Journal of Insect Science, (2022) 22(6): 3; 1–6
https://doi.org/10.1093/jisesa/ieac066
Research

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Assessing Fungal Diversity and Abundance in the Black Soldier Fly and its Environment

Tzach Vitenberg[1,2] and Itai Opatovsky[1,2,3,]

[1]Department of Nutrition and Natural Products, Laboratory of Insect Nutrition and Metabolism, MIGAL - Galilee Research Centre, Kiryat Shmona, Israel, [2]Department of Animal Science, Faculty of Sciences and Technology, Tel-Hai College, Upper Galilee, Israel, and [3]Corresponding author, e-mail: itaio@migal.org.il

Subject Editor: Phyllis Weintraub

Received 12 July 2022; Editorial decision 27 October 2022.

Abstract

Detritivorous insects that flourish in decaying environments encounter microorganisms throughout their life cycle. However, it is not clear whether the microbial composition of the decaying environment affects the microbial composition of the insect gut, or whether the opposite is true, with the microorganisms that are adapted to the insect's digestive system being dispersed by the insects to new habitats, thereby becoming more and more common in the environment. To test these questions the fungal composition of the black soldier fly (BSF) (Stratiomyidae; *Hermetia illucens* Linnaeus) larval gut and its surrounding decaying environment (household compost bins) were analyzed using amplicon sequencing. Constancy in the dominance of the genus *Candida* (Debaryomycetaceae) in most of the environments and larval guts was found. This finding may suggest a 'core' structure to the fungal community of the BSF. In locations where nutrient composition of the environment had higher fiber content, the *Candida* was not dominant and the most common fungi were the genus *Gibberella* (Nectriaceae) and the family Dipodascaceae. The later was dominant also in the larval gut and the former was replaced with Meyerozyma (Debaryomycetaceae), which may suggest a selection process by the insect's gut. Little is known about the ecological interactions of insects with eukaryotic microorganisms, such as yeast-like fungi. As their metabolic complexity and ability is intense, they have the potential to dramatically affect the physiological condition of the insect.

Key words: *Candida*, *Meyerozyma*, Dipodascaceae, household compost, *Hermetia illucens*

The black soldier fly (BSF) (Stratiomyidae; *Hermetia illucens* Linnaeus) is native to the tropical and subtropical regions of America and is currently found worldwide (Kaya et al. 2021), mostly in dumpsters and compost bins in urban areas (Diclaro and Kaufman 2012). The adult fly hardly feed, while the larvae feed on a variety of decaying organic materials and complete their life cycle in two months in an ideal environment (Makkar et al. 2014). Furthermore, the larvae were found to reduce pathogens in waste, e.g., *Escherichia coli* (Liu et al. 2008). Therefore, rearing *H. illucens* has been an efficient way to dispose of organic wastes by converting them into a protein- and fat-rich biomass suitable for various purposes, including animal feed, biodiesel, and chitin production (Makkar et al. 2014) and large-scale production of BSF adults and larvae are being conducted worldwide (Cortes Ortiz et al. 2016).

As the BSF is found in decaying environments, which contain a plethora of microorganisms, some beneficial and some pathogenic, it encounters these microorganisms throughout its life cycle. These microorganisms can enter the BSF's body cavity (hemocoel) either by being consumed and penetrating the insect's gut or through the insect's cuticle. Therefore, the BSF can harbor a vast variety of microorganisms, some providing nourishment while others are pathogenic or pass through the alimentary tract unaffected (Nayduch and Burrus 2017, Rotheray 2019). In addition, these environments are highly changeable in their biotic and abiotic condition as the decay heads towards assimilation (Ceustermans et al. 2010). These changes affect the composition of the microbial community that the insects encounter (Jiang et al, 2019). However, it is not clear whether the microbial composition in the decaying environment affects its composition in the insect gut, or whether the opposite is true, with the microorganisms that are adapted to the insect's digestive system being dispersed by the insects to new habitats, thereby becoming more and more common in the environment.

