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ROLE OF NECTAR-INHABITING FUNGI FOR EGG PARASITOIDS IN THE CONTEXT OF CONSERVATION BIOLOGICAL CONTROL

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Summary

Nectar-producing flowering plants are incorporated in agricultural landscapes as a conservation measure to preserve and promote parasitoids as the biological control of insect pests. This strategy of pest management is a classic example that falls under the principle of Conservation Biological Control (CBC), by which parasitoids are provided with crucial resources, like floral nectar, to support fitness and maximize efficacy. Adults of many hymenopteran parasitoids are dependent on floral nectar as the main source of energy necessary to perform their daily tedious activities, such as host location, foraging, flight and oviposition. In order for the CBC programs to be effective, flowering plants should possess attractive traits to anticipate recurrent visitations. Thus, floral visitors like hymenopteran parasitoids exploit plant-emitted volatiles to locate nectar rewards. However, this bipartite interaction between flowering plants and insects has recently advanced into a more complex tripartite interaction mediated by nectar-inhabiting microbes. Nectar is a sugary secretion in the flower that is ubiquitously colonized by microbes. These microbes are linked to the alteration of nectar traits, thereby affecting the olfaction behavior of insects, as well as their performance. So far, members of bacteria and yeasts are described to be widespread in the floral nectar of several plant species. Although yeasts in the *Metschnikowia* clade are commonly known to influence pollinator behavior and performance, there is inadequate information about how these microbes affect the other important hymenopteran wasps, like the egg parasitoids. Also, other microbes that can potentially exist in nectar environments, like filamentous fungi, have been overlooked in the extensive investigation of nectar microbial ecology.

In this dissertation, the hypothesis that nectar-inhabiting fungi (filamentous and yeasts) could influence the selection behavior and performance of egg parasitoids via nectar fermentation was investigated. To address this, an experimental system that consisted of two egg parasitoids, *Trissolcus basalis* and *Ooencyrtus telenomicida*, of the southern green stink bug *Nezara viridula* was established. These egg parasitoids are biological control agents of stink bugs, and they are reported to co-exist in Southern Italy, where their host is considered a key pest of several vegetable crops. On the other hand, nectar specialist yeasts *Metschnikowia gruessii* and *M. reukaufii* isolated in the nectar of *Symphytum officinale*, and the filamentous fungi from the nectar of buckwheat *Fagopyrum esculentum* were used to ferment synthetic nectar. Furthermore, microbe-fermented nectars were tested in the egg parasitoids, and their response behavior and performance were measured accordingly.

In Chapter 2, we have indicated that fermentation by nectar specialist yeasts can positively elicit the preference behavior of two stink bug egg parasitoids without any measurable negative impact on their adult longevity and survival rate. Here, specialist yeasts were used to ferment synthetic nectar. After fermentation, nectars were filtered to produce cell-free fermented nectar and then tested in egg parasitoids. It was found that nectar fermented by *M. gruessii* was significantly preferred by both egg parasitoids in contrast to the control synthetic nectar. On the other hand, nectar fermented by *M. reukaufii* did not elicit any significant responses in both wasp species. In the case of adult longevity and survival rate of egg parasitoids, no yeast-mediated effects were found. Analysis of the volatile profile of the fermented nectars in comparison with the control synthetic nectar

revealed clear qualitative and quantitative differences, which may contribute to explaining the contrasting attractiveness of nectar yeasts toward egg parasitoids.

In Chapter 3, it was demonstrated that aside from bacteria and yeasts, filamentous fungi could also affect the preference behavior of egg parasitoids via changes in nectar traits. Here, filamentous fungi isolated from buckwheat nectar were used to ferment synthetic nectar and then underwent filtration to produce fungus-free fermented nectar. In the olfactometer bioassay, it was found that out of 6 synthetic nectar fermented by filamentous fungi, 2 fermented nectars elicited positive response behavior in both egg parasitoids. Interestingly, the females of *T. basalis* were only attracted to *Cladosporium* sp. SAAF 22.2.11-fermented nectar, but *O. telenomicida* displayed a preference toward nectars fermented by both *Cladosporium* spp. SAAF 22.2.11 and SAAF 22.3.29. Volatile profile analyses showed that there were 36 volatile organic compounds detected across the different fermented nectar in contrast with the control synthetic nectar. Moreover, the analysis revealed 7 compounds that have VIP scores > 1.5, namely, phenol, 3,4-dimethyl; 2,4-dimethyl glutarate; α-terpineol; hydroxyacetophenone; 2,6-di-tert-butyl-p-benzoquinone; methyl benzoate; and 2-methyl benzaldehyde. These compounds may help elucidate the possible mechanism that drives the contrasting responses in the egg parasitoids.

Chapter 4, however, investigated the impact of the microbial bodies themselves on the olfaction behavior of the egg parasitoid *T. basalis* via nectar fermentation. Thus, after the fermentation, microbial bodies were suspended evenly in the nectar solution by vortexing and directly tested in the olfaction bioassay involving the egg parasitoids. Results revealed that *T. basalis* only preferred the odors emitted by fermented nectar with fungal bodies of *Cladosporium* sp. SAAF 22.2.11 in contrast with the control nectar. This corroborated with the previous results in Chapter 3 that, indeed, this particular nectar-inhabiting filamentous fungus produced nectar that was attractive to the parasitic wasp. However, other filamentous fungi neither caused attraction nor repellence behavior in the wasps. On the other hand, when wasps were exposed simultaneously to fermented nectar with and without fungal bodies, *T. basalis* generally preferred odors emitted by fungus-free nectar over its counterpart, especially the ones fermented by *Aspergillus* sp. SAAF 22.3.26 and *Stachybotrys* sp. SAAF 22.1.15. Our results suggested that aside from the fermentation products, direct effects due to microbial bodies in nectar should be highly considered in investigating the role that nectar-inhabiting microbes play in flowering plant-insect interaction.

Lastly, chapter 5 demonstrated the impact of the fermentation product of nectar-inhabiting filamentous fungi on the adult longevity and survival of egg parasitoids *T. basalis* and *O. telenomicida*. Performance bioassay revealed that no significant microbe-mediated effect was found in terms of the adult longevity of two wasp species. In contrast to the survival rates of *O. telenomicida*, which was not affected by the nectar filamentous fungi, *T. basalis* fed on nectar fermented by *Cladosporium* spp. SAAF 22.2.12 and SAAF 22.3.29 had reduced survival rates when compared to those wasps fed on control nectar. These results suggest that, aside from yeasts and bacteria, filamentous fungi could also play a crucial role in the complex interactions between flowering plants and egg parasitoids by means of altering the quality of nectar rewards.

Overall, nectar-inhabiting fungi, both yeasts and filamentous species, mediate the interactions between flowering plants and egg parasitoids of insect pests without causing any deleterious effect on the general performance, at least in the case of those fungi that elicited positive behavioral responses. Our results, however, are not only essential to understanding the complexity of microbe-mediated plant-parasitoid interactions but also relevant from a practical standpoint as nectar-rich flowering plants such as buckwheat are widespread in agricultural landscapes to promote conservation biological control of insect pests. In that sense, an additional tool that involves nectar fungi could be potentially used to augment the attractiveness of the nectar-producing flowering plants toward egg parasitoids by way of providing honest microbial signaling that could facilitate insects in locating nutritious nectar rewards and consequently improving insect fitness and efficacy in pest regulation.

Chapter 1

General Introduction

1.1. Introduction

With the advent of chemical pest control along with its countless side effects on the environment, farmers have to shift toward more sustainable and eco-friendly pest management approaches (Shields et al., 2019). Among the various tactics available, the use of natural enemies is a promising option for the farmers. However, in intensified agricultural landscapes, resources are tremendously scarce and may cause starvation of natural enemies (Sentil et al., 2022; van Rijn & Wäckers, 2016), thus, they are greatly compromised, rendering such a tactic ineffective. To combat the challenges brought about by chemical pesticide applications and crop intensification on the natural enemies of arthropod pests, conservation biological control has been one of the many solutions available (Shields et al., 2019). The overall principle of CBC is to manipulate the agroecosystem, making it a more favorable habitat for the resident natural enemies with the expectation of enhancing their efficiency in suppressing pest populations (Landis et al., 2000).

Planting non-crop flowering plants around the field that produce and offer basic resources crucial for natural enemies, like floral nectar, has been a widespread practice in agroecosystems (Foti et al., 2017; Kowalska et al., 2022). Hymenopteran parasitoids are among the insect guilds that visit flowers to take advantage of the nectar (Jervis et al., 1993). Floral nectar is rich in sugar (fructose, glucose, and sucrose), proteins, free amino acids, lipids, vitamins, and essential minerals, among other compounds that may support insect fitness (Jervis et al., 1993; Lievens et al., 2015). It is one of the primary sources of nutrition for the parasitoids, enabling them to execute energy-demanding activities like foraging, flight, host searching and oviposition (Araj et al., 2011; Cusumano & Lievens, 2023; Nafziger & Fadamiro, 2011; Takasu & Lewis, 1995).

Several plant traits have been considered in choosing the best representative flowering plants in conservation biological control (Wäckers & van Rijn, 2012). For instance, candidate plants should not only have accessible nectar for the natural enemy but the nectar should also be nutritious and sufficient enough to sustain their entire presence in the field (van Rijn & Wäckers, 2016; Wäckers, 2004). Thus, it is highly encouraged to select flowering plants that are characterized by longer anthesis. In addition, the flowering plants should possess attractive flowers in order to guarantee frequent visitations by natural enemies (Wäckers, 2004; Wäckers & van Rijn, 2012). Several flower visitors are known to be attracted to the bouquet of volatiles that flowering plants release (Colazza et al., 2023; Raguso, 2008; Rusman et al., 2024). These floral volatiles are considered signals that help flower visitors locate and feed on floral nectar (Bianchi & Wäckers, 2008; Foti et al., 2017; Raguso, 2008; Schiestl, 2015). While parasitoids may be drawn to attractive flowering plants, the nectar rewards are sometimes not enough to support insects' fitness (Belz et al., 2013; Colazza et al., 2023). On the other hand, for flowering plants that possess better nectar rewards, volatile cues that would otherwise facilitate visitations may be less attractive to the parasitoids (Colazza et al., 2023). Thus, for conservation biological control to be effective, flowers must not only offer high-quality nectar rewards but, at the same time, should be attractive to the parasitoids for successful and quick location (Foti et al., 2017).

However, in the past decades, this research field of chemical ecology has shifted drastically from merely an interplay between flowering plants and insect visitors into more complex interactions mediated by nectar-inhabiting microbes (Colazza et al., 2023; Sobhy et al., 2018). Floral nectar is considered an "open habitat" that can be colonized by a multitude of microorganisms, with bacteria and yeasts being the most dominant taxa recorded so far (Good et al., 2014; Rering et al., 2018; Vannette, 2020). In fact, bacterial cell densities could reach approximately 10^7 -10^9 per milliliter of nectar, whereas yeasts could naturally occur in floral nectar at 10^5 - 10^6 cell densities per cubic millimeter (Christensen et al., 2021; de Vega et al., 2009; Fridman et al., 2012; Herrera et al., 2009). Multiple bacterial species under the different phyla (e.g., Actinobacteria, Bacillota, Pseudomonadota) were reported to be common inhabitants of the floral nectar from various plant species (Álvarez-Pérez et al., 2012; Fridman et al., 2012; von Arx et al., 2019). Moreover, culture-dependent bacteria were found to be more widespread, as much as twice the occurrence of yeasts in floral nectar (Vannette et al., 2021). More recently, an investigation of the bacterial community of nectar from the flowering plant *Fagopyrum esculentum*, a common plant species used in CBC, described 14 bacterial strains, wherein the members of Firmicutes being the most prevalent (Cusumano et al., 2023). On the other hand, the ascomycete yeast in the genus *Metschnikowia* has also been frequently reported from across the globe to be associated with floral nectar. For example, *M. reukaufii* and *M. gruessii* were recorded from the nectar of various Mediterranean plants (Álvarez-Pérez & Herrera, 2013). Also, these two species of nectar yeasts were found to be associated with the nectar of ant-pollinated plant *Cytinus hypocistis*, and surprisingly, they appeared in almost 85% of all the microbes isolated from the nectar of 24 different plant species in Southern Spain (de Vega & Herrera, 2012; Pozo et al., 2011). In other parts of Europe, diverse yeast species were also described in the floral nectar of numerous bee-pollinated plant species across different habitats in Belgium and Germany (Bartlewicz et al., 2016; Brysch-Herzberg, 2004; Jacquemyn et al., 2013). Furthermore, several microbiological surveys were conducted in other parts of the world, where they also reported the presence of different yeast species associated with floral nectar, such as in Asia (Tsuji & Fukami, 2018; Yang et al., 2019), Africa (de Vega et al., 2009) and North America (Belisle et al., 2012; Canto et al., 2017; Dhami et al., 2018; Klaps et al., 2020; Schaeffer et al., 2014, 2015; Vannette et al., 2021; Vannette & Fukami, 2017). While there is an increasing body of evidence regarding the presence of bacteria and yeasts in floral nectar, other microbial taxa that can potentially colonize nectar are sparingly documented. In fact, filamentous fungi from the genera *Cladosporium* and *Aspergillus* were reported to colonize sugar-rich environments such as floral nectars (Ehlers & Olesen, 1997; von Arx et al., 2019). These reports suggest that filamentous fungi deserve attention when investigating nectar ecology.

