**The invasion of the holo-organism - The importance of the mycobiome for the invasion of arthropods**

1. **Scientific background**

Invasive species, species that have successfully been introduced, established, and spread beyond their native range, are a major feature of the Anthropocene period, due to the increased movement of people and goods (Le Roux, 2022). One of the main groups of invasive species that affect dramatically the native species composition (Kenis et al., 2009) and cause high economic costs due to damage to the food supply and human health (Diagne et al., 2021) are insects. In the US alone, invasive insects caused estimated damage of US$21.4 billion per year in 2017 (Diagne et al., 2021). The invasive insect characteristics are 1) Generalist feeding habits: many invasive insects exhibit a wide range of feeding preferences, allowing them to utilize diverse food sources. They often possess adaptations that enable them to feed on a variety of plants or prey on different organisms (Snyder and Evans, 2006). This versatility increases their chances of finding suitable resources in their new environment. 2) in addition, invasive species have a more efficient resource use for consumption: Invasive insects tend to utilize resources more efficiently than native species. They may have specialized physiological or behavioral adaptations that allow them to extract maximum energy or nutrients from limited resources, giving them a competitive edge (Shik and Dussutour, 2020). 3) Environmental tolerance: Invasive insects can tolerate a wide range of environmental conditions, including temperature, humidity, and soil types. This adaptability enables them to thrive in diverse habitats, increasing their chances of survival and spread (Renault et al., 2017). 4) Reduced natural predators: Invasive insects are often introduced to regions where they have no natural predators or where their predators are absent, ineffective or they can evade them. Without these natural control mechanisms, they can experience reduced pressure and face fewer constraints on their population growth (Fortuna et al., 2022). 5) In addition, they have high dispersal and reproduction rates that allow them to colonize novel environments (Renault et al., 2017).

To become invasive and establish a proliferating population in a novel habitat invasive species often exhibit phenotypic plasticity, meaning they can adapt their traits and behaviors to different environmental conditions (Le Roux, 2022). This flexibility allows them to exploit various resources and adapt to changing circumstances, giving them a competitive advantage over native species. An additional factor that contributes to phenotypic plasticity and is mostly neglected is the holobiome (Renault et al., 2017), which consists of the organism and its associated microorganisms (Zilber-Rosenberg and Rosenberg, 2008). As these microorganisms can affect the host genome through horizontal gene transfer (Renault et al., 2017), they can also influence its phenotypic plasticity. For example, microbes can provide the host with nutrients that will allow it to consume a more generalist diet, e.g. by detoxifying plant defensive allelochemicals for herbivorous insects (Oliver and Martinez, 2014) or utilize the diet more efficiently and therefore become a better competitor (Oliver and Martinez, 2014). in addition, in some cases, the microorganisms can affect their host’s ability to survive in different a-biotic conditions, e.g. low and high temperatures (Heyworth et al., 2020). Therefore, the presence and composition of microorganisms and their effect on their host’s ability to become invasive is a novel research direction that should be further investigated.

Insects are known to have a comprehensive world of interactions with microorganisms. Insects can harbor microorganisms on their outer skeleton, in the gut, within their cells, or within specialized organs called mycangia or bacteriocytes (Douglas, 2015). The microorganisms can be transferred between the generations in a vertical transmission or can be acquired by each generation from the environment or other individuals (Bright and Bulgheresi, 2010). The microorganisms are known to provide essential nutrients that are lacking in the insect’s diet, such as specific amino acids, vitamins, and sterols (Douglas, 2009). This phenomenon is common in insects with a homogenous diet, such as aphids, termites, and planthoppers (Douglas, 2015, 2009). In addition, microorganisms can assist in the digestion of indigestible plant materials such as lignin and cellulose and increase the nutrient intake efficiency of their host (Douglas, 2015, 2009). Usually, insects that use microorganisms to degrade plant cell walls will be equipped with fermentation chambers or projecting papillae in their hindguts (Douglas, 2015, 2009). Microorganisms can assist herbivorous insects by detoxification of plant secondary metabolites as seen in *Lasioderma serricorne* beetles that eliminate allelochemicals using the yeast-like symbiont *Symbiotaphrina kochi* (Douglas, 2015). The microorganism can protect its host against natural enemies, e.g., the symbiont *Hamiltonella defensa* confers pea aphid resistance to the parasitoid *Aphidius ervi* (Douglas, 2015). In addition, microorganisms can affect the production of pheromones that can influence the insect’s behaviors (aggregation, oviposition site ect’) (Engl and Kaltenpoth 2018). Therefore, due to the vast interactions between insects and microorganisms, I expect that these microorganisms affect the invading abilities of insects by improving the insect’s fitness and competition abilities (Lu et al., 2016).

