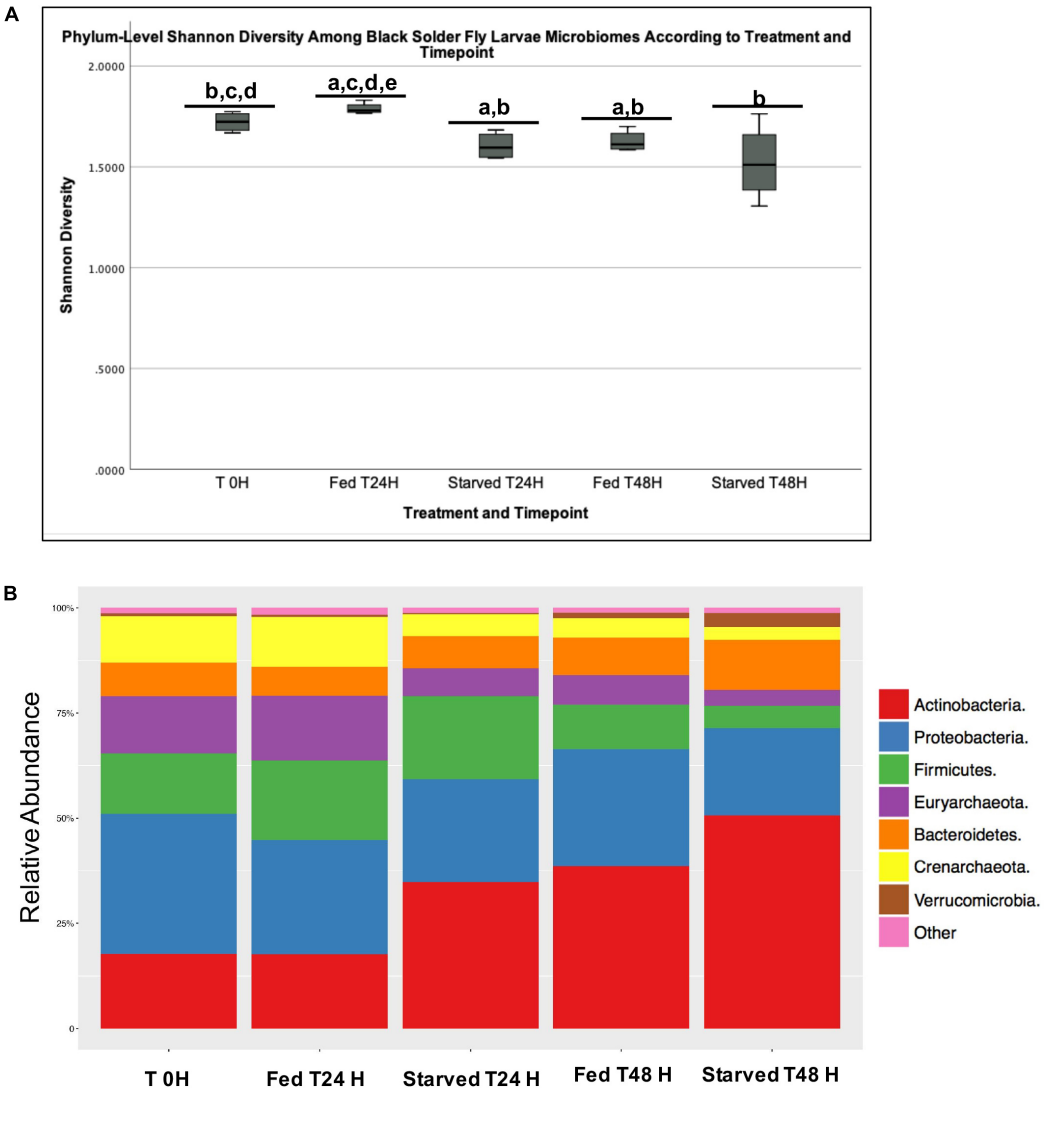

FIGURE 2 | Phylum level Shannon diversity **(A)** and relative abundance **(B)** across treatments and timepoints.

TABLE 1 | Phyla with significantly different relative abundances between Fed and Starved groups.

Phylum	Mean Fed[a]	Mean Starved[b]	*q*-value
Actinobacteria	0.230	0.433	<0.001
Firmicutes	0.178	0.082	<0.001
Euryarchaeota	0.135	0.061	<0.01
Bacteroidetes	0.067	0.107	<0.01
Crenarchaeota	0.096	0.040	<0.001
Verrucomicrobia	0.005	0.027	<0.001
Planctomycetes	0.002	0.001	<0.05

[a]*Mean abundance among all Fed samples at T0H, T24H, and T48H.*
[b]*Mean abundance among all starved samples at T24H and T48H.*

the other hand, Firmicutes, Euryarchaeota, Crenarchaeota, and Planctomycetes (found within "other" in **Figure 2B**) were decreased within the Starved cohort. Notably, all had decreased by 50% or more (**Table 1**).

Genus-Level Differences Between Treatments and Timepoints

Analysis of alpha diversity using the Shannon Diversity Index showed no significant differences between the T0 H black soldier fly larval microbiomes with any other treatment or timepoint at the genus level (**Figure 3A**). Overall, the Fed group increased in Shannon diversity over time, while the Starved group decreased in diversity over time at the genus level. For instance, the Fed T24 H group showed significantly lower Shannon diversity at the genus level than Starved group at T24 H (p = 0.044) and Fed group at T48 H (p = 0.008). The Starved group at T24 H was significantly lower in alpha diversity compared to the Fed group at T48 H group, but was significantly higher than the Starved T48 H group. Bray Curtis Distances were computed at the genus level and showed individual samples clustering together according to treatment and timepoint (**Figure 3B**). Samples from the Fed group at T24 H clustered closest to the T0 samples, while the T24 H Starved group clustered closest to the T48 H Fed group. Individual samples from the Starved group at T48 H clustered with each other, but further away from the other samples. The single Feed sample showed greatest separation from the larval samples (**Figure 3B**). But, analysis of clustering across and between cohorts using PERMANOVA with inclusion of Bonferroni adjustment was not statistically significant.

Taxa with relative abundance greater than 5% across all Fed and Starved groups and timepoints are shown in **Figure 3C** (individual relative abundance in each sample across treatments and timepoints are shown in **Supplementary**

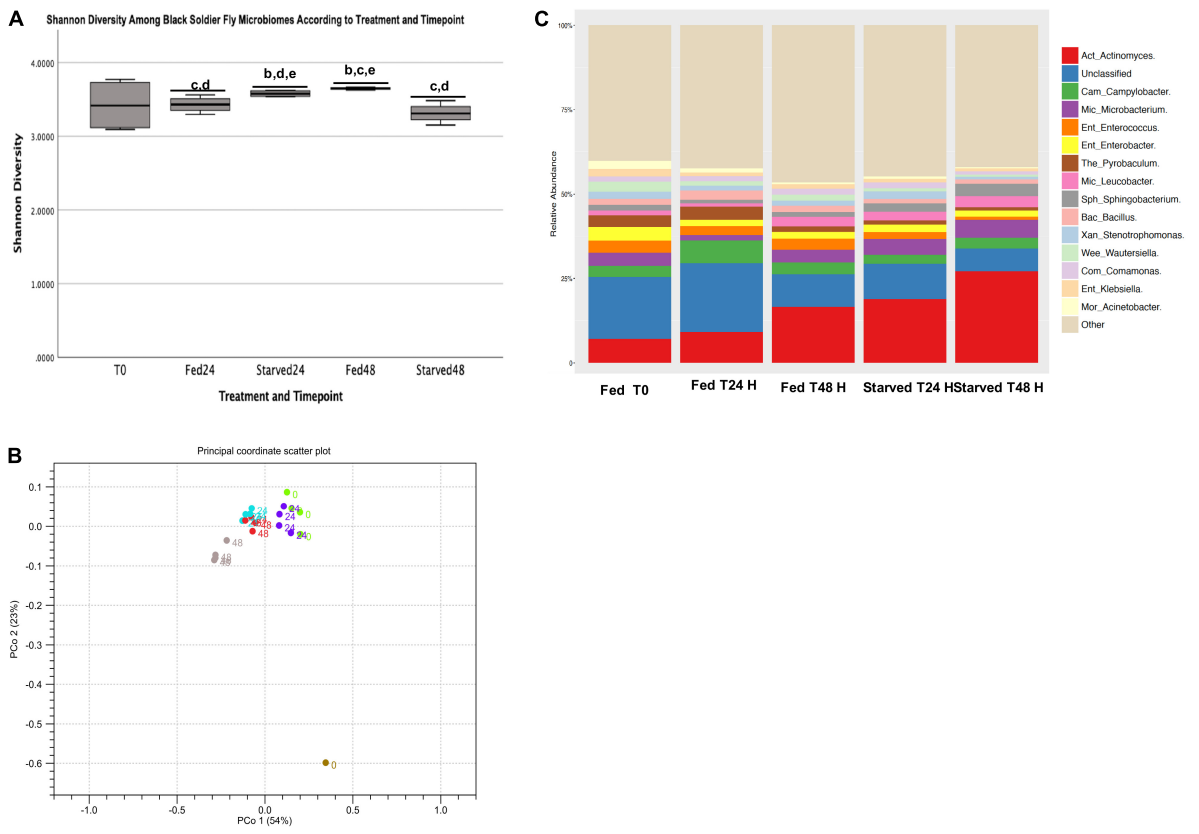

FIGURE 3 | Genus level Shannon diversity and Relative Abundance. **(A)** Alpha diversity using Shannon index. **(B)** Principal coordinate plots using Bray-Curtis distances of individual fed and starved black soldier fly microbiome samples and a single feed sample. **(C)** Genus leval taxa with relative abundance greater than 5% across Fed and Starved Groups at T0, T24 H, and T48 H.

Figure 1 and **Supplementary Table 1**). Of these, *Enterobacter, Stenotrophomonas, Wautersiella, Klebsiella* and *Comamonas* were not statistically different between Starved and Fed groups at either T24 H or T48 H timepoints. These genera also had mean relative abundances of 2.0, 1.5, 1.2, and 1.5%, respectively, across the timepoints.

Statistically Significant Differences in Black Soldier Fly Microbiome Relative Abundances at Either the Twenty-Four or Forty-Eight Hour Timepoints

A total of 56 identified genera had significantly ($q < 0.05$) different abundances between treatments at only the T24 H, and 39 genera at only the T48 H timepoint (**Supplementary Tables 1**, **2**). Among the most abundant taxa (greater than 5% across treatments and timepoints), *Campylobacter* relative abundance doubled between T0 and T24 H in the Fed samples, but then decreased to initial abundance, comparable also to the Starved samples (**Figure 3C**). However, there was a statistically significant difference in *Campylobacter* relative abundance between Fed and Starved at the 24 H timepoint (6.8% versus 3.2%, $p = 0.0006$). *Pyrobaculum* relative abundance increased from 3.3% at T0 to 3.9% at T24 H in relative abundance in the Fed group and was statistically higher in relative abundance than the Starved group ($p < 0.001$), which decreased from 1.3% at the 24 H timepoint to 0.95% at the 48 H timepoint in the Starved Group (**Figure 3C**). *Bacillus* relative abundance in the Fed group had an initial increase between T0 and T 24 H (from 1.8 to 2.7%) that was statistically higher than the Starved group ($p = 0.0012$), but then decreased to 1.7% at T 48 H. *Bacillus* relative abundance in the Starved group remained relatively constant (1.7 and 1.3%, respectively). Finally, *Acinetobacter* decreased across all timepoints in both treatments from 2.4 at T0 to 1.2 and 0.55%, in the Fed group and to 0.7 and 0.3%in the Starved group at T24 H and T48 H), but was statistically higher in the Fed group than in the Starved group at T24 H ($p < 0.001$). Unclassified taxa accounted for statistically significant higher (10.5–22.3%, $p < 0.001$) relative abundance in the Fed group, with only 8.3–11.1% in the Starved group across timepoints (**Figure 3C**). While there were statistically significant differences between treatments at the T48 H timepoint (**Supplementary Table 2**), taxa represented relative abundances less than 5% across all timepoints.

Statistically Significant Differences in Black Soldier Fly Microbiome Relative Abundances at Both Twenty-Four and Forty-Eight Hour Timepoint

Five genera with a minimum abundance of 5% at any treatment or timepoint were identified as significantly different at both the T24 H and T48 H (**Figures 3**, **4**). *Actinomyces* was identified as the most abundant genus with an average abundance of 15.8% across all samples. *Actinomyces* increased abundance over time in larval samples, no matter the treatment (**Figure 4A**). Additionally, *Actinomyces* was statistically significantly higher in the Starved treatment (19%, $p < 0.001$, **Figure 4A**) than the Fed (9.2%) at the 24H timepoint and, and also at the T48 H timepoint (27% Starved versus 17% fed, $p < 0.001$). *Microbacterium* was also significantly

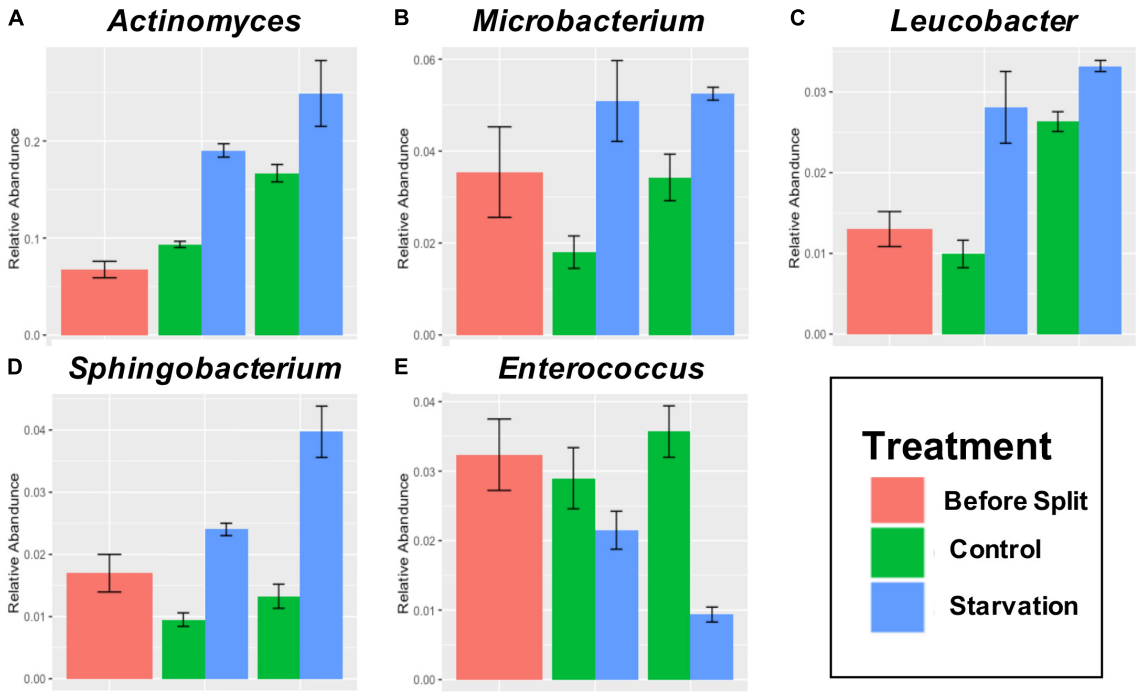

FIGURE 4 | Mean relative abundance greater than 5% and significantly different ($q < 0.05$) abundance at both T24 H and T48 H for Genera *Actinomyces* **(A)**, *Microbacterium* **(B)**, *Leucobacter* **(C)**, *Sphingobacterium* **(D)**, and *Enterococcus* **(E)**. T0 is the initial timepoint prior to division into Fed and Starved treatments.

higher in the Starved group at T24 H (4.6% in Starved versus 1.5% in Fed, $p = 0.0005$) and at T48 H (5.3% in the Starved versus 3.7% in Fed, $p < 0.001$, **Figure 4B**). *Leucobacter* relative abundance was also statistically significantly different between the treatments at the T24 H and T48 H timepoints with higher relative abundance in the Starved group. *Leucobacter* relative abundance was 0.98% in Fed versus 2.6% in Starved at T24 H ($p = 0.0008$), and was 2.8% in Fed versus 3.3% in Starved at T48 H ($p < 0.001$, **Figure 4C**). *Sphingobacterium* relative abundance was also significantly higher in the Starved group at both timepoints ($p = 0.0003$ at T24 H and $p < 0.0001$ at T48 H) where it increased from 2.5% at T 24 H to 3.7% at T 48 H versus 1.1 and 1.5% in the Fed group at T24 H and T 48 H, respectively (**Figure 4D**). *Enterococcus* remained relatively constant across the Fed group from T24 H to T 48 H (2.6, and 3.3%, respectively), but was significantly higher ($p = 0.0084$ and $p < 0.001$ at T24 H and T48 H, respectively, **Figure 4E**) than the Starved group that decreased to 2.0% at T24 H and further decreased to 0.9% at T48 H.

Functional Prediction

The functions of black soldier fly larval microbiomes were predicted using PICRUSt analysis. With this, 16S rRNA gene sequencing data were categorized into 6,709 KEGG functional pathways (**Supplementary Table 3**). Pathways present in <10% of all samples were removed, leaving 5,905 KEGG pathways for comparison. The DESeq2 package identified 4,401 predicted gene abundances that were statistically different ($q < 0.05$) between Starved and Fed groups (**Supplementary Table 4**). We focused on predicted genes with log2 fold change cut-offs yielding fold changes of at least 1, and identified the 10 predicted genes down regulated and 10 predicted genes upregulated (**Table 2** and **Supplementary Table 4**) in the Starved group. Of those upregulated, three showed predicted function in carbohydrate metabolism, two were predicted to be involved in metabolism of other amino acids, and three were predicted to be involved in metabolism of cofactors, terpenoids, or methane, respectively. Many of these predicted genes are also known to be involved in microbial metabolism in diverse environments. The remaining two of the ten significantly upregulated had predicted functions in environmental information processing and signal transduction (**Table 2**).

Three of the ten predicted genes that were significantly downregulated in KO abundance in the Starved group were placed within the genetic information processing category and included genes predicted to be involved in DNA replication, chaperones, and general stress response (**Table 2**). Four additional downregulated genes were predicted to be involved in signaling and cellular processes as part of secretion systems. The remaining three significantly downregulated were predicted to be involved in environmental information processing, energy metabolism, and carbohydrate metabolism, respectively.

Further analyses of mean abundance of predicted genes classified 16S rRNA sequences into 31 BRITE hierarchies. The random forest algorithm was applied to determine whether these predicted BRITE hierarchies could differentiate between Fed and Starved groups. The mean decrease in accuracy of BRITE hierarchy attributes showed the most relevant descriptors were associated with cell growth and death, transport and catabolism, cancers/human diseases, biosynthesis of other secondary metabolites, and metabolism of terpenoids and polyketides (**Figure 5**). BRITE hierarchy features that were statistically significantly predicted to be most associated with Starved or Fed groups are summarized in **Table 3**.

DISCUSSION

Our data demonstrate that starvation impacts microbial community structure in the larvae. Such impacts were demonstrated across taxonomic scale (i.e., phylum and genus) and predicted gene function. This study represents not only the first to demonstrate such impacts on black soldier fly larvae but is also among only a few on insects in general.

As discussed below, research on starvation has primarily focused on vertebrates. Understanding such influences across taxa is critical for deciphering the mechanisms regulating these responses as a means to develop strategies for combating health-related benefits. From an industrial perspective of insect farming, once described, such processes could be manipulated to enhance production of the targeted insect, such as the black soldier fly, or the inverse- prolong development as a means to enhance waste conversion. Both outcomes could be massively important for stabilizing the industry as they could lead to greater efficiency in production or maintenance of a larval 'bank' that can be used when organic streams surpass facility needs.

Starvation could impact final product output. Inabilities to feed black soldier flies at a consistent rate throughout larval development could impact waste conversion and growth patterns. Previous reviews discuss the impact of diet nutrition on the protein and fat composition of harvested black soldier fly larvae (see reviews by Barragan-Fonseca et al. (2017), Gold et al. (2018), and Surendra et al. (2020). And, of equal importance, and discussed in the previous reviews cited, nutrient composition of resulting larvae could also be impacted. Given the black soldier fly larvae are produced for their protein and fat, failure to optimize the process could result in product not meeting regulatory or industrial standards.

While many studies have focused on the impact of adding nutrients into a system on associated gut microbiome structure, a literature review revealed only limited investigations of bacterial community shifts during host starvation (Xia et al., 2014; Davis, 2016; Seitz et al., 2019; Cornide-Petronio et al., 2020; Golonka et al., 2020; Jing et al., 2020; Schroder et al., 2020). In fact, little is known about the impact of fasting on the microbiome of invertebrates. Tinker and Ottesen (2016) demonstrated stability in the microbiome of the gut of the American Cockroach, *Periplaneta americana* (Blattodea: Blattidae) when starved (Tinker and Ottesen, 2016). Interestingly, their results contrast with those demonstrated in the current study; however, it is hypothesized that such differences could be due to life-history differences with the American roach having a longer life-span (e.g., 12 months) with adults feeding, and the black soldier fly adult have a comparatively short life-span with little

TABLE 2 | The top 10 significantly upregulated and downregulated predicted genes in the Starved treatment with log2 fold changes greater than 1 (means), along with their KEGG annotations (listed in order of q-value followed by log2 fold change).