Although it remains controversial whether microbes have already colonized floral nectar before anthesis, there are three possible pathways on how microbes are introduced into the floral nectar (Vannette, 2020). Microbes were already occupying the floral tissues even before the opening of flowers and became the source of inocula that later colonized the floral nectar (Vannette, 2020; von Arx et al., 2019). The most obvious pathway of microbial colonization of floral nectar is through the activities of nectar feeders and other flower visitors alike

that may act as dispersal agents (Belisle et al., 2012; Brysch-Herzberg, 2004; Morris et al., 2019). These animals may have transported the microbes in their body parts acquired during feeding and flower visitations and later inoculated into the nectar of different flowers of the same or another plant species (Pozo et al., 2018). As a consequence, the population growth of the microbes in nectar environments continues to proliferate. On the other hand, abiotic factors like rain and air are also thought to influence the dispersal and proliferation of nectar-inhabiting microbes in the landscape (Jacquemyn et al., 2013). Regardless of the method of introduction of microbes into floral nectar, it is clear that microbes ubiquitously colonize nectar.

Nectar-inhabiting microbes generally modify various nectar traits including, but not limited to, the nectar volume (Vannette & Fukami, 2018), nectar acidity (Vannette et al., 2013), sugar or amino acid proportions and compositions (de Vega & Herrera 2013; Herrera et al. 2008; Vannette et al., 2013), or even the occurrence of secondary metabolites (Vannette & Fukami, 2016) and dynamics in nectar temperature (Herrera & Pozo, 2010). Nectar microbes are also thought to influence the scent profile of flowers due to the production of microbial volatile organic compounds (mVOCs) emitted from the fermented nectar (Cusumano et al., 2022; Golonka et al., 2014; Rering et al., 2018; Sobhy et al., 2018). All these microbe-mediated effects on flower nectar could greatly influence how natural enemies like parasitoids locate suitable nectar sources and the frequency of flower visitations. Furthermore, the fact that metabolic activities of microbes cause changes in nectar quality, rewards provided by flowering plants to parasitoids could also be affected. This potential effect on nectar driven by microbes could further enhance or may jeopardize the suitability and effectiveness of flowering plants used to recruit parasitoids in agricultural landscapes (Cusumano & Lievens, 2023). Thus, it is of great importance to take into consideration the microbial component of nectar in CBC programs. Overall, we can assume that such alteration in nectar by microorganisms may have an impact on the foraging behavior of flower visitors as well as on their performance (Sobhy et al., 2018).

1.1.1. Microbe-mediated impact on insect olfaction

Studies have highlighted the role of volatiles emitted by nectar-inhabiting microbes in insect foraging behaviors. For such instance, nectar specialist yeasts *Metschnikowia* spp. induce a positive response in pollinators, like in honey bees (Rering et al., 2018; Vannette & Fukami, 2016) and bumblebees (Schaeffer et al., 2014; Schaeffer et al., 2017; Herrera et al., 2013). Also, a few bacterial species that are natural or inoculated in flowers may enhance nectar attractiveness to flower-visiting insects (Rering et al., 2018). However, there are also reports that bees may manifest avoidance of nectar colonized by bacteria (Junker et al., 2014; Good et al., 2014). Studies on the chemical ecology of nectar microbes were mostly focused on their impact on nectar-feeding pollinator guilds, while less attention has been given to other flower visitors, like insect parasitoids. Adult parasitoids feed on sugar-rich resources such as floral nectar, and thus, it is reasonable to assume that nectar-inhabiting microbes could also influence their interaction with the flowering plants. Yeasts and bacteria are thus far the reported microbes found to elicit an olfactory response in parasitoids. For example, volatiles from nectar specialist yeasts *M. gruessii* and *M. reukaufii* were attractive to the parasitoid *Aphidius ervi* (Sobhy

et al., 2018) and were found to influence insect learning and memory (Sobhy et al. 2019). Also, it has been reported that the egg parasitoid *Trissolcus basalis* displayed attraction behavior toward nectar bacteria isolated from buckwheat flower, a flowering plant that is widely integrated into CBC programs (Cusumano et al., 2023).

1.1.2. Microbe-mediated impact on insect performance

Besides the preference behaviors observed in multiple pollinator and parasitoid species toward nectar-inhabiting microbes, previous studies have also demonstrated that feeding on nectar colonized by microbes resulted in contrasting performances among the model insects tested. For example, while no bacterial-mediated effects were observed in terms of consumption rates of nectar, it was found that adult longevity of *A. ervi* had increased after feeding on *Lactococcus* sp.-fermented nectar, in contrast to those insects that fed on *Asaia* sp.-fermented nectar (Lenaerts et al., 2017). In the case of nectar generalist yeasts, it was observed that *A. ervi* consumed less nectar fermented by *Aureobasidium pullulan* and *Sporobolomyces roseus*, which was also reflected in their reduced longevity (Sobhy et al., 2018). On the other hand, consumption rates by honey bees were not significantly affected by both *M. reukaufii* and *Asaia astilbes* or even by their combinations in nectar (Rering et al., 2020). Another study conducted with bumblebees showed that while *M. reukaufii* induced attraction, no yeast-mediated effects were observed in terms of pollen consumption and egg deposition (Schaeffer et al., 2017). Moreover, nectar fermented by *M. reukaufii* and *M. gruessii* did not cause significant differences in adult longevity and survival rate of *A. ervi* when compared with those wasps fed on nectar without microbial inoculation (Sobhy et al., 2018). With all these results from previous studies, much is still needed for our understanding of the nectar-inhabiting microbes in order to draw generalizations about their impact on the insects' performance. Thus, further research that would investigate the responses of other insect species to nectar microbes, taking into consideration other life-history parameters, is highly warranted. Moreover, it would also be better to look into how feeding on living nectar microbes would affect insect performance, as microbes themselves could provide crucial benefits to the general health of insects (Gibson & Hunter, 2010; Stefanini, 2018).

1.2. Main objective and research questions

The overall aim of this doctorate thesis is to elucidate the underlying mechanisms of how nectar-inhabiting fungi may influence the volatile emission of flowers via nectar fermentation, with a particular focus on its cascading effects on the biological control agents of insect pests. This work took a unique approach by focusing on the impact of nectar-inhabiting fungi on egg parasitoids, floral visitor species that have received less attention among previous studies. The work utilized the nectar-inhabiting fungi (filamentous and yeast) isolated from wild flowers and buckwheat to ferment synthetic nectars, which were then tested on the egg parasitoids of stink bugs. Olfactometer and performance bioassays were conducted to identify which fungal strain has an impact on the foraging behavior of the egg parasitoids and, consequently, how consumption of nectars fermented by these fungi affects adult longevity and survival rates. Finally, volatile profile analyses of fermented synthetic nectars were also carried out to identify further which chemical compounds or their combinations were responsible for explaining the biological observations taken from the bioassays.

The following research questions have been addressed accordingly in the succeeding chapters:

- Do the nectar specialist yeasts affect the foraging behavior and performance of egg parasitoids of stink bugs, and what particular volatile organic compounds are being emitted by the yeast-fermented synthetic nectars? (Chapter 2)
- Other than yeasts and bacteria, what are the potential microbes inhabiting the nectar of flowers, especially of those plant species that are widely utilized in conservation biological control? If there are, what could be their implications for the foraging behavior of egg parasitoids of stink bugs, and what microbial volatile organic compounds influence such behaviors? (Chapters 3 and 4)
- Does the nectar fermentation by the nectar-inhabiting filamentous fungi affect the performance of the egg parasitoids? (Chapter 5)

1.3. Study systems

To shed some light on the research questions mentioned above, we established a plant-microbe-insect study system in the context of conservation biological control (Figure 1). In this study system, we chose a flowering plant species (Buckwheat, *Fagopyrum esculentum*) widely used in conservation biological control (CBC). This flowering plant species was also utilized for the isolation of the different culturable nectar-inhabiting filamentous fungi that were used throughout the different experiments in this paper. While we were not able to isolate common specialist yeasts from the nectar, we used nectar-inhibiting yeast isolates from the wild comfrey plant, *Symphytum officinale*. This was done to investigate if the specialist yeasts commonly reported in the literature for their role in insect olfaction and fitness would also have effects on the foraging behavior and performance of the egg parasitoids.

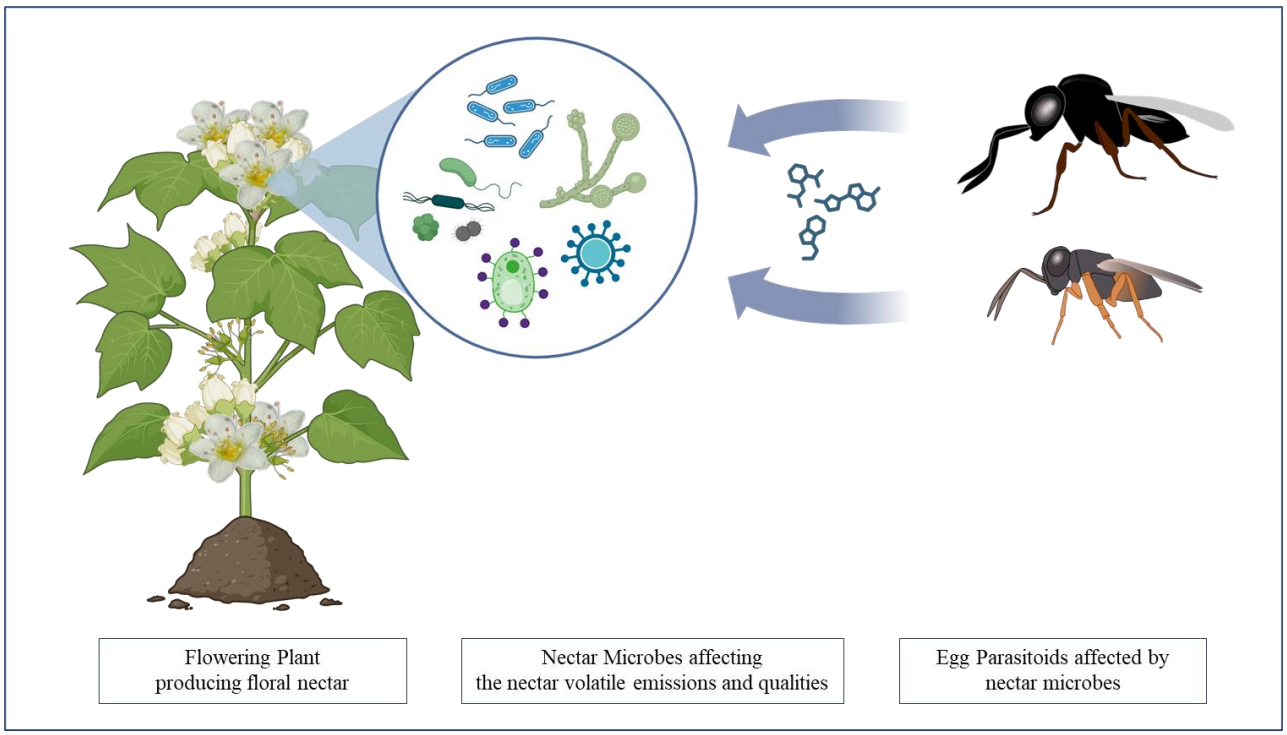

Figure 1. The representation of the model system comprising the tri-trophic interactions among the flowering plants, the nectar-inhabiting microbes and the egg parasitoids *Trissolcus basalis* and *Ooencyrtus telenomicida*. The flowering plant species *Fagopyrum esculentum* was used for the isolation of nectar-inhabiting filamentous fungi (*Cladosporium* spp., *Aspergillus* spp. and *Stachybotrys* sp.), whereas wild comfrey plant *Symphytum officinale* was used for the isolation of nectar specialist yeasts (*Metschnikowia gruessii* and *M. reukaufii*).