The black soldier fly (*Hermetia illucens*; BSF) is a good model organism to test for the influence of microorganisms on the invasive abilities of their insect host. The BSF is of Nearctic origin and it is presently distributed in the majority of the warmer parts of the world due to its highly invasive abilities (Ståhls et al., 2020). The BSF completes its life cycle within 60 days (Makkar et al., 2014). The larvae have detritivorous feeding habits and grow in rotten organic material, from household compost to animal feces and carcasses (Makkar et al., 2014). At the 6th instar stage (pre-pupa) the larvae change their color to black and desert the organic material to pupate. The adults that emerge do not feed but reproduce and the female lays about 500-1000 eggs near a patch of organic material (Booth and Sheppard, 1984). The larvae encounter in the rotten organic material a wide array of microorganisms, some can be beneficial, and some pathogenic. The bacteria present in the BSF gut are composed of a core community that changes according to the substrate composition (De Smet et al., 2018; Klammsteiner et al., 2020) and are hypothesized to assist in hydrolytic degradation of complex substrates (Callegari et al., 2020) and increase efficiency of substrate utilization (Li et al., 2022).

In addition, the mycobiome composition of the BSF gut and environment is also composed of the main core community (Boccazzi et al., 2017; Vitenberg and Opatovsky, 2022), which is suggested to assist in amino-acids and vitamin B6 metabolism (Kannan et al., 2023). In addition, as the BSF is now widespread throughout the Palearctic region, it has to deal with wide temperature gradient and survival during the diapause in the cold season. The dominant fungi that were found in Italy and Russia (Boccazzi et al. 2017, Kuznetsova et al. 2022; temperate regions) were *Pichia kudriavzevii*, in northern Israel (Vitenberg & Opatovsky 2022, Mediterranean region) the dominance was of *Pichia Tropicalis* and in the South of Israel (Semi-arid region, preliminary results) the dominant species was *Kluyveromyces marxianus*. This difference in dominance can be due to the adaptation of the yeast or some beneficial advantage of these yeasts in different temperatures. In addition, plants guard themselves against herbivory using primary and secondary metabolites, such as Polysaccharides (cellulose, pectin, and lignin) and polyphenols (tannins). For insects that consume rotten decaying vegetative materials, such as the BSF, degradation of these components can facilitate the digestion process. As fungi are known to degrade these metabolites, especially for wood-eating insects (Dowd 1992.; Geib et al., 2008; Itoh et al., 2018) the presence of these fungi can improve the nutrition ability of the invasive species and therefore facilitate their invasion. Preliminary results support that *C. tropicalis* increase the carbohydrates in the substrate that contains fiber (preliminary results). These results suggest that *C. tropicalis* may digest the indigestible fiber. This research concentrates on the ability of the BSF’s mycobiome to improve the invasive abilities of the insect. The effect of the gut fungal composition in insects has been hardly studied and is expected to have a vast influence due to their intense metabolic complexity and ability, therefore they have the potential to dramatically affect the physiological condition of the insect.

As the BSF colonizes novel and temporal habitats it should have a strong competitive ability. These abilities provide a potential for invasiveness for the BSF. In 1959 Furman et al. (Furman et al., 1959) argued that in manure where multiple larvae of BSF larvae were abundant, *Musca domestica* (L.) flies were scarce. This observation (also observed personally) may indicate the strong competitive abilities of the BSF larvae. However, Direct consumption of *M. domestica* was not observed. In our preliminary work, the survival of *M. domestica* larvae was tested after exposure to BSF larvae (to test for direct consumption), exposure to the substrate after BSF consumption (to test for indirect harmful extrusions by the BSF larvae), and to *Candida tropicalis* (common yeast-like fungi from the BSF gut and environment) that were added to the feeding substrate. A significant reduction in survival was found in the *M. domestica* larvae that were exposed to the fungi (Figure 1 and Preliminary results section).



Figure 1: Survival of house fly larvae in a diet with BSF larvae (BSF larvae), in the substrate after BSF feeding (Substrate after BSF), in a diet with *Candida tropicalis* (CT), and standard diet (Control).

This led me to the main hypothesis of the research proposal, that the BSF transfers microorganisms to novel habitats through its gut system or that it helps the colonization of specific microorganisms and these microorganisms help it to compete and colonize these habitats (and also to invade new habitats). This process can be achieved by 1) Directly harming other competing species, 2) Changing the environment making it less suitable for other organisms, 3) Improving the nutrient utilization of the BSF from the substrate or 4) Helping the BSF to deal better with new a-biotic conditions. However, for these interactions to occur deliberately, the BSF has to be able to identify and be attracted by these microorganisms and to help colonize these microorganisms in the novel environment.