In recent years the bacterial composition of the BSF has been extensively studied and was found to have a core bacterial community composition (Klammsteiner et al. 2020, Greenwood et al. 2021), which is influenced from the diet source, the insect's developmental stage, and location in the insect's digestive tract (De Smet et al. 2018, Bruno et al. 2020, Wynants et al. 2019, Callegari et al. 2020, Khamis et al. 2020, Klammsteiner et al. 2020) and the host genetics (Khamis et al. 2020, Greenwood et al. 2021). However,

knowledge regarding the fungal composition of the gut is lacking. A study that tested the fungal composition of the BSF larval gut under different diets found that the major fungal spp. were *Geotrichum candidum*, *Candida tropicalis*, *Pichia fermentans*, *P. kluyveri*, and *P. kudriavzevii*(Boccazzi et al. 2017). However, the fungal composition of the BSF larval gut is not known in populations from the natural environment, nor is it known whether the insect's fungal composition is correlated to the fungal composition in the surrounding decaying environment.

To test these questions, natural populations of BSF from household composts in Israel were chosen. The household compost was chosen due to the successive input of organic material, which is suggested to maintain a constant state of decomposing material and therefore create stable conditions for fungal community succession. Therefore, this study tests whether the fungal community in the insect gut is affected from the environmental composition or whether there is a selection for specific species by the insect.

Materials and Methods

Larvae Collection

Larvae at 5th instar stage were collected from the upper layer of household composts in Israel, which contained fresh waste that was not composited, aerobic conditions, and similar waste characteristics (only vegetarian households): Kiryat Tiv'on – KT (32° 43′05″ N 35° 07′39″ E); Timrat – T (32° 42′12″ N 35° 13′31″ E); Kfar HaHoresh – KH (32° 42′06″ N 35° 16′24″ E); Misgav Am – MA (33° 14′51″ N 35° 32′54″ E); Metula – M (33° 16′38″ N 35° 34′41″ E); She'ar Yashuv – S (33° 13′36″ N 35° 38′47″ E). The larvae were placed in 95% ethanol upon collection and were kept on ice until storage at –20°C prior to further analysis.

Nutrient Analysis of the Compost

To determine whether the yeasts in the environment were affected by the environmental conditions, the mega-nutrient composition of the compost was analyzed in three samples from each compost site. The samples were freeze-dried. Protein content was measured using the Kjeldahl method for analyzing total nitrogen and then calculating the protein ratio (WHO/FAO 2003). Total lipid content was determined by extraction with hexane in a Soxhlet apparatus following the technique reported by Bligh and Dyer (Bligh and Dyer 1959). Fiber and mineral contents were measured after digestion with H_2SO_4 (1.25%) and NaOH (1.25%) and burning the remains at 600°C in laboratory furnace (Bifartherm).

Amplicon Sequencing of the Fungal Composition of the BSF Larval Gut and of the Environment

For fungal samples from the insect larval gut, the larvae were rinsed in ethanol 70% and washed with distilled water. They were then dissected in laminar hood (Ivgel 9, A.D.S LAMINAR) and the gut was removed, rinsed with distilled water, and crushed with sorbitol buffer that contained 200 U lyticase (Lyticase from *Arthrobacter luteus*, Sigma-Aldrich). Each sample contained one larva (five larvae per site) and each collecting location had five replicates. In order to isolate fungi from the compost material, 5 g compost material was vortexed with 10 ml distilled water; 1 ml of the supernatant was collected and centrifuged at 11,000 rpm. The pellet was collected and mixed with a sorbitol buffer that contained 200 U lyticase (lyticase from *Arthrobacter luteus*, Sigma-Aldrich). Genomic DNA (gDNA) was extracted from the BSF samples using a Qiagen DNeasy blood and tissue kit. The ITS region of the rRNA gene was amplified using the primer set ITS1-ITS2 [(ITS1: TCCGTAGGTGAACCTGCGG; ITS2: GCTGCGTTCTTCATCGATGC (White et al. 1990)]. The libraries were sequenced on Illumina MiSeq platform, paired-end reads, 2X150bp. Raw sequence data were processed to remove adapters, primers (truncation of 29 bp), and primer free reads using R (R Core Team 2021). The package DADA2 (Callahan et al. 2016) was used to denoise the reads and remove chimeric sequences. Error rates were learned by alternating between sample inference and error rate estimation until convergence using the DADA2 package. The dereplicated sequences were clustered into operational taxonomic units (OTUs) with the UNITE (ver.8 04.02.2020, Nilsson et al. 2018) reference database based on a 97% consensus threshold.