KEGG orthology	log2 fold change	*q*-value	Gene description	KEGG BRITE functional classifications
K14448	1.92	3.93E-31	(2*S*)-methylsuccinyl-CoA dehydrogenase	Carbohydrate metabolism; Glyoxylate and dicarboxylate metabolism; Microbial metabolism in diverse environments
K03851	1.95	2.33E-29	taurine-pyruvate aminotransferase	Metabolism of other amino acids; Taurine and hypotaurine metabolism
K04036	1.88	2.33E-29	divinyl protochlorophyllide a 8-vinyl-reductase	Metabolism of cofactors and vitamins; Porphyrin and chlorophyll metabolism
K14451	2.03	2.73E-29	(3*S*)-malyl-CoA thioesterase	Carbohydrate metabolism; Glyoxylate and dicarboxylate metabolism; Microbial metabolism in diverse environments
K09847	2.01	2.56E-27	spheroidene monooxygenase	Metabolism of terpenoids and polyketides; Carotenoid biosynthesis
K08927	1.62	5.14E-24	light-harvesting complex 1 beta chain	Environmental Information Processing; Signal transduction
K14083	1.32	6.37E-26	trimethylamine—corrinoid protein Co-methyltransferase	Methane metabolism; Energy metabolism; Microbial metabolism in diverse environments/methanogenesis
K05915	1.78	7.62E-26	(hydroxymethyl)phosphonate/2-amino-1-hydroxyethylphosphonate	Metabolism of other amino acids; Phosphonate and phosphinate metabolism
K14449	1.60	3.08E-25	2-methylfumaryl-CoA hydratase	Carbohydrate metabolism; Glyoxylate and dicarboxylate metabolism; Microbial metabolism in diverse environments
K07772	−1.35	9.39E-24	two-component system, OmpR family, torCAD operon response regulator TorR	Environmental Information Processing; Signal transduction
K03346	−1.00	2.33E-13	Replication initiation and membrane attachment protein	Genetic information processing; DNA replication proteins
K12269	−2.43	2.11E-11	Accessory secretory protein Asp2	Signaling and cellular processes; Secretion system
K12270	−2.32	3.16E-11	Accessory secretory protein Asp3	Signaling and cellular processes; Secretion system
K07683	−1.65	3.27E-11	Two-component system, NarL family, sensor histidine kinase NreB	Environmental Information Processing; Two-component system, NarL family, sensor histidine kinase NreB
K02828	−1.30	3.88E-11	Cytochrome aa3-600 menaquinol oxidase subunit III	Energy metabolism; Cytochrome aa3-600 menaquinol oxidase subunit III
K03697	−1.04	4.97E-11	ATP-dependent Clp protease ATP-binding subunit ClpE	Genetic information processing; Chaperones and folding catalysts
K10984	−1.03	5.08E-11	Galactosamine PTS system EIIB component	Carbohydrate metabolism; Galactose metabolism
K02245	−1.02	5.98E-11	Competence protein ComGC	Signaling and cellular processes; Type II secretion system
K02240	−1.02	5.98E-11	Competence protein ComFA	Signaling and cellular processes; Type II secretion system
K07570	−1.01	6.44E-11	General stress protein 13	Genetic information processing; Translation

reliance on adult nutrition (Rau, 1940; Tomberlin et al., 2002; Bertino-Grimaldi et al., 2013).

Clearly, in situations where nutrients are deficient, the host can experience starvation depending upon the circumstances. And, it can be expected that in such situations, similar downstream effects are observed for the commensal microbes. This impact has been well-documented for vertebrate models. For instance, Costello et al. (2010) demonstrated an abundance of Bacteroidetes during fasting that shifted toward a post-prandial abundance of Firmicutes in the Burmese python. Similarly,

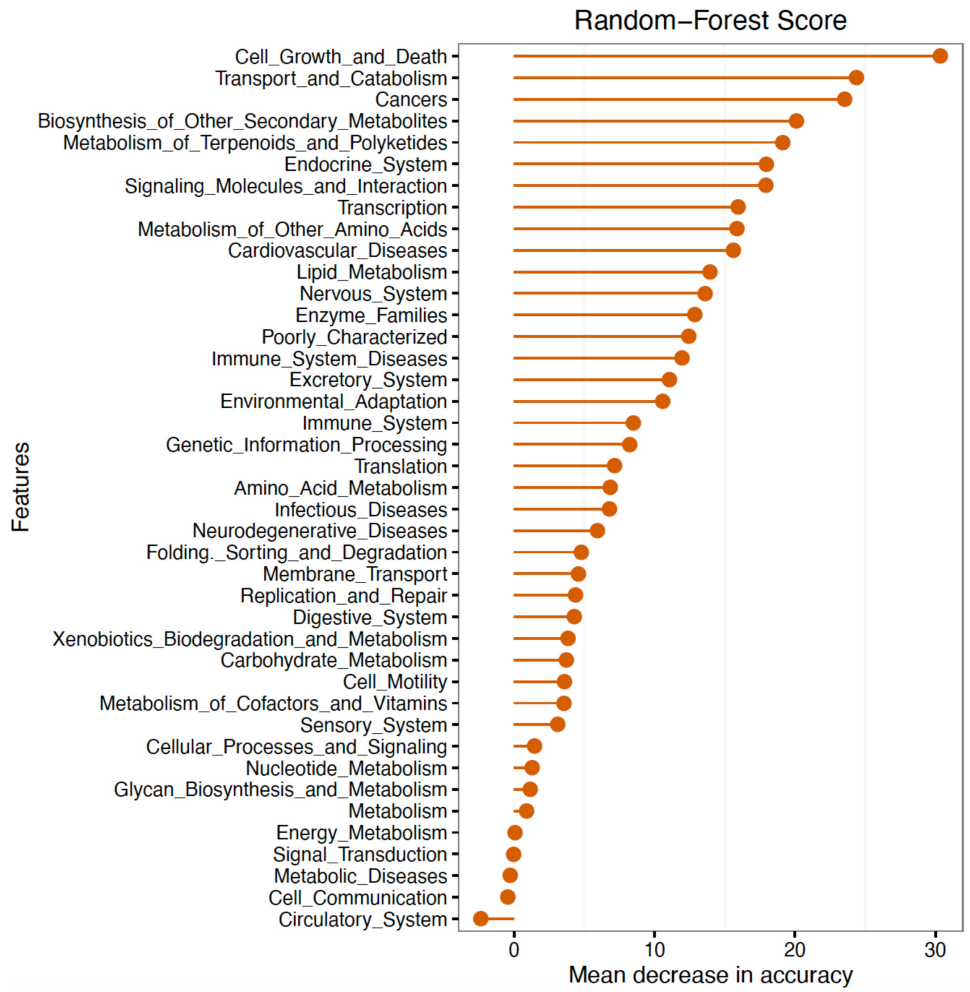

FIGURE 5 | Random forest classification of BRITE hierarchies as most important predictors of Fed versus Starved Groups.

Crawford et al. showed similar results when starving mice for 24 H (Crawford et al., 2009). Our results demonstrate similar patterns indicating mechanisms of microbiome responses to fasting (i.e., starvation) are potentially convergent across taxa. Such mechanisms, once deciphered, could be put into practice to enhance, or at minimum stabilize, black soldier fly production associated nutrient content.

As mentioned initially in the discussion, achieving such advancements are critical for optimizing black soldier fly farming for more accurate projections in production. For example, one possible approach to optimize production is to keep black soldier fly larvae hungry, by maintaining a microbiome that is associated with host hunger (i.e., predicted function). In such a system, "hunger-related" microbes (i.e., *Enterococcus* species within the current study) found internally or externally, might serve as signaling agents for hunger induction in the larval host. This hunger trigger would presumably promote increased feeding, and ultimately waste to protein conversion, meeting farmers' goals for higher biomass per capita; especially in the black soldier fly larvae production industry. Similarly, microbial community members may be identified that provide added nutritional value and utility in waste conversion.

Starvation could also reduce microbial community structure, as seen in our study (Fed v Starved, **Figures 2**, **3**) allowing for easier manipulations for greater production. Decreased microbial diversity, which tends to be associated with larger populations of the same species (i.e., microbes in gut of insect), often result in individual species having more energy and resources and a higher capacity for host manipulation, because fewer resources are spent on competition. In this instance, bacterial cross-feeding, wherein one species of bacteria provides nutrients for another species, may also be occurring (Smith et al., 2019). This concept agrees with other studies that showed bacterial metabolites and cross-feeding can influence host satiety pathways, and have extensive effects on appetite and feeding behavior (Fetissov, 2017; Yang and Tomberlin, 2020).

Starving larvae did not impact the presence of some key microbial phyla. Actinobacteria was the most dominant metabolically active phylum shared by samples from both Fed and Starved groups, but was found in higher abundance in the

TABLE 3 | BRITE hierarchy features statistically significantly associated with Starved or Fed groups using the Wilcoxon Rank Sum Test with Benjamini Hochberg (BH) *p*-value correction.

BRITE hierarchy	Mean Fed	Mean Starved	Standard Deviation Fed	Standard deviation Starved	Wilcoxon-BH
Cell growth and death	4.38E-03	4.84E-03	3.49E-04	1.08E-04	6.16E-05
Biosynthesis of other secondary metabolites	8.65E-03	9.35E-03	1.91E-04	3.67E-04	6.16E-05
Transport and catabolism	2.87E-03	3.42E-03	2.36E-04	1.98E-04	6.16E-05
Endocrine system	3.44E-03	4.14E-03	3.61E-04	3.22E-04	6.16E-05
Signaling molecules and interaction	1.66E-03	1.95E-03	2.01E-04	1.11E-04	8.82E-05
Cancers	1.22E-03	1.47E-03	1.37E-04	7.99E-05	8.82E-05
Lipid metabolism	3.04E-02	3.25E-02	1.71E-03	1.65E-03	2.81E-04
Metabolism of other amino acids	1.68E-02	1.76E-02	8.10E-04	3.37E-04	2.82E-04
Environmental adaptation	1.24E-03	1.44E-03	1.32E-04	1.38E-04	3.31E-04
Transcription	2.68E-02	2.57E-02	1.16E-03	3.61E-04	3.92E-04
Poorly characterized	5.51E-02	5.18E-02	3.13E-03	1.14E-03	4.66E-04
Metabolism of terpenoids and polyketides	1.87E-02	1.94E-02	5.29E-04	2.01E-04	5.57E-04
Translation	5.48E-02	5.02E-02	4.69E-03	2.82E-03	3.28E-03
Genetic information processing	2.67E-02	2.50E-02	1.64E-03	5.33E-04	3.83E-03
Amino acid metabolism	9.99E-02	1.02E-01	2.14E-03	1.31E-03	5.58E-03
Enzyme families	1.93E-02	1.89E-02	7.24E-04	3.36E-04	5.83E-03
Neurodegenerative diseases	2.25E-03	2.42E-03	2.55E-04	1.92E-04	6.80E-03
Infectious diseases	4.04E-03	3.75E-03	4.21E-04	2.74E-04	1.95E-02
Glycan biosynthesis and metabolism	1.81E-02	1.92E-02	1.12E-03	1.27E-03	2.67E-02
Xenobiotics biodegradation and metabolism	2.64E-02	2.78E-02	2.20E-03	1.70E-03	2.90E-02
Metabolism of cofactors and vitamins	4.10E-02	4.15E-02	1.41E-03	8.36E-04	2.90E-02

Starved cohort. Additionally, Bacteroidetes and Verrucomicrobia were also found with higher abundance in the Starved cohort. On the other hand, Planctomycetes, Firmicutes, Euryarchaeota, and Crenarchaeota, showed higher relative abundance among the Fed cohort. Planctomycetes were previously considered to be only found in marine environments, however recent data has shown members of the phylum to be associated with insect and human microbiomes (Franzini et al., 2016).

Euryarchaeota and Crenarchaeota are archaeal phyla. Archaea represent a large proportion of the Earth's ecosystem, with the capability of residing in diverse and extreme environments (Eme and Doolittle, 2015). Many archaea are substantial components of complex microbial communities, including in plant and animal microbiomes (Lurie-Weinberger and Gophna, 2015; Moissl-Eichinger et al., 2018). And, archaeal sequences have been identified in association with microbiomes of black soldier flies raised on low bioburden diets, though at low relative abundances, suggesting that these phyla are normal black soldier fly residents, aiding in digestion (Klammsteiner et al., 2020). Our shotgun data showed highest archaeal resolution at the order level, with four archaeal orders, including Halobacteriales, Methanobacteriales, Methanomicrobiales, and Thermoproteales (**Supplementary Table 1**). All of the orders showed a statistically significant reduction in relative abundance in the Fed group from T24 H to T48 H with even lower relative abundance in the Starved group. There was a statistically significantly lower relative abundance in the Starved group than Fed group at T24 H for all detected archaeal orders, however there was no statistically significant difference between the two groups at T48 H (**Supplementary Table 1**, and data not shown). With the exception of Methanobacteriales, the mean relative abundances ranged from 0.13 to 3.6% across all treatments and timepoints, and were at very low relative abundances or absent from the single feed sample (**Supplementary Table 1**). Methanobacteriales had a mean relative abundance of 14.7% at T0 and 14.6% at T24 H in the Fed group, decreasing to 5.9% at T48 H. The Starved group had a mean relative abundance of 6.4% Methanobacteriales, decreasing to 4.2% at T48 H. The single feed sample showed a 6.7% relative abundance of Methanobacteriales. Methanomicrobiales and Methanobacteriales are well defined within the Euryarchaeota phylum, and are hydrogenotrophic organisms that carry out methanogenesis. These archaea are capable of entering into syntrophic relationships with other gut microbes, and likely stimulate symbiotic digestion of plant material, and the process of methanotrophy used by other consortial microbes for sources of carbon and energy (Mitsumori et al., 2002; Reuss et al., 2015). Members of Methanobacteriales also have a limited range of catabolic substrates, utilizing H_2, CO_2, CO, formate, and C_1-metyhlated compounds (Bonin and Boone, 2006). Although methane emission was not measured in this study, the high relative abundance of methanogenic archaea in the Fed group at T0 and T24 H is interesting, especially with regards to several reports of low methane emission rates by black soldier fly larvae (Mertenat et al., 2019; Pang et al., 2020; Parodi et al., 2020). Indeed, differences in methanogen abundance would affect methane production, but it remains also possible that differences in microbial composition and

functioning that result in perturbation of hydrogen metabolism or accumulation may also impact methanogenesis and methane emissions (Sugimoto et al., 1998; Sollinger et al., 2018; Aguilar-Marin et al., 2020). Notwithstanding, the higher relative abundance of Methanomicrobiales in the black soldier fly larval microbiomes within the early timepoints of the Fed group could be an indication of the reduced gut environment, as well as conditions of the external (i.e., fresh substrate with very little frass) and consumed substrate (i.e., within the gut), including mixed alcohol and other products resulting from digestion and fermentation of plant polymers (Lurie-Weinberger and Gophna, 2015; Moissl-Eichinger et al., 2018). The presence of metabolically active methanogens in the feed sample could be due to the addition of water for saturation prior to sampling that might allow for fermentation and a more reduced environment to occur, and products that could be conducive for methanogen enrichment. However, a more meaningful assessment of saturated Gainesville diet with a sample size conducive for increased power would be necessary in order to further, or confirm this hypothesis. Halobacteriales and Thermoproteales are both fron the Crenarchaeota phylum and have been identified in human and insect microbiomes (Borrel et al., 2014; Gaci et al., 2014; Douglas, 2015; Prasad et al., 2018; Kim et al., 2020).

Enhancing the taxonomic resolution to genus level revealed overall microbial structure was impacted by starvation. Among genera with at least 5% mean relative abundance across all treatments and timepoints, *Actinomyces*, *Microbacterium*, *Enterococcus*, *Sphingobacterium*, and *Leucobacter* showed significantly different relative abundance at both time points, with higher relative abundances in the Starved group. This suggests that starvation resulted in some microbial members becoming dominant and outcompeting those not able to tolerate starvation stress. These dominating bacteria may also aid in host adaptation to starvation. For instance, *Actinomyces* is known to produce enzymes for degradation of chitin and plant material, and is also known to produce antibiotics against pathogenic fungus (Sharma, 2014). These microbial characteristics could help the host survive during prolonged nutrient deprivation. Taxa were identified that were found in both Starved and Fed groups, as well as those significantly associated individually with either group. Taxa identified in both groups, but not found in the feed, could constitute members of a core microbiome, though their specific role in host biology needs further investigation. And, as the feed sample was only a $N = 1$, additional studies using multiple replicates would be necessary. Indeed, a recent study by Wynants et al. showed that black soldier fly larval microbiomes and substrate-associated microbes differed substantially, but also that there were some shared taxa between larvae, despite differences in rearing conditions (Wynants et al., 2019). Many of the genera in our study were also found in their study. Further, another study found that *Actinomyces* and *Enterococcus* were among those making up the core microbiome (Klammsteiner et al., 2020).

Our microbiome data differ from those generated in other black soldier fly studies. For instance, Zheng et al. (2013) surveyed standard grain diet fed 7-d-old black soldier fly larvae associated symbionts, and found, that Bacteroidetes (54.4%) was the most abundant phylum, followed by Firmicutes and Proteobacteria. Jeon et al. (2011) surveyed three groups of 8-d-old black soldier fly larval gut microflora fed with restaurant waste, cooked rice, and calf forage, and found that the microbial community structures were directly influenced by the intake feed. For example, those fed restaurant waste showed Bacteroidetes (67.4%) and Proteobacteria (18.9%) as predominant phyla, whereas Proteobacteria (54%) and Firmicutes (42.3%) were dominant when black soldier fly larvae fed on cooked rice. Larvae who fed on calf forage had a more evenly distributed community with Proteobacteria (31.1%), Actinobacteria (24.6%), Firmicutes (23.5%), and Bacteroidetes (20.5%) (Jeon et al., 2011). In both of these previous studies, Bacteroidetes was found to be the most abundant. In our studies, black soldier fly larvae fed Gainesville diet at T0 H (11-d-old), had gut microbiomes predominated by Proteobacteria (31.4%), followed by Euryarchaeota (16.9%) and Actinobacteria (16.6%). These differences underscore the importance of diet considerations in selective pressure for influence on resident gut microbiomes. Other contributors, such as larval age, rearing environment, host genetics, and early microbial exposure may also account for some differences. It is also worth mentioning that these previous studies used 16S rRNA targeted amplicon pyrosequencing techniques, while our study used a total RNA-based shotgun sequencing technology, which provides greater resolution and deeper analysis for strain diversity, and profiled the metabolically active community, potentially explaining some of the community profile differences. It is also likely, as has been shown in other studies, that larval density, diet, temperature, and other abiotic and biotic factors in our study may be driving microbial richness and evenness (Bruno et al., 2019; Wynants et al., 2019; Klammsteiner et al., 2020). Furthermore, differences in microbial richness, evenness, and relative abundance could also be accounted for in differing locations of the midgut. We sampled the internal and external microbiome of black soldier fly larvae, but recent studies have shown that structural, biological and functional differences exist in the midgut that drive microbial diversity (Bruno et al., 2019; Bonelli et al., 2020).

Additionally, bacteria are likely to show a group stress response under starvation conditions (Li and Tian, 2012). In fact, a growing body of knowledge has shown that quorum-sensing molecules are responsible for inter-kingdom communication (Hughes and Sperandio, 2008). As such, microbes possibly, through mechanisms of modulated host or microbe genes, hijacking host signals, or production of microbial chemical signals, induce host dysphoria to induce host hunger and consequently, nutrient intake. Therefore, besides the information of bacterial structure, information of functional potential according to host nutritional status is also important. PICRUSt utilizes characterized bacterial genomes and phylogenetic relationships to predict the functional genomes of other bacteria within the constructed phylogeny based on 16S marker gene data. PICRUSt has been validated by the Human Microbiome Project and has been utilized for functional prediction across a diverse array of microbiome datasets (Fazlollahi et al., 2018; Ferreira et al., 2018;

Reyes-Sosa et al., 2018; Wilkinson et al., 2018; Miao et al., 2020). But, it is important to note that functional prediction is not the same as true function. Indeed PICRUSt infers functional potential based on 16S rRNA sequences, and as such may over predict the number of pathways with significant difference over time and treatment compared to true metatranscriptome datasets because predictions are based on microbial community structure (Wilkinson et al., 2018). It will be important and informative to determine the level of accuracy of PICRUST in comparison to true functionality, versus over or under inflated predictions of pathways between genes.

Starvation impacted predicted microbial function associated with black soldier fly larvae. For instance, our data showed differences in black soldier fly larvae microbiome functional potential between microbiomes of Starved or Fed black soldier fly larvae. Three of the top 10 predicted downregulated KO abundances were associated with genetic processing. Predicted genes included those for a replication initiation and membrane attachment protein, an ATP-dependent Clp protease ATP-binding subunit ClpE that is a chaperone or folding catalyst, and for a general stress protein involved in translation (**Table 2**). A predicted gene encoding a galactosamine PTS system EIIB component was also predicted to be downregulated in the Starved group. These genes typically function in carbohydrate metabolism and have beta galactosidase activity (UniProt Consortium, 2018). The *qoxC* gene was also predicted to be downregulated. This gene is involved in energy metabolism (UniProt Consortium, 2018). Four of the top 10 significantly lower KO abundances were predicted genes involved in signaling and cellular processes and were part of secretion systems. And finally, one predicted gene with significantly lower KO abundance in the Starved group was involved in Environmental Information Processing and was part of a two-component system, NarL family, sensor histidine kinase NreB involved with dissimilatory nitrate reduction. Overall, predicted downregulation in genetic information processing and metabolism in the Starved cohort, indicates not only a microbial structure shift, but also that some bacteria in the starved host might have entered a dormant stage in response to the lack of nutrients, and have also altered metabolic pathways in response to changing carbon and energy sources (**Tables 2**, **3**). Furthermore, downregulation in secretion systems and energy metabolism suggests an means to conserve energy.

On the other hand, three of the 10 significantly predicted upregulated genes were those for glyoxylate and dicarboxylate metabolism. These genes are involved in carbon fixation pathways in prokaryotes that leads to the biosynthesis of carbohydrates from fatty acids or two-carbon precursors. These genes are also active during microbial metabolism in diverse environments (UniProt Consortium, 2018). Other predicted upregulated genes include those for carotenoid synthesis, methane metabolism, a gene encoding a light-harvesting complex 1 beta chain involved in signal transduction and a gene involved in metabolism of cofactors and vitamins. Finally, two predicted upregulated genes included those for taurine and hypotaurine metabolism and phosphonate and phosphinate metabolism, respectively. These as well as significantly different BRITE hierarchies (**Tables 2**, **3**) suggests shifts in the metabolically active microbial community structure to those organisms utilizing these functions, but also suggests a shift to a more diverse metabolism and utilization of carbon sources as well as to functional stress responses. Therefore, predicted functional responses may reflect selective pressures applied by the host but may also denote a competitive survival strategy for these bacteria to adapt to different environmental conditions. Whether predictive gene regulation shown here are demonstrative of responses to specific host signals in this context is unclear, and will be an interesting avenue for further investigation.