1.3.1. Buckwheat, ***Fagopyrum esculentum*** (Moench, 1794)

Common buckwheat (Polygonales: Polygonaceae) is a flowering plant that has been widely used in CBC programs to enhance the attraction and fitness of natural enemies. Flowers of buckwheat were found to be attractive to *T. basalis,* and provision in cages led to an increase in wasp fecundity (Foti et al., 2017). Moreover, in a field experiment, it was observed that planting buckwheat along the margins of tomato fields increased

stink bug egg parasitism rates by *T. basalis* (Foti et al., 2019). Additionally, volatiles emitted from buckwheat flowers were also found to elicit positive responses in *Peristenus spretus*, which also resulted in improved parasitism rates in the field (Xia et al., 2023). Apart from that, several studies have also reported the benefit of buckwheat in terms of the performance of various parasitoids such as *Microplitis mediator* (Géneau et al., 2012), *Diadegma semiclausum* (Lavandero et al., 2005), and *Ascogaster quadridentate* (Mátray & Herz, 2022), among others. Recently, a microbial investigation of buckwheat nectar described 14 bacterial strains, 4 of which elicited positive behavioral responses in an egg parasitoid of stink bugs (Cusumano et al., 2022). This has paved the way to assess further the other microbial assemblages that might be present in buckwheat nectar and investigate their ecological relevance in the CBC context.

1.3.2. Nectar-inhabiting fungi

1.3.2.1. Nectar-specialist yeast

As mentioned, several authors have reported that nectar-inhabiting yeasts mediated interactions between flowering plants and pollinators. However, to date, there are only two studies that investigated how nectar-inhibiting yeasts affect the olfactory responses and performance of parasitoid species of insect pests. Thus, further studies are warranted in order to elucidate the role played by nectar-inhabiting yeasts in the foraging behavior and, consequently, on the performance of egg parasitoids. The nectar specialist yeasts *Metschnikowia gruessii* (isolate ST12.14/016) and *M. reukaufii* (ST12.14/017) used in Chapter 2 were isolated from the floral nectar of the wild comfrey plant, *Symphytum officinale* (Sobhy et al. 2018). These yeast species are under the family Metschnikowiaceae of the Phylum Ascomycota.

1.3.2.2. Nectar-inhabiting filamentous fungi

So far it has not been investigated how filamentous fungi associated with floral nectar mediate the interactions of flowering plants and egg parasitoids. Bacteria and yeasts are thus far the most widely documented nectar-inhabiting microbes and are well-studied for their importance in flowering plant-insect interactions. Yet, no study has evaluated how other microbial taxa, like the filamentous fungi, may interplay in such interactions. Here, we isolated the culturable filamentous fungi from the nectar of buckwheat to investigate how such microbial taxa could contribute to the interactions between flowering plants and egg parasitoids. The cultured filamentous fungi underwent identification through molecular analyses of the internal transcribed spacer regions of the fungal ribosomal DNA. We identified 6 isolates of nectar-inhabiting filamentous fungi belonging to 3 families (Stachybotryaceae, Cladosporiaceae, and Aspergillaceae) of the Phylum Ascomycota. In the family of Stachybotryaceae, we found 1 strain of *Stachybotrys* sp. SAAF 22.1.15. On the other hand, 3 strains under Cladosporiaceae were isolated, namely the *Cladosporium* spp. SAAF 22.2.11, SAAF 22.2.12, and SAAF 22.3.29. Lastly, two *Aspergillus* spp. (SAAF 22.3.26 and SAAF 22.4.40) belonging to the family Aspergillaceae were also found in the buckwheat nectar.

1.3.3. Egg parasitoids

Here, we use the two egg parasitoids of the southern green stink bug *Nezara viridula*, a cosmopolitan sucking-insect pest capable of causing economic losses to several vegetable crops. *Trissolcus basalis* (Wollaston, 1858) (Hymenoptera: Scelionidae), a solitary egg parasitoid, is one of the main egg parasitoids of *N. viridula* (Colazza & Bin, 1995), which co-occurs together with *Ooencyrtus telenomicida* (Vassiliev, 1904) (Hymenoptera: Encyrtidae) in southern Italy (Peri et al. 2014). These two egg parasitoids engage in intrinsic and extrinsic interspecific competition for possession of host resources (Cusumano et al. 2022; Peri et al. 2011). Interestingly, the two egg parasitoid species also display divergent nutritional needs because *O. telenomicida* performs concurrent host feeding while *T. basalis* does not (Cusumano et al. 2015). Such behavior, which consists of feeding on host resources before laying an egg (Jervis & Kidd 1986), is displayed by some parasitoid species like *O. telenomicida,* which produce yolk-rich eggs and are expected to have high nutritional demands, particularly in terms of proteins necessary for egg development and maturation (Cusumano et al. 2015).

1.4. Outline of the thesis

In **Chapter 1**, I presented the general introduction, taking into consideration the important literature relevant to this project. Also, I presented briefly the model system, the main objectives, and the research questions.

In **Chapter 2**, I investigated whether microbial fermentation by the nectar specialist yeasts *M. gruessii* and *M. reukaufii* mediates preference behaviors and performances of *T. basalis* and *O. telenomicida,* two egg parasitoid species associated with the southern green stink bug *N. viridula*. To do that, synthetic nectar was produced, inoculated with the individual yeast species, and underwent fermentation. Following the removal of the yeast cells, I conducted a two-choice bioassay to assess the indirect yeast-mediated effects on the olfactory responses of the egg parasitoids when exposed simultaneously to yeast-fermented synthetic nectar and non-inoculated control nectar. Indirect yeast-mediated effects are described as the effects of yeasts via nectar fermentation without taking into account the yeast cells themselves as they were filtered out after fermentation. The volatile profile of the yeast-fermented nectar was further assessed to elucidate the potential compounds that may contribute to explaining the olfactory responses of the egg parasitoids. Finally, I also carried out a no-choice feeding bioassay to investigate whether consuming yeast-fermented synthetic nectar impacted the longevity and survival rates of the two stink bug egg parasitoids.

In **Chapter 3**, the ecological role of nectar-associated microbes beyond yeasts and bacteria was explored. Here, culturable filamentous fungi were isolated and characterized from the floral nectar of buckwheat (*Fagopyrum esculentum*). Subsequently, I also investigated how fermentation by these filamentous fungi would impact the nectar scent and, consequently, the olfactory responses of the egg parasitoids *T. basalis* and *O. telenomicida*. Finally, in order to identify which compounds could be responsible for the parasitoid olfactory responses, the chemical nature of the volatile organic compounds emitted by the fungus-fermented nectars was analyzed.

In **Chapter 4**, I studied how nectar-inhabiting filamentous fungi directly mediate interactions between flowering plants and egg parasitoids. Based on the literature, most laboratory studies have only shown the indirect effects that microbial fermentation may have on parasitoids, but few have investigated the direct role played by the microbial bodies themselves on the insect foraging behavior. Here, I investigated the direct fungus-mediated effects of fermented nectar on the foraging behavior of the egg parasitoid *T. basalis*. I utilized similar strains of microbes isolated from buckwheat to ferment synthetic nectars, and their direct impact on parasitoid olfaction was studied. At the end of this chapter, I demonstrated that direct effects due to microbial bodies in nectar should be considered prior to generalizing the potential role that nectar-inhabiting microbes may play in flowering plant-insect interaction in laboratory assays instead of just mere indirect effects due to microbial fermentation.

In **Chapter 5**, I investigated the impact of the fungus-fermented synthetic nectar on the performance of two egg parasitoids. Here, the isolated nectar-inhabiting filamentous fungi from buckwheat were used to test the hypothesis that fungi affect the longevity and survival of the stink bug egg parasitoids *T. basalis* and *O. telenomicida* via changes in nectar quality. The individual wasp was isolated and fed with the fermented

synthetic nectar until death (longevity). I also analyzed the percentage of the wasps that were alive at a given point after being fed with the different fungus-fermented synthetic nectar (survival rate). Since buckwheat has been widely used in conservation biological control, findings of this experiment are relevant for sustainable agriculture practices by taking into account the role of untapped nectar microbes in the complex flowering plant-parasitoid interactions, which may translate into better and more effective pest management.

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Chapter 2

The indirect effect of nectar-inhabiting yeasts on olfactory responses and longevity of two stink bug egg parasitoids

Contents in this chapter can be accessed in:

Ermio, J.D.L., Peri, E., Bella, P. Rostas, M., Sobhy, I.S., Wenseleers, T., Colazza, S., Lievens, B. and Cusumano, A. The indirect effect of nectar-inhabiting yeasts on olfactory responses and longevity of two stink bug egg parasitoids. *BioControl* (2024). https://doi.org/10.1007/s10526-023-10237-y

Abstract

Adult parasitoids are well known to feed on sugar-rich resources such as floral nectar. Recently, an increasing body of evidence has shown that nectar is ubiquitously colonized by microorganisms and, as a consequence, microbial metabolic activity can affect several traits of floral nectar. Yet, how the fermentation of nectar by yeasts impacts the olfactory responses and performance of parasitoids is largely understudied, especially in the case of egg parasitoids. In this study, we investigated whether fermentation by the nectar yeasts *Metschnikowia gruessii* and *M. reukaufii* affects the olfactory responses of *Trissolcus basalis* and *Ooencyrtus telenomicida,* two egg parasitoid species associated with the southern green stink bug *Nezara viridula*. We also investigated how yeast fermentation affects the longevity and survival of the egg parasitoids. Results of static four-chamber olfactometer tests showed that nectar fermented by *M. gruessii* (but not by *M. reukaufii*) was attractive to both egg parasitoid species, whereas no significant yeast-mediated effects were found in terms of wasp longevity. Gas chromatography coupled with mass spectrometry (GC-MS) showed a clear separation of the volatile profiles among *M. gruessii*, *M. reukaufii*, and non-fermented control nectar, supporting the results of the insect bioassays. The results of our study highlight the need to consider the role of microbes when studying interactions between flower nectar and egg parasitoids and could have implications from a conservation biological control perspective.

Keywords: *Trissolcus basalis* • *Ooencyrtus telenomicida* • *Metschnikowia gruessii* • *Metschnikowia reukaufii* • *Nezara viridula* • Floral nectar yeast

2.1. Introduction

In agroecosystems, flowers represent essential niches that are frequently visited by a multitude of insect species that play different ecological functions (pests and beneficials) (Géneau et al., 2012; Olesen et al., 2007; Vannette, 2020). Hymenopteran parasitoids are among the common insect visitors of flowers because they largely feed on nectar as adults (Jervis et al., 1993). In fact, adult parasitoids depend highly on floral nectar to sustain their energetic requirements (Sobhy et al., 2018; Winkler et al., 2009), as nectars are rich in sugars, amino acids, vitamins, and essential minerals (Lievens et al., 2015). Highly energetically demanding activities, such as host location and oviposition, require a continuous provision of sugar-rich resources to support parasitoid fitness and enhance efficiency (Araj et al., 2011; Nafziger & Fadamiro, 2011; Takasu & Lewis, 1995). In agricultural landscapes, flowers can be scarce, and therefore, resident parasitoids may be starved (Sentil et al., 2022; van Rijn & Wäckers, 2016). Thus, it is a common practice in conservation biological control to incorporate non-crop flowering plants along peripheries or within crop rows in the field, enabling easy-access floral nectar for the parasitoids (Foti et al., 2017; Kowalska et al., 2022).

Despite the clear importance of floral nectar from a biological control perspective, we still have a long way to go in order to fully understand floral nectar ecology (Colazza et al., 2023). Floral nectars are rich in resources, and thus they also represent alluring habitats for diverse microorganisms (Lievens et al., 2015; Pozo et al., 2014; Vannette, 2020). Nectar-inhabiting microbes are regarded as omnipresent colonizers of flowers, yet their role in conservation biological control is largely overlooked (Klaps et al., 2020). Nectar yeasts and bacteria are among the most frequently reported microbes that colonize floral nectars, where they could grow into high densities (Herrera et al., 2009; Lievens et al., 2015). Nectar-inhabiting microbes generally modify various nectar traits, including, but not limited to, the nectar volume (Vannette & Fukami, 2018), nectar acidity (Vannette et al. 2013), sugar or amino acid ratios and compositions (de Vega & Herrera, 2013; Herrera et al., 2008; Vannette et al., 2013), or even the occurrence of secondary metabolites (Vannette & Fukami 2016) and shifts in nectar temperature (Herrera & Pozo, 2010). Nectar microbes are also thought to influence the scent profile of flowers due to the production of microbial volatile organic compounds (mVOCs) emitted from the fermented nectar (Cusumano et al., 2022a; Golonka et al., 2014; Rering et al., 2018; Sobhy et al., 2018). All these microbe-mediated effects on flower nectar could alter the foraging behavior of insect parasitoids and also their performance (Sobhy et al., 2018).