Data Analysis

Differences in the nutrient composition of the compost material were analyzed using Kruskal–Wallis nonparametric analysis. The OUT's data was normalized by scaling with ranked subsampling (SRS) (Beule and Karlovsky 2020) prior for diversity analysis using the SRS package in R (Heidrich et al. 2021). Alpha diversity of the fungal community was calculated using the Simpson index. Differences in the taxonomic diversity and richness were analyzed using two-way ANOVA analysis. Beta diversity was measured using non-metric multidimensional scaling (NMDS) with Bray-Curtis dissimilarity analysis. Redundancy analysis (RDA) with log transformation was used to test the correlations between nutrient composition and community composition in the compost material. All statistical analyses were conducted using PAST (Hammer 2001) and R software, version 4.1.2 (R Core Team 2021).

Table 1. Content (percent by dry weight) of fat, protein, fiber and minerals in household composts from six locations

	Fat		Protein		Fiber		Minerals	
Location	Average	SD	Average	SD	Average	SD	Average	SD
Kiryat Tiv`on (KT)	30.05A	0.66	12.72B	0.09	11.07B	0.72	0.75B	0.95
Timrat (T)	26.16A	7.64	10.90BC	0.48	16.58AB	8.37	5.02B	3.64
Kfar HaHoresh (KH)	10.89B	4.63	12.00B	0.26	24.13A	1.86	1.3B	0.17
Misgav Am (MA)	25.13A	0.15	9.23C	0.05	16.55AB	2.31	2.79B	0.49
Metula (M)	10.29B	1.18	6.92D	0.09	11.90B	1.90	15.43A	7.03
She`ar Yashuv (S)	20.79A	3.32	14.40A	0.15	12.59B	0.84	0.73B	0.42

Capital letters represent significant differences among the locations ($p < 0.05$).

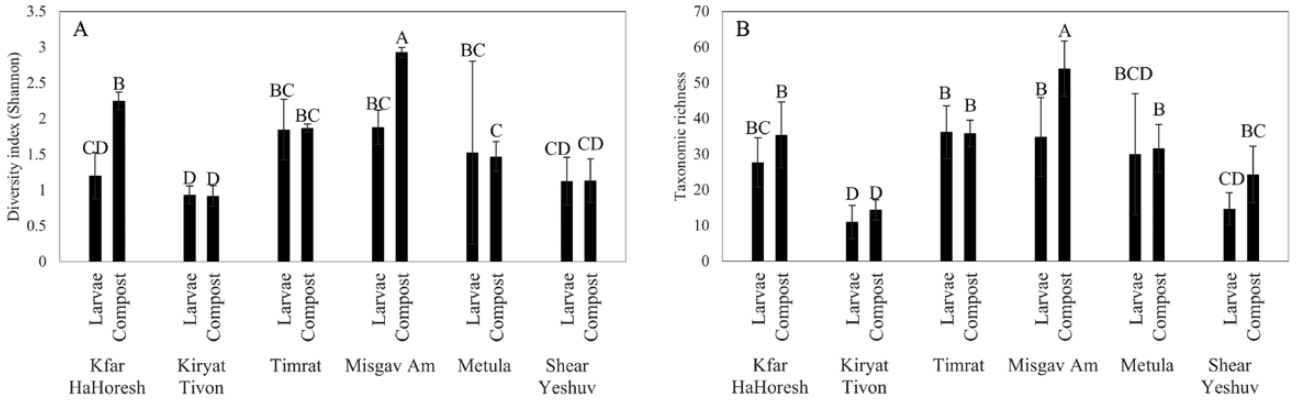

Fig. 1. Simpson diversity index (A) and taxonomic richness at the genus level (B) of the fungal community composition in the BSF larvae and its surrounding environment (compost) from six locations. Significant differences are represented by different capital letters.