Finally, our taxonomic resolution was able to identify specific genera; however, only 17.7% sequences were identified on the species level, indicating these sequences belonged to poorly characterized microbial lineages associated with black soldier fly larvae. Species identification within our samples will be necessary to achieve any targeted manipulation as well as to determine probiotic versus pathogenic potential. For instance, though many *Weissella* strains have both pre- and probiotic properties, some are opportunistic pathogens to humans, and *W. ceti* is pathogenic to farmed rainbow trout (*Oncorhynchus mykiss*) (Figueiredo et al., 2012). Further investigations are required to determine if such a community structure containing possible pathogenic bacteria in black soldier fly larvae would cause animal infections. In addition, *Campylobacter* was found in both Fed and Starved larvae (**Supplementary Table 1**). Some *Campylobacter* ssp. are human pathogens causing diarrhea, cramping, abdominal pain, and fever in those infected, and is the most common cause of diarrheal illness in the United States (CDC, 2014). *Campylobacter* organisms are present in livestock such as chicken flocks, and can be transmitted through common water sources and contact with infected feces, and most *Campylobacter* can be easily killed through proper handling (CDC, 2014). Recently, *Campylobacter* was detected in abundance greater than 1.5% in larvae from two large-scale rearing facilities, but whether these represent commensals, transient microbes, or pathogenic organisms remain unknown (Wynants et al., 2019). Thus these findings merit more in-depth study.

In conclusion, our data revealed that starvation of the black soldier fly larval host led to a dramatic shift in the metabolically active microbiomes. Although the biological meaning of such a shift remains undetermined, investigating the functions of those specific taxa associated with nutrient rich or replete states are valuable for elucidating inter-kingdom communication and contribution of microbes in waste conversion and nutrient uptake. This increased knowledge will allow utility for designing methods and strategies for improved black soldier fly bioconversion efficiency through microbial manipulation to evolve into a solution of increasing protein production and waste reduction.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

FY and HJ designed and performed the experiment, analyzed the data, and wrote the draft. JT designed the experiment and wrote the draft. HJ organized the communication. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.601253/full#supplementary-material

Supplementary Figure 1 | Genus-level relative abundances of individual samples across treatments and timepoints.

Supplementary Table 1 | Relative abundance of taxa across samples.

Supplementary Table 2 | DESeq2 results of genera with significantly different abundances between Starved and Fed larvae at T24 H and T48 H. A total of 82 genera had significantly ($q < 0.05$) different abundance between treatments within the T24 H time point, and 64 genera had significant differences in abundances between treatments within the T48 H time point. Highlighted cells indicate the top six genera that were statistically significantly different in abundance across both time points.

Supplementary Table 3 | Predicted KEGG Orthology based on hylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt).

Supplementary Table 4 | Significantly different Predicted Gene Abundances Based on DeSEQ2 Analyses.

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Cottonseed Press Cake as a Potential Diet for Industrially Farmed Black Soldier Fly Larvae Triggers Adaptations of Their Bacterial and Fungal Gut Microbiota

Dorothee Tegtmeier[1], Sabine Hurka[2], Patrick Klüber[1], Karina Brinkrolf[3], Philipp Heise[1] and Andreas Vilcinskas[1,2]*

[1] Department of Bioresources, Fraunhofer Institute for Molecular Biology and Applied Ecology, Giessen, Germany, [2] Institute for Insect Biotechnology, Justus Liebig University Giessen, Giessen, Germany, [3] Department of Bioinformatics and Systems Biology, Justus Liebig University Giessen, Giessen, Germany

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****Correspondence:***
Dorothee Tegtmeier
dorothee.tegtmeier@ime.fraunhofer.de

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Black soldier fly larvae (*Hermetia illucens*, Diptera: Stratiomyidae) are used for the bioconversion of organic side products into valuable compounds such as proteins, lipids and chitin. However, the economic competitiveness of farmed insects compared to conventional protein production systems in agriculture and aquaculture depends on the availability of large quantities of inexpensive insect feed. Cottonseed press cake (CPC) is a side-stream of cotton production that is rich in proteins and lipids but unsuitable as feed for several farmed animals, except ruminants, due to the presence of the anti-nutritional sesquiterpenoid gossypol. Here, we tested CPC as a feed for black soldier fly larvae and studied the impact of this diet on the gut microbiome. Larvae reared on CPC developed normally and even showed a shorter life-cycle, but were smaller at the end of larval development than control larvae reared on chicken feed. The adaptability of the larvae to different diets is mediated by their versatile gut microbiome, which facilitates digestion and detoxification. We therefore used amplicon sequencing to analyze the bacterial and fungal communities associated with larvae reared on each diet, revealing differences between the larval guts and frass (residual feed substrate) as well as differences between the two diet groups. For example, *Actinomycetaceae* and Aspergillaceae were significantly enriched in guts of the CPC diet group and may help to metabolize compounds such as gossypol. Potentially probiotic yeasts and beneficial *Enterobacteriaceae*, which presumably belong to the core microbiota, were detected in high relative abundance in the gut and frass, indicating a functional role of these microbes, especially the protection against pathogens. We conclude that CPC may be suitable as an inexpensive and environmentally sustainable feed for the industrial rearing of black soldier flies.

Keywords: black soldier fly larvae, amplicon sequencing, bacteria, fungi, cottonseed press cake, core microbiome, insect development, gossypol

INTRODUCTION

The larvae of the black soldier fly (*Hermetia illucens*) are economically the most important farmed insects used for the bioconversion of organic waste into valuable compounds such as proteins, lipids and chitin (van Huis, 2013; Müller et al., 2017). These fast-growing insects can recycle large amounts of organic carbon into edible proteins and oils rather than breaking it down into CO_2 and methane (Perednia et al., 2017). Black soldier fly larvae (BSFL) have already been approved in the EU as a feed substrate in aquaculture, and are also being considered as livestock feed.

The gut microbiota plays a key role in the adaptability of BSFL to different diets, and is regulated by a panel of antimicrobial peptides that are expressed in a diet-dependent manner (Vogel et al., 2018). Although the BSFL gut microbiome remains stable during development when larvae are reared on a uniform diet (Cifuentes et al., 2020), its composition can change significantly when the diet is varied, as shown for BSFL reared on food waste, cooked rice or calf forage (Jeon et al., 2011) and vegetable or fish meal (Bruno et al., 2019). The BSFL microbiome is shaped by diet and other biotic and abiotic factors, explaining substantial differences between the microbiomes of larvae reared in different locations (Wynants et al., 2019).

The bacterial community of BSFL has been studied in detail but the fungal community has been largely overlooked. However, the fungal community also plays an important role in digestion, nutrient supply and the breakdown of toxic metabolites in insect guts (Starmer et al., 1986; Dowd, 1992; Vega and Dowd, 2005). Nevertheless, fungi can also produce toxins (Bennett and Klich, 2003) and their numbers must therefore be controlled during the farming of insects as food and feed. Currently, there is only one comprehensive study available which evaluated the fungal microbiome of BSFL. This study has shown that also the fungal community composition was strongly dependent on the diet (Varotto Boccazzi et al., 2017).

The adaptability of the BSFL gut microbiome allows this insect to convert diverse industrial side-streams and waste products into valuable proteins. Cottonseed press cake (CPC) is an abundant industrial side-stream generated during cottonseed oil production. Its global production is about 14.7 million tons per year (Heuzé et al., 2019). Whereas most agricultural side products are rich in cellulose, pectin, lignin and other indigestible fibers, CPC is highly nutritious, comprising 27–51% protein and 8% fat as a proportion of dry matter (He et al., 2015; Kumar et al., 2015). However, cottonseeds also contain the toxic polyphenolic compound gossypol, at a concentration of 0.4–1.5% free gossypol and 2–4% bound gossypol (Pons and Eaves, 1967). This is a natural insecticide, protecting the plant from herbivores (Li et al., 2016). Accordingly, CPC is generally unsuitable as feed because it causes growth depression, infertility and disorders of digestion (Herman, 1970; Berardi and Goldblatt, 1980; Liener et al., 1990; Francis et al., 2001). Thus far, only ruminants have been shown to tolerate CPC because they produce rumen proteins that detoxify gossypol by sequestration into inactive complexes (Reiser and Fu, 1962) prior to degradation by microbes, which use the gossypol as a carbon source (Zhang et al., 2018). Reflecting this microbial activity, fermented cottonseed meal therefore has lower levels of bound and free gossypol and is enriched with vitamins and enzymes (Zhang et al., 2006, 2007; Khalaf and Meleigy, 2008; Lim et al., 2010; Mageshwaran et al., 2017).

CPC has not been tested as a feed for insect larvae, but given that the versatility of BSFL reflects the presence of an adaptable microbiome, we hypothesize that CPC would be suitable as a feed because the microbial community would adapt in order to digest and detoxify compounds such as gossypol. We therefore evaluated the impact of CPC on the development and physiological performance of BSFL and analyzed the microbial communities of the BSFL gut and frass (residual feed substrate) in populations reared on CPC compared to those reared on the standard chicken feed diet. Illumina high-throughput sequencing of the bacterial 16S rRNA gene and the fungal internal transcribed spacer (ITS) was used to characterize the microbial population in detail and to identify diet-specific differences.

MATERIALS AND METHODS

Black Soldier Fly Breeding

Black soldier flies were obtained from Bio.S Biogas (Grimma, Germany). The flies were kept in mesh cages (Aerarium 60 × 60 × 90 cm, Bioform, Nuremberg, Germany) in a greenhouse at 25 ± 1°C, 40 ± 10% RH, and 12:12 (L:D) h according to Klüber et al. (2020) with slight modifications. Water was provided by water-soaked paper towels in a polypropylene container. Additionally, the flies were sprayed daily with water *ad libitum*. Eggs were harvested from oviposition sides (stacks of three wooded boards separated by washers) using plastic spatulas, placed in plastic boxes (19.5 × 16.5 × 9.5 cm) sprayed with water and closed with lids (200 mg eggs = ∼ 9,000 eggs per box). When ∼50% of the larvae had hatched, the lid was replaced with a fine mesh. Larvae were reared in a climate room at 27 ± 1°C and 65 ± 5% RH in the dark according to Klüber et al. (2020) with slight modifications.

As control diet we used Golddott Eierglück chicken feed (Raiffeisen, Münster, Germany). CPC (from mechanically extracted *Gossypium hirsutum* seeds) was obtained from a cotton ginning and oil mill factory (Kafantaris-Papakostas, Karditsa, Greece) where it was stored in a dry roofed warehouse for a few days to a few months until shipment. The concentration of free gossypol in the CPC constitutes about 0.06%. After delivery, the dry chicken feed and dry CPC (moisture content of 6–8%) were stored at room temperature in separate plastic lidded barrels (60 L). No transgenic feed was used in this study.

Prior to feeding to the larvae, the chicken feed and CPC were ground to an approximately similar particle size (range of 100–1,500 μm), using a Mockmill 200 grain mill (Wolfang Mock, Otzberg, Germany) and an EGK200 spice and coffee mill (Rommelsbacher, Dinkelsbühl, Germany), respectively. Particle size was measured with analytical sieves (Retsch, Haan, Germany). Freshly hatched larvae were initially fed with 10 g grinded feed. Boxes with larvae were checked daily for their need of water and feed.

Additional water and feed were provided *ad libitum* until larvae reached the prepupal stage. Moisture content of the substrate was measured with a TMT-MC-7828S soil moisture meter (OCS.tec, Neuching, Germany) and adjusted to approximately 70% by spraying and mixing the substrate with water. Disposable nitrile gloves were worn throughout the experiments. Separate populations of BSFL were reared continuously on chicken feed (established in July 2018) or CPC (established in February 2019) as the only diet for several generations.

Developmental and Physiological Parameters

Larval growth on the different diets was investigated with three replicate boxes per diet. Larvae used for the experiment were from generation 17 (chicken feed) and generation 13 (CPC). Briefly, 150 mg of eggs (same date of harvest; collected within 24 h) were placed in each box and the hatching date was recorded. Once larvae reached 3–4 mm in length (L3 larvae), pools of 50 larvae per box were weighed at 2 day intervals for all replicate boxes until 50% of the larvae had reached the prepupal stage (dark color). Subsampling was performed for each replicate box by removing larvae separately from the substrate, removing substrate particles from the larval surface with spring steel tweezers, subsequent drying with a paper tissue, and collecting 50 individuals in a weighing bowl.

The pH of the frass was measured in 2 day intervals (at the same time as larval weight measurement) by inserting pH-Fix pH 4.5–10.0 indicator strips (Macherey-Nagel, Düren, Germany). The temperature of the substrate was monitored in 2 day intervals with a TFX410 precision thermometer (Ebro, Ingolstadt, Germany). The final weight and length of 50 L5 larvae, prepupae and pupae were documented for each box. Larval length was measured for each individual under a VHX-2000 digital stereomicroscope (Keyence, Osaka, Japan) and the larval instar was determined by measuring the width of the head capsule according to Barros et al. (2019). Successful development of the stages larva–prepupa, prepupa–pupa and pupa–imago were determined in triplicates with 100 individuals each in 18.5 × 16.0 × 8.5 cm polypropylene containers. Feed was provided only for the evaluation of the development from larvae to prepupae, since prepupae and pupae do not feed. The developmental period was recorded as soon as 50% of the individuals reached the next developmental stage. Imagoes were collected within 24 h after emerging with spring steel tweezers and cold-inactivated on ice to determine their sex-specific weight and length.

Individual larval weight and gut weight were recorded 13 and 22 days after hatching. The pH of the gut was determined by placing L5 larvae on feed mixed with either 0.2% phenol red or 0.2% bromophenol blue (Bruno et al., 2019) in three replicate plastic boxes (11.5 × 9.5 × 6 cm, with holes) per diet. After incubation for 24 h, larvae were washed, and the guts were dissected under a stereomicroscope and photographed. The pH was determined by observing the coloring of the different gut sections with phenol red (pH $\geq$ 8.2 intensive red; pH $\leq$ 6.8 yellow) and bromophenol blue (pH $\leq$ 3.0 yellow; pH $\geq$ 4.6 blue). For intermediate values, a gradual color transition was observed for both indicators. Bacterial and fungal cell counts in the guts of BSFL were determined by homogenizing five guts of L5 larvae reared on each diet in a 16 ml culture tube with 5 ml phosphate-buffered saline and 1 g of glass beads (2 mm) by vortexing for 10 min. The gut homogenate was serially diluted and 200 µL of the dilutions (10^{-4}–10^{-8}) were plated on nutrient agar (CM0957, Oxoid, Wesel, Germany) and yeast glucose agar with chloramphenicol (Carl Roth, Karlsruhe, Germany). After incubation at 27°C for 4 days, bacterial and fungal colony forming units (CFU) on the respective agar were counted.

Nutritional Parameters of BSFL and Feed Substrates

L5 larvae (pool of 100 g per diet) and feed (20 g per diet) were ground with a mortar under liquid nitrogen and then lyophilized for 72 h. The following analyzes relate to dry matter. 0.5 g of lyophilized larvae and 1 g of lyophilized feed were used to determine crude protein and fat. Total nitrogen was determined following the Kjeldahl method (Kjeldatherm, Vapodest 500, Gerhardt, Königswinter, Germany) to calculate the crude protein value using conversion factors <6.25 (Lenaerts et al., 2018). For crude fat determination samples were manually disintegrated for 30 min in 2 mol·L^{-1} HCl, filtered, washed neutral and dried for 2 h at 105°C. The fat was extracted automatically with n-hexane in a Soxtherm system (Gerhardt, Königswinter, Germany) and the content was determined gravimetrically.

Sample Collection and DNA Extraction

L5 larvae from generation 12 (chicken feed) and generation 9 (CPC) were collected at the breeding facility and transferred to the laboratory. Frass samples were collected from the top left, middle and bottom right of same breeding containers and pooled. Feed samples were collected at three different time points within 1 week (days 1, 4, and 7) to identify microbes already present in the feed and to compensate for variations of the microbiota in the feed over time.

L5 larvae with a fresh weight of 100–150 mg were collected from one box per diet, washed with sterile water and rinsed twice with 70% ethanol. Separate vessels with water and ethanol were used for larvae from the different diets. Guts were dissected with sterile forceps under a stereomicroscope. Forceps were washed with sterile water and ethanol after each dissection. Single guts (nine replicates per diet for 16S rRNA gene sequencing and six replicates for ITS sequencing), 100 mg of fresh chicken feed and CPC (three replicates per diet), and 100 mg of frass (three replicates per diet) were disrupted by applying two cycles of bead beating in a FastPrep-24 (MP Biomedicals, Solon, OH, United States) for 45 s at 6.5 ms^{-1}. DNA was extracted with the NucleoSpin soil kit (Macherey-Nagel) according to the manufacturer's instructions, and checked for quantity and purity by spectrophotometry

on a Take 3 plate reader (BioTek Instruments, Winooski, VT, United States).

Analysis of the Microbial Community by Amplicon Sequencing

Libraries were prepared and sequenced by LGC Genomics (Berlin, Germany) using primers U341F (5′-CCT AYG GGR BGC ASC AG-3′) and U806R (5′-GGA CTA CNN GGG TAT CTA AT-3′) (Sundberg et al., 2013) to amplify variable region V3–V4 of the 16S rRNA gene of bacteria and archaea, or primers fITS7 (5′-GTG ART CAT CGA ATC TTT G-3′) (Ihrmark et al., 2012) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) (White et al., 1990) to amplify the fungal ITS2 region. The libraries were sequenced on an Illumina MiSeq V3 (San Diego, CA, United States) to generate ∼20,000 paired-end reads per sample with a read length of 300 bp. Samples were multiplexed and pooled for sequencing.

Demultiplexing of samples and the clipping of adapters and primers were carried out by LGC Genomics using bcl2fastq 2.17.1.14 software (Illumina, Inc., San Diego, CA, United States). Reads were analyzed using QIIME 2020.6 (Bolyen et al., 2019). ITS sequences were also trimmed if the synthesized strand reached the second sequencing adapter (read-through) using the cutadapt plugin (Martin, 2011) before further analysis. Only forward reads of the ITS amplicons were analyzed. We used the DADA2 plugin (Callahan et al., 2016) for error correction, quality control, filtering of chimeric sequences, and the creation of an amplicon sequence variant (ASV) table containing the number of sequences for each observed ASV per sample.

Bacterial taxonomic classification was carried out using a self-trained naïve Bayes classifier on SILVA 132 QIIME-compatible release with 99% sequence identity (Quast et al., 2013). We also trimmed the reference sequences to the specific region of the 16S rRNA gene targeted with the amplicon primers (Werner et al., 2012). Confidence was set to 0.7 as recommended (Bokulich et al., 2018). Fungal taxonomic classification was carried out using a self-trained naïve Bayes classifier on UNITE 8.2 QIIME-compatible release with 99% sequence identity (Abarenkov et al., 2020) and a minimum confidence of 0.94 (Bokulich et al., 2018). Sequences originating from mitochondria or chloroplasts were removed from the classified datasets. Alpha diversity was based on Faith's phylogenetic diversity (Faith, 1992) and observed ASVs. Beta diversity was determined using UniFrac distance metrics (Lozupone and Knight, 2005), and we rarefied the 16S rRNA and ITS data to equal sequencing depths of 5,484 and 2,639 reads, respectively.

Statistical Analysis

The linear relationship between weight gain and pH of the frass was calculated using Pearson product-moment correlation (Hilgers et al., 2019). Significant differences in developmental and physiological parameters were tested with Student's *t*-test. Differences in relative abundances of certain taxa between the two diet groups were determined by applying Student's or Welch's *t*-test (Welch, 1947) depending on the homogeneity of variance verified by a Levene's test (Levene, 1960) with significance at 0.05. For statistics and graphics we used Microsoft Excel and R v4.0.3 with packages qiime2R, tidyverse, car, dplyr, plyr, scales, ggpubr, and ggplot2.

RESULTS

Black Soldier Fly Developmental, Physiological, and Nutritional Parameters

The life cycle of black soldier flies reared on CPC showed many similarities to that of the flies raised on chicken feed, but the total developmental cycle was significantly ($p < 0.001$; df = 4; $t = 2.13$) shorter on the CPC diet (43.4 ± 1.8 days) compared to the chicken feed diet (47.3 ± 3.5 days). Hatching time, larval development and the transition from prepupa to pupa were similar on both diets, whereas intrapuparial metamorphosis was significantly ($p < 0.001$; df = 4; $t = 2.13$) faster in the CPC diet group. The rates of successful development (larva–prepupa and pupa–imago) were also similar for both diets. Only the developmental success from prepupa to pupa was significantly ($p < 0.000005$; df = 4; $t = 2.13$) lower on the chicken feed diet (90.7%), whereas 100% of the prepupae in the CPC diet group reached the pupal stage (**Table 1**).

We also observed major differences in larval growth between the two diets. Weight gain was similar for the first 11 days on both diets. However, whereas larvae reared on chicken feed gained weight continuously until day 19, those reared on CPC gained little weight from days 11 to 15, then experienced a 2 day growth phase followed by a longer plateau lasting 5.5 days until ≥50% prepupae were present (**Figure 1**). A pause in larval growth was also observed in a repeating trial only with larvae reared on CPC but not with larvae reared on chicken feed (**Supplementary Figure 1**). The final larval weight was significantly ($p < 0.00001$; df = 10; $t = 1.83$) lower in the CPC diet group and the weight of imagoes reared on CPC was lower than that of imagoes reared on chicken feed. In contrast, the average gut weight of larvae reared on CPC was significantly higher ($p < 0.00001$ after 13 days and $p < 0.03$ after 22 days; df = 43; $t = 1.68$) than corresponding samples in the chicken feed diet group. Therefore, the proportion of the gut to the total larval body was also higher for larvae reared on CPC compared to larvae reared on chicken feed after 13 days and after 22 days (**Table 1**).

The pH of the frass showed a similar trend during larval development on both diets, but the pH of the CPC frass was slightly higher than that of the chicken feed frass. Furthermore, we observed a moderate negative correlation between growth and pH from days 9 to 15 ($r = -0.47$) only with larvae reared on CPC. The increase in pH of the frass goes along with the slowdown of growth on days 11–15, followed by a decrease in pH accompanied by the resuming of larval growth (**Figure 1**). The temperature of the substrate mixed with larvae was similar in each replicate box. Furthermore, there were only minor differences in temperature between the boxes with larvae reared on chicken feed (30.8 ± 2.36°C) and the boxes with larvae reared on CPC (29.5 ± 0.84°C) throughout the period of measurement

(**Supplementary Table 1**). The luminal pH of the various gut sections was similar for larvae reared on both diets. The lumen of the anterior midgut was slightly acidic (pH $\leq$ 6.8). The lumen of the middle midgut contained a strongly acidic region (pH $\leq$ 3.0), whereas the lumen of the posterior midgut was alkaline (pH $\geq$ 8.2). The lumen of the hindgut was neutral to alkaline (pH $\geq$ 6.8) given that some individuals showed orange coloring (pH 6.8–8.2) and some individuals showed intensive red coloring of the hindgut (pH $\geq$ 8.2) when incubated with phenol red, regardless of diet (**Supplementary Figure 2**).