1. **Research objective and expected significance**

**The overall objective of the proposed study is to obtain direct evidence for the effect of mycobiome composition on the invasiveness abilities of their insect host and the mechanisms in which this effect occurs**. The specific objectives of this study are: 1) To test for vertical transmission of fungi in the BSF life cycle; 2) To test for colonization of specific fungal species by the BSF; 3) To test the ability of the black soldier fly to detect specific fungi species 4) To evaluate the influence of microorganisms on adapting to new environments (a-biotic conditions). 4) To evaluate the influence of microorganisms on adapting to new environments (biotic conditions). 5) To evaluate the effect of the mycobiome on the community composition and colonization of habitats by the BSF. The **significance** and **novelty** of this research lie in providing knowledge regarding the interaction between insects and their fungal gut microorganisms, a research topic that is hardly been studied, in addition, the research will provide knowledge regarding the effect of microorganisms on the invasion process of insect, which is a topic lack of information. This research will provide basic knowledge of unstudied interactions and effects between microorganisms and their host. This knowledge can be further applied to other systems of invading insects (such as agricultural systems) or other invading organisms (non-insects). In addition, this research will provide knowledge regarding the influence of microorganisms on interaction within the multi-trophic web, by using laboratory experiments to test for specific effects and then combining this data into a multi-species environment using machine learning methods to construct the complex experimental setting.

A summary of the research design is presented below (Figure 2):



Figure 2: A summary of the research design, research objectives are marked in bold, and planned tests are presented in boxes.

1. **Detailed description of the proposed research**

C.1. General working hypothesis

My general hypothesis is that the fungal microorganisms in the insect gut increase the invasiveness of their insect host in mutual interaction. The effect on the host is due to changes in the environment and making it more suitable for the insects, increasing the ability of the host to deal with different a-biotic conditions or reducing the competitiveness of the competitors. On the other hand, the insect host provides a vector for dispersal or helps the microorganisms to colonize the habitat. To test this, I will combine molecular and ecological tools to test the dispersal and colonization of insects and microorganisms within the individual, population, and community scales.

C.2. Objective 1: Test for vertical transmission of specific fungi throughout the BSF lifecycle

C.2.1. Specific working hypothesis

The fungal species that were found common in the BSF gut and environment (Boccazzi et al., 2017; Vitenberg and Opatovsky, 2022) are expected to be vertically transmitted throughout the life cycle of the insect and therefore colonize new patches by the BSF adults. This hypothesis will be tested by identifying and quantifying the fungal load throughout the different life stages of BSF both on the outer cuticle and within the gut. This experiment will provide knowledge of whether the fungi are transferred to the adults from the pupa and whether the adults transfer the fungi through the eggs.

C.2.2. Identifying fungal community composition and quantifying the fungal load throughout the BSF lifecycle

The presence of microorganisms and their composition will be tested on the outer and inner parts of the adults, eggs, 1st instar, 5th instar, and pupa of the BSF. The outer surface will be washed with buffer and DNA will be extracted using a DNA extraction kit (from the washed buffer). For the inner parts, the outer surface will be sterilized and the gut (or whole content in the case of the eggs and small larvae) will be used to extract DNA using a DNA extraction kit. In both cases, the ITS region of the rRNA gene will be amplified using the primer set ITS1-ITS2 [(ITS1: TCCGTAGGTGAACCTGCGG; ITS2: GCTGCGTTCTTCATCGATGC (White et al., 1990)]. The libraries will be sequenced on the Illumina MiSeq platform, paired-end reads, 2X150bp. Raw sequence data will be processed to remove adapters, primers, to denoise the reads and remove chimeric sequences using R package DADA2 as described at (Vitenberg and Opatovsky, 2022). The dereplicated sequences will be clustered into operational taxonomic units (OTUs) with the UNITE reference database (Nilsson et al., 2019). This analysis will provide data regarding changes in the community composition. To test for changes in the fungal abundance (in a quantified method) a q-PCR with specific primers based on the ITS region.

For testing the fungal transfer between adults and larvae: adult BSF that hatched from the pupa of larvae that were reared on the whole life cycle on a basic diet that contains all nutrients (including casein, sugar, potato starch, canola oil, mineral mix, vitamin mix, sawdust, and water) (Kannan et al., 2023) with supplemental yeast (*C. tropicalis*, *P.* *kudriavzevii* that were isolated form the BSF gut and *S. cerevisiae* that will be purchased– separately) will be used. The specific yeast amount (% of diet) that should be added to achieve effect, will be tested beforehand. These adults will be taken to lay eggs on a sterile diet and the presence of fungi on the eggs' surface and content will be tested. Based on the presence of the yeast in the eggs, a further experiment for testing the fungal transfer within the larvae stages and to the adults will be conducted. If the eggs contain fungi, they will be placed in sterile substrate and the presence of the fungi will be tested in the different larval stages, pupa, and adults. If the eggs do not contain fungi, they will be placed in substrates that include yeast. From each yeast and life cycle, five individuals will be examined and compared to individuals from a diet without supplemental yeast.