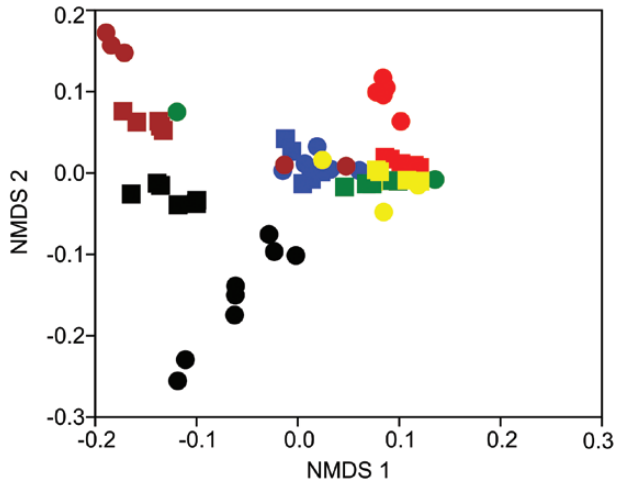

Fig. 2. NMDS using Bray-Curtis dissimilarities of the fungal composition in BSF larval gut (circle) and compost samples (square) from the different locations: Kfar HaHoresh (KH; black), Kiryat Tiv'on (KT; red), Timrat (T; blue), Misgav Am (MA; brown), Metula (M; green) and She'ar Yashuv (S; yellow).

Results

Compost Composition

Two sites, KH and M, had the lowest fat content ($X^2_{df=5}$ = 15.8, p = 0.007). Protein content was highest in S and lowest in M ($X^2_{df=5}$ = 16.6, p=0.005). Fiber content was highest in KH but not significantly higher than in MA and T ($X^2_{df=5}$ = 12.4, p = 0.03). Mineral content was highest in M ($X^2_{df=5}$ = 16.7, p = 0.005) (Table 1).

Taxonomic Diversity and Richness

Taxonomic diversity and richness was highest at the compost of MA (Diversity: 2.9, locations: $F_{df=5,50}$ = 32.4, p < 0.001, gut/compost: $F_{df=1,50}$ = 29.6, p < 0.001, locations*gut/compost: $F_{df=5,50}$ = 8.3, p < 0.001; Richness: 54, locations: $F_{df=5,50}$ = 27.3, p < 0.001, gut/compost: $F_{df=1,50}$ = 16.1, p < 0.001, locations*gut/compost: $F_{df=5,50}$ = 2.2, p = 0.7; Fig. 1A and B). The lowest taxonomic diversity and richness was found in KT's larvae and compost (diversity: 0.9, 0.9; richness: 11, 14; respectively). The NMDS using Bray-Curtis dissimilarities showed three groups: most of MA's larvae and compost that were clustered together, second cluster was the compost of KH, and the third cluster was the rest of the larvae and compost site from the other locations (Fig. 2).

Amplicon Sequencing of the Fungal Composition of the BSF Larval Gut and the Compost Material

In total, 697,802 OTUs were identified in the analysis (62 samples, Supp Table 1 [online only]). Comparison of fungal composition of the compost and the larval gut at the different locations showed several patterns: locations that were dominated by *Candida spp*. Berkhout (Debaryomycetaceae) in both the compost and the BSF larval gut. These locations were KT, T, M, and S, with *Candida* abundance was 76%, 46%, 68%, and 76%, respectively, in the compost, and 60%, 55%, 50%, and 68%, respectively, in the larval gut (Fig. 3 and Supp Table 1 [online only]). Locations where the fungal composition was not dominated by *Candida* showed different patterns: in MA, were the compost had diverse species composition with low abundance, the gut was dominated by Dipodascacea (33% in the larva gut and 14% in the compost). KH that had higher abundance of *Gibberella* Sacc (Nectriaceae), *Candida*, and *Meyerozyma* Kurtzman & M. Suzuki (Debaryomycetaceae) in the compost (abundance of 27%, 20%, and 15%; respectively; Fig. 3), was dominated in the larvae gut by *Meyerozyma* (52%; Fig. 3).