The count of bacterial CFU was 2.6×10^7 mg^{-1} gut in BSFL reared on CPC and 6.8×10^6 mg^{-1} gut in BSFL reared on chicken feed. The count of fungal CFU was much lower than the count of bacterial CFU, with 5.5×10^4 mg^{-1} gut and 5.0×10^5 mg^{-1} gut in BSFL reared on CPC and chicken feed, respectively. Crude protein of the larvae reared on CPC was higher than that of larvae reared on chicken feed whereas for crude fat the opposite was the case (**Table 1**).

Analysis of the Bacterial and Archaeal Communities by Amplicon Sequencing

We generated 711,390 read pairs for the 30 samples (nine samples of individual guts, three feed samples, and three frass samples per diet). Quality control, including the removal of chimeric sequences, reduced the total to 605,424 read pairs. After joining forward and reverse reads, the average read length was 419 bp. Thereby, the numbers of reads per sample ranged from 5,484 to

TABLE 1 | Developmental and physiological parameters of black soldier flies reared on chicken feed (CF) and cottonseed press cake (CPC).

	Sample size	CF	CPC
Hatching time (d)	≥50%	3.0 ± 0.8	3.0 ± 0.8
Larval development (d)[a]	≥50%	22.3 ± 0.5	23.7 ± 0.5
Prepupa–pupa (d)	*n* = 300	11.3 ± 1.7	10.0 ± 0.0
Intrapuparial metamorphosis (d)[b]	*n* = 300	10.7 ± 0.5	6.7 ± 0.5
Total development (d)[c]	*n* = 300	47.3 ± 3.5	43.4 ± 1.8
Successful development larva–prepupa (%)	*n* = 300	100.00 ± 0.00	99.33 ± 0.94
Successful development prepupa–pupa (%)	*n* = 300	90.67 ± 0.47	100.00 ± 0.00
Successful development pupa–imago (%)	*n* = 300	97.00 ± 2.45	99.67 ± 0.47
Final larval weight (mg)	*n* = 300	285.14 ± 26.40	160.36 ± 16.08
Final larval length (mm)	*n* = 150	25.96 ± 0.07	18.65 ± 0.18
Weight prepupa (mg)	*n* = 150	219.60 ± 18.69	101.80 ± 9.59
Length prepupa (mm)	*n* = 150	23.43 ± 0.12	16.82 ± 0.40
Weight pupa (mg)	*n* = 150	168.97 ± 10.74	93.89 ± 4.91
Length pupa (mm)	*n* = 150	22.65 ± 0.08	16.95 ± 0.17
Weight imago ♂ (mg)	*n* = 90	89.42 ± 8.51	40.18 ± 3.98
Weight imago ♀ (mg)	*n* = 150	103.57 ± 8.29	59.09 ± 3.56
Weight imago, total (mg)	*n* = 255	98.05 ± 8.02	50.64 ± 3.50
Length imago ♂ (mm)[d]	*n* = 90	16.76 ± 0.25	14.41 ± 0.19
Length imago ♀ (mm)[d]	*n* = 150	17.50 ± 0.25	15.77 ± 0.24
Length imago, total (mm)[d]	*n* = 255	17.20 ± 0.24	15.15 ± 0.23
Weight 13 d larvae (mg)	*n* = 25	124.17 ± 19.49	118.87 ± 9.66
Weight 22 d larvae (mg)	*n* = 25	247.00 ± 38.51	162.36 ± 25.65
Gut weight 13 d larvae (mg)	*n* = 25	25.43 ± 7.75	35.73 ± 5.01
Proportion of gut to total body of 13 d larvae (%)	*n* = 25	20.48 ± 6.24	30.06 ± 4.21
Gut weight 22 d larvae (mg)	*n* = 25	42.70 ± 19.50	52.14 ± 13.78
Proportion of gut to total body of 22 d larvae (%)	*n* = 25	17.29 ± 7.89	32.11 ± 8.49
pH anterior midgut	*n* = 10	≤6.8	≤6.8
pH middle midgut	*n* = 10	≤3.0	≤3.0
pH posterior midgut	*n* = 10	≥8.2	≥8.2
pH hindgut	*n* = 10	≥6.8	≥6.8
Crude protein of larvae (%DM)	*n* = 300	38.60 ± 1.24	47.43 ± 0.08
Crude protein of feed (%DM)	–	18.47 ± 0.18	24.76 ± 0.62
Crude fat of larvae (%DM)	*n* = 300	33.94 ± 1.09	23.34 ± 1.23
Crude fat of feed (%DM)	–	2.49 ± 0.03	8.65 ± 0.51

Values are means ± standard deviation.
[a] *Period from hatching to ≥50% prepupal populations.*
[b] *Period until ≥50% of the adults emerged from the pupae.*
[c] *Period from oviposition to adults emerging.*
[d] *Distance between cranial antennae attachment points and abdominal appendages.*

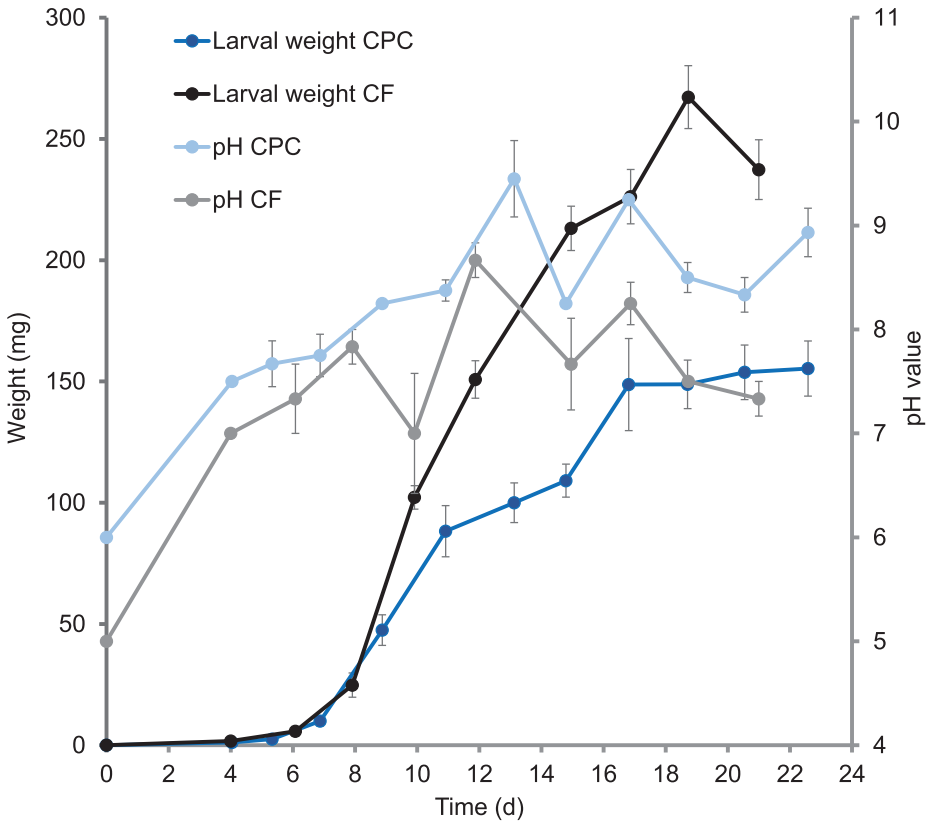

FIGURE 1 | Growth curve of larvae reared on chicken feed (CF) and cottonseed press cake (CPC), and the pH of the corresponding frass. The average body weight per BSFL and average pH values of the frass are shown. Symbols are means (+ SEM) of measurements with three replicates (containers with BSFL + substrate).

40,144. Rarefaction curves suggested the sequencing depth was sufficient (**Figure 2**).

After using DADA2 and our self-trained naïve Bayes classifier based on SILVA 132 followed by removing mitochondrial and chloroplast sequences we identified 170 unique amplicon sequence variants (ASVs) across our 30 samples. The same classifier assigned taxonomic labels down to the genus level for 146 of the 170 ASVs. All classified ASVs belonged to the domain Bacteria; no Archaea were found. The detailed results of the classification on all taxonomic levels (from phylum to species) and relative abundances of ASV counts are shown in **Supplementary Table 2**.

For all feed samples, the sum of ASV counts was very low (maximum 428 counts per sample). No bacteria were found in two of the three chicken feed samples, and only 21 counts were detected in the remaining sample. These were classified as *Enterobacteriaceae* (*Pantoea*), *Pseudomonadaceae* (*Pseudomonas*), and *Microbacteriaceae* (*Curtobacterium*). In contrast, CPC feed samples featured a more diverse bacterial community dominated by different groups of *Enterobacteriaceae, Pseudomonadaceae,* and *Bacillaceae*, which were present in all replicate feed samples (**Figure 3**). *Enterobacteriaceae* were the dominant family in all gut samples. However, the relative abundance was significantly ($p < 0.05$) higher in guts of larvae reared on chicken feed. Furthermore, *Enterococcaceae* and *Actinomycetaceae* were present in all replicate gut samples from both diet groups. Interestingly, *Actinomycetaceae* were significantly ($p < 0.002$) enriched only in the guts of larvae reared on CPC. *Lachnospiraceae* were present at a low abundance in almost all gut samples, regardless of the diet. *Burkholderiaceae*, *Rhizobiaceae*, *Erysipelotrichaceae,* and *Clostridiales* Family XI were only found in gut samples of larvae reared on CPC (at least five of nine replicates) whereas *Corynebacteriaceae* and *Lactobacillaceae* were only present in gut samples of larvae reared on chicken feed (at least three of nine replicates) (**Figure 3**).

We observed pronounced differences between the two diet groups at the genus level. *Morganella* was the most prominent genus (67.2–95.6% in eight of nine replicates) in the guts of larvae reared on chicken feed, whereas *Providencia* was more abundant in the CPC diet group (≥32% in seven of nine replicates, compared to ≤2% in all replicates in the chicken feed group) (**Supplementary Table 2** and **Supplementary Figure 3**). Almost all bacterial families found in gut samples were also present in frass samples, although the relative abundance varied. However, we also observed pronounced differences between the gut and frass microbiomes, especially in the CPC group where families such as *Sphingobacteriaceae*, *Paenibacillaceae*, *Clostridiaceae* 2, and *Caulobacteraceae* were found in the frass but were completely absent from the gut. CPC frass was dominated by *Burkholderiaceae* (mostly classified as

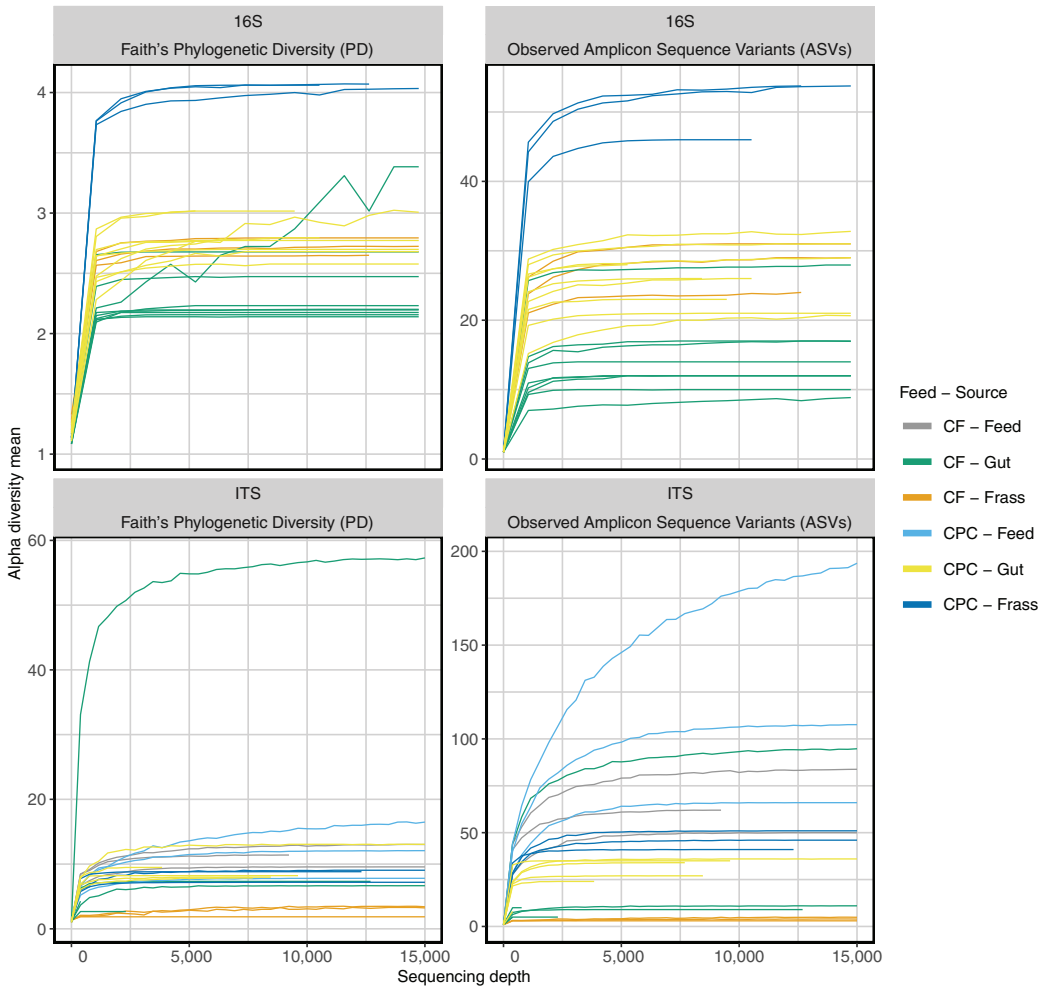

FIGURE 2 | Rarefaction curves of 16S rRNA gene and ITS sequencing for all samples. Curves reach a plateau for nearly all samples of the chicken feed (CF) and cottonseed press cake (CPC) diet groups after ∼10,000 reads. Even samples with fewer reads reach a plateau for ITS and 16S rRNA gene sequencing. Therefore, higher sequencing depth does not influence microbial diversity (Faith's phylogenetic diversity and observed amplicon sequence variants) found in the sequencing data.

Alcaligenes, 44–54%), *Rhizobiaceae* (7–18%), *Carnobacteriaceae* (7–11%), *Microbacteriaceae* (4–7%), and *Bacillaceae* (4–5%) in all replicates, whereas chicken feed frass was dominated by *Corynebacteriaceae* (44–57%), *Lactobacillaceae* (30–33%), and *Enterobacteriaceae* (3–9%). Furthermore, *Staphylococcaceae* and *Aerococcaceae* were present at a lower abundance in chicken feed frass but were completely absent from the gut samples. The most abundant families in the CPC frass were also present in some replicates of the feed samples, but were absent or scarce in the corresponding gut samples (**Figure 3** and **Supplementary Table 2**).

When larvae were reared on CPC, most of the gut and frass samples showed a higher alpha diversity of bacteria (Faith's phylogenetic diversity and observed ASVs) when compared to those raised on chicken feed (**Figure 2** upper part; **Figures 4A,B**). Principal coordinate analysis (PCoA) of weighted UniFrac distances (**Figure 4A**) revealed that some gut samples of larvae reared on both diets clustered together, whereas PCoA of unweighted UniFrac distances (**Figure 4B**) indicated more differences between the two diet groups. Little similarity was observed between gut samples and frass samples on either diet (**Figure 4A**), but the differences were greater for the CPC diet group. We also observed a large difference between CPC and chicken feed frass samples (**Figures 4A,B**).

Analysis of the Fungal Community by Amplicon Sequencing

We generated 535,559 raw reads for ITS sequencing from the 24 samples (six samples of individual guts, three feed samples, and three frass samples per diet), with 488,426 reads remaining after quality control (540–99,232 per sample). After trimming, the read length range was 83–273 bp. The average read length was 259 bp. Rarefaction curves suggested the sequencing depth was sufficient (**Figure 2**). DADA2 identified 854 unique ASVs across 24 samples, 413 of which were classified to the genus level using

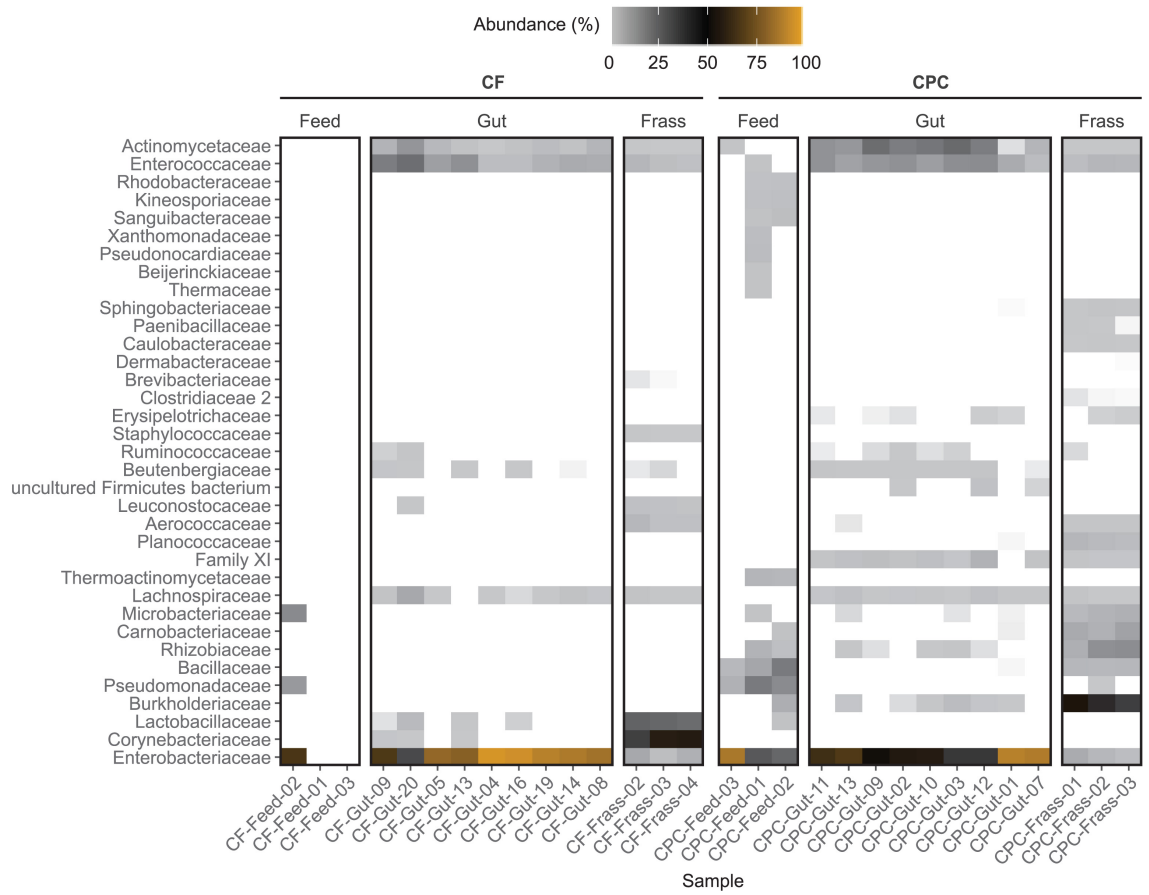

FIGURE 3 | Heat map showing the family-level composition of the bacterial community of feed, BSFL guts and BSFL frass in the chicken feed (CF) and cottonseed press cake (CPC) diet groups based on 16S rRNA gene amplicon sequencing. The relative abundances of amplicon sequence variant (ASV) counts are collapsed to the family level for all replicates. Undefined families are not shown. Percentages of relative abundances are displayed by coloring from light gray over black to orange representing the highest abundance. Families depicted in white were not detected in the sample. Only the top 35 families are shown, measured by the mean counts per family level for all samples.

our naïve Bayes classifier based on UNITE 8.2 with a minimum confidence of 0.94. The detailed results of the classification on all taxonomic levels (from phylum to species) and relative abundances of ASV counts are shown in **Supplementary Table 3**.

The ITS amplicon sequencing revealed higher sums of ASV counts in the feed samples (9,579–99,232 counts per sample) compared to the 16S amplicons. Most of the classified ASVs in chicken feed samples represented the families Cladosporiaceae (all *Cladosporium*), Pleosporaceae (*Alternaria* and *Stemphylium*), Aspergillaceae (mostly *Xeromyces* and *Aspergillus*), and Nectriaceae (mostly *Fusarium*) and were found in all replicates. These families were also found in all replicates of the CPC feed samples but their relative abundance was on average lower (**Figure 5** and **Supplementary Figure 4**). However, most of the ASV counts in the CPC feed samples (38–68%) could not be classified, whereas only a minority of sequences in the chicken feed samples (1–11%) remained unclassified.

In all gut and frass samples (regardless of diet) less than 21% of the ASV counts remained unclassified (**Supplementary Table 3**). The fungal gut microbiota of larvae reared on chicken feed and CPC was dominated by the family Trichosporonaceae (49.6–90.4% in four samples of larvae reared on chicken feed and five samples in larvae reared on CPC). Furthermore, the family Cladosporiaceae (all *Cladosporium cladosporioides*) and Saccharomycetales family *incertae sedis* (all *Diutina rugosa*) were relatively abundant in at least five replicates of both diets. *Trichosporon* (all classified as *Trichosporon asahii*) and *Diutina* (all classified as *Diutina rugosa*) were the only two genera found in frass samples of larvae reared on chicken feed, whereas frass samples of larvae reared on CPC showed a highly diverse community of many different fungal groups (**Figure 5** and **Supplementary Table 3**).