The fungal species will be taken from fungal isolates that are present in Opatovsk’s laboratory. The fungi will be reared on yeast extract peptone dextrose medium (YPD) and will be concentrated using a centrifuge to be added to the insect’s diet. The adult flies will be reared in 1X1X1 cages in a rearing room (30oC, 70% humidity, and 12/12 L/D) in Opatovsky’s laboratory and let to lay eggs in cardboard that will be placed over plastic cases with diet. Larvae will be reared in 250 ml plastic cases with 100 gr of insect diet and 100 larvae in a rearing chamber (NUVE, TK-600; 27oC, 70% humidity, and 12/12 L/D)

 C.2.3 Expected results and pitfalls

 The experiment will provide data on whether the fungi that are common in the BSF gut are acquired at the larval stage and transferred to the next generation through the adults.

C.3. Objective 2: colonization of specific fungal species by the BSF

C.3.1. Specific working hypothesis

I hypothesize that BSF has selectivity to several yeast species which allow them to reproduce and colonize the environment or transmit to novel environments. If a vertical transmission is detected, colonization of the fungi in novel environments by the BSF adults will be tested. If no vertical transmission is detected, an increase in the abundance of the specific fungi due to the BSF will be tested. In case there is vertical transmission but no colonization of fungi by the BSF adults will be detected, changes in the yeast abundance in the environment will be also tested

C.3.2. Dispersal of specific fungi to novel patches

To test for the dispersal of fungi to novel patches by the BSF, adults who were reared on a diet with supplemented fungi will be exposed to sterile substrate for egg laying. The presence and abundance of the fungi in the substrate will be measured and will be compared to a substrate that was exposed to adults that were reared on a sterile substrate without supplemental fungi. The adults will be hatched and reproduce in net cages in the rearing room and will be provided with plastic cases with diet and cardboard for egg laying. After laying eggs the eggs will be hatched in a petri dish in a rearing chamber and the larvae will be reared in a rearing chamber with a 500 gr diet in a 2 liter plastic case (500 larvae in each case, 5 replicates for each treatment). The presence and abundance of the yeasts will be tested using qPCR with general primers for yeast (YEASTF 5′-GAGTCGAGTTGTTTGGGAATGC-3′; YEASTR 5′-TCTCTTTCCAAAGTTCTTTTCATCTTT-3′; Hierro et al. 2006) and specific primers for the tested yeast (e.g. for *C. tropicalis* - Garcia-Martines et al. 2010). The samples (1 gr) will be taken from different locations and depths of the substrate (three samples from the center of the pile – upper part, middle, and bottom and the same sample from the edge of the pile) every five days from encountering of the hatched larvae (five replicates for each treatment).

C.3.3. Dispersal of specific fungi within the patches

To test for the assistance of the BSF in the colonization of the fungi within the patch, newly hatched larvae (1st instar) will be placed in substrates that are supplemented with yeasts that will be placed in the middle of the pile (500 gr of diet with 500 larvae placed in 2 L plastic pox in rearing chamber’ 5 replicated for each treatment). A preliminary experiment’ in which the effect of different amounts of yeast (0.001% and 0.01% from the diet weight) on the establishment by the BSF should be conducted. The presence and abundance of the yeast will be tested in the substrate as explained before (section C.3.2) and will be compared to substrates without supplemental yeast.

C.3.4. Dispersal of specific fungi within the population

In addition, the dispersal of fungi to other BSF larvae within the population will be tested. The experiments will be based on the results of the previous section (C.2.2.). If the hatched larvae contain the yeast, 10 larvae (1st instar) with yeast will be placed in a sterile substrate with 90 sterile larvae. Every five days 30 larvae will be taken and analysed for the presence of the yeast using specific and general primers (as described in section C.3.2.). Five replicated will be conducted. If the hatched larvae do not contain the yeast, sterile larvae will be placed in substrates with supplemented yeasts (0.001% and 0.01% from the diet weight) that will be placed in the middle of the pile. The percentage of larvae that contain the yeast will be sampled as described before.