Effect of Nutrient Contents on Taxonomic Composition in the Compost

The first two RDA axes explained 36% and 22% of the variance, respectively. The macronutrient composition of the environment significantly (p < 0.05) influenced the fungal community composition of the compost and larval samples. The fungal community composition was separated on the first axis that was influenced mainly by fiber versus fat, protein, and minerals contents and the second axis that was influenced by protein and fiber versus fat content (Fig. 4). The fungal composition in Misgav AM (MA) and Hfar Ha`Horesh (KH) showed a different patterns. These sites were influenced manilt by the fiber content. However, MA was affected also from the fat content and KH was influenced also from the protein content (Fig. 3).

Discussion

Few studies have analyzed the fungal composition of BSF larvae; most of them studied laboratory colonies that were reared on different diets (Boccazzi et al. 2017, Kuznetsova et al. 2021, Tanga et al. 2021). This study presents a novel insight into the fungal composition of the BSF larval gut and its environment from samples that were collected from natural habitats. Although the microbial

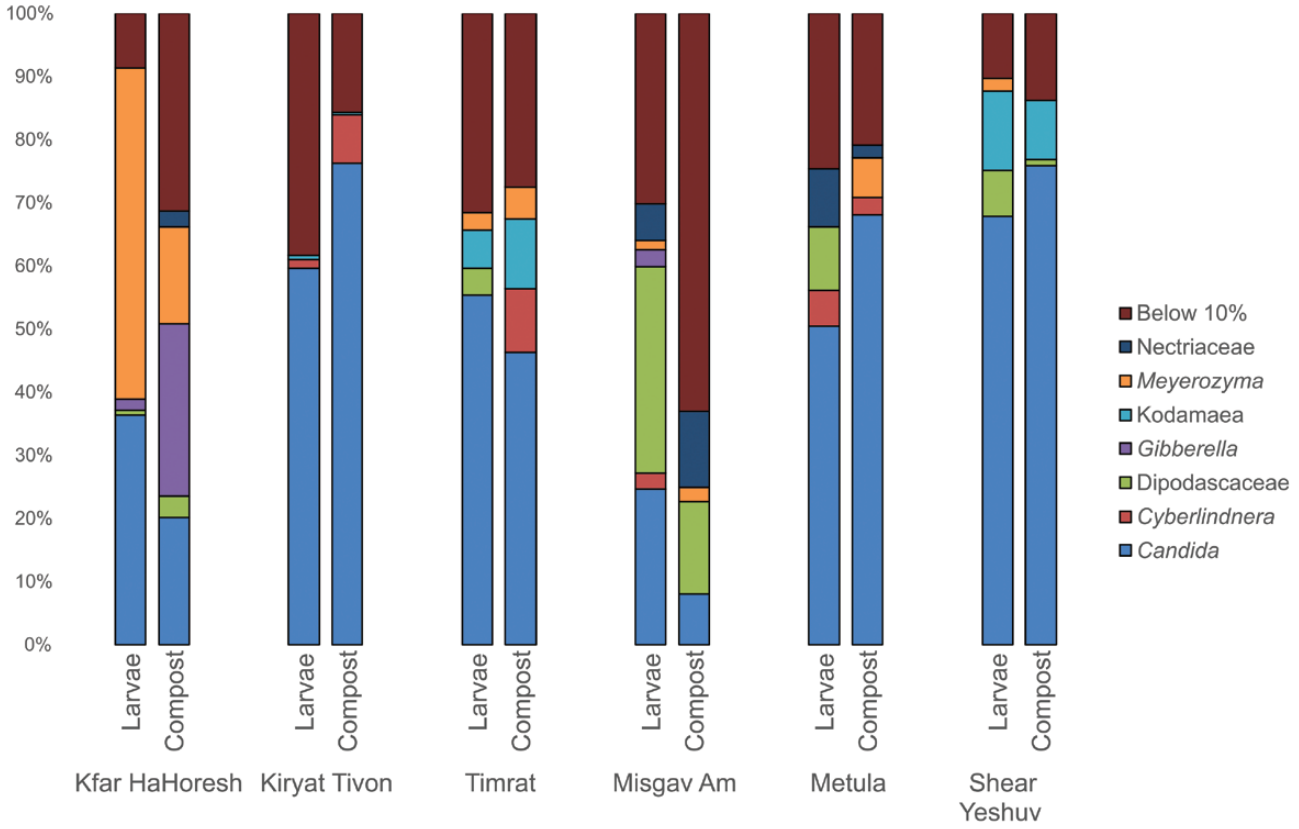