Although the relative abundance of the Aspergillaceae was higher in the feed samples of both diets (2.5–38.8%), this family was only present in the guts of larvae reared on CPC (5.5–17.9% in five of six replicates) and was completely absent from the guts of larvae reared on chicken feed (**Figure 5**). Most Aspergillaceae in the gut were assigned to the genus *Aspergillus*. Most of them were not classified to species level, except *Aspergillus glaucus*, *A. penicillioides,* and *A. protuberus.* The remaining Aspergillaceae

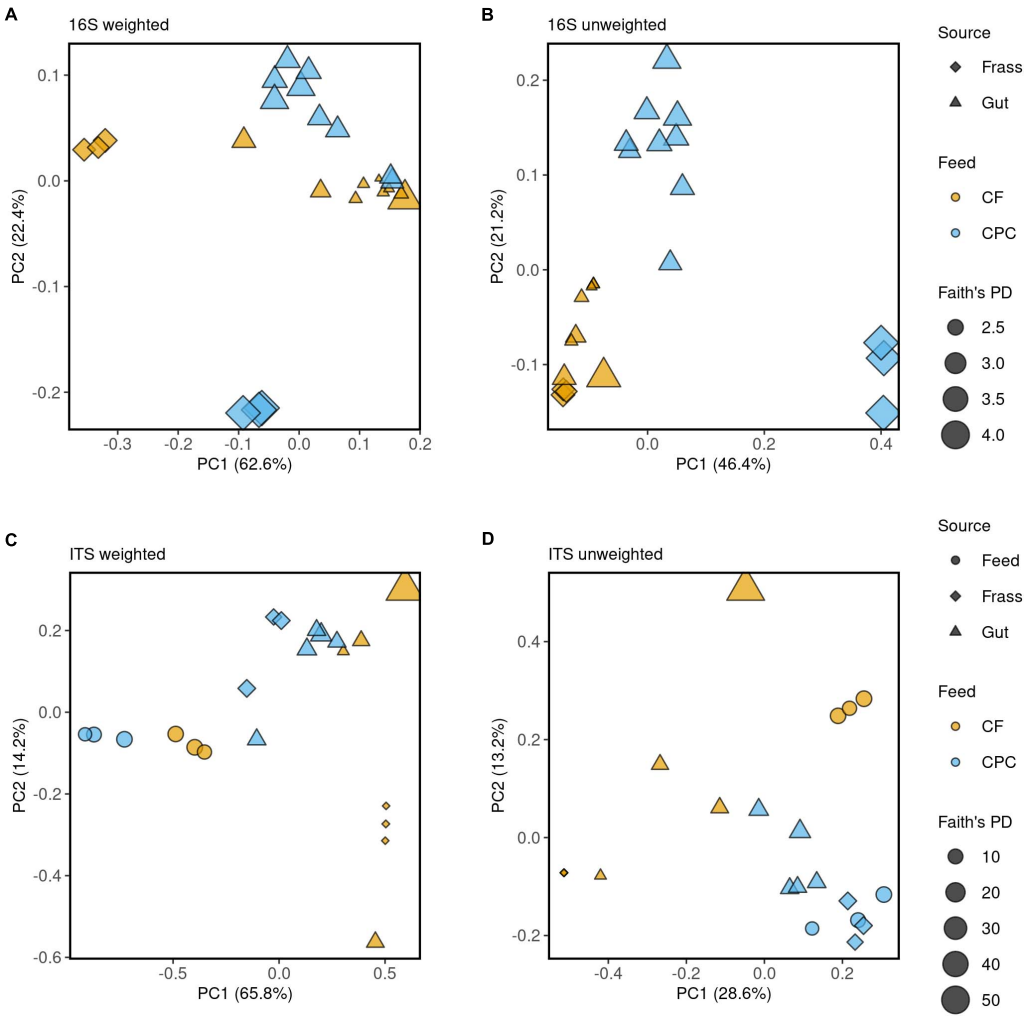

FIGURE 4 | Principal coordinates analysis (PCoA) of the microbial community structure among chicken feed (CF) and cottonseed press cake (CPC), BSFL guts and BSFL frass samples. **(A,B)** PCoA of bacterial communities calculated on weighted and unweighted UniFrac distance metrics, respectively. **(C,D)** PCoA of fungal communities calculated on weighted and unweighted UniFrac distance metrics, respectively. The 16S rRNA and ITS data were rarefied to an equal sequencing depth 5,484 and 2,639, respectively. The source of the sample is indicated by symbols color coded by feed source (CF and CPC), as displayed in the legend on the right side of the figure. Faith's phylogenetic diversity (PD) is indicated by the size of the symbol.

were mostly classified as *Xeromyces bisporus,* which showed a higher relative abundance (1–12%) in four of six samples of larvae fed on CPC. Furthermore, Trichocomaceae and Microascaceae (all classified as *Acaulium acremonium*), which were present in most of the replicates of larvae reared on CPC, were not detected in any replicates of larvae reared on chicken feed (**Figure 5** and **Supplementary Table 3**).

When larvae were reared on CPC, most gut and frass samples showed a higher alpha diversity of fungi (Faith's phylogenetic diversity and observed ASVs) compared to chicken feed (**Figure 2** lower part; **Figures 4C,D**). In contrast to the bacterial microbiome, there was more similarity between the fungal communities in the gut and frass samples of larvae reared on CPC (**Figures 4C,D**). Few similarities between gut samples of larvae reared on the different diets were observed in the PCoA of unweighted UniFrac distances, whereas the PCoA of weighted UniFrac distances showed that some gut samples representing the two diet groups clustered closer together. Like the bacterial microbiome, the fungal community in the CPC frass differed substantially from that in the chicken feed frass (**Figures 4C,D**).

DISCUSSION

Developmental, Physiological, and Nutritional Parameters of Black Soldier Flies

Here, we show that BSFL can be reared on CPC despite the presence of the normally insecticidal secondary metabolite gossypol. This is corroborated by the significantly shorter

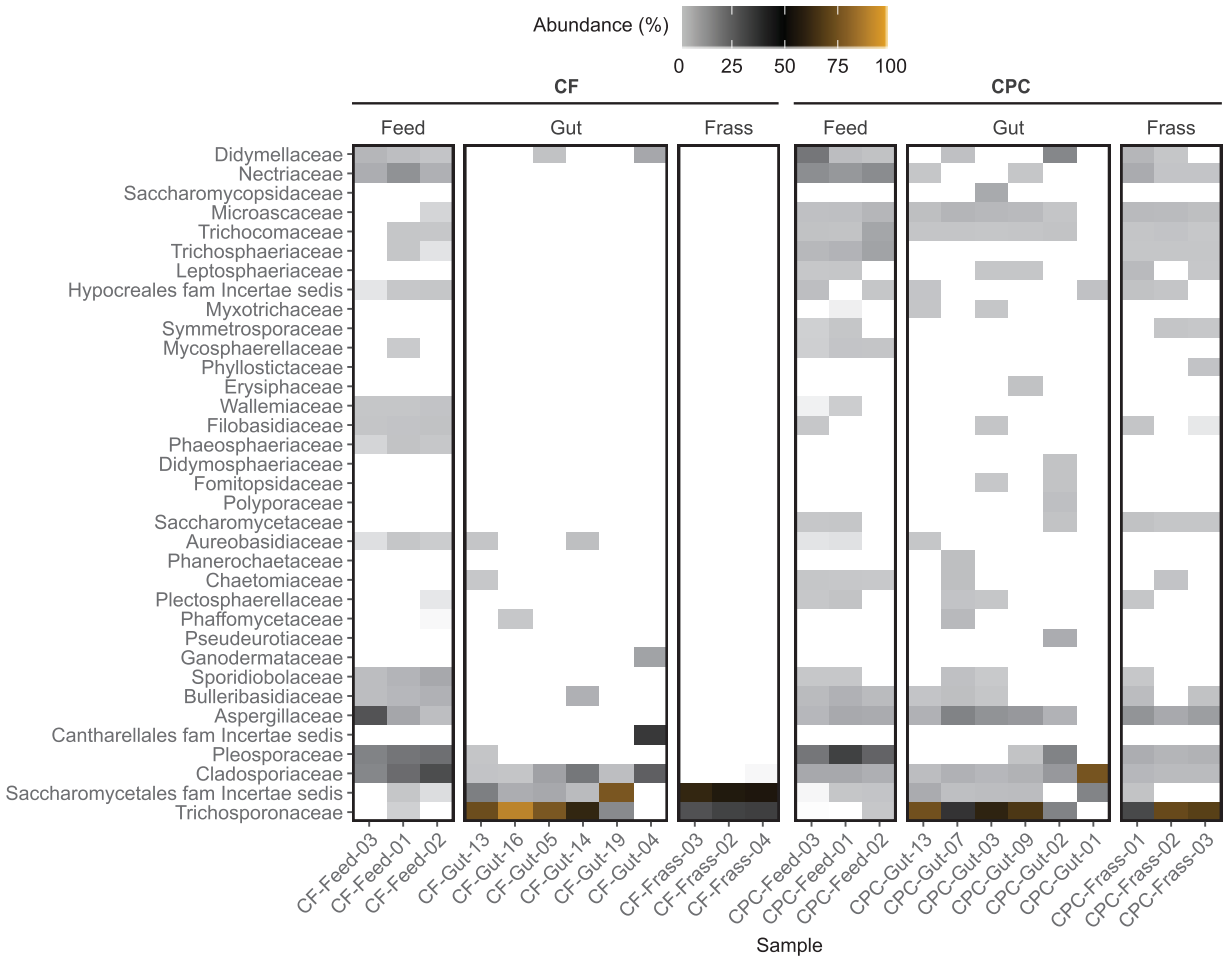

FIGURE 5 | Heat map showing the family-level composition of the fungal community of feed, BSFL guts and BSFL frass in the chicken feed (CF) and cottonseed press cake (CPC) diet groups based on ITS amplicon sequencing. The relative abundances of amplicon sequence variant (ASV) counts are collapsed to the family level for all replicates. Undefined families are not shown. Percentages of relative abundances are displayed by coloring from light gray over black to orange representing the highest abundance. Families depicted in white were not detected in the sample. Only the top 35 families are shown, measured by the mean counts per family level for all samples.

developmental time and the higher rates of successful development of prepupa to pupa of BSF reared on CPC. In contrast, several other insects show lower rates of successful development and a prolonged developmental time when subjected to gossypol (Bottger and Patana, 1966; Meisner et al., 1978; Stipanovic et al., 2006). The significantly shorter intrapuparial metamorphosis might be related to differences in microbial community composition in BSF reared on CPC, since microbial symbionts can produce hormones that influence insect signaling pathways to promote development and metamorphosis of the host (Paludo et al., 2018; Hammer and Moran, 2019; Wu et al., 2020). The accelerated development and higher rate of successful development of BSFL fed on CPC observed in this small-scale BSF-breeding facility may also apply to industrial insect farming and might be profitable for large-scale production.

The similar early growth performance of BSFL reared on CPC in comparison to the control group reared on chicken feed is remarkable, indicating that freshly-hatched larvae rapidly convert the substrate and are not harmed by the toxic components of CPC during this time period. However, the subsequent slowdown of larval growth for several days contributed to a significantly lower final weight. In the Lepidoptera gossypol also caused a lower weight of larvae and pupae (Bottger and Patana, 1966; Stipanovic et al., 2006) which is attributed to an inhibition of digestive enzymes (Meisner et al., 1978). However, such a negative effect on the insect's digestion would also cause a significant delay in development and a decrease in rates of successful development as also shown by Meisner et al. (1978), which is not the case for BSFL. The brief pause in growth observed in the CPC diet group after 11 days may be caused by other effects of gossypol e.g., by an increased energy-consuming detoxification activity due to an accumulation of this compound at this time point. However, so far we do not know the exact concentration of gossypol of this particular CPC. According to the manufacturer the concentration of free gossypol

in the CPC constitute about 0.06% but can vary depending on humidity conditions of the cotton when harvested. Since most of the gossypol in the seeds is bound to proteins (2–4% bound gossypol; Pons and Eaves, 1967), we assume that the amount of bound gossypol is also higher than the amount of free gossypol in the CPC used in this study.

The high protein and fat content of CPC suggests that interruption of growth and the lower final larval weight of BSFL reared on CPC are likely not a result of malnutrition. However, such a growth pause or a period of low weight gain would be unfavorable for large-scale production. Since BSFL reared on CPC showed highest weight gain in the period from days 7 to 11, an early harvest of the larvae when industrially reared should be considered. Although the CPC feed showed a much higher fat content as chicken feed, larvae fed with CPC even showed a lower fat content than larvae fed with chicken feed. The low fat and high protein content of these larvae might also be beneficial for future food and feed use. However, detailed measurements, including the quantification of gossypol in the feed, larvae and frass at different time points, as well as a more detailed evaluation of the nutritional value of the CPC and the larvae, would be required to confirm our hypotheses.

Growth inhibition might also be related to the increase in pH, implied by the negative correlation between growth and pH from days 9 to 15 for the larvae reared on CPC. The increase in pH during the rearing on nitrogen-rich substrates is caused by the formation of ammonia (Green and Popa, 2012; Klammsteiner et al., 2020; Pang et al., 2020; Parodi et al., 2020), which can also inhibit gowth of dipteran larvae (Wang and Leung, 2015; Dias et al., 2019). However, also other so far unknown effects, e.g., other incompatible components of the CPC diet may cause a pause of larval growth.

The pH of the BSFL gut showed a remarkable transition from the acidic region ($\leq$3.0) in the middle midgut to the alkaline region ($\geq$8.2) in the posterior midgut, as previously reported (Bruno et al., 2019). Although we and others (Klammsteiner et al., 2020) found that the type of diet influences the pH of the substrate in which the larvae grow, the pH of the gut lumen appears to be largely unaffected by the diet and instead reflects the specific morphofunctional features of the dipteran midgut. Like the BSFL, other dipteran larvae such as those of the housefly (*Musca domestica*) and the fruit fly (*Drosophila melanogaster*) feature an acidic region ($\leq$3.0) in the middle midgut (Lemos and Terra, 1991). The low pH is generated by specialized midgut cells, so-called copper cells (McNulty et al., 2001; Dubreuil, 2004; Shanbhag and Tripathi, 2009), which were recently also found in BSFL (Bonelli et al., 2019). Our data suggest that the luminal gut pH is host-specific and is not strongly affected by diet-dependent shifts in the microbiome. However, the luminal pH of the insect gut probably influences the colonization of the gut by certain microbes based on their functional pH range. Populations of *Salmonella enterica* and *Escherichia coli* in chicken manure, were reduced by BSFL which is attributed to changes in pH of the manure (Erickson et al., 2004). Furthermore, passage through the acidic and alkaline regions of the gut leads to the selection of certain microorganisms which colonize the gut and also the substrate.

The significantly higher gut weight of larvae reared on CPC might be caused by a higher feed intake and a higher microbial colonization density, in order to boost the metabolic activity (e.g., gossypol degradation) within the gut. Studies with germ-free cockroaches have also shown that individuals raised under aseptic conditions showed a significantly lower gut weight when compared to normal cockroaches with an abundant gut microbiota (D. Tegtmeier, unpublished data). This suggests that the microbial load probably also affects the gut weight of BSFL. The higher gut weight of larvae reared on CPC is further corroborated by the higher bacterial CFU counts in the guts of these larvae. However, these numbers only represent the proportion of BSFL gut bacteria from both diet groups, that can be cultured on standard medium, while the total quantity is likely higher.

The BSFL Bacterial and Archaeal Communities

The presence of *Enterobacteriaceae* (mostly *Morganella* and *Providencia*), *Enterococcaceae,* and *Actinomycetaceae* in all individual gut samples regardless of diet indicates that these groups belong to the BSFL core microbiome. The dominance of *Morganella* in gut samples from larvae in the chicken feed group and in most corresponding samples from the CPC group agrees with studies by Wynants et al. (2019), who also found a dominance of this genus in BSFL reared at two different locations. Furthermore, *Morganella* was prominent in BSFL reared on chicken feed (Cifuentes et al., 2020) and calf forage, and it was also present (albeit with lower relative abundance) when larvae were fed other diets (Jeon et al., 2011). Besides *Morganella*, also the genera *Providencia* and *Enterococcus* (*Enterococcaceae*) were previously found to be abundant in BSFL guts regardless of diet, rearing conditions or developmental stage, suggesting they form the core microbiome (Zheng et al., 2013; Wynants et al., 2019; Cifuentes et al., 2020). Also, a cultivation-dependent study has shown that the majority bacteria cultivated from BSFL guts belong to the *Enterobacteriaceae* and a large proportion of the isolates were identified as *Providencia*, *Morganella,* and *Enterococcus* (Callegari et al., 2020). *Providencia* may even be vertically transmitted, because it was detected in imagoes and eggs (Zheng et al., 2013). This is a reasonable assumption because *Providencia* and *Enterococcaceae* found in the guts samples in our experiments were completely absent in feed samples and may therefore be vertically transmitted via the eggs or taken up by the larvae from the surface of the eggs.

The high relative abundance of *Enterobacteriaceae* (in particular *Morganella* and *Providencia*) indicates a functional role of these bacteria within the BSFL gut. *Providencia rettgeri* and *Morganella morganii* are also abundant endogenous members of the gut microbiome of the burying beetle (*Nicrophorus vespilloides*), both in adults (Vogel et al., 2017; Heise et al., 2019) and in larvae (Vogel et al., 2017; Wang and Rozen, 2017), and were reported to protect larvae against pathogens (Wang and Rozen, 2018). Furthermore, *P. rettgeri* and *M. morganii*

produce extracellular bacteriolytic enzymes that can degrade components of *E. coli* and *Pseudomonas aeruginosa* cell walls (Branca et al., 1996; Janda and Abbott, 2015). The high relative abundance of *Morganella* and *Providencia* in L5 larvae may explain why we did not detect *Pseudomonas* in any of the gut samples, although it showed a high relative abundance in chicken feed and CPC samples. This may also explain our frequent observation of *Pseudomonas* contamination, revealed by a green biofilm on top of the substrate and a characteristic odor of linden blossom only present during the initial rearing phase (2–6 days). This contaminant is usually eliminated by the larvae when they gain weight and reach a certain size. Furthermore, *Pseudomonas* was absent in BSFL samples from rearing locations with a high relative abundance of *Morganella*, but was present in samples where *Morganella* was scarce (Wynants et al., 2019). The reanalysis of published data (Cifuentes et al., 2020), revealed a negative correlation between the occurrence of *Pseudomonas* and *Morganella* + *Providencia* ($r = -0.26$) suggesting that these two genera help BSFL to resist colonization by pathogens.

Morganella and possibly other *Enterobacteriaceae* in BSFL guts likely contribute to the digestion of the protein-rich substrate and the breakdown of uric acid, as *Morganella morganii* possesses genes for proteases (Chen et al., 2012; Zamaliutdinova et al., 2014) and exhibits urease activity (Janda and Abbott, 2015). Furthermore, *Morganella*, and other *Enterobacteriaceae* are known for oxidative deamination of various amino acids and nitrate reduction (Janda and Abbott, 2015). In the course of nitrogen metabolism several *Enterobacteriaceae* (including *M. morganii*) generate ammonia (Özoğul, 2004). Therefore, ammonia emissions during the BSFL rearing process, are attributed (at least partially) to the abundant genera *Morganella* and *Providencia* in the guts of BSFL.

Most of the bacteria detected in the feed were not found to be abundant in gut samples of L5 larvae, suggesting the feed is unlikely to be the major source of inoculum for establishing the bacterial community in the gut. However, we found that some groups present in the CPC feed were also present in the frass. The abundance of certain bacterial groups only in the feed and frass but not in the gut indicates that many ingested bacteria find the physiochemical conditions in the gut unwelcoming (especially the extreme pH conditions) or are displaced by other microbes. This observation also explains the pronounced differences between the bacterial communities of the frass and the gut (Jiang et al., 2019; Wynants et al., 2019; Cifuentes et al., 2020).

The higher bacterial alpha diversity in BSFL reared on CPC may reflect the greater complexity of the diet, as previously shown for BSFL (Jeon et al., 2011) and other insects (Huang et al., 2013). In contrast to our results, most previous studies of the BSFL microbiome did not identify *Actinomycetaceae* as an abundant component, despite the use of diverse substrates including the Gainesville diet, different types of food waste, vegetables, and fish meal (Zheng et al., 2013; Bruno et al., 2019; Jiang et al., 2019; Wynants et al., 2019). *Actinomycetaceae* have been reported as part of the BSFL gut microbiome when larvae are reared on chicken feed, albeit with a low abundance (Cifuentes et al., 2020) similar to our results obtained with BSFL reared on chicken feed. In our study, the relative abundance of *Actinomycetaceae* was significantly higher in the larvae reared on CPC, suggesting that this bacterial family may play a key role in the metabolism of CPC-specific components such as gossypol. *Actinomycetales* are known for the degradation of complex molecules such as lignocellulose (McCarthy, 1987; Wang et al., 2014) and a variety of pesticides including many phenolic compounds (Schrijver and Mot, 1999). Gossypol is a phenolic pesticide (Li et al., 2016) which supports the potential role of these bacteria in its degradation. Other families present only in larvae reared on CPC (*Burkholderiaceae*, *Rhizobiaceae*, *Erysipelotrichaceae,* and *Clostridiales* Family XI) may also be involved in the degradation of such compounds, but little is known about gossypol degradation in bacteria. The only gossypol-degrading bacterial isolate known thus far is a *Bacillus subtilis* strain isolated from the cow rumen, but this is not yet available in a culture collection (Zhang et al., 2018).

Changes in the BSFL microbiome could affect greenhouse gas emissions, especially the production of methane, which is exclusively attributed to the Archaea (Enzmann et al., 2018). Therefore, also studies about the archaeal microbiota are of major importance in the context of large-scale insect farming. Methane emission from BSFL farms is generally low but varies between locations (Ermolaev et al., 2019; Mertenat et al., 2019; Parodi et al., 2020). When BSFL are reared on chicken feed mixed with 0.3% rabbit manure, 28.54% of the carbon initially present within the system is lost to the atmosphere in the form of CO_2 along with negligible amounts of methane (Perednia et al., 2017). Variations in methane emission are likely attributed to differences in the feed source and the gut microbial community, which was not investigated in the studies mentioned above.

Amplicon sequencing has shown that only ∼0.02% of all sequences obtained from BSFL guts belong to archaea, most of them methanogens (Klammsteiner et al., 2020). However, there is no evidence that this small population includes metabolically active residents of the gut. Indeed, we used primers designed to include both Bacteria and Archaea, but none of the sequences we recovered were classified as Archaea. This suggests that BSFL reared on CPC or chicken feed do not produce significant amounts of methane, which is favorable for large-scale insect farming.