C.3.5. Expected results and pitfalls

If the fungi are vertically transmitted in the BSF life cycle, I expect to see that the BSF helps to colonize the fungi in new habitats. If the fungi are not vertically transmitted I hypothesize that the fungi arrive at the patch individually but the BSF larvae help the fungi to colonize and take over the patch. Therefore, I expect to see an increase in the abundance of fungi in the patch. In case I don’t any effect of the BSF larvae on the colonization of the fungi will test this in different diets: 1) standard substrate for Dipteran (Gainesville diet contains alfalfa, wheat bran, and corn meal) (Hogsette, 1992). 2) a Diet that is composed of fruits and vegetables (that resemble household composts – habitat that is being colonized naturally by BSF) and 3) Chicken manure (habitat that is being colonized naturally by BSF)

C.4. Objective 3: the ability to identify specific microorganisms by the BSF

C.4.1. Specific working hypothesis

I hypothesize that if the microorganisms provide an advantage for the insect they will be able to identify them in the environment. The identification will be either at the adult stage and will affect the decision of the adults where to lay eggs or at the larval stage and affect the consumption pattern causing for consumption of patches of food with microorganisms.

C.4.2. Identification of selected fungi by the BSF adults

This test will be composed of two experiments: the direct effect of the microorganism volatiles on adult attraction using an olfactometer and the effect of the microorganisms on the oviposition behavior of the fly that will be tested in cage experiments.

The olfactometer experiments will be conducted on the olfactometer that will be purchased for this matter. In each set of experiments, there will be a comparison in the fly behavior between two samples: one sample of the diet without yeast and one sample of the diet with yeast. Four types of diet will be tested to find different effects of the substrate itself and the interactions between the microorganisms and the substrate: 1) Basic diet (Kannan et al., 2023). 2) standard substrate for Dipteran (Gainesville diet) (Hogsette, 1992). 3) a Diet that resembles household composts and 4) Chicken manure. In these diets, the effect of the fungi *C. tropicalis* *P. kudriavzevii* and *S. cerevisiae* will be tested (eight replicates from each combination). In case an effect is present, the minimum abundance of yeast that produces the effect will be tested. In each experiment, one BSF adult female (two days after hatching – mated female) will be used.

The cage experiment will be conducted in 1X1X1 m net cages that will be placed in a rearing room (30oC, 70% humidity, and 12/12 L/D). At the beginning of the experiment, 40 BSF pupae will be placed in each cage. In each experiment, the best combination of diet and yeast will be tested (according to the olfactometer experiments). If no effect will be observed in olfactometer experiments the four types of diet (mentioned before) will be tested. In each cage 200 ml plastic container will be placed with each treatment, eight replicates for each cage (the different diets will be tested in separate experiments). In each plastic container, the cardboard will be placed above the substrate for oviposition. The effect on the oviposition will be measured by opening the cardboard, collecting the eggs, and comparing their weight (using \_\_\_\_\_\_).

C.4.3. Identification of microorganisms by the BSF larvae

This test will be composed of two experiments: the direct effect of the microorganism volatiles on larvae attraction using an olfactometer and the effect of the microorganisms on the feeding behavior of the larvae will be tested in plate experiments.

The olfactometer experiments will be conducted and described for the BSF female adults (same diets and yeast combinations) with 3rd instar (five days larvae) and 5th instar (eight replicates each).

The plate experiments will be conducted in Petri dishes (size 20 cm) that will contain a thin layer of the substrates with half of the plate containing the supplemental microorganism (substrate and yeast described before). The larvae movement will be observed and recorded using \_\_\_\_\_\_\_ and the movement will be analyzed using Ethovision XT software (Noldus). The time spent in each area will be calculated for every half an hour from the placement of the larvae. the experiment will be conducted with the 3rd instar (five days old larvae) and 5th instar (eight replicates each).

C.4.4. Expected results and pitfalls

It is expected to find an effect of the yeast on the substrate preference of the BSF adults or larvae (or both). However, it could be that no preference will be found by the insect. This finding could mean that the insect contributes to the colonization of the fungi without identifying them in the environment.

On the other hand, if there is no effect on the BSF preference, various aspects should be tested: the cage experiments with the BSF adults could be affected by volatile masking within the cage. Therefore, separate experiments with isolated treatment – each cage with specific treatment (or using sealed cages) and egg weight will be compared between the treatments. If no effect on the larvae movement is detected, a "cafeteria" experiment with different lumps of food will be conducted (as done by (Shishkov et al., 2019). In addition, the effect of the presence or absence of yeast in the adult and larvae gut on their preference should be tested.

C.5. Objective 4 – Influence of selected fungi on adapting to a new environment (a-biotic conditions)

 C.5.1. Specific working hypothesis

The environmental fungi are expected to provide an environmental advantage to the BSF that harbors them. I will test these advantages in two conditions that favor an invasive species into rotten organic material: dealing with high and low temperatures and the presence of the plant’s indigestible materials in the substrate (polysaccharides and tannins).