To date, there are only a few studies that have illustrated how fermentation by nectar microbes affects the foraging behavior and performance of insect parasitoids. These studies have focused on yeasts of the ascomycete genus *Metschnikowia* which are specialist nectar-inhabiting microbes documented to be associated with nectar globally (Brysch-Herzberg, 2004; Canto et al., 2017; de Vega et al., 2009; Pozo et al., 2011), and thus they are of particular interest when studying plant-parasitoid interactions. mVOCs from nectar fermented by *M. gruessii* and *M. reukaufii* were found to be attractive to *Aphidius ervi*, a generalist parasitoid of aphids (Sobhy et al., 2018; Sobhy et al., 2019). Also, fermentation by these nectar specialist yeasts enhanced adult parasitoid performance compared to the generalist yeast species isolated from floral nectar (Sobhy et al., 2018).

However, our current knowledge on the role of microbial fermentation by nectar-inhabiting yeasts in plant-parasitoid interactions is restricted to only this case study with an aphid parasitoid species. Therefore, further research efforts must be conducted to generalize parasitoid responses to microbe-fermented nectar.

In order to start filling this gap, we investigated the indirect effects of fermentation by the nectar yeasts *M. gruessii* and *M. reukaufii* on the olfactory responses, longevity, and survival of two egg parasitoids, *Trissolcus basalis* and *Ooencyrtus telenomicida*, both of which are associated with eggs of the southern green stink bug, *Nezara viridula*. The southern green stink bug is a cosmopolitan pest species responsible for serious economic losses in a wide variety of vegetable crops. *Trissolcus basalis* is the main biological control agent of *N. viridula* worldwide, which co-occurs together with *O. telenomicida* in southern Italy (Peri et al., 2014). These two egg parasitoids are known to feed on floral nectar and engage in intrinsic and extrinsic interspecific competition for possession of host resources (Cusumano et al., 2022b; Peri et al., 2011). Interestingly, the two egg parasitoid species also display divergent nutritional needs because *O. telenomicida* performs concurrent host feeding while *T. basalis* does not (Cusumano et al., 2015). Such behavior, which consists of feeding on host resources before laying an egg (Jervis & Kidd, 1986), is displayed by some parasitoid species like *O. telenomicida,* which produce yolk-rich eggs and are expected to have high nutritional demands, particularly in terms of proteins necessary for egg development and maturation (Cusumano et al., 2015). Thus, we could hypothesize that these two stink bug egg parasitoids would have different responses toward yeast-fermented nectar.

To shed light on whether microbial fermentation by the nectar yeasts *M. gruessii* and *M. reukaufii* mediates preference behaviors and performances of egg parasitoids of insect pests, we produced synthetic nectar, inoculated with the individual yeast species, and underwent fermentation. Following the removal of the yeast cells, we conducted a two-choice bioassay to assess the indirect yeast-mediated effects on the olfactory responses of the egg parasitoids when exposed simultaneously to yeast-fermented synthetic nectar and unfermented, non-inoculated control nectar. We also carried out a no-choice feeding bioassay to investigate whether consuming yeast-fermented synthetic nectar (YFSN) impacted the longevity and survival of the two stink bug egg parasitoids. The results of these experiments provide an assessment of the impact that microbial fermentation by specialist yeasts may have on the nectar selection behavior and fitness of stink bug egg parasitoids with potential application for biological control of insect pests.

2.2. Materials and Methods

2.2.1. Study Organisms

2.2.1.1. Nectar yeasts

The nectar yeasts *Metschnikowia gruessii* (isolate ST12.14/016) and *M. reukaufii* (ST12.14/017) (Saccharomycetales: Metschnikowiaceae) used throughout this study were isolated from the floral nectar of the wild comfrey plant, *Symphytum officinale* (Sobhy et al., 2018).

2.2.1.2. Egg parasitoids

The egg parasitoids *Trissolcus basalis* (Hymenoptera: Scelionidae) and *Ooencyrtus telenomicida* (Hymenoptera: Encyrtidae) were reared on egg masses of the southern green stink bug, *Nezara viridula* (Hemiptera: Pentatomidae). Southern green stink bug populations were initially established from field-collected adults throughout Palermo, Sicily, Italy (38°03'57"N, 13°28'10"E). Colonies were maintained in insect netted cages (47.5 × 47.5 × 47.5 cm, BugDorm-44545 MegaView Science Co. Ltd, Taichung, Taiwan) inside an experimental chamber (24 ± 2°C, 70 ± 5% RH, and 16L:8D photoperiod). Nymphs of different instars and adults were reared in separate cages. Fresh seasonal vegetables supplemented with sunflower/soybean seeds were provided *ad libitum*, and replenished every 2-3 days. Water dispensers in Petri dishes with cotton pads were also provided and crumpled or stripped tissue papers were either hung or scattered inside the adult cages to serve as the substrate for oviposition. The presence of eggs was checked every 2-3 days and collected when present for the maintenance of the stink bug population and the egg parasitoids.

Egg masses of *N. viridula* were individually exposed to five females of *T. basalis* or *O. telenomicida* for two days. Then, parasitized egg masses were isolated and incubated in 55-ml glass tubes (25 x 150 mm) closed with cotton plugs until parasitoid emergence. Drops of diluted organic honey (proportion honey:water 80:20 v:v) on parafilm were used to feed the parasitoids. Female egg parasitoids used in the olfactometer bioassay were between 2 to 5 days old and considered naïve (*i.e.* with no prior exposure to synthetic nectar odors). Female egg parasitoids used for the longevity and survival bioassays were newly emerged (≈ 24 h old) and fed only with water.

2.2.2. Inoculation and fermentation of synthetic nectar

To produce yeast-fermented synthetic nectar (referred to as YFSN hereafter), synthetic nectar solutions were inoculated with either of the nectar yeasts, *M. gruessii* or *M. reukaufii*. We used synthetic nectar that mimicked the composition of many natural nectars and was produced by mixing sucrose and casamino acid, as described by Vannette & Fukami (2014). Specifically, synthetic nectar was prepared by mixing filter sterilized 50% w/v sucrose solution (Carlo Erba Reagents S.A.S., Val-de-Reuil, France) and 3.16 mM casamino acid (OmniPur, Merck KGaA, Darmstadt, Germany) (Vannette & Fukami, 2014).

For the inoculation and fermentation of synthetic nectar, following the procedures described in Sobhy et al. (2018), yeast isolates were streaked on YPDA (Yeast Potato Dextrose Agar) and incubated for two days at

25°C. Next, a single colony was inoculated in 10 ml YPDB (Yeast Potato Dextrose Broth) in a sterile 50-ml conical centrifuge tube and incubated overnight in a rotary shaker (150 rpm) with a temperature set at 25°C. Then, cultures were washed twice with sterile physiological water (sodium chloride solution 0.9%) and suspended in the same buffer to obtain an optical density of 1 (OD600). Twenty milliliters of sterile synthetic nectar in 50-ml centrifuge tubes were then inoculated with 100 µl of cell suspension of either yeast strain and subjected to static incubation at 25°C for five days. The incubation period was based on the approximate floral lifespan of many plant species (Lenaerts et al. 2016, 2017; Peay et al., 2012; Vannette & Fukami, 2014). Non-inoculated sterile synthetic nectar was also included during the fermentation to represent the control treatment. After fermentation, YFSN and the control were centrifuged at 10,000 rpm for 15 minutes and filtered using a 0.20-µm syringe filter to obtain cell-free cultures. Aliquots of filtered YFSN were streaked on YPDA to check potential yeast growth or contamination. Cell-free YFSN was then aliquoted to 1-ml amber glass vials and stored at -80°C until the subsequent bioassays. The freezing method is regarded to have no adverse effects on the general chemistry and quality of the YFSN (Peay et al., 2012).

2.2.3. Impact of yeast-fermented nectars on parasitoid olfactory responses

A static olfactometer with four equally divided chambers was used to assess the olfactory response behavior of the egg parasitoids toward the YFSN. The olfactometer was made of acrylic glass formed into a cylinder-shaped enclosure with a height of 4.5 cm and 20 cm in diameter, as described by Steidle & Schoeller (1997). The static four-chamber olfactometer was fitted with a removable gauze cover (0.5 mm mesh) supported by an acrylic glass rim (0.9 mm high), which served as a walking arena for the insects. A netted lid was also laid on the top of the olfactometer to prevent the insects from escaping while ensuring vertical diffusion of odor in order to prevent saturation of volatiles inside the chamber. Two hundred µl of either test solution (YFSN, fermented by either *M. gruessii* or *M. reukaufii*) or control solution (non-fermented synthetic nectar) was pipetted on a filter paper disc (3.8 cm diameter, Whatman No. 1) placed in a Petri dish. The same test treatments were placed in two diagonal olfactometer chambers, while the other two chambers were for the control. About 24 h before performing the bioassays, female parasitoids were individually put in small vials (1.5 × 5 cm) without food to induce starvation. One insect was released in the center of the walking arena in every run, and the insect was given one minute to be acclimatized. After that, the insect olfactory response was evaluated, and the residence time spent in each chamber was recorded for 5 minutes with the aid of a video camera (Logitech C920 HD Pro Webcam) using JWatcher V 0.9 software (https://www.jwatcher.ucla.edu/). In each assay, 40 naïve female parasitoids for each species were used, and each of them was only used once and assigned as one independent replicate. The olfactometer was rotated quarterly after one replication to avoid positional bias. The filter paper disc with synthetic nectar was replaced with every replicate. After each bioassay, the olfactometer was cleaned with 90% ethyl alcohol and tap water and left overnight to dry up at room temperature. The experiment was performed in a rack covered with a black curtain with a white fluorescent lamp (Philips, TLD 58 W/640) situated approximately 0.5 m just above the center of the static four-chamber olfactometer. Windows in the olfactometer room were also covered with cardboard to eliminate

any visual bias due to light coming from the outside. The experiments were carried out between 09:00 and 17:00 with a room temperature maintained at 21 ± 5°C and 50 ± 10% RH.

2.2.4. Impact of yeast-fermented nectars on parasitoid longevity and survival

To determine whether the changes in synthetic nectar due to microbial fermentation affect parasitoid fitness, a longevity and survival bioassay was carried out. Longevity was considered as the length of time that the adult parasitoid lived from emergence up until the point of death, while survival was considered as the percentage of parasitoid individuals that are still alive in a given time after being exposed or fed on the different synthetic nectars. Egg parasitoids were isolated individually in 5-ml glass vials. Drops of YFSN (fermented by *M. gruessii* and *M. reukaufii*) were provided *ad libitum* at the glass vial's side and regularly replenished. As a comparison, unfermented synthetic nectar was included in the experiment, while water was included as a negative control. The bioassay was conducted at 24 ± 2°C, 70 ± 5% RH, 16 h:8 h L:D photoperiod. For each treatment, 15 mated female parasitoids were used. Dead insects were scored daily, and the experiments were concluded when all insects had died.

2.2.5. Chemical analysis of yeast-fermented nectars

To assess whether yeast fermentation affected the VOC profiles, nectars were analyzed by headspace solid phase micro extraction gas chromatography followed by mass spectrometry detection (HS-SPME-GC-MS) as described previously (Goelen et al., 2020). Briefly, GC-MS analyses were performed with a Thermo Trace 1300 GC system equipped with a MXT-5 column (30 m length × 0.18 mm inner diameter × 0.18 µm film thickness; Restek Bellefonte, Pennsylvania, USA) and compounds were subsequently detected by a ISQ mass spectrometer (Thermo Fisher Scientific). The HS-SPME volatile collection was conducted using a 50/30 µm DVB/CAR/ PDMS coating fiber (Supelco, Bellefonte, Pennsylvania, USA). After five min of equilibration, the SPME fiber was exposed to the headspace sample for 30 min. Splitless injection was used with an inlet temperature of 320 °C, a split flow of 9 ml/min, a purge flow of 5 ml/min and an open valve time of 3 min. To obtain a pulsed injection, a programmed gas flow was used whereby the helium gas flow was set at 2.7 ml/min for 0.1 min, followed by a decrease in flow of 20 ml/min to the normal 0.9 ml/min. The GC oven was programmed as follows: the temperature was initiated at 30 °C, held for 3 min and then raised to 80 °C at 7 °C/min. Next, the temperature was raised to 125 °C at 2 °C/min, and finally the temperature was raised to 270 °C at 8 °C/min for 15 min to thermally desorb the compounds that were trapped on the fiber in the injection port of the chromatograph. Mass spectra were recorded in centroid mode using a mass acquisition range of 33–550 atomic mass units, a scan rate of 5 scans/s and an electron impact ionization energy of 70 eV. A mix of linear n-alkanes (from C7 to C23; Supelco) was injected into the GC-MS under identical conditions to serve as external retention index markers. Compounds were identified and quantified as described in Reher et al. (2019). Chromatograms were analyzed with AMDIS 32 to deconvolute overlapping peaks. Obtained spectra were subsequently annotated using the NIST MS Search v2.0g software, using the NIST2011, FFNSC and Adams libraries. Peak areas were compared to the background signal, which was identified by running a GC-

MS with a sample obtained from demineralized water. The background signal was then subtracted from the peak areas of the corresponding tentatively identified compounds. When the difference in peak area fell below a set threshold of 1,000, compounds were eliminated from further analysis. To further extract and integrate the compound elution profiles, a file was used with all our target compounds containing the expected retention times and spectrum profiles. Extraction was performed for every compound in every chromatogram over a time-restricted window using weighted non-negative least square analysis (Lawson and Hanson, 1995), and for every compound, the peak areas were computed from the extracted profiles.