The BSFL Fungal Community

We found that the fungal community of the BSFL gut was dominated by Trichosporonaceae (*Trichosporon asahii*) in both diet groups. The same fungus was also dominant in another study of BSFL reared on chicken feed and present (albeit in lower abundance) in BSFL reared on other diets (Varotto Boccazzi et al., 2017) suggesting this species is part of the core microbiome. Interestingly, the frass of larvae reared on chicken feed exclusively contained two fungal species (*T. asahii* and *Diutina rugosa*) whereas frass from the CPC group contained a diverse fungal community. The high relative abundance of *T. asahii* in gut and frass samples (both diets) and *D. rugosa* in gut samples (both diets) as well as in chicken feed frass, indicates that these yeasts are functionally associated with BSFL

and may have a probiotic effect. Mutualistic associations between yeasts and insects are common, especially in the orders Diptera, Hymenoptera and Coleoptera (Vega and Dowd, 2005; Vogel et al., 2017; Madden et al., 2018; Stefanini, 2018). The genus *Diutina* was also found in *Drosophila* spp. (Phaff et al., 1956; Camargo and Phaff, 1957) and ants (Ba and Phillips, 1996). Moreover, some yeasts can bind to chitin (Ishijima et al., 2017) via cell-surface adhesion proteins known as flocculins (Brückner and Mösch, 2012). This may allow the attachment of yeasts to the cuticle in the foregut and hindgut, and to the peritrophic membrane in the midgut, thus promoting stable colonization.

The probiotic effect of *D. rugosa* has been shown to increase body weight and feed conversion ratios in poultry (Wang et al., 2019a,b). *D. rugosa* probably helps to establish a healthy gut and substrate microbiome in BSFL and, if not present naturally in farmed populations, could be applied as a feed supplement in the future. *T. asahii* may also help to establish a healthy microbiome, given its antimicrobial activity against the pathogenic yeasts *Candida glabrata* and *C. lusitaniae* (Varotto Boccazzi et al., 2017). *T. asahii* and *D. rugosa* may also protect BSFL from *Fusarium* species, which are pathogenic to many insects, including the Diptera (Santos et al., 2020). We detected abundant *Fusarium* species in all feed samples but these potential entomopathogens were completely absent from the guts of BSFL reared on chicken feed and scarce in the guts of BSFL reared on CPC. *Trichosporon* spp. may be beneficial for BSFL development and the establishment of a healthy gut microbiome, but their numbers must be controlled in BSFL farms because they are also opportunistic human pathogens that cause infections in immunocompromised patients (Colombo et al., 2011). Similarly, the presence of Cladosporiaceae (all *Cladosporium*) in almost all samples was anticipated because this mold fungus is ubiquitous, but the numbers must be controlled in BSFL farms because these fungi can cause allergic reactions (Bavbek et al., 2006).

Like the bacterial microbiome, the fungal gut microbiome was diet-dependent, as shown by the higher alpha diversity in BSFL reared on CPC, which may reflect the characteristic features of the substrate including complex compounds such as gossypol. The Aspergillaceae (most belonging to the genus *Aspergillus*) were abundant in the guts of larvae reared on CPC but completely absent in the guts of larvae reared on chicken feed, despite their presence in the feed samples. The Aspergillaceae are known for their ability to break down gossypol (Yang et al., 2011, 2012; Mageshwaran et al., 2017; Grewal et al., 2020) suggesting they may metabolize this compound in the BSFL gut. However, the Aspergillaceae also produce aflatoxins and gliotoxins (Bennett and Klich, 2003). Although black soldier flies tolerate high levels of aflatoxins and do not accumulate aflatoxin B1 (Bosch et al., 2017), the potential accumulation of other mycotoxins should be investigated in more detail before using CPC routinely as insect feed. Furthermore, Trichocomaceae and Microascaceae were found in larvae from the CPC diet group but not the chicken feed diet group, suggesting these families may also contribute to the degradation of gossypol.

CONCLUSION AND OUTLOOK

Here we could show, that BSFL can be reared on CPC as the sole diet for several generations without any problems. The shorter life cycle and higher rates of successful development of black soldier flies reared on CPC might also be beneficial for large-scale farming and colony maintenance. Nevertheless, the growth pause and the lower final weight would be disadvantageous for a large-scale production. BSFL appear to tolerate certain amounts of gossypol and might be capable of detoxifying the substrate by degrading this compound. However, this needs to be confirmed by analytical gossypol quantification of BSFL and frass samples in the future. Furthermore, it needs to be tested if the developmental, physiological and nutritional properties of BSF reared at this small scale can apply for industrial insect farming. Also, variations in nutritional value, gossypol concentration and the overall quality of CPC, depending on the cottonseed variety, manufacturing process and storage conditions must be considered.

Amplicon sequencing of bacterial 16S rRNA genes and fungal ITS sequences revealed a functional core microbiome that may protect BSFL from pathogens, but also highlighted pronounced diet-dependent differences in the microbial community of the BSFL gut and frass. There was little similarity between the bacterial communities of the gut and frass, but more similarity between the corresponding fungal communities, especially those representing the chicken feed diet group. The high relative abundance of yeasts such as *D. rugosa* and *T. asahii* suggests these fungi might play a beneficial role during the rearing of BSFL.

The high relative abundance of the Aspergillaceae and *Actinomycetaceae* only in larvae reared on CPC revealed diet-dependent differences in the gut microbiome possibly indicating specific dietary adaptations. The Aspergillaceae are known for their ability to metabolize gossypol and we detected them only in larvae reared on CPC, suggesting they may also degrade gossypol within the BSFL gut. Our ITS data may reveal other gossypol-metabolizing fungi that could be added as feed supplements in large-scale insect farms using CPC as a major substrate. Bacterial gossypol degradation is still generally sparsely investigated and it is unclear whether it occurs in the BSFL. This can only be confirmed by establishing pure cultures and testing them directly for their metabolic activity *in vitro* accompanied by comparative genomics. Our amplicon data provide essential information for our on-going isolation and screening of potentially gossypol-degrading bacteria and other beneficial microorganisms which might be exploited for industrial applications in the future.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The amplicon sequencing data can be accessed at NCBI (https://www.ncbi.nlm.nih.gov) under the BioProject PRJNA674583, which includes the 16S rRNA gene and ITS sequences with the following accession numbers: SRX9457342–SRX9457395.

AUTHOR CONTRIBUTIONS

DT, SH, KB, PK, and AV designed the experiments. DT performed molecular work, processed the samples, collected developmental and physiological data, evaluated, visualized and discussed the data, and wrote the manuscript. SH analyzed Illumina sequences, evaluated, discussed and visualized the data, and wrote the manuscript. PK collected developmental, physiological and nutritional data, analyzed and discussed the data. KB, PH, and AV evaluated and discussed the data and edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.634503/full#supplementary-material

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A Molecular Survey of Bacterial Species in the Guts of Black Soldier Fly Larvae (*Hermetia illucens*) Reared on Two Urban Organic Waste Streams in Kenya

Marwa Shumo[1,2,3,4], Fathiya M. Khamis[3], Fidelis Levi Ombura[3], Chrysantus M. Tanga[3], Komi K. M. Fiaboe[3,5], Sevgan Subramanian[3], Sunday Ekesi[3], Oliver K. Schlüter[1], Arnold van Huis[6] and Christian Borgemeister[2]*

[1] Leibniz-Institute for Agricultural Engineering Potsdam-Bornim (ATB), Potsdam, Germany, [2] Department of Ecology and Natural Resources Management, Center for Development Research (ZEF), Bonn, Germany, [3] Plant Health Unit, International Centre of Insect Physiology and Ecology (ICIPE), Nairobi, Kenya, [4] Hermetia Baruth GmbH, Insect Technology Center (ITC), Berlin, Germany, [5] IPM Department, The International Institute of Tropical Agriculture, Yaoundé, Cameroon, [6] Laboratory of Entomology, Wageningen University & Research, Wageningen, Netherlands

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***Correspondence:**
Marwa Shumo
mshumo@atb-potsdam.de

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Globally, the expansion of livestock and fisheries production is severely constrained due to the increasing costs and ecological footprint of feed constituents. The utilization of black soldier fly (BSF) as an alternative protein ingredient to fishmeal and soybean in animal feed has been widely documented. The black soldier fly larvae (BSFL) used are known to voraciously feed and grow in contaminated organic wastes. Thus, several concerns about their safety for inclusion into animal feed remain largely unaddressed. This study evaluated both culture-dependent sequence-based and 16S rDNA amplification analysis to isolate and identify bacterial species associated with BSFL fed on chicken manure (CM) and kitchen waste (KW). The bacteria species from the CM and KW were also isolated and investigated. Results from the culture-dependent isolation strategies revealed that *Providencia* sp. was the most dominant bacterial species detected from the guts of BSFL reared on CM and KW. *Morganella* sp. and *Brevibacterium* sp. were detected in CM, while *Staphylococcus* sp. and *Bordetella* sp. were specific to KW. However, metagenomic studies showed that *Providencia* and *Bordetella* were the dominant genera observed in BSFL gut and processed waste substrates. *Pseudomonas* and *Comamonas* were recorded in the raw waste substrates. The diversity of bacterial genera recorded from the fresh rearing substrates was significantly higher compared to the diversity observed in the gut of the BSFL and BSF frass (leftovers of the rearing substrates). These findings demonstrate that the presence and abundance of microbiota in BSFL and their associated waste vary considerably. However, the presence of clinically pathogenic strains of bacteria in the gut of BSFL fed both substrates highlight the biosafety risk of potential vertical transmission that might occur, if appropriate pre-and-postharvest measures are not enforced.

Keywords: organic waste treatment, black soldier fly larvae (BSFL), gut microbiota, insect rearing, feed safety, food security

INTRODUCTION

The expanding world population, rapid urbanization, and growing welfare have increased the demand for animal products (FAO, 2009; UN, 2014). However, the inclusion of more animal-based products constitute a major challenge for the global food production system in terms of sustainability due to the high ecological footprint associated with the production of meat and dairy (Gerber et al., 2013; Springmann et al., 2018; Kim et al., 2020). Therefore, access to affordable and innovative feed is a prerequisite to establish profitable and sustainable livestock and fisheries production systems and to ensure food security, especially in the developing world.

Recently, protein-rich edible insects have been recognized as innovative protein alternatives due to their ability in decomposing and valorizing different organic wastes (Nowak et al., 2016; Van Huis and Tomberlin, 2017; Surendra et al., 2020). Moreover, insects are rich in micronutrients, energy and fatty acids (Sheppard et al., 1994; Finke, 2013; Nowak et al., 2016). For instance, black soldier fly larvae (BSFL) *Hermetia illucens* L. (Diptera: Stratiomyidae) have been identified as a promising feed ingredient for poultry, pigs, and aquaculture (Makkar et al., 2014; Lock et al., 2018; Dörper et al., 2020; Veldkamp and Vernooij, 2021). Black soldier fly (BSF), originally traced to the Americas, is present in most tropical and temperate regions of the globe (Sheppard et al., 1994). Crude protein constitutes about 35 to 49% of the total dry weight of the BSFL while fat accounts for about 29–35% of their total dry weight and their amino acid profile is of a similar quality to that of fishmeal (Renna et al., 2017; Dabbou et al., 2018; Spranghers et al., 2018; Gasco et al., 2019). Though naturally occurring in chicken, pigs and cow manure, BSFL have been successfully reared on other organic waste streams such as catering waste, urban municipal organic waste, fish viscera, vegetable remains, coffee bean pulp, straw, and dried distillers grains with solubles (Nguyen et al., 2015; Leong et al., 2016; Van Huis and Tomberlin, 2017; Meneguz et al., 2018; Surendra et al., 2020). Moreover, BSF breeding has recently been developed at industrial scale in order to reduce large amounts of wastes produced in facilities (Miranda et al., 2020; Scala et al., 2020).

Previous studies have also reported the potential of BSFL to reduce *Escherichia coli* and *Salmonella enterica* loads in chicken and cow manure (Erickson et al., 2004; Lalander et al., 2013, 2015; Čičková et al., 2015; Nguyen et al., 2015). However, several studies revealed a considerable influence of the rearing substrate on the gut microflora of BSFL (Dillon and Dillon, 2004; Jeon et al., 2011; Engel and Moran, 2013; EFSA Scientific Committee, 2015; Boccazzi et al., 2017; Klammsteiner et al., 2018; Khamis et al., 2020). Therefore, BSFL gut may assimilate pathogens present in rearing substrates or may proliferate by inappropriate processing and storage (Bruno et al., 2019; Khamis et al., 2020). This may subsequently cause diseases in animals fed with BSFL-derived feed, highlighting the importance of selecting safe rearing substrates for the successful production of feed (Erickson et al., 2004; Čičková et al., 2015; Wang and Shelomi, 2017).

Several studies investigated the influence of rearing substrates on the dynamics of BSFL gut microflora during rearing in either a laboratory setting or in production facilities (De Smet et al., 2018; Bruno et al., 2019; Jiang et al., 2019; Wynants et al., 2019; Cifuentes et al., 2020). However, these studies were conducted in the developed world where strict regulations are imposed on production facilities (Jeon et al., 2011; Zheng et al., 2013; Boccazzi et al., 2017; Bruno et al., 2019; Wynants et al., 2019). On the other hand, little is known about the safety of BSFL-derived feeds in Africa. Therefore, the current study sought firstly to investigate the variability of the bacterial species associated with the guts of BSFL and two readily available rearing substrates in the Kenyan capital Nairobi [chicken manure (CM) and kitchen waste (KW)]. Although, both waste streams are not allowed in the European Union (Insects as Feed EU Legislation – Aquaculture, Poultry & Pig Species), their suitability and utilization for rearing BSFL is widely accepted in Africa (EFSA Scientific Committee, 2015; Nakimbugwe et al., 2020). Secondly, to investigate the bacterial species associated with BSFL frass, given their importance as organic fertilizer for crop production (Lalander et al., 2015; Oonincx et al., 2015; Beesigamukama et al., 2020).

MATERIALS AND METHODS

The study was undertaken at the International Centre for Insect Physiology and Ecology (ICIPE), Kasarani, Nairobi, Kenya (S 01_13′14.6″; E 036_53′44.5″, 1,612 m.a.s.l.).

Stock Colony

The ICIPE Animal Rearing and Containment Unit (ARCU) maintains a population of BSF adults which acted as the stock colony for this study. The stock colony was established following methods described by Booth and Sheppard (1984) and Sripontan et al. (2017).

The adult BSF were housed in metal framed cages with screen (1.8 × 1.8 × 1.8 m with 1.5 mm mesh) with strong access to daylight spectra, while temperatures were maintained at 28 ± 5°C to encourage mating. Flies were supplied with water to prolong their life, and corrugated cardboard and some spent grain (SG) were placed within the cage to stimulate oviposition. The eggs were hatched on wheat bran and harvested for the experiment after day 5.

Preparation of Substrate and Larvae Feeding

The two feeding substrates, CM and KW, were both sourced locally. Chicken manure was collected from a broiler poultry farm in the greater Nairobi area and fermented for 1-week to enhance moisturization. Kitchen waste was a mixture of potato peeling, carrot remains and peelings, rice and bread debris collected from a local restaurant in Nairobi. Kitchen waste materials were chopped into fine pieces and fed directly to the larvae. The feedstock was hydrated to approximately 60 ± 5% moisture by weight.

One hundred (100) 5-days neonatal BSFL obtained from ICIPE's stock colony were placed carefully in 23 × 15 cm plastic containers containing 2 kg of the substrates. During the rearing process, the temperature was maintained at 28 ± 2°C and relative humidity at 65 ± 5%. Distilled water was sprinkled on

the substrate to ensure 65–70% moisture content. The relative humidity was confirmed using a moisture sensor with two 12 cm long probes (HydroSenseTM CS620; Campbell Scientific, Inc., Logan, UT, United States). Once the substrate had been broken down by the larvae, the dried peels or coarse particles were removed. The rearing time ranged between 16 and 21 days. The fifth instar larvae were harvested and stored at −20°C to avoid any changes in the BSFL microflora until further analysis. Samples of both fresh rearing substrates (prior to the exposure to BSFL) and BSF frass (left-overs of waste substrates after exposure to BSFL) were also harvested and stored at −20°C to avoid any changes in their microflora until further analysis.

Isolation and Morphological Characterization of Bacterial Cultures

The isolation of the larval guts was performed following aseptic techniques and under a closed sterile Purifier Logic + Class II, Type A2 Biosafety Cabinet (Labconco, Kansas City, MO, United States). The exterior of each BSF was washed once in 70% ethanol and then in 0.9% sterile phosphate-buffered saline (PBS). The entire BSFL gut was extracted using fine-tipped forceps and homogenized in a 2.0 ml microcentrifuge tubes containing 1.5 mL of sterile 0.9% PBS. Guts of a total of 50 CM fed and 50 KW fed BSFL were successfully extracted. To isolate culturable bacterial strains, aliquots of 0.1 mL from each extracted gut were spread onto agar plates containing either Nutrient Agar, MacConkey Agar or Violet Red Bile Agar (VRBA) then incubated at 37°C for 48 h. MacConkey was used to isolate coliforms and other gut pathogens particularly members of the family Enterobacteriaceae and the genus *Pseudomonas*. VRBA on the other hand was used to isolate, detect, and enumerate coli-aerogenes bacteria with emphasis being on *Enterobacter aerogenes, Escherichia coli, Salmonella* Enteritidis, *Salmonella* Typhimurium, and *Staphylococcus aureus* due to the nature of the BSF rearing substrates. Three replicates were prepared for each medium type. Selected discrete bacterial colonies were then aseptically removed by a sterile inoculation loop and sub-cultured 3 to 4 times on the same agar medium for 48 h at 37°C. The isolates were morphologically identified using traditional bacterial methods: handbooks, identification keys based on colony characteristics (size, shape, color, margin, and elevation) and microscopic morphology.

Molecular Characterization of Bacterial Cultures

Extraction and Amplification of 16S rDNA

Bacterial isolates were aseptically harvested by scraping discrete bacterial colonies off the surface of cultures with a sterile inoculation loop. The genomic DNA was extracted using ISOLATE II Genomic DNA Kit (Bioline, London, United Kingdom). The resultant extracted DNA quality and quantity was assessed using NanoDrop 2000/2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States). The 16S rDNA of each bacterial isolate was amplified in 30 μL volume PCR mix containing 10X PCR buffer (GenScript USA Inc, New Jersey, United States), 0.5 pmol μl^{-1} of each primer (27F 5′- AGAGTTTGATCMTGGCTCAG -3′ (Lane, 1991) and 1492R 5′- GGTTACCTTGTTACGACTT -3′ (Turner et al., 1999), 0.25 mM $MgCl_2$, 0.0625 U μl^{-1} Taq DNA polymerase (GenScript USA Inc, New Jersey, United States), and 20 ng μl^{-1} of DNA template. PCR reactions were set up in a PTC 100 thermocycler (MJ Research, Gaithersburg, MD, United States). The cycling conditions involved an initial denaturation step at 95°C for 10 min, 35 cycles of a denaturation step at 94°C for 1 min, an annealing step of 52°C for 1 min and an extension step at 72°C for 1 min, followed by a final extension at 72°C for 10 min. The expected product size was 1,500 bp.

DNA Purification and Sequencing

The PCR products were resolved through 1% agarose gel for 1 h at 70 V (Bio-Rad model 200/2-0 power supply and wide mini-sub cell GT horizontal electrophoresis system, Bio-Rad laboratories, Inc., Hercules, CA, United States). The DNA was then visualized under UV-illumination. A KODAK Gel Logic 200 Imaging System software (Raytest GmbH, Straubenhardt, Germany) was used to photograph, analyZe, and document the gel. Following the manufacturer's instructions, the QuickClean 5M Gel Extraction Kit II from GenScript (GenScript Corporation, Piscataway, NJ, United States) was used to purify the PCR products which were then sent to Macrogen Europe BV for bi-directional sequencing.

16S rDNA Amplification, MinION Library Preparation and Sequencing

Genomic DNA extraction from BSFL, microbial cultures of the raw substrates, bacterial plates and frass was done using the Isolate II DNA extraction kit (Bioline, London, United Kingdom) as per the manufacturer's protocol. The purity and concentration of the resultant DNA was determined using a Nanodrop 2000/2000c spectrophotometer (Thermo Fischer Scientific, Wilmington, NC, United States). Library preparation was performed using the Ligation Sequencing Kit (SQK-LSK108) and Native Barcoding Kit (EXP-NBD103) for genomic DNA, according to the standard 1D Native barcoding protocol provided by the manufacturer (Oxford Nanopore Technologies, Oxford, United Kingdom). The constructed library was loaded into the Flow Cell R9.4 (FLO-MIN106) of a MinION device (Oxford Nanopore Technologies, Oxford, United Kingdom), which was run with the SQK-LSK108_plus_Basecaller script of the MinKNOW1.7.14 software, for 4 h.

Morphological Data Analysis

Stata (version 15.1) was used for analyses. Bacterial isolates occurrence was expressed as a percentage of the total number of dissected BSFL. A two-sample test of proportions (Z-test) for non-parametric data was used to compare the occurrence of bacterial isolates obtained from larvae guts of BSF previously reared on CM and KW substrates.

Molecular Data Analysis

The sequences were assembled and edited using Chromas version 2.13 (Technelysium Pty Ltd, Queensland, Australia). Consensus sequences from both the forward and reverse strands

were generated and were then queried through BLASTN in the GenBank database hosted by the National Center of Biotechnology Information (NCBI)[1] for identification purposes and to check for similarity with organisms already identified. Any isolate exhibiting 97–100% sequence similarity to NCBI strains were considered as the correct species for that isolate. Moreover, the consensus sequences were aligned using ClustalX version 1.81. Sequences' similarities and identities was done using SIAS (Sequence Identity And Similarity)[2]. Further phylogenetic analyses that were conducted using MEGA X (Kumar et al., 2018). Out of 24 nucleotide models, Kimura-2 parameter was considered the best fit model (K2 + G) for our data set since the model posted the lowest Bayesian Information Criterion. The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model (Kimura, 1980). The tree with the highest log likelihood (−18732.10) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 53 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Non-coding. There were a total of 1977 positions in the final dataset.

Metagenomic Data Analysis

Low quality sequence reads, primers and adapters were trimmed from the sequences using the trim.seqs script in Mothur (1.30.2) (Schloss et al., 2009). Reads were formatted and chimeras removed using chimera.slayer function in Mothur and the paired- end reads merged using FLASH (v1.2.7) (Magoc and Salzberg, 2011). High- quality sequences were aligned using INFERNAL aligner at the Ribosomal Database Project (RDP) website with a threshold bootstrap of 80% (Cole et al., 2009). Resulting data were analyzed using QIIME 2 where the sequences were used to pick Operational Taxonomic Units (OTUs) at 97% similarity threshold (Caporaso et al., 2010) and taxonomy assigned using RDP classifier using Greengenes database as the reference database (McDonald et al., 2012). Alpha diversity runs were done to calculate species richness and species diversity using the Shannon indexing (Spellerberg and Fedor, 2003) with rarefaction curves generated to describe OTU abundance and diversity in QIIME. Stacked bar plot was used to do quantitative comparison on abundance at genus level with a minimum abundance of 0.1% cut off being set to select the most abundant taxa in each sample. Any taxa with read counts below the 0.1% threshold was collapsed and lumped into the "others" category. In a community, if the bacterial species numbers (both culturable and non-culturable) are large and diversified, viewing at genus level (higher levels) provides better picture as compared to lower levels (i.e., species level). For microbial comparison between the different groups of samples, Beta diversity was calculated using UNIFRAC metrics (Lozupone et al., 2011). Principle Coordinate Analysis (PCoA) was used to visualize the differences between the microbial communities and heat maps and/or rarefaction curves generated.