C.5.2. Effect on adaptations to various temperatures

To test for the influence of the selected fungi on the ability to adapt to different temperatures, the larvae will be reared from 1st stage on a basic diet (Kannan et al., 2023) with and without supplemental fungi (*P. kudriavzevii*, *C. tropicalis*, *K. marxianus* and *S. cerevisiae* as reference fungi). Yeast inoculum will be extensive (1% of total diet weight) and after identifying an effect, minimal yeast inoculums will be tested. The experiment will be conducted in a rearing chamber, in 2 L plastic cages with 250 gr of diet and 250 larvae. Five temperatures will be tested: optimal 30oc, minimal 25Oc, maximal 35Oc, and extreme cold 15oc and hot 40oc (Yong-Chia et al. 2018; 5 replicates for each temperature). The time of development (until the pupa stage), survival (at each life stage) hatching rates, and body weight (of 5th instar larvae and adults) will be measured.

C.5.3. Effect on digestion of indigested plant materials

To test for the influence of the selected fungi on the digestion of indigestible plant materials, larvae will be reared on a basic diet (Kannan et al., 2023) with additional components. To test the effect of the selected fungi on polysaccharide digestion, sawdust will be added to the diet (25%), and four yeast species (*P. kudriavzevii*, *C. tropicalis*, *K. marxianus,* and *S. cerevisiae* as reference fungi, 1% of diet weight). The yeast will be supplemented in two ways: one group will be added one week before inoculation with the larvae and the second group will be supplemented while adding the larvae to the experimental setting (flask of 100 mg of diet with 100 larvae; 5 replicates). At the end of the experiment, when 50% of the larvae change color and become pre-pupa) the larvae's weight will be measured. Differences in the amounts of remaining polysaccharides will be tested by digestion with H2SO4 (1.25%) and NaOH (1.25%) and burning the remains at 600°C in a laboratory furnace (Bifartherm) to exclude the amount of minerals in the samples.

to test for detoxification of the tannin, tannin will be added to the basic diet (0.01%, 0.1%, and 1%; Sigma-Aldrich). The four yeast species that were mentioned above will be supplemented in two ways: one group will be added one week before inoculation with the larvae and the second group will be supplemented while adding the larvae to the experimental setting (flask of 100 mg of diet with 100 larvae; 5 replicates). At the end of the experiment when 50% of the larvae change color and become pre-pupa) the larvae weight will be measured. The amount of the remaining tannins will be measured using a specific kit (Abbexa Ltd.).

 C.5.4. Expected results and pitfalls

The experiments are expected to reveal the influence of gut fungi on the growth rate and survival under different temperatures. In case such influence would not be found, similar experiments under an insufficient diet, e.g. low protein level (as conducted by Yong-Chia et al. 2018). The experiments with the indigestible plant materials should show the effect of the specific fungi on the growth of the larvae through the digestion of harmful or indigestible materials. In case providing the yeast before adding the larvae will be harmful to the larvae due to fermentation products of the larvae, adding different amounts of yeast, in different periods before the larvae or adding larvae at different life stages should be tested.

C.6. Objective 5 - Influence of selected fungi on adapting to a new environment (biotic conditions)

 C.6.1. Specific working hypothesis

The environmental fungi are expected to provide an advantage to the BSF that harbors them. I will test these advantages in two biotic conditions that an invasive species into rotten organic material may encounter: dealing with pathogens (entomopathogenic fungi) and competitors (other dipteran larvae). the effect on other Diptera species will be tested directly (consumption), indirectly (due to BSF larvae execution in the substrate), or due to the fungi present in the substrate

C.6.2. Influence on pathogen resistance

To test the effect of the selected fungi on the ability of the BSF larvae to deal with pathogens, BSF larvae will be exposed to entomopathogenic microorganisms with and without the presence of the selected gut fungi. The experiment will be conducted in plastic flasks with a 100gr simple diet and 100 larvae. the diet will be supplemented with one of the selected fungi (described previously). The pathogens will be added after one week of larvae rearing. The pathogens are *Beauveria* spp. and *Metarhizium* spp. – fungi; *Bacillus thuringiensis* – bacteria (separately) and will be compared to substrates without the pathogens. At the end of the experiment after 50% of the larvae will reach the pre-pupa stage the abundance of selected gut fungi and pathogenic microorganisms will be tested in the substrate and the larvae using RT-PCR with specific primers. In addition, the weight and the survival ratio of the larvae will be tested, and the hatching ratio of the adults.