2.2.6. Statistical analysis

Olfactometer, longevity and survival data were analyzed with the R software, version R 4.1.3 (R Core Team, 2022). Initially, the normality assumption of the data on parasitoid residence time above the static olfactometer chambers was assessed using the Shapiro-Wilk test. The analysis revealed that the normality assumption was not violated ($p > 0.05$). Consequently, a parametric analysis was performed using a *t*-test for dependent samples.

Longevity data were analyzed using Generalized Linear Models (GLMs), fitting a gamma error distribution and a reciprocal link function (Crawley, 2007). Tukey's test was used for post hoc comparisons of means, given that means among different treatments were significantly different. The survival of the adult egg parasitoids fed on the different nectars was analyzed using the Kaplan-Meier estimator with the log-rank test of the Benjamini-Hochberg procedure for pairwise comparisons. Consequently, survival probability curves were generated for both egg parasitoids.

Multivariate data analysis (Principal Component Analysis - PCA) was used to analyze peak areas of the VOCs detected. Therefore, peak areas were first log-transformed, mean-centered and subsequently scaled to unit variance before they were subjected to the analysis using MetaboAnalyst (Xia et al., 2009). The results of the analysis were visualized in score plots, which reveal the sample structure according to model components, and loading plots, which display the contribution of the variables to these components.

2.3. Results

2.3.1. Impact of yeast-fermented nectars on parasitoid olfactory responses

Trissolcus basalis females preferred the odors emitted by *M. gruessii*-fermented synthetic nectar to unfermented synthetic nectar (t = -3.112, df = 39, p = 0.003). However, no differences in terms of residence time were found when wasps were given a choice between *M. reukaufii*-fermented synthetic nectar and unfermented synthetic nectar (t = -0.083, df = 39, p = 0.934) (Figure 2A). The same trend was found in the response of *O. telenomicida* to YFSN. Parasitoids showed a preference for odors emitted by *M. gruessii*-fermented synthetic nectar over unfermented synthetic nectar (t = -3.068, df = 39, p = 0.004), whereas no preferences were displayed between *M. reukaufii*-fermented synthetic nectar and the control treatment (t = -0.284, df = 39, p = 0.778) (Figure 2B).

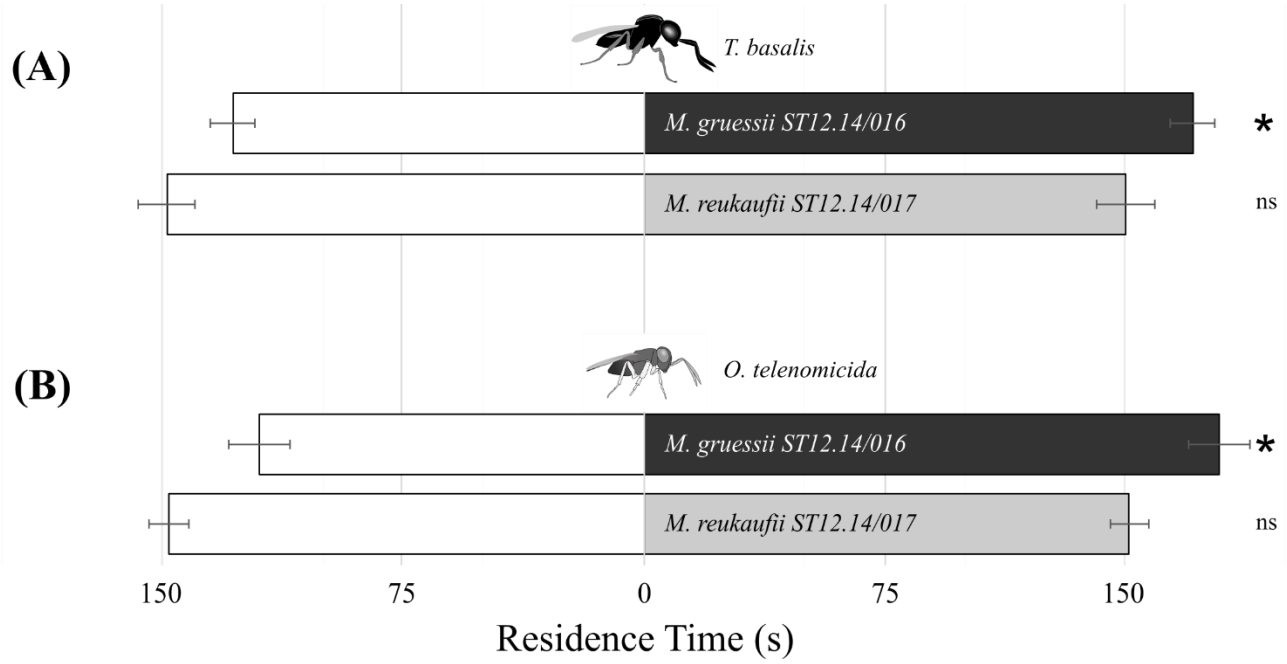

Figure 2. Olfactory responses of the female egg parasitoids (**A**) *Trissolcus basalis* and (**B**) *Ooencyrtus telenomicida* assessed in terms of residence time in two-choice assays over an observation time of 300s. Test treatments represent synthetic nectars fermented by the yeasts *Metschnikowia gruessii* (black bars) and *Metschnikowia reukaufii* (light gray bars). Unfermented synthetic nectar was used in all the pairwise comparisons as a control (white bars). Each treatment was replicated 40 times (paired t-tests for dependent samples, * = p <0.05; ns = not significant). Error bars represent Standard Error (SE).

2.3.2. Impact of yeast-fermented nectars on parasitoid longevity and survival

When testing the effects of the different YFSNs, non-fermented synthetic nectar and water (control) on the longevity of *T. basalis*, only water had a statistically significant effect among all treatments (F = 112.25, df = 3,56 p < 0.001) (Figure 3A). Parasitoids provided only with water died after 3-5 days. No differences were found when wasps were fed with unfermented synthetic nectar compared with wasps fed with synthetic nectar

fermented by the two *Metschnikowia* strains. Longevity of wasps was above 90 days in the three treatments. The same pattern was found for the survival curves of *T. basalis* (χ^2 = 78.1, df = 3, p < 0.0001) (Figure 3B). Likewise, only the water treatment had a significant effect on the longevity of *O. telenomicida* when compared with the synthetic nectars (F = 72.064, df = 3,56 p < 0.001). No differences were detected between parasitoid wasps fed on synthetic nectar fermented by the two *Metschnikowia* strains and wasps fed on unfermented synthetic nectar (Figure 4A). The same trend was found for the survival curves of *O. telenomicida* (χ^2 = 70.4, df = 3, p < 0.0001) (Figure 4B).

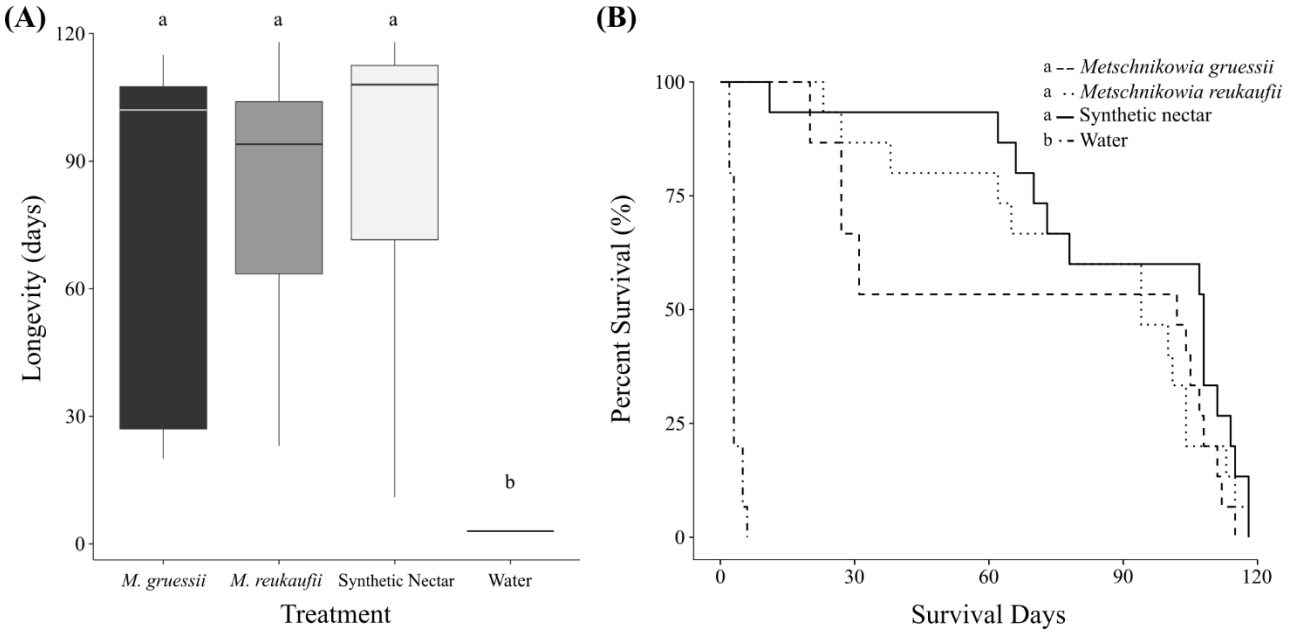

Figure 3. (**A**) Median longevity and (**B**) Kaplan–Meier survival curves for the adult female egg parasitoid *Trissolcus basalis* fed on: synthetic nectar fermented by *Metschnikowia gruessii*; synthetic nectar fermented by *Metschnikowia reukaufii*; unfermented/uninoculated synthetic nectar (reference) and water (negative control). Each treatment was replicated 15 times. Different letters in (**A**) indicate significant differences among treatments (GLM, p 0.05). Bold horizontal lines inside the box show medians, boxes contain the 25th, 50th percentiles, and whiskers that show the upper and lower quartiles. Black box: *M. gruessii*, Light gray: *M. reukaufii*, Off-white: Unfermented synthetic nectar, and Blank: Water. In (**B**), different letters indicate significant differences among parasitoid survival curves according to the pairwise log-rank post hoc tests with the Benjamini-Hochberg correction (p <0.05).

2.3.3. Volatile analysis of yeast-fermented nectars

Analysis of the VOCs that were collected from the different yeast-fermented nectars and the unfermented synthetic nectar revealed large differences in the qualitative and quantitative chemical composition of the VOC blends (Figure 5A). In total, 38 VOCs were consistently detected across the three biological replicates for *M. gruessii,* 33 VOCs were detected for *M. reukaufii,* and 21 compounds were found in the unfermented synthetic nectar (Table 1). A PCA of the VOCs showed that the first component accounted for 58.8% of the total variation