Fig. 3. Composition of the main fungal groups (above 10% abundance) in the BSF larval gut and the surrounding environment (compost) from the different locations.

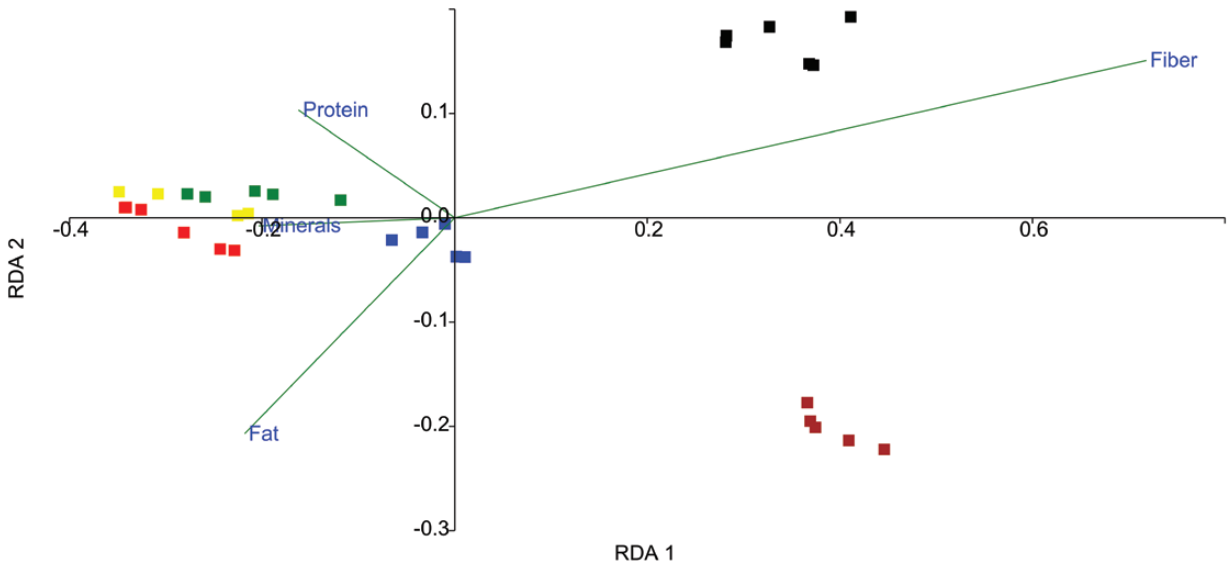

Fig. 4. RDA of the fungal composition in BSF larval gut and compost samples from the different locations: Kfar HaHoresh (KH; black), Kiryat Tiv'on (KT; red), Timrat (T; blue), Misgav Am (MA; brown), Metula (M; green) and She'ar Yashuv (S; yellow). The significant nutrient components of the compost are indicated and marked in green arrows.

composition may change in this type of environment through time due to the composting process (Jiang et al. 2019), we found constancy in the dominance of *Candida* in most of the environments and larval guts. These results contradict previous analyses of the gut fungal community composition of BSF larvae that were fed on agricultural waste (Boccazzi et al. 2017, Tanga et al. 2021) and of the community composition of the compost environment that was treated with BSF (Kuznetsova et al. 2021). In these cases, the most abundant fungal genera in the BSF were *Pichia* and *Cyberlindnera*, which were not abundant in our study. This study suggests that the fungal community structure is affected from a 'core' fungal community as found in related studies of the bacterial community

composition of the BSF (Klammsteiner et al. 2020, Greenwood et al. 2021).