C.6.3. Influence on other species

The influence of the BSF and the selected fungi on other Dipteran species, that the BSF may encounter in the natural environment, will be tested in plastic flasks with 25 gr of Gainesville diet suited for Diptera (Hogsette, 1992). At the first stage, the different Diptera species (*Musca domestica, Drosophila melanogaster,* and BSF*)* will be reared from 1st instar to pupa in the diet alone to test for survival in the diet (25 larvae for each species, 5 replicates). At the end of the experiment (when all larvae become pupa), the body sizes of the pupa, time to reach pupa, and survival will be measured. In addition, to test for competition 12 larvae (BSF and each of the species) will be grown together and survival, time to reach pupa, and body weight of the pupa will be measured at the end of the experiment (when they will reach the pupa stage) Second, the Diptera species will be grown from 1st instars together with 1st instars of BSF (12 larvae each species). Due to different growth rates, the survival of the larvae will be measured every 5 days until the BSF reaches the pupa stage. Also, due to the different growth rates and body sizes, the time of supplementing each species will be tested, e.g. adding the BSF larvae after a week of larval growth of the other species. These experiments will provide information on whether the BSF larvae directly consume the larvae of the other Diptera species. Third, the development of the Dipteran species will be tested on the Gainesville diet that was consumed by the BSF larvae (provided to the Diptera species after the BSF larvae completed their lifecycle). These experiments will provide information on whether the BSF larvae extract metabolites that harm the competitors (e.g. ammonia or uric acid). Fourth, the Gainesville diet supplemented with the selected fungi (described previously, 1% of the diet weight from each species) will be provided to the Diptera species, and the survival, time to reach pupation and body weight of the pupa will be measured at the end of the experiment. This experiment will provide information on whether the fungi are harmful to the other species. In case the effect of the fungi on the species will be observed another set of experiments to detect the minimal abundance of fungi in the environment that causes a reduction in the survival of the Diptera species.

 C.6.4. Expected results and pitfalls

The supplemented gut fungi are expected to affect the survival or body size of the BSF larvae that encounter entomopathogenic microorganisms. If no effect is observed, the experiment will be conducted with a less nutritious diet (e.g., low protein levels) to weaken the larvae and its immune system and to see a larger effect of the gut fungi.

In the interaction between the BSF larvae and other Diptera species, first of all, I expect to see the effect of competition, e.g. reduction in survival or body mass when both species are present together compared to when they are alone. If such an effect is not seen, it may be that the diet is enough for both species and it is not a limiting factor. In that case, an increase in the number of both species will be tested (as conducted in Chase and Berlovsky 1994).

In addition, I expect to see an effect of the fungi in the substrate on the survival or body size of the Diptera species. If no such effect is detected, another set of experiments will be conducted. In these experiments the BSF larvae will be fed with the fungi and the remaining substrate (after BSF larvae reach pupation) will be used to rear the Diptera species. In this case, the interaction between the BSF and their gut fungi and its effect on competitors will be tested.

C.7. Objective 6 – Testing the predictions on the community level

C.7.1. Working hypothesis

Affect testing the different factors that affect the BSF life cycle and the benefit that the gut fungi provide them, multi-species experiments that test the invading potential of the BSF with its gut fungi will be constructed. To construct such an experimental setting, the use of machine learning models will be used based on the results of the previous sections.

 C.7.2 Machine learning model

I will use ECHO, which is a generic simulator that was developed by Holland (Holland 1992) and designed to explore interactions among large numbers of different adaptive agents (AA). It provides populations of evolving, reproducing agents distributed over geography with different inputs of renewable resources at various sites. This adaptive agent (AA) model provides a realistic framework for ecosystem simulation, evolving ecosystem structures and behaviors by emerging, submerging, interacting, and evolving ecological entities. This individual-based AA proves applicable to a spatially explicit simulation of highly simplified terrestrial food webs (Recknagel 2006).

C.7.3 Multi-species experiments

The experiment will be conducted in net cages (1X1X1 m) in a rearing room (according to the a-biotic conditions that will be found relevant). In the cages, 1 Kg of Gainesville diet will be placed in plastic containers and the Diptera eggs or larvae will be placed according to the model (3 replicates to each condition). The experiment will be conducted over a period that will allow the development of several generations of the Diptera (2 months). During the experimental period, every 10 days the number of larvae, pupa, and adults will be measured and the presence and abundance of the different fungi species will be measured using RT-PCR will specific primers (in a 1 gr substrate sample; 3 replicates from each substrate).

 C.7.4 Expected results and pitfalls

**D. Preliminary results**

The interaction of BSF larvae with house fly larvae was tested in plastic flasks containing 25 grams of Gainesville diet. The treatments that were tested are: 1) Rearing 25 black soldier fly larvae together with 25 house fly larvae (to test for direct predation). 2) Rearing 50 house fly larvae on a substrate consisting of the remaining material from BSF larvae consumption (to test for active metabolites that were extracted by the BSF). 3) Rearing 50 house fly larvae on a with the addition of *Candida* (1 gr, to test the effect of the fungi. 4). Rearing 50 house fly larvae without any supplements (control). Five replicates from each treatment were conducted. All the treatments were incubated for 4 days in a rearing chamber (29oC, 70% humidity). At the end of the experiment, live larvae were counted in order to calculate the survivability in the different treatments, and the average weight of 3 larvae was measured. The survival of the house fly larvae was lower in the control and Candida treatments compared to the treatment with BSF larvae and the BSF remains (Hdf=3,21=31.6, p<0.01, Figure 1).