For the microbiome data, differences in intrinsic parameters such as OTU richness and diversity parameters were analyzed using ANOVA or ANOSIM (analysis of similarity) in R Studio (version 3.2.5) (R Development Core Team, 2016). For all tests a significant level of 0.05 was considered. Non-metric multidimensional scaling (NMDS) was also done using R package vegan (v.2.43) to create dissimilarity matrixes using the Bray-Curtis dissimilarity method. All raw data were deposited in the NCBI database as BioProject:PRJNA728669 and BioSample SAMN19093411.

RESULTS

Morphological Identification of Bacterial Isolates

A total of five bacteria isolates belonging to the genera *Providencia*, *Morganella*, *Brevibacterium*, *Staphylococcus* and *Bordetella* were obtained from the 50 dissected BSFL reared on CM and KW. *Providencia* was the most abundant genus in the guts of BSFL reared on CM (59.5%) and KW (51.2%) [95% confidence interval (CI) 49.0–70.0% and 95% CI 40.5–61.9%, respectively]. Both *Morganella* and *Brevibacterium* occurred only in BSFL reared on CM at 27.4% [95% CI 17.9–36.9%] and 13.1% [95% CI 5.9–20.3%], respectively. Moreover, *Staphylococcus* and *Bordetella* both occurred only in BSFL reared on KW at 30.9% [95% CI 21.1–40.8%] and 17.9% [95% CI 9.7–26.1%], respectively (**Figure 1**). We found a significant difference in the occurrence of *Providencia* obtained from BSFL reared on CM in comparison to both *Morganella* ($p = 0.001$) and *Brevibacterium* ($p < 0.001$). Moreover, there was a significant difference between the occurrence of *Morganella* and *Brevibacterium* ($p = 0.035$). In addition, the occurrence of *Providencia* obtained from BSFL reared on KW fed in comparison to both *Staphylococcus* ($p = 0.036$) and *Bordetella* ($p < 0.001$) differed significantly. On the other hand, no significant difference was found between the occurrence of *Staphylococcus* and *Bordetella* ($p = 0.080$).

GenBank accession numbers provided for the nucleotide sequences of the bacterial isolates are as follows: *Providencia* sp. MSB6 = MK276967, *Providencia* sp. MSB9 = MK276968, *Providencia* sp. MSB12 = MK276969, *Providencia* sp. MSB22 = MK276974, *Morganella* sp. MSB27 = MK276976, *Brevibacterium* sp. MSB14 = MK276970, *Staphylococcus* sp. MSB18 = MK276972, *Bordetella* sp. MSB17 = MK276971, *Bordetella* sp. MSB21 = MK276973, and *Bordetella* sp. MSB24 = MK276975.

Molecular Characterization of Bacterial Isolates

The isolated bacterial species depicting isolate species identities with 97–98% similarity and 0.0 E values after sequencing have been molecularly identified (**Table 1**). Like the morphological

[1]http://www.ncbi.nlm.nih.gov

[2]http://imed.med.ucm.es/Tools/sias.html

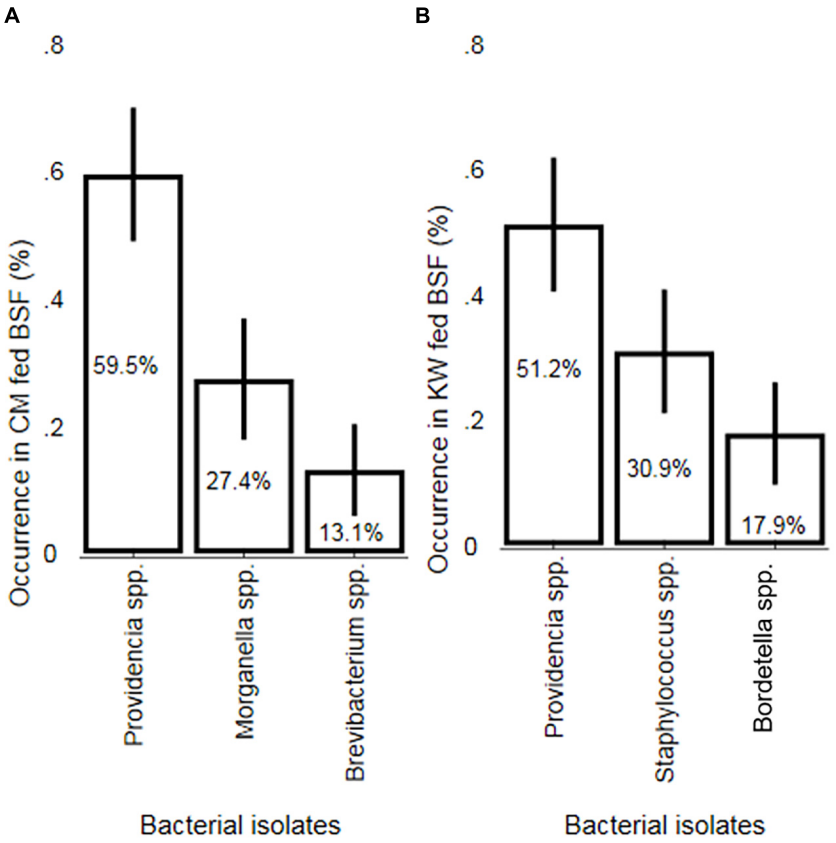

FIGURE 1 | A two-sample test of proportions (Z-test) for non-parametric data depicting the occurrence of bacterial isolates obtained from larvae guts of BSF previously reared on CM and KW substrates. Mean bacterial isolates occurrence (in%) in the gut of **(A)** chicken manure (CM) and **(B)** kitchen waste (KW) fed black soldier fly larvae (BSFL). Error bars represent 95% confidence intervals.

TABLE 1 | Summary of the identified bacterial species using 27 F and 1492 R primers.

Bacterial isolates codes	Bacterial isolates sources	ID from GeneBank	ID %	Species GenBank accession number
MSB6	BSF reared on CM	*Providencia rettgeri* strain ALK417 16S	97	KC456547.1
MSB9	BSF reared on CM	*Providencia rettgeri* strain ALK417 16S	97	KC456547.1
MSB12	BSF reared on CM	*Providencia rettgeri* strain ALK417 16S	98	KC456547.1
MSB22	BSF reared on KW	*Providencia rettgeri* strain ALK417 16S	98	KC456547.1
MSB27	BSF reared on CM	*Morganella morganii* subsp. *morganii* strain ALK057 16S	97	KC456563.1
MSB14	BSF reared on CM	*Brevibacterium luteolum* partial 16S rRNA	97	LT222061.1
MSB18	BSF reared on KW	*Staphylococcus* sp. strain 82584 16S	98	KX525724.1
MSB17	BSF reared on KW	*Bordetella* sp. BAB-4401 16S	98	KP751929.1
MSB21	BSF reared on KW	*Bordetella* sp. BAB-4401 16S	97	KP751929.1
MSB24	BSF reared on KW	*Bordetella* sp. BAB-4401 16S	98	KP751929.1

identification, the molecular characterization also yielded five isolate species identified as *Providencia rettgeri* (isolates MSB6, MSB9, MSB12, and MSB22), *Morganella morganii* (isolate MSB27), *Brevibacterium luteolum* (isolate MSB14), *Staphylococcus* sp. (isolate MSB18) and *Bordetella* sp. (isolates MSB17, MSB21, and MSB24) (**Supplementary Table 2**). Thus, the molecular identification of the bacterial isolates corroborates that of the morphological study (**Figure 1** and **Table 1**).

The evolutionary history of the bacterial isolates was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0.52129017 is presented in **Figure 2**. In this figure the percentage of replicate trees in which the associated taxa clustered together in the

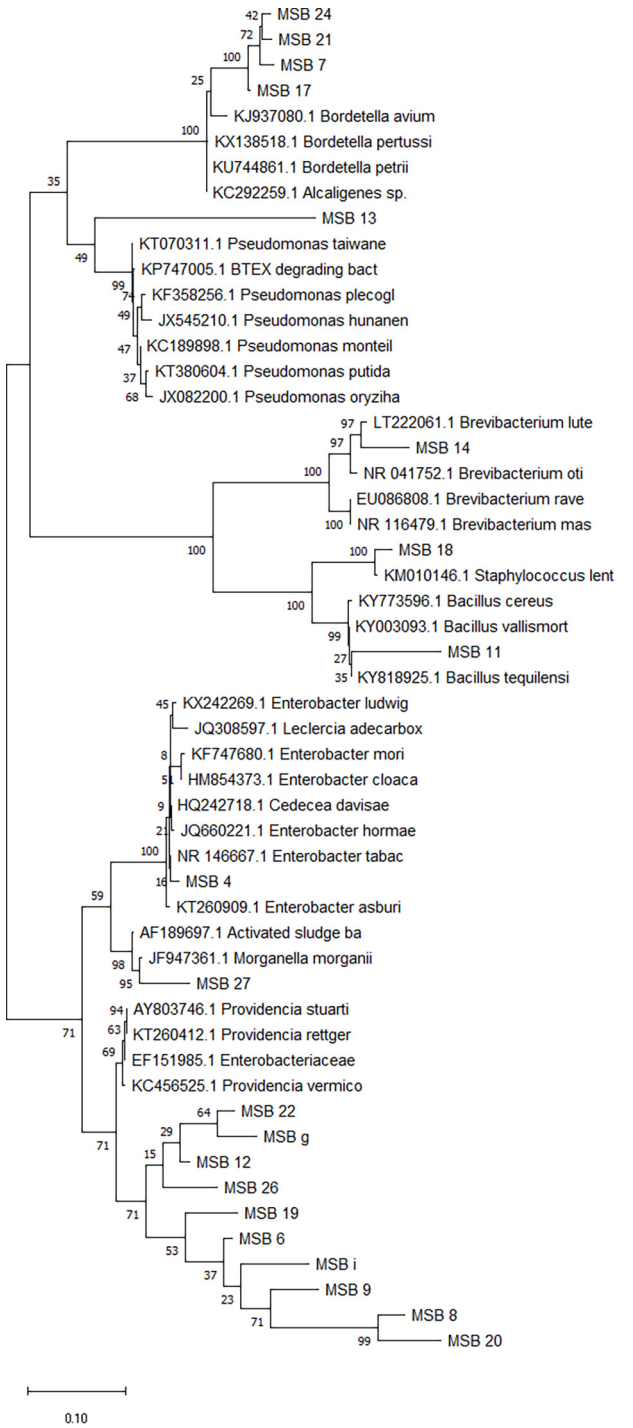

FIGURE 2 | Phylogenetic tree by Maximum Likelihood Method showing the evolutionary relationships of bacterial isolates from chicken manure (CM) and kitchen waste (KW) fed black soldier fly larvae (BSFL) and isolates from GenBank.

bootstrap test (500 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree (**Figure 2**). The analysis involved 15 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Non-coding. All positions containing gaps and missing data were eliminated. There was a total of 966 positions in the final dataset. Five groups resulted from this analysis (**Figure 2**). The first group consisted of the *Providencia* sp. isolates where each of the samples MSB12, MSB22, MSB6, and MSB9 isolates branched separately. Furthermore, all the *Providencia* isolates linked to *Providencia rettgeri* of accession number KC456547.1 during blasting (**Table 1**). The second group consisted of *Morganella* sp. isolate MSB27 which linked to *Morganella morganii* of accession number KC456563.1 during blasting. The third group consisted of *Brevibacterium* sp. isolate MSB14 which linked to *Brevibacterium luteolum* of LT222061.1 during blasting. The last two clusters of the phylogenetic tree consisted of *Staphylococcus* sp. isolate MSB18 and *Bordetella* sp. isolates MSB17, MSB21, and MSB24, respectively (**Figure 2**). The *Staphylococcus* sp. isolate linked to *Staphylococcus* sp. accession number KX525724.1 while all the *Bordetella* isolates linked to *Bordetella* sp. of accession number KP751929.1 during blasting (**Table 1**).

Library Size and Cumulative Abundance

The insect extraction had the largest 16S metagenomic library size of 70,518 reads, followed by the fresh waste substrates (55,184 reads) then finally the BSF frass exposures at 38 reads (**Figure 3**). The taxonomic composition at genus levels of the samples and the cumulative abundance of the bacterial genomes is detailed in **Figure 4**. *Providencia* (42.28%) and *Bordetella* (27.75%) were the most abundant genera in the direct insects' extraction batch (non-cultured samples). In the raw waste substrates, *Pseudomonas* (19.3%) and *Comamonas* (18.7%) were most abundant genera while *Providencia* (18.42%) and *Bordetella* (15.8%) were most abundant genera in the BSFL frass (**Figure 4**).

Alpha Diversity and Beta Diversity Analyses

The fresh waste substrates had the highest species richness (30), followed by the direct BSFL extracts (26) and the BSFL frass (15) (**Figure 4** and **Supplementary Table 1**). In terms of homogeneity, BSFL frass had the highest evenness index (**Figure 4** and **Supplementary Table 5**) while BSFL extract was the most heterogeneous.

The Shannon diversity index showed that raw waste substrates had the highest diversity of bacterial genera (2.67), followed by BSFL frass (2.5), and direct BSFL extract (1.5) (**Figure 5**). The effective diversity (True-Shannon) followed the same trend and showed that the raw waste substrates had the highest effective number of species (14.38), followed by BSFL frass (12.22), and BSFL extract (4.54) (**Figure 5** and **Supplementary Table 3**). A heatmap (**Figure 6**) depicting the differential abundance of microbial taxa that varied among sample groups at FDR < 0.05 was generated.

According to the Bray Curtis dissimilarity index, the interpopulation diversity index between BSFL extracts,

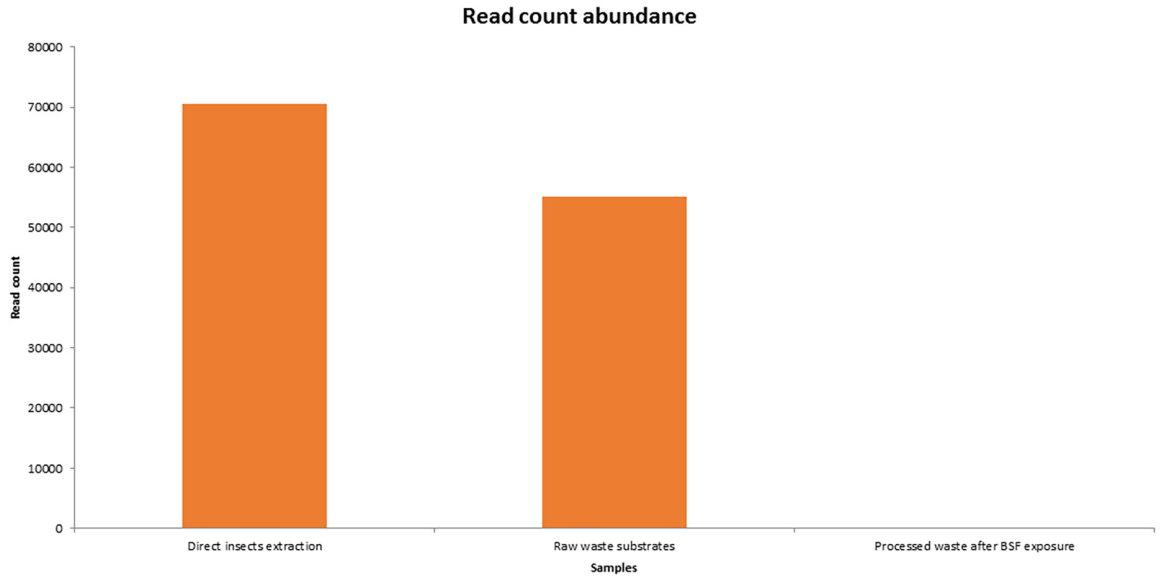

FIGURE 3 | Overview of 16S metagenomic library size generated by MinION sequencing of BSF and its substrates. There were three pooled sample categories i.e., direct insects extraction batch, raw waste substrates, and the processed waste substrates after BSF exposures.

and BSFL frass at its lowest was 89.62% (**Figure 7** and **Supplementary Table 6**).

DISCUSSION

Chicken manure and kitchen waste are two common organic waste streams in Nairobi and arguably also in other megacities in the world (Shumo et al., 2019b). We reared BSFL on these waste streams and used both a culture-dependent and independent sequence-based approach to survey the bacterial species in the larval gut. The effect of these waste streams on the bacterial communities of BSFL and the BSFL frass were also evaluated.

Bacteria of the genus *Providencia* are Gram-negative opportunistic pathogens have been isolated from a wide variety of environments and organisms, ranging from humans to insects, sea turtles and shark mouths (Ami et al., 2010; Galac and Lazzaro, 2011; Hamden et al., 2013; Augustinos et al., 2015). In addition, *Providencia* spp. are associated with a wide range of human infections, and they show harmful and pathogenic effects on their hosts. This may have an economic impact on the food safety industry. However, *Providencia* are known to be vertically transmitted in BSF and can stimulate oviposition (Galac and Lazzaro, 2011; De Smet et al., 2018). *Morganella* sp. belonging to the phylum Proteobacteria and *Brevibacterium* spp. belonging to the phylum Actinobacteria were both detected only in CM fed BSFL. In contrast, *Staphylococcus* sp. (phylum Firmicutes) and *Bordetella* sp. (phylum Proteobacteria) were only detected in KW fed BSFL. This reconfirms the results reported by Osimani et al. (2021) which detected an influence of the rearing substrate on of the relative abundance of *Morganella* sp. in BSFL. *Staphylococcus* sp. are Gram-positive bacteria and are commonly found as symbionts in the guts of different insect species like the common fruit fly *Drospholia melanogaster* Meigen (Diptera: Drosophilidae), the southern house mosquito *Culex quinquesfasciatus* Say (Diptera: Culicidae), *Analeptes trifasciata* Fabr. (Coleoptera: Cerambycidae) and the drosophila parasitoid wasp *Asobara tabida* (Nees) (Hymenoptera: Braconidae) (Zouache et al., 2009; Oyedokun and Adeniyi, 2016). *Staphylococci* are well known for developing antibiotic resistance as well as causing food-borne diseases and nosocomial infections (Kadariya et al., 2014).

Our metagenomics study confirms the presence of several species including *Morganella*, *Enterococcus*, *Pseudomonas* and *Providencia* that were previously reported (Klammsteiner et al., 2018; Bruno et al., 2019; Khamis et al., 2020; Tanga et al., 2021). The presence of universal bacterial species across studies, substrate and locations may indicate the existence of conserved members of the BSF larval gut microbiota. However, no studies have investigated the interactive roles of these conserved members with their associated BSFL host. Obtaining such information would be important in determining whether the BSFL have a core gut microbiota, that might be important in enhancing their performance or vice versa. However, what remains puzzling to us is the absence of *Dysgonomonas*. Previous studies reported *Dysgonomonas* as one of the top three most abundant members of the BSFL gut microbiota (Klammsteiner et al., 2018; Bruno et al., 2019; Khamis et al., 2020; Tanga et al., 2021). *Dysgonomonas* sp. is widely known for its essential role in the gut of termites during the degradation of recalcitrant lignocellulose (Yang et al., 2014; Sun et al., 2015). Bruno et al. (2019) reported that *Dysgonomonas* plays a significant role in the digestion of complex polysaccharides. This was reconfirmed as Jiang et al. (2019) reported that *Dysgonomonas* sp. obtained from the gut of BSF is directly associated with genes for sulphate, carbohydrate, and nitrogen metabolism. Moreover, a

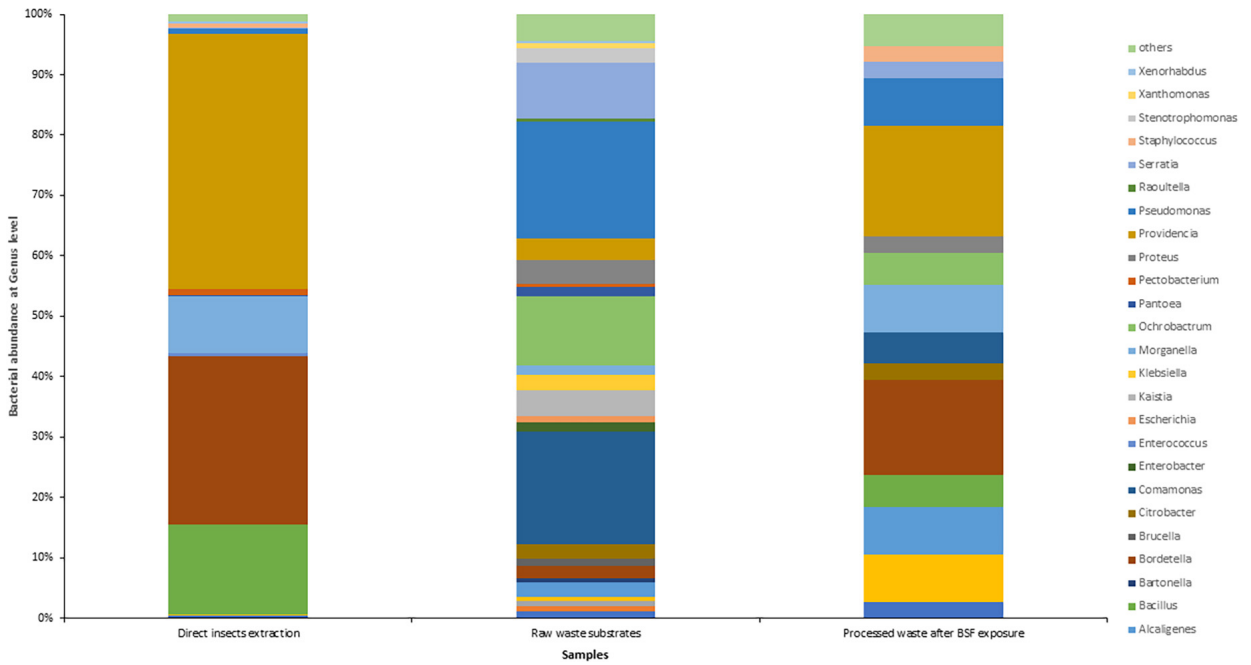

FIGURE 4 | Taxonomic composition and percentage of reads of bacteria at the genus level, in the black soldier fly larvae (BSFL) and its substrates, using Stacked Bar plot. For clarity, taxa with cumulative read counts below the cut-off value of 0.1% were summed into "Others" category.