The mechanism behind the dominance of *Candida* in these environments may be mutualistic interactions between the BSF larvae and *Candida*, as the larvae provide nutrients and mechanical defense for the fungi and the fungi provide supplementary metabolites or nutrients for the insects through direct consumption. Such mutualistc interactions are known from other dipterans, such as *Drosophila spp*., in which the fungus is known to affect insect survival and developmental time (Anagnostou et al. 2010). High abundance of *Candida* has also been reported in several *Drosophila* species (Chandler et al. 2012). Conversely, the interaction may be commensalism, in which *Candida* does not provide any benefits to the insect, while the insect provides a vector for fungal dispersal to new resource patches and increases the probability of outbreeding (Madden et al. 2018). However, these dispersal 'services' can have an indirect effect on the insect due to colonization of the insect's environment by neutral fungi, which compete with entomopathogenic fungi and reduce their ability to increase in population size (Stefanini 2018) or due to secretion of mycotoxins that harm pathogenic fungi (Boccazzi et al. 2017).

Given the fungal–insect interaction, we must also consider what determines the fungal composition of the insect environment and gut. *Candida spp*. may be common in the environment, and therefore, highly abundant in the insect gut. Alternatively, these species may be adapted to the insect gut and provide the insect with a benefit that allows the insect to disperse *Candida spp*. to new resource patches and increase their abundance in the environment. We must also determine whether the most important factor affecting fungal composition is the insect diet (Li et al. 2021) or the environmental composition (Cohen et al. 2020). It seems that in the locations that were not dominant by the *Candida* (MA and KH) the fungal community composition was influenced by the fiber content of the compost. In Misgav Am (MA) the fungal diversity was highest and the fungal composition was diverse without any dominant groups. The insect gut from this location had higher abundance of Dipodascaceae, which was one of the abundant groups in the compost. Therefore, it seems that dominance of different species in the environment affects their dominance in the insect gut. On the other hand, in Kfar Ha`Horesh (KH), the most common genus in the insect gut at this location was *Meyerozyma*, whereas the compost had higher abundance of *Gibberella*. Therefore, it seems that the insect shows selectivity for one of the fungal groups, maybe due to the abiotic condition of its gut, e.g., pH. Moreover, consumption of a high fiber diet can increase the production of anti-microbial peptides (AMP) by the insect (Vogel et al. 2018). These AMP are part of the insect's immune system and can affect the microbial composition of the insect's environment and gut. Therefore, in cases where dominant species are not present, the important factor determining the fungal composition of the insect gut may be its diet.

To conclude, the fungal community of the BSF is dominated by *Candida*. However, in environment rich in fiber, other fungal genus are more common. In general, the dominated fungal group in the environment will dominate the insects gut, although in some cases the insect gut affects selectively the colonization of the fungi in the gut. Understanding the effect of these fungi on the BSF lifecycle is still a black box. Little is known about the ecological interactions of insects with eukaryotic microorganisms, such as yeast and yeast-like fungi and molds. As their metabolic complexity and ability is intense, they have the potential to dramatically affect the physiological condition of the insect. Revealing these interactions may help to increase the rearing efficiency of insects, such as the BSF, which is currently being reared at large scales as an alternative protein source (Kooienga et al. 2020).

Acknowledgments

We would like to thank Llzar M. and Sherf G. for assisting with the NGS analysis and three anonymous reviewers who helped to improve this manuscript. This research was support by the Israel Science Foundation (1167/21).

Author Contributions

TV: Conceptualization, Investigation, Formal analysis, writing – original draft. IO: Funding Acquisition, Supervision, Writing – review & editing.

Supplementary Data

Supplementary data are available at *Journal of Insect Science* online.

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