Fungal isolation from BSF larvae’s gut that was collected from different locations (Shear Yeshov- 33°13′35″N 35°38′48″E, Kiryat Tivon - 32°42′56″N 35°07′36″E, Timrat - 32°42′13″N 35°13′31″E, Kfar Ha`Horesh - 32°42′04″N 35°16′22″E, Ruhama - 31°29′51″N 34°42′19″E) showed a decrease in the abundance of *Candida tropicalis* and an increase in the abundance of *Kluyveromyces marxianus* toward the south of Israel (Figure 3). The colonies were isolated from five larvae on YPD agar. Ten colonies from each larva were identified using Sanger sequencing using the primers ITS1-ITS2 (ITS1: TCCGTAGGTGAACCTGCGG; ITS2: GCTGCGTTCTTCATCGATGC) (White). The sequencing was conducted in HyLabs (Rehovot, Israel).



Figure 3: Proportion of colonies isolated from five BSF larvae form different locations

Testing the effect of entomopathogenic fungi (*Beauveria bassiana*) on theBSF larvae was tested by rearing BSF larvae (1st instar) on Gainesville diet for 4 days; then the larvae were transferred to the treatments with the Bioveria formulation (LAM international, Butte, Montana, United States of America). The fungal conidia were counted using a hemocytometer and one high concentration of 107 conidia/ml was selected for use as reported by Lecocq et al. (2021). We set up three treatments as follows: 1. Control (no fungus); 2. Dipping the larvae in Bioveria formulation for one minute and transferring them to the Gainesville diet (BD); and 3. Feeding the larvae on the Gainesville diet treated with the Bioveria formulation (BF). After 10 days of fungal treatment, larval body weight was measured. The larvae were then left to grow until adult emergence. The number of adults emerging was counted and adult body weight was measured. The *B. bassiana* feeding treatment (BF) significantly reduced BSF larval body weight (0.63 ± 0.04 g), compared to the control (2.52 ± 0.08 g) and the dipping treatment (BD) (2.34 ± 0.06 g) (p = 0.0001). Furthermore, the BF treatment significantly decreased the percentage of adult emergence (8 ± 5.83%), compared to the control (76 ± 14.89%) and BD (38 ± 9.69%) (p = 0.02) (Figure 1B). Additionally, the BF treatment significantly reduced BSF adult fly body weight (28 ± 3.54 mg) compared to the control (94 ± 4.71 mg) and BD (90.3 ± 5.69 mg) (p = 0.014). There was no significant difference observed in larval body weight, adult emergence, or adult body weight between the control and BD (Kannan et al. under review).

To test the BSF larvae’s preference for fungi in the substrate, YPD substrate was made with the proportions of 65 grams of YPD powder and 1 liter of DDW. The mix was autoclaved and cooled down until it reached to 50 C°. 10-30 ml of YPD mix was poured into each petri dish. When the YPD was totally solid in the petri dish, a 100 ml sample of yeast *(S. cerevisiae*) was spread on one half of the treatment group of the petri dish and then all the petri dishes were left in the incubator for 24 hours so the yeast cells could grow into large colonies (fig 1). Then, 1 larvae was added to each petri dish for a period of 4 hours (Figure 4). Videos of the larvae's movements were taken with a camera that was assembled above the petri dishes, and the data was processed using a computer program called Ethovision. There was no preference for one of the Petri dish parts by the larvae. it may be that the substrate should contain a thin layer of larvae’s diet and other fungi should be tested.

**E. Personnel and facilities**

My laboratory is well-equipped for molecular biology work, including thermoshakers and centrifuges, a PCR machine (T100 Thermal Cycler, BioRad), online quantitative PCR (BioRad CFX384); and for rearing and handling of insects and fungi: a temperature-controlled rearing room, incubator for controlled insect rearing and dissecting stereomicroscope (Zeiss, Stemi 508) and biological hood, shakers and incubators for rearing yeasts. In order to perform the necessary work a rearing room for the adult fly will be dedicated for this grant. A post-doctoral fellow (100%) and a part-time technician will be recruited for this project. Illumina, and nanopore sequencing will be conducted at the Research Resources Center, University of Illinois at Chicago (see support letter from Dr. Stefan J. Green), and Dr. \_\_\_\_\_ from the MIGAL will assist with the machine learning models (see support letter).