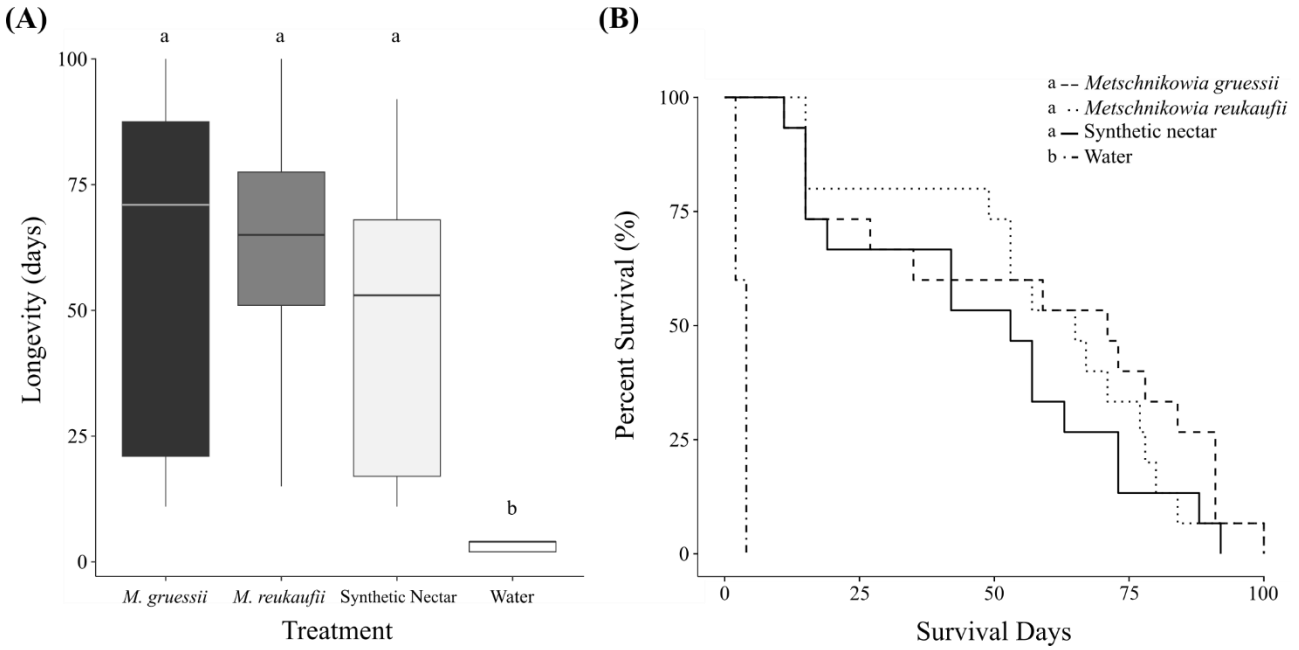

Figure 4. (**A**) Median longevity and (**B**) Kaplan–Meier survival curves for the adult female egg parasitoid *Ooencyrtus telenomicida* fed on: synthetic nectar fermented by *Metschnikowia gruessii*; synthetic nectar fermented by *Metschnikowia reukaufii*; unfermented/uninoculated synthetic nectar (reference) and water (negative control). Each treatment was replicated 15 times. Different letters in (**A**) indicate significant differences among treatments (GLM, p 0.05). Bolded horizontal lines inside the box show medians, boxes contain the 25th, 50th percentiles, and whiskers that show the upper and lower quartiles. Black box: *M. gruessii*, Light gray: *M. reukaufii*, Off-white: Unfermented synthetic nectar, and Blank: Water. In (**B**), different letters indicate significant differences among parasitoid survival curves according to the pairwise log-rank post hoc tests with the Benjamini-Hochberg correction (p <0.05).

in volatile profiles whereas the second component accounted for 18.9% of the variation (Figure 4a). The five greatest loadings of PC1, in descending order of absolute values, were for Ethyl-(E)-cinnamate (ID 37: 0.1975), 2-Butanone (ID 4: -0.1964), 3-Methyl-2-hexanol (ID 15: 0.1948), Propyl acetate (ID 9: 0.1947) and Pentyl-octanoate (ID 35: 0.1940), whereas the greatest loadings of PC2 were for 2-Ethyl-1-hexanol (ID 23: 0.3259), Benzyl alcohol (ID 21: 0.3211), Isoborneol (ID 31: -0.3080), Prenyl isobutyrate (ID 25: -0.2788) and γ-Terpinene (ID 26: 0.2676) (Figure 5B).

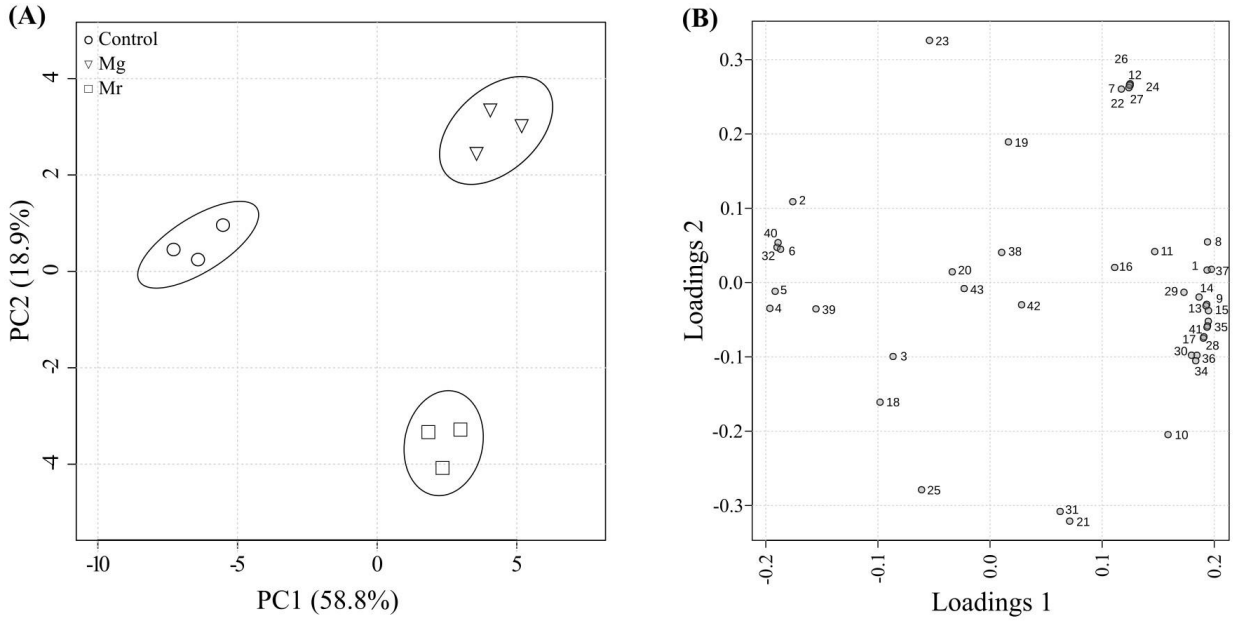

Figure 5. Principal component analysis (PCA) of synthetic nectar fermented by *Metschnikowia gruessii* and *Metschnikowia reukaufii* and non-fermented synthetic nectar. All biological replicates (N = 3) indicate cell-free nectars. (**A**) Score plot visualize the grouping pattern of the samples according to the first two principal components (PCs) with the explained variance in parenthesis. Ellipses indicate 95% confidence interval. (**B**) Loading plot of the first two PCs showing the contribution of each compound to the two PCA components. See Table 1 for the full list of compound IDs.

Table 1. Volatile organic compound (VOC) composition of nectars fermented by the specialist yeasts *Metschnikowia reukaufii* and *M. gruessii*, and the unfermented control.

ID	Volatiles	RT	Synthetic Nectar*		
			Cont.	M.r.	M.g.
	Alcohols				
2	Isopropyl alcohol	1.109	8.94 ± 0.65	5.16 ± 0.10	5.75 ± 0.41
3	Ethanol	1.201	2.24 ± 0.26	1.62 ± 0.08	1.40 ± 0.59
7	1-Propanol	1.918	ND	ND	0.86 ± 0.12
15	3-Methyl-2-hexanol	9.683	ND	0.42 ± 0.03	0.46 ± 0.04
19	5-Methyl-2-furanmethanol	11.690	25.22 ± 4.55	20.71 ± 4.12	30.20 ± 6.82
20	1-Octen-3-ol	11.996	2.62 ± 0.39	2.43 ± 0.03	2.48 ± 0.28
23	2-Ethyl-1-hexanol	12.707	103.70 ± 11.04	ND	126.17 ± 20.13
21	Benzyl alcohol	13.707	ND	0.97 ± 0.11	ND
29	Phenethyl alcohol	16.669	0.54 ± 0.03	1.18 ± 0.17	1.38 ± 0.41
30	4-Methyl-1-pentanol	17.666	ND	89.57 ± 19.20	67.33 ± 18.76
31	Isoborneol	17.815	ND	170.38 ± 111.40	ND
	Aldehydes				
13	Furfural	8.044	ND	0.44 ± 0.03	0.57 ± 0.18
18	Benzaldehyde	11.268	6.51 ± 1.55	6.63 ± 3.62	2.77 ± 1.37
	Alkanes				
27	Undecane	15.394	ND	ND	0.55 ± 0.09

	Benzenoids				
14	Styrene	8.646	0.61 ± 0.20	1.73 ± 0.16	1.88 ± 0.15
28	Guaiacol	15.836	ND	21.03 ± 2.07	19.12 ± 4.34
39	E-methyl isoeugenol	36.375	33.37 ± 1.39	26.49 ± 3.92	22.71 ± 0.54
	Esters				
5	Ethyl Acetate	1.765	1.80 ± 0.09	1.15 ± 0.11	1.00 ± 0.05
8	Ethyl propanoate	4.238	ND	3.68 ± 0.28	8.48 ± 0.71
9	Propyl acetate	4.918	ND	1.93 ± 0.22	1.94 ± 0.03
10	Ethyl isobutyrate	5.643	ND	2.97 ± 0.59	0.69 ± 0.06
6	Isobutyl acetate	6.163	4.40 ± 1.48	ND	ND
11	Ethyl butyrate	6.758	2.29 ± 0.15	2.81 ± 0.24	3.02 ± 0.31
16	Amyl acetate	10.391	0.22 ± 0.14	0.27 ± 0.07	0.30 ± 0.02
22	Isopentyl butanoate	12.652	ND	ND	135.82 ± 35.40
25	Prenyl isobutyrate	14.261	1.51 ± 0.53	2.12 ± 0.26	0.44 ± 0.10
35	Pentyl-octanoate	34.311	ND	1.88 ± 0.25	1.82 ± 0.13
37	Ethyl-(E)-cinnamate	34.892	ND	1.27 ± 0.05	2.06 ± 0.11
41	Ethyl dodecanoate	38.644	ND	9.34 ± 1.80	7.96 ± 0.84
43	Isopropyl hexadecanoate	42.644	1.01 ± 0.10	1.10 ± 0.47	0.86 ± 0.12
	Ketones				
4	2-Butanone	1.575	30.36 ± 2.34	10.42 ± 1.33	6.94 ± 0.65
	Terpenoids				
24	(E)-β-Ocimene	14.166	ND	ND	0.63 ± 0.08
26	γ-Terpinene	14.649	ND	ND	1.04 ± 0.19
33	1-α-Terpineol	20.400	ND	2.73 ± 0.28	2.70 ± 0.30
34	α-Guaiene	32.052	ND	1.32 ± 0.39	0.84 ± 0.21
32	Valencene	34.583	52.53 ± 21.28	ND	ND
36	9-epi-(E)-Caryophyllene	34.726	ND	3.62 ± 0.59	2.73 ± 0.69
38	trans-Calamenene	36.127	27.96 ± 3.63	30.21 ± 13.08	32.24 ± 3.93
40	2E,6Z-Farnesol	40.606	1.24 ± 0.54	ND	ND
42	14-Hydroxy-α-humulene	40.892	4.31 ± 3.11	3.27 ± 0.94	3.39 ± 1.30
	Unknowns				
1	Unknown 1	0.878	0.59 ± 0.02	0.94 ± 0.05	1.07 ± 0.04
12	Unknown 2	7.806	ND	ND	0.73 ± 0.05
17	Unknown 3	10.833	ND	0.88 ± 0.21	1.03 ± 0.05

*Values on the table are means of absolute peak areas ($\times 10^9$) ± SEM of three biological replicates (n = 3) of synthetic nectars fermented by two nectar specialist yeasts (M.g. *Metschnikowia gruessii*; M.r.; *M. reukaufii*) or unfermented control (Cont.). Volatile organic compound(s) in each chemical class are ordered in accordance with their increasing retention time (RT) in a gas chromatograph. VOCs were tentatively identified based on spectra, Kovats retention index, and NIST 17 library matches. ND means not detected.

2.4. Discussion

In this study, we investigated the indirect effect of two nectar specialist yeasts in the genus *Metschnikowia* on the olfactory responses and longevity and survival of two egg parasitoids, *T. basalis* and *O. telenomicida*. Results revealed that both parasitoids responded positively to the odors of the synthetic nectars fermented by the yeast *M. gruessii*, while no significant differences were found in the case of *M. reukaufii* in the two-choice bioassays. Despite the differential yeast-mediated effects on the olfactory responses of the two egg parasitoids, continuous consumption of *M. gruessii*-fermented nectar did not result in differences in adult longevity and survival compared to *M. reukaufii*-fermented nectar. To date, this is the first evidence documenting the relevant - yet overlooked - role of nectar-inhabiting yeasts on egg parasitoids.

Interestingly, it has been demonstrated that a variety of insects prefer flowers colonized by *Metschnikowia* yeasts and that they forage and remove more nectar from yeast-colonized flowers than from uncolonized flowers (Herrera et al., 2013; Schaeffer et al., 2014, 2017; Schaeffer & Irwin, 2014). Specialized nectar microbes, such as *Metschnikowia* yeasts, are thought to alter nectar traits to lure flower-visiting insects, which then serve as vectors for fungal dispersion to new environments (Christiaens et al., 2014; Rering et al., 2018). So far, it is only widely known that nectar-inhabiting yeasts attract pollinators (Pozo et al., 2014), but interestingly, a few recent studies also suggest that carnivorous insects are similarly attracted to the volatiles emitted by specialist yeasts. In fact, we found that synthetic nectar fermented by *M. gruessii* attracted both *T. basalis* and *O. telenomicida,* and the mVOCs from the same *Metschnikowia* strains were also found to attract the aphid parasitoid *A. ervi* (Sobhy et al., 2018; 2019). The fact that only fermentation by *M. gruessii* (but not *M. reukaufii*) elicited a behavioral response in both egg parasitoids suggests that the nectar odors between the two closely related yeasts would be different, although it is not clear, from an evolutionary perspective, why the egg parasitoids displayed such preferences. The GC-MS analyses showed that nectar fermentation strongly affected the chemical composition of the nectar odors, and the PCA clearly separated the two different yeast treatments, corroborating our findings in the olfactometer. According to the PCA model, the compounds that are mostly responsible for the chemical differences are ethyl-(*E*)-cinnamate, 2-butanone, 3-methyl-2-hexanol, propyl acetate, and pentyl-octanoate. Further experiments with the aid of synthetic standards are required to confirm the role of these compounds in mediating egg parasitoid attraction towards odors from yeast-fermented nectars.