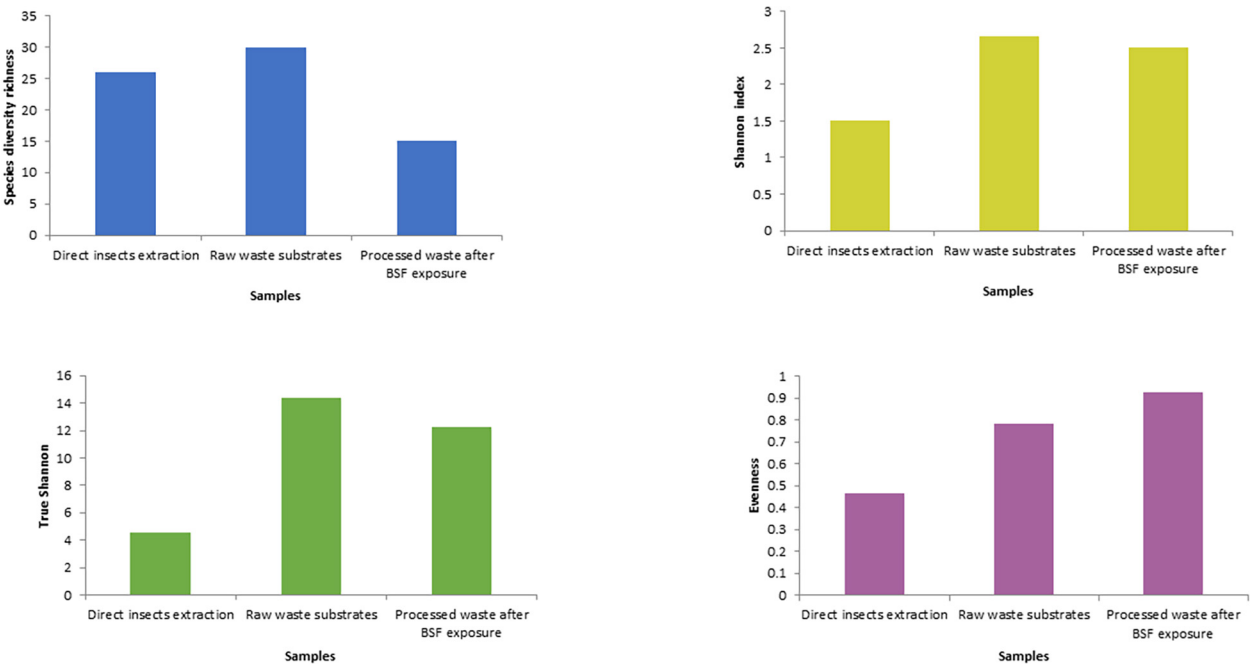

FIGURE 5 | Alpha-diversity measures using evenness, Shannon diversity index, and species richness at the genus level in the black soldier fly larvae (BSFL) and its substrates. The samples were composed grouped into three pooled categories (insects' extraction, raw waste substrates, and the BSF frass) and are represented on the *X*-axis, and their estimated diversity is represented on the *Y*-axis.

metagenomic analysis of the BSFL gut traced the origin of a new α-galactosidase gene that facilitates the breaking up of α-galactoses abundant in non-digestible plant carbohydrates to a specific *Dysgonomonas* strain (Lee et al., 2018). Additionally, *Dysgonomonas* sp. may contribute to the biodegradation of pharmaceutical products like ciprofloxacin when appearing

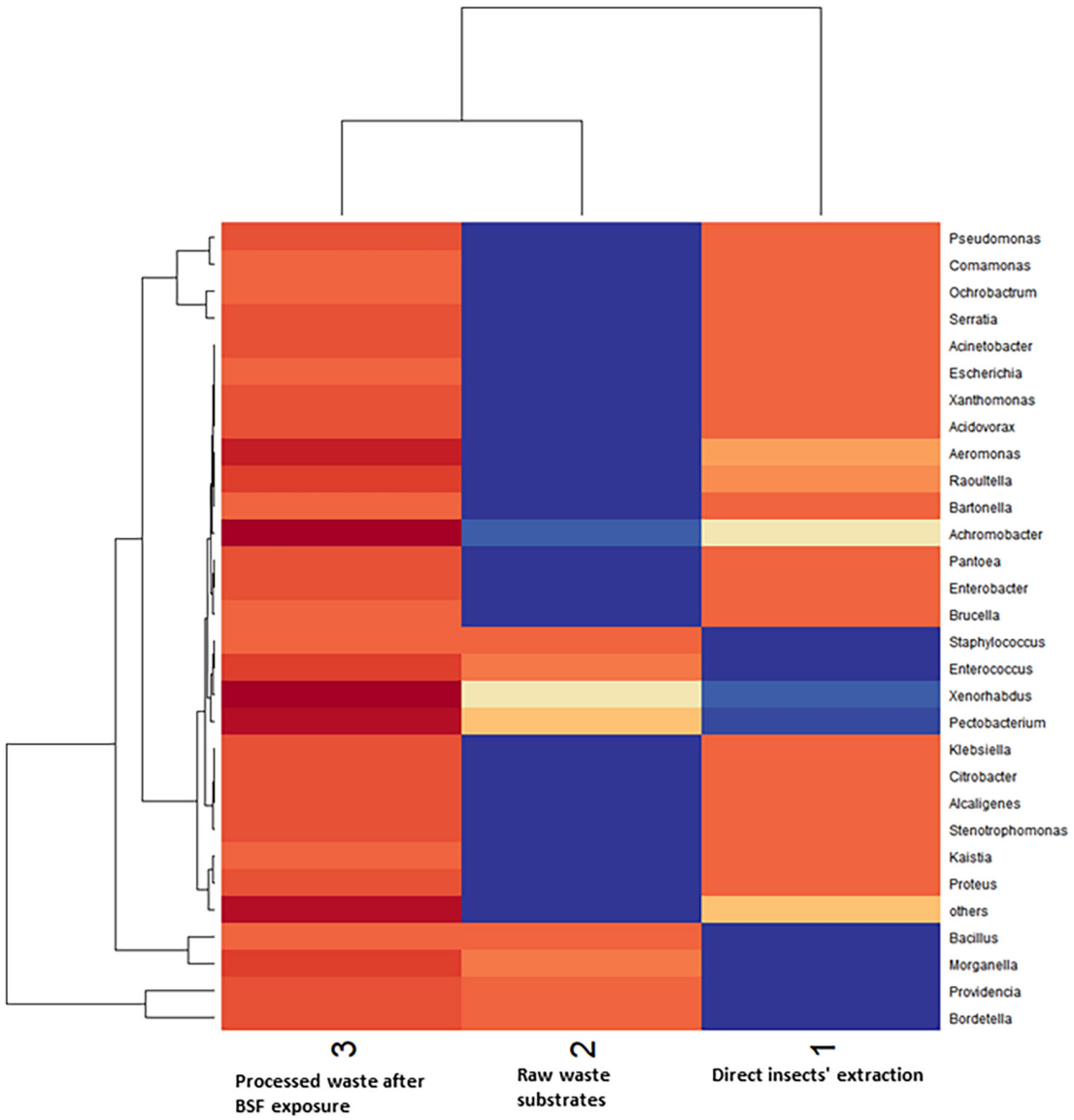

FIGURE 6 | A heatmap depicts the differential abundance of microbial taxa that varied among sample groups at FDR < 0.05. Rows (microbial taxa at genus level) and columns (samples) were ordered by hierarchical clustering. The top dendrogram shows which environmental parameters (samples) have the most similar responses. The side dendrogram shows which genera are behaving most similar.

in a consortium with other bacteria-like microorganisms, for instance, *Actinomyces* sp., underlining the potential for further biotechnological applications (Martins et al., 2018).

In our study, *Bacillus* sp. was observed in BSFL extracts and members of this genus are known for their wide range of physiologic characteristics and their ability to produce enzymes, antibiotics, and metabolites. That explains why they have been widely used in many medical, pharmaceutical, agricultural, and industrial processes (Logan, 2012; EFSA Panel on Biological Hazards [BIOHAZ], 2016; Celandroni et al., 2019). In addition, they produce nutraceuticals such as vitamins such as riboflavin, cobalamin, and inositol as well as carotenoids. For that reason, they are used in the production of several health supplements for human consumption (Mohammed et al., 2014; Tanaka et al., 2014; Takano, 2016).

Although the BSFL used in this study and the ones used in previous study by Tanga et al. (2021) were obtained from the same stock colony, bacterial species reported in both studies showed a high degree of variability. This suggests that despite the possibility of the presence of a BSF core gut microbiota, other factors may have contributed to the microflora present. These factors may include the organic waste streams used (diet), biotic and abiotic factors. For instance, Tanga et al. (2021) reported the presence of gastrointestinal pathogens *Campylobacter*, which might be attributed to external contamination of the rearing substrates before the BSFL were introduced. Likewise, Jeon et al. (2011)

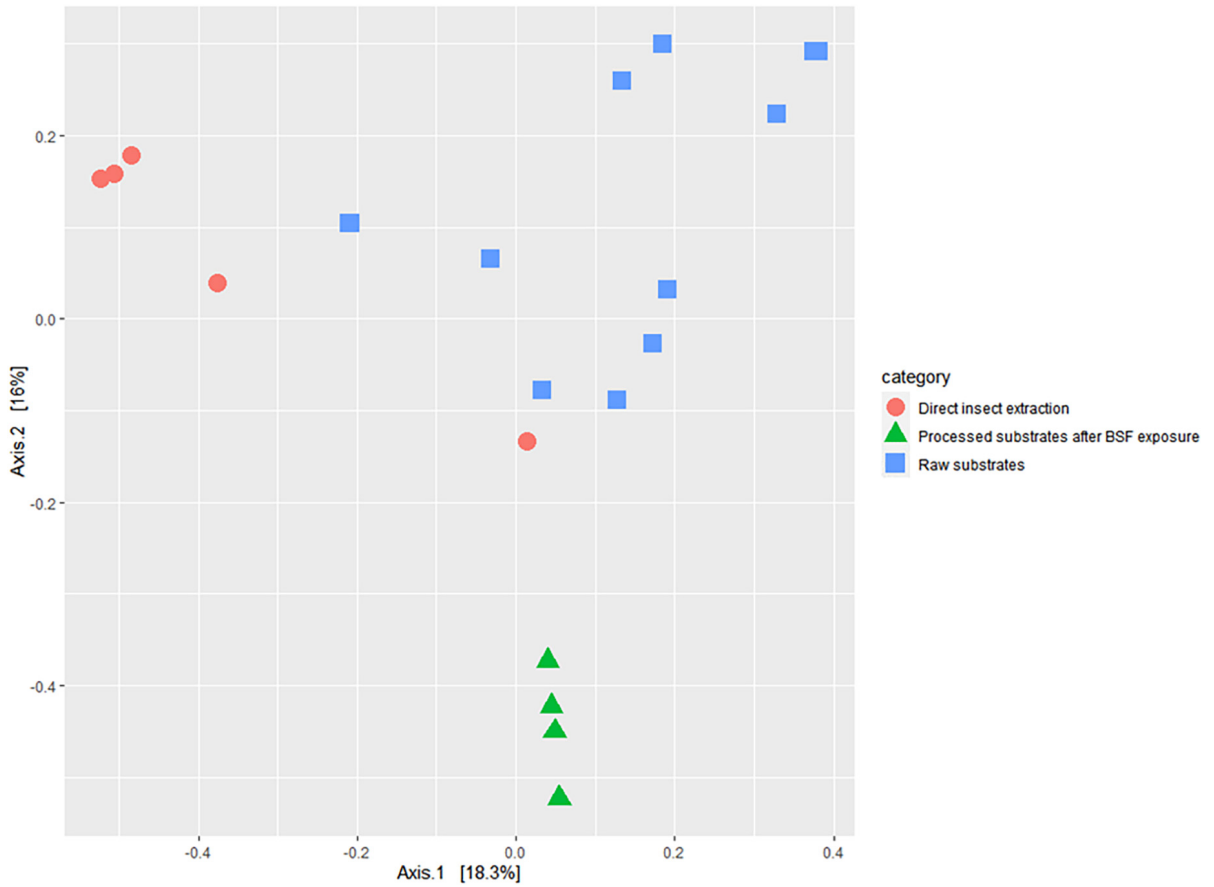

FIGURE 7 | Two-dimensional principal coordinate analyses plot of the beta diversity of bacterial genera in the black soldier fly larvae (BSFL) and its substrates, estimated using the Bray Curtis dissimilarity index showing.

reported a higher bacterial diversity of food waste fed larvae in comparison to ones reared on cooked rice or calf forage; the bacterial variability was attributed to the influence of the rearing substrates. Moreover, environmental conditions probably play an important role in shaping the microbial communities in the guts of BSFL. For instance, oxygen variability can influence the gut shape which in return influences the microbial communities inhabiting the gut (Hoback and Stanley, 2001; Ke et al., 2010). Moreover, the pH content of the rearing substrates possibly facilitates the presence of certain bacterial species and inhibits the growth of others. Erickson et al. (2004) observed a reduction in pathogenic bacterial populations in alkaline BSFL rearing substrates such as CM in comparison to acidic ones like cow or hog manure. Moreover, temperature may have an influence on the bacterial composition of the rearing substrates and consequently on the BSFL gut microflora. Studies on the potential of BSFL in the reduction of *Escherichia coli* and *Salmonella* spp. showed that both the nature of the rearing substrate as well as the temperature may influence the effectiveness of such a reduction. For instance, Erickson et al. (2004) reported that the effectiveness of BSFL in the reduction of *Escherichia coli* and *Salmonella* spp. increased with increasing temperatures as they observed higher reduction rates at temperatures of 27°C and 32°C in comparison to 23°C. Likewise, Liu et al. (2008) demonstrated an increase in the effectiveness of BSFL in the reduction of *Escherichia coli* and *Salmonella* spp. in cow manure accompanied with a 100% mortality of BSFL at a temperature of 35°C. However, they observed a similar increase in the reduction of bacterial counts in cow manure controls not containing BSFL at the same temperature. Yet, Shumo et al. (2019a) found that BSFL were capable of surviving at higher temperatures (up to 35°C), which is an indication of phenotypic plasticity. This has also been reported by Moczek (2010) and Kelly et al. (2012). Therefore, further studies are needed to verify whether phenotypic plasticity influences the dynamics of microbial communities associated with the guts of BSFL.

The presence of certain bacterial species may also be linked to vertical transmission as reported by Su et al. (2010), who demonstrated the presence of both *Providencia* spp. and *Morganella morganii* in the gut of newly emerged adult house flies. This indicates that certain microorganisms are vertically transmitted and can be carried over in the gut from the larval to adult stage. Moreover, Zheng et al. (2013) recorded *Providencia*

spp. in both eggs and adult BSF. Insects have developed different mechanisms for vertical transmission to ensure that offspring acquires the necessary microbial symbionts (Engel and Moran, 2013). Symbiotic microorganisms are important for host survival and reproduction as they perform essential metabolic roles such as nutrient digestion (Engel and Moran, 2013). For example, *Providencia* spp. and *Morganella morganii* can express urease which in turn leads to the production of high levels of biogenic amines (Hu et al., 1990; Zogul and Zogul, 2004). Biogenic amines neutralizes acidic digestive fluids in the host's gut and therefore prevents the hydrolysis of certain bacterial species (Kawahara et al., 1999). Moreover, the presence of bacteria may be influenced by shifts in the insect life cycle. In our study, the BSFL guts were examined only at the fifth instar larval stage. Holometabolous insects such as BSF experience metabolically dynamic and complex processes during their transition from larvae to adults (Kohl et al., 2013), and gut microbial communities experience significant changes during metamorphosis and in the adult stage (Moll et al., 2001). Likely the gut undergoes a sterilization process during metamorphosis and acquires new microbiota in the adult stage (Kohl et al., 2013). Yet, Chen et al. (2016) showed that *Enterococcus* spp. such as *Enterococcus mundtii* survived metamorphosis in the gut of the cotton leafworm *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) and were carried over to the adult stage. Moreover, Zheng et al. (2013) reported shifts in the gut bacterial composition of BSF reared on identical rearing substrates during different development stages. Finally, Su et al. (2010) reported a carry-over of several bacterial taxa through larval stages to newly emerged adult house flies. Such observations suggest that though the composition of microbial communities associated with the BSF gut undergoes changes during metamorphosis, certain bacterial species are carried over regardless of life stages development. The wide-spread presence of these bacterial genera in different BSFL from different locations suggest the possible existence of core microbiota in BSF. Yet, the abundance of these species seems highly variable depending on the abiotic and biotic factors in the rearing system, and possibly even the insect strain (Wynants et al., 2019).

In our study, the diversity of bacterial species in BSFL extract was relatively low in comparison to fresh rearing substrates and BSF frass. Additionally, *Staphylococcus* sp. was present in limited amounts in BSFL extracts in comparison to fresh rearing substrates and BSF frass. *Staphylococcus* spp. is a genus of gram-positive bacteria that colonizes a variety of animal species (Enright et al., 2002; Sung et al., 2008; Sakwinska et al., 2011). Almost all staphylococcal species have been identified as causes of opportunistic infections (Otto, 2004). However, certain *Staphylococcus* species are recurring and are considered threatening pathogens (Weese, 2010). For instance, *Staphylococcus aureus* is a dangerous pathogen that can cause severe and life-threatening human diseases including severe sepsis, pneumonia, toxic shock syndrome and endocarditis (Lowy, 1998). Moreover, *Staphylococcus aureus* can cause infections in animals including mastitis in dairy-producing animals and bumblefoot in chickens (McNamee and Smyth, 2000; Dufour et al., 2012; Zecconi and Scali, 2013). However, the significance of *Staphylococcus aureus* in terms of public health is caused by its ability to develop resistance to antimicrobials (Weese, 2010). The low diversity and limited abundance of bacterial species including *Staphylococcus* sp. that we observed in our study may be attributed to the expression of antimicrobial peptides (AMPs) in BSFL. AMPs are pathogen-targeting peptides that are produced in fat and blood cells and secreted to hemolymph through the activation of humoral immunity in response to pathogenic infections (Boman, 1995; Bulet et al., 1999; Brogden, 2005). AMPS which are small cationic molecules composed of 10 to 100 amino acids exhibit activities against bacteria, fungi, viruses and parasites (Lavine and Strand, 2002). Moreover, certain AMPS exhibit a cytotoxic behavior toward cancer cells (Hoskin and Ramamoorthy, 2008). Previous studies reported that insects including Coleoptera, Diptera, Hymenoptera and Lepidoptera can abundantly produce AMPs (Casteels et al., 1990; Bulet et al., 1999; Hoffmann and Reichhart, 2002; Goo et al., 2008a,b; Vetterli et al., 2018). For instance, a study by Choi et al. (2012) reported that a methanol extract of BSFL demonstrated antibacterial effects against gram-negative bacteria. Moreover, rearing BSFL on contaminated organic waste streams in our study may have enhanced the production of AMPs. Previous studies confirmed that the production of AMPs in insects is enhanced by bacterial infections (Lemaitre et al., 1997; Bulet et al., 1999; Hoffmann and Reichhart, 2002; Wang et al., 2016; Wu et al., 2018). Specifically, several studies reported a significant increase in the expression of AMPs in BSFL infected with bacteria (Vieira et al., 2014; Park et al., 2016; Lee et al., 2020). These results suggest that BSFL may be used in the commercial production of natural antibiotics that can replace synthetic ones in the future (Bahar and Ren, 2013). The replacement of synthetic antibiotics with natural ones can put an end to antibiotics resistance and antibiotics related environment pollution (Lee et al., 2020). Therefore, future research should focus on the possibility of developing cost effective and biologically stable BSFL- derived AMPs extraction and production methods.

CONCLUSION AND OUTLOOK

Even though this study has demonstrated the influence of rearing substrates on the gut microbial community of BSFL and indicated the potential of BSFL to up-take, among others, pathogens from contaminated rearing substrates, further assays need to be undertaken over a period to ascertain the trends observed. Moreover, the dominant presence of *Providencia* spp. in the guts of BSFL reared on both substrates highlights the existence of a core microbiota in their guts, irrespective of the rearing substrates used and needs to be explored further over time. The presence of some clinically pathogenic bacteria in the gut of BSFL is an indication that the selection of safe organic waste streams for industrial production of BSFL production for the wide market is crucial. This scenario is not common to the African continent alone, therefore global

insect-based feed policies should consider such findings. To conclude, policies and regulations that govern the production and use of emerging insect meal as an alternative to conventional meal sources as well as appropriate safety hygiene and quality control standards needs to be developed.

DATA AVAILABILITY STATEMENT

The data for morphological identification of bacterial isolates presented in the study are deposited in the GenBank of the National Center for Biotechnology Information (NCBI) repository (https://www.ncbi.nlm.nih.gov/nuccore/). Accession numbers provided for the nucleotide sequences of the bacterial isolates are as follows: *Providencia* sp. MSB6 = MK276967, *Providencia* sp. MSB9 = MK276968, *Providencia* sp. MSB12 = MK276969, *Providencia* sp. MSB22 = MK276974, *Morganella* sp. MSB27 = MK276976, *Brevibacterium* sp. MSB14 = MK276970, *Staphylococcus* sp. MSB18 = MK276972, *Bordetella* sp. MSB17 = MK276971, *Bordetella* sp. MSB21 = MK276973, and *Bordetella* sp. MSB24 = MK276975. The data for molecular data analysis presented in the study are deposited in the NCBI database repository under accession numbers PRJNA728669 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA728669) and SAMN19093411 (https://www.ncbi.nlm.nih.gov/biosample/?term=SAMN19093411).

AUTHOR CONTRIBUTIONS

MS, FK, FO, CT, KF, SS, SE, and CB conceived and designed the study, and discussed the results. MS performed the experiments, analyzed the data, and did the original draft preparation. FK, FO, CT, KF, SS, SE, OS, AH, and CB reviewed and edited the article. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.687103/full#supplementary-material

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