To date, it is not clear how microbes affect the nectar quality of parasitoids (Cusumano & Lievens, 2023). On the one hand, it is reasonable to assume that microbial fermentation may remove or reduce the amount of key sugars/amino acids (Vannette & Fukami, 2018) and thus reduce nectar quality for parasitoids (Colazza et al., 2023). On the other hand, microbial fermentation cannot simply be considered a mere removal of key sugars/amino acids because microbes can also produce compounds, including vitamins, in the nectar media, and there are studies showing that microbial fermentation can positively affect parasitoid longevity (Lenaerts et al., 2017). Our study supports the latter hypothesis since we found that fermentation by *Metschnikowia* spp.

did not reduce the longevity and survival of *T. basalis* and *O. telenomicida* compared with unfermented synthetic nectar.

Surprisingly, the two egg parasitoid species performed in a way contrary to our expectations, despite their differential dietary requirements. Given that female *O. telenomicida* must produce yolk-rich eggs and therefore have high protein requirements (Cusumano et al., 2015), we hypothesized that *O. telenomicida* females would perform poorly when fed on YFSN. However, results revealed otherwise, suggesting that yeast fermentation could yield nectar that is capable of fulfilling diverse nutritional needs across parasitoid species. It is worth noting that our study aimed at assessing the indirect effects that microbial fermentation *per se* may have on egg parasitoids. Future research efforts should be directed towards understanding the role played by the yeast cells themselves since microbes may have a direct contribution to insect nutrition and well-being (Crotti et al., 2009; Sobhy et al., 2018). This could be particularly important in the case of *O. telenomicida*, which requires more protein, and microbes can act as an additional protein source for floral visitors.

Our results corroborate the findings of previous reports, showing that nectar specialist yeasts are unlikely to affect the performance of floral visitors and may provide honest signals for food resource location. For example, *A. ervi* parasitoids fed on synthetic nectar fermented by *M. gruessii* and *M. reukaufii* did not reduce their longevity and survival compared with unfermented synthetic nectar (Sobhy et al., 2018). While we found no effects of yeast fermentation on the longevity and survival of egg parasitoids, we cannot exclude that other fitness-related traits, such as egg-laying capacity, can be negatively affected because there are parasitoid species that may require more proteins for egg development, such as the *O. telenomicida*. Although it has been reported that *M. reukaufii* did not negatively affect egg laying and the number of eggs produced by pollinators (Schaeffer et al., 2017), to date, no studies have looked at the effect of microbial fermentation on the reproductive capacity of egg parasitoid species.

In summary, we have demonstrated that fermentation by nectar yeasts can positively influence the olfaction of two stink bug egg parasitoids without any detectable negative effect on their longevity and survival. Our findings indicate that specialist nectar yeasts hold the potential to develop ecological strategies that can attract parasitoids in the field or help in locating high-quality resources through honest microbial signaling which could lead to improved parasitoid fitness and effectiveness in pest control. However, in order to fully understand the potential applications that nectar-inhabiting microbes such as *Metschnikowia* spp. may have for biological control, future research should be carried out to evaluate not only the indirect effects due to microbial fermentation but also the direct effects due to microbial cells naturally thriving in floral nectar. Once both direct and indirect effects on parasitoids are unraveled, we believe that microbe-mediated approaches targeting floral nectar could represent additional tools that biological control practitioners may use to improve insect pest management programs based on conservation biological control.

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Chapter 3

Neglected microbes in floral nectar: Influence of filamentous fungi on nectar scent and parasitoids olfactory responses

Abstract

Floral nectar is a sugar-rich resource which is ubiquitously inhabited by a wide array of microorganisms. Microbial fermentation by nectar-inhabiting microbes can change several nectar traits, including nectar scent, via the production of microbial Volatile Organic Compounds (mVOCs). Although there is growing evidence on how yeasts and bacteria influence the foraging behavior of pollinators and parasitoids, the potential role of other microbial taxa that can colonize nectar has been largely neglected. In this study, we investigated how filamentous fungi isolated from the floral nectar of buckwheat, *Fagopyrum esculentum*, affect nectar scent and the olfactory responses of two co-occurring egg parasitoid species, *Trissolcus basalis* and *Ooencyrtus telenomicida*. Among nectar-feeding insects, parasitoids are common visitors of flowers as they depend on sugar-rich resources to satisfy their energetic and nutritional needs. In olfactometer assays, we found that 2 out of 6 fungal strains, namely *Cladosporium* sp. SAAF 22.2.11 and *Cladosporium* sp. SAAF 22.3.29 induced attraction in the egg parasitoid species. In particular, *O. telenomicida* displayed olfactory responses to both *Cladosporium* spp.-fermented nectars, while *T. basalis* only responded to nectar fermented by *Cladosporium* sp. SAAF 22.2.11. Gas chromatography coupled with mass spectrometry (GC-MS) highlighted both qualitative and quantitative differences in the VOCs emitted by different fungus-fermented nectars. By demonstrating that parasitoids exhibit olfactory responses to nectar fermented by filamentous fungi, we contribute to uncovering interactions between flower-visiting insects and flower-associated microbes, extending beyond what is known for yeasts and bacteria.

Keywords: *Trissolcus basalis* • *Ooencyrtus telenomicida* • *Cladosporium* • *Fagopyrum esculentum* • *Nezara viridula* • Filamentous fungi

3.1. Introduction

Floral nectar is a sugar-rich aqueous substance that stems from the phloem and is secreted by most flowering plants across the angiosperm communities (De la Barrera & Nobel, 2004; Parachnowitsch et al., 2019). It has been recognized as an essential factor in mediating mutualistic interactions between flowering plants and nectar-feeding insects (Parachnowitsch et al., 2019; Schaeffer et al., 2017). Nectar is loaded with carbohydrates, amino acids, minerals, and vitamins essential for the insects' intense metabolic activities (Kowalska et al., 2022; Lievens et al., 2015; Sobhy et al., 2018; Winkler et al., 2009). Floral nectar is visited by many insects that take advantage of the nectar, including pollinators such as honeybees and bumblebees, as well as natural enemies of insect pests like parasitoids (Géneau et al., 2012; Jervis et al., 1993; Olesen et al., 2007; Vannette, 2020). To the pollinators, nectar becomes its reward as a consequence of the pollination services to plants (Kowalska et al., 2022; Olesen et al., 2007; Parachnowitsch et al., 2019; Vannette, 2020), whereas, for the adults of many hymenopteran parasitoids, nectar is an essential resource used for demanding activities such as host searching (Géneau et al., 2012; Kowalska et al., 2022; van Rijn & Wäckers, 2016). Conventionally, in intensive, less diverse agricultural landscapes with meager nectar resources, non-crop habitats comprising nectar-rich flowering plants are structured and built with the aim of improving the effectiveness of natural enemies as biological control agents (Araj et al., 2011; Foti et al., 2017; Nafziger & Fadamiro, 2011; Takasu & Lewis, 1995).

It is well known that flowering plants release plumes of volatiles to attract flower visitors (Colazza et al., 2023; Raguso, 2008; Rusman et al., 2024). These floral volatiles are considered signals that help flower visitors locate and feed on floral nectar (Bianchi & Wäckers, 2008; Foti et al., 2017; Raguso, 2008; Schiestl, 2015). However, it has been more evident in recent decades that floral nectar is ubiquitously inhabited by an array of microbes of different guilds that reshape the flowers' distinct odor signature as a product of microbial metabolism and may have an influence on the foraging behaviors of floral visitors (Klaps et al., 2020; Lievens et al., 2015; Pozo et al., 2014; Steffan et al., 2024; Vannette, 2020). It has been suggested that nectar-inhabiting microbes have a dual effect on the composition of Volatile Organic Compounds (VOCs) associated with floral nectar: first, they can modify the constitutive blends of VOCs emitted by sterile floral nectar; second, their microbial metabolic activity can lead to the emission of de novo volatiles in nectar, also known as 'microbial volatile organic compounds' (mVOCs) (Cusumano & Lievens, 2023). Aside from modifying nectar odors, nectar microbes may also be responsible for several alterations in other nectar traits, such as variations in the composition and proportion of different sugars and amino acids, changes in pH, reduction of secondary metabolites, and even the formation of warmer nectar (De Vega & Herrera, 2013; Herrera et al., 2008; Herrera & Pozo, 2010; Vannette et al., 2013; Vannette & Fukami, 2016, 2018). Considering all these microbe-mediated changes in nectar traits, it is important to consider nectar-inhabiting microbes as "hidden players" that can affect the complex interactions between flowering plants and their associated insect visitors.

Bacteria and yeasts in nectar are thus far the widely studied microorganisms known to affect pollinators' and parasitoids' foraging behavior via changes in the nectar scent (Cusumano et al., 2023; Herrera et al., 2009;

Lievens et al., 2015; Sobhy et al., 2018). For instance, the nectar specialist yeasts belonging to the genus *Metschnikowia* spp. are often reported to elicit positive responses in pollinators, especially for bumblebees (Herrera et al., 2013; Schaeffer et al., 2014, 2017). On the contrary, bacterial colonization can either reduce (Good et al., 2014; Junker et al., 2014) or enhance (Rering et al., 2018) nectar attractiveness to pollinators. In the case of parasitoids, volatiles from nectar specialist yeasts *Metschnikowia* spp. were reported to be attractive to the aphid parasitoid *Aphidius ervi* (Sobhy et al., 2018) and the egg parasitoids *Trissolcus basalis* and *Ooencyrtus telenomicida* (Ermio et al., 2024). More recently, it has also been reported that *T. basalis* displayed positive response behavior toward nectar-inhabiting bacteria (Cusumano et al., 2023).

While an increasing body of evidence is accumulating on how yeasts and bacteria affect pollinators' and parasitoids' foraging behavior, the role of other microbial taxa that can potentially colonize nectar has been largely overlooked. For example, filamentous fungi from the genera *Cladosporium* and *Aspergillus* have also been reported to colonize sugar-rich environments such as floral nectars (Ehlers & Olesen, 1997a; von Arx et al., 2019a), yet the role played by filamentous fungi is generally neglected in the interactions between flowering plants and insect visitors. Taking into account that filamentous fungi are closely related to yeasts, as both belong to the Phylum Ascomycota, it is possible to hypothesize that filamentous fungi can also alter the properties of nectar in the flower via metabolic fermentation, with consequences for the community of floral visitors. Yet, because true fungi also have distinctive traits compared with yeasts, it is also possible to hypothesize that the way they affect nectar traits and the foraging behavior of flower-visiting insects might differ compared with yeasts.

In order to better explore the ecological role of nectar-associated microbes beyond yeasts and bacteria, we have first isolated and characterized the culturable filamentous fungi inhabiting the floral nectar of buckwheat (*Fagopyrum esculentum*), a flowering plant widely used to enhance parasitoid effect as natural enemies of insect pests in agricultural landscapes (Gurr et al., 2017). In fact, despite the importance of buckwheat in biological control, the microbial community of fungi, including yeasts, has not been investigated. Subsequently, we have also investigated how fermentation by these filamentous fungi would impact the nectar scent and, consequently, the olfactory responses of two egg parasitoids, *Trissolcus basalis* and *Ooencyrtus telenomicida*. Both parasitoids are well known to feed on floral nectar, and thus, they are also suitable organisms for investigating how the preference behaviors of insects are affected by microbes that colonize floral nectars. The egg parasitoid *T. basalis* is considered the major natural enemy of the southern green stink bug *Nezara viridula*, a highly destructive sucking insect pest for a multitude of vegetable crops globally (Esquivel et al., 2018). The egg parasitoid *O. telenomicida* is also associated with the stink bug *N. viridula*, and it has been reported to coexist with *T. basalis* in Southern Italy (Peri et al., 2014). Finally, in order to identify which compounds could be responsible for the parasitoid olfactory responses, we analyzed the chemical nature of the volatile organic compounds emitted by the fungus-fermented nectars.

3.2. Materials and Methods

3.2.1. Plant rearing and floral nectar sampling

Buckwheat seedlings were initially cultivated indoors (24 ± 2°C, 45 ± 10% RH, 12L:12D photoperiod) using a commercial multipurpose potting medium (Supernutrient Vegetable Soil, Virgoplant, Piacenza, Italy) before exposure to the natural fields from May to October of 2021. Briefly, buckwheat seeds were sown in seed trays, and 1-week-old germinated seedlings were transplanted to bigger plastic pots with 1-liter volume. After the seedling establishment (~1 week), potted plants were transferred to the experimental fields (University of Palermo, Italy) and exposed to naturally occurring microorganisms in the area to maximize the diversity of microbial taxa that can be sampled from floral nectar.

Twenty samples of nectar suspension taken from random buckwheat flowers were prepared for the isolation of filamentous fungi. Here, nectars were collected from fully opened flowers of 4- to 5-week-old buckwheat plants. To do this, potted plants from the field were transported early in the morning (0700 – 0930 H) to the laboratory on every collection date, and ten inflorescences per plant were sampled. Nectar samples were drawn from these flowers using 0.5 µl glass capillary tubes under a sterile laminar flow cabinet using the procedure described by Cawoy et al. (2008). Finally, collected nectars (approximately 0.05 µl of nectar/flower) were accumulated in 1.5 ml Eppendorf tubes containing 100 µl of sterile distilled water.

3.2.2. Isolation and identification of filamentous fungi from buckwheat nectar

Diluted flower nectar of 100 µl volume was inoculated and spread evenly onto the Petri plate with yeast extract glucose agar (YGC; Sigma-Aldrich, Steinheim, Germany) supplemented with 0.5 g/l chloramphenicol, a universal growth media, to determine the presence of filamentous fungi. Then, inoculated plates were kept in the incubator at 25°C for 7-15 days. Afterward, morphologically distinct fungal colonies from the different plates were subcultured in separate plates using the same media. Monosporic cultures of the fungal isolates were stored in test tube slants with potato dextrose agar (PDA) at 4°C until further use.

All isolates were examined and identified morphologically and further confirmed through molecular analysis of the internal transcribed spacer regions of the fungal ribosomal DNA (ITS rDNA). Fungal DNA extraction began with the harvesting of the mycelia and conidia from the fungal masses by gently scraping the surface of PDA plates with the aid of a disposable sterile plastic wire loop. Harvested fungal bodies were then put in 2-ml Eppendorf tubes with glass beads and vortexed to facilitate the smashing of fungal cells. Then, 600 ml CTAB extraction buffer (pH 8-8.4) was added to the tubes and vortexed afterward for 5-10 min. Next, the suspension was incubated in a thermal block at 65°C for 1 h and mixed by vortexing every 10-15 min. After incubation, 300 µl of phenol plus 300 µl of chloroform: isoamyl alcohol (24:1) solution were added, and the tube was inverted several times. All tubes were centrifuged for 10 min at 10,000 rpm. The supernatant was then transferred to a 1.5-ml Eppendorf tube containing 600 µl chloroform: isoamyl alcohol (24:1) solution. The resultant solution was mixed briefly and centrifuged for 10 min at 10,000 rpm. The supernatant after the centrifugation was transferred to another 1.5-ml Eppendorf tube, and 600 µl of isopropanol was added and

shaken briefly. Afterward, tubes were incubated at -20°C for 10-20 min and subsequently centrifuged for 10 min at 14,000 rpm. The supernatant was discarded, and the remaining DNA pellets were washed twice with 70% alcohol. DNA pellets in tubes were then left in the laminar flow cabinet to dry up, which later dissolved in 100 µl sterile distilled water instead of the 1x TE (Tris and EDTA) buffer. Finally, all DNA samples were measured in a NanoDrop® ND-1000 spectrophotometer and stored at -20°C for later analysis.

Fungal ITS rDNA was amplified using the primer pairs ITSF1 (CTTGGTCATTTAGAGGAAGTAA) and ITS4 (5'-TCCTCC GCTTATTGATATGC-3'). Polymerase chain reaction (PCR) was run in a 25-µl volume PCR mixture with 1x of the GoTaq® G2 colorless master mix, 2 µM of ITSF1 forward primer, 2 µM of ITS4 reverse primer, 1.5 µl of water and 1 µl of fungal DNA template. The reaction was carried out using the MultiGene OptimaxTM Gradient thermal cycler (Labnet International, Inc.) programmed with an initial denaturation at 90°C for 5 min, 35 cycles of denaturation-annealing-extension reactions at 94-50-70°C for 30-60-60 sec, and the last cycle at 72°C for 10 min for the final extension. Gel electrophoresis (1.0% agarose gel) was also performed to check the correct amplification of the PCR products. PCR samples, including the forward and reverse primers, were then prepared with the required amount and sent to BMR Genomics (Padova, Italy, https://www.bmr-genomics.it/) for DNA sequencing. Finally, fungal DNA sequences were edited and compared with the already available sequences based on the type strains at the GenBank standard database with the help of BLASTN (Basic Local Alignment Search Tool, https://www.ncbi.nlm.nih.gov/). Fungal isolates were assigned to the closest homologous strain according to the highest sequence identity percentage results. The obtained sequences were deposited in GenBank, and the accession numbers were assigned (see Table 1). Sequences were aligned using Clustal W in MEGA XI software, and the evolutionary history of the fungi was analyzed using the Neighbor-joining (NJ) method based on the Jukes-Cantor model.

To confirm if the isolated filamentous fungal strains were truly associated with the floral nectar, we conducted a growth trial using synthetic nectar solutions with different concentrations of sucrose (15%, 30%, and 50%) added with casamino acids in accordance with the protocol of the production and fermentation of synthetic nectar (see section below). Fungal growths were checked visually after the fermentation and plated on PDA to confirm the viability of the fungi. In the end, all fungal strains used in the succeeding experiments were proven to be growing and thriving in the nectar solution during and after the fermentation period regardless of the sucrose concentrations. Based on these observations, we ruled out the likelihood that these strains were opportunistic microbes.

3.2.3 Synthetic nectar solutions

The production of synthetic nectars began with the preparation of synthetic nectar solutions by mixing filter-sterilized 50% (w/v) sucrose (Carlo Erba Reagents S.A.S., Val-de-Reuil, France) solution and 3.16 mM casamino acid (OmniPur, Merck KGaA, Darmstadt, Germany) as outlined by Vanette and Fukami (2014). This concentration models several floral nectars found in nature, including buckwheat nectar (Cawoy et al., 2006). To produce fungus-fermented synthetic nectars, synthetic nectar solutions were individually inoculated with

the six filamentous fungi. In brief, fungal strains from the mother cultures were plated on PDA and incubated for 7 days at 25°C. Then, the fungal conidia were harvested using sterile distilled water with the aid of a disposable sterile plastic wire loop. The resulting fungal suspension was subsequently filtered using sterile cloth gauze to eliminate the other fungal bodies and just retain the conidia in the suspension. The number of conidia was counted using the Thoma hemocytometer to ensure the consistency of the conidial counts across the different fungal suspensions for nectar inoculation. Fresh 100 µl of 10^6 conidia per ml fungal suspension of each fungal strain was inoculated to 20 ml of sterile synthetic nectar solution. After that, all tubes were incubated at 25°C for 5 days, which is the approximate floral lifespan of many plant species (Lenaerts et al., 2016, 2017; Peay et al., 2012; Vannette & Fukami, 2014). After the fermentation/incubation period, the fungus-fermented synthetic nectars were centrifuged, and the supernatants were filtered using 0.20-micron filters. To confirm that the synthetic nectars were fungus-free, streaking in PDA with filtered fungus-fermented synthetic nectars was carried out, and possible growth of fungi was observed for another 3 to 5 days. Finally, fungus-fermented synthetic nectars were aliquoted to 1-ml amber vials and directly stored in a -80°C freezer for further use and subsequent volatile analyses.

3.2.4. Parasitoid rearing

The egg parasitoids *Trissolcus basalis* (Hymenoptera: Scelionidae) and *Ooencyrtus telenomicida* (Hymenoptera: Encyrtidae) were maintained using fresh *Nezara viridula* egg masses obtained as described below. Diluted organic honey (80:20 v/v) was provided for the adult wasps. The *N. viridula* colony was reared in the laboratory by feeding fresh organic vegetables (carrots, cabbages, tomatoes and green beans) and sunflower/soybean seeds. Nymphs and adults of stink bugs were reared separately in insect net cages (47.5×47.5×47.5 cm, BugDorm-44545 MegaView Science Co. Ltd, Taichung, Taiwan) at the following environmental conditions: 24±1°C; 70±5% RH and 14L:10D photoperiod. To obtain egg masses of stink bugs, tissue papers were either hung or scattered inside the net cages as an oviposition substrate. Egg masses were collected three times a week and were used to maintain both the stink bug and egg parasitoid colonies. All insect colonies were originally established from material collected around Palermo, Italy (38°03'57"N, 13°28'10"E).

N. viridula egg masses were exposed to 2-3 gravid females of either egg parasitoids for two days and incubated in 55 ml test tubes (25×150 mm) until adult emergence. Following the emergence, adults of both sexes were kept in the same test tube to allow mating. All individuals used in the experiment were females which were assumed to be mated, naïve to the odors of synthetic nectars, and tested only once. About 24 h before the experiments, newly emerged wasp females were individually put in small vials (1.5 × 5 cm) without food to induce starvation.

3.2.5. Olfactory response of egg parasitoids toward fungus-fermented synthetic nectar

A four-chamber static olfactometer was used to investigate the microbe-mediated effects on the olfaction behavior of stink bug egg parasitoids when exposed to odors of fungus-fermented synthetic nectars. The cylindrical body was made of acrylic glass (4.5 × 20 cm) with four equal divisions supported by vertical plates to isolate the odor sources, as described by Steidle and Schoeller (1997). To separate the wasps from the odor sources, a walking arena (1.5 × 20 cm) made of plastic gauze (0.5 mm mesh) fitted with an acrylic rim was placed on the top of the chambers. The olfactometer was also covered with a netted lid (~ 0.5 mm mesh) to confine the wasps inside the walking arena while also facilitating the vertical diffusion of odors to avoid possible volatile saturation in the chamber. Throughout the experiment, the olfactometer was placed on a rack covered with a blackout curtain equipped with a white fluorescent light (Philips, TLD 58 W/640) on the top. The same type of test odor sources (i.e., fungal-fermented nectar) was positioned diagonally to each other in the olfactometer, while non-fermented synthetic nectar (control odor) was always allocated in the other two chambers. Previous results have shown that *T. basalis* is attracted by odors from the raw nectar of buckwheat over non-fermented synthetic nectar (Cusumano et al., 2022). In each bioassay, 200 µl of either fermented or non-fermented nectar was pipetted in sterile filter paper (3.8 cm diameter, Whatman No. 1) on a Petri plate and placed in the odor chambers. One egg parasitoid was released inside the walking arena at a time and given at least 1 min to get accustomed to the arena. Subsequently, the behavior of the egg parasitoid was assessed and recorded for 5 min with the aid of a video camera (Logitech C920 HD Pro Webcam). The residence time of the egg parasitoid above on each of the chambers was then analyzed using the event-recording software J Watcher V0.9 (https://www.jwatcher.ucla.edu/).

For each of the pairwise comparisons, 40 egg parasitoids were tested using a fully randomized experimental design. As the VOC composition of all five biological replicates of nectar was highly similar (see results), the olfactory response was determined for one of the five biological replicates. To avoid unforeseen biases in the set-up, the olfactometer was rotated quarterly after testing every insect. Also, the filter paper disks with the synthetic nectar were changed every replication. The olfactometer was cleaned using 90% ethanol and tap water, followed by drying overnight at room temperature. All olfaction bioassays were carried out in a room with temperature and relative humidity kept at 21 ± 5°C and 50 ± 10%, respectively, between 09h00 and 17h00.

3.2.6. Volatile analysis of fungus-fermented synthetic nectar

The volatile organic compounds (VOCs) from the fungus-fermented synthetic nectars were collected using the headspace solid-phase microextraction (HS- SPME) technique and analyzed by gas chromatography-mass spectrometry (GC-MS) using an Agilent 6890 GC system equipped with a DB5-MS column and MS5973 quadruple MS. The system was programmed in a splitless injection mode with a continuous flow of helium gas as the carrier. Moreover, the inlet port of the GC ran at 260°C with the detector temperature of 280°C. The initial temperature in the GC oven was at 40°C and held for 5 min with a gradual increase of 10°C/min until it

reached 250°C for 30 min. The mass spectra were then recorded between 40 and 550 atomic mass units using an electron impact ionization energy of 70 eV.

The fiber used for the experiment was a Carbowax–divinylbenzene (CW-DVB), 65 µm (Supelco, Bellefonte, PA, USA) that, prior to use, was conditioned in the GC inlet port for 30 min with a temperature of 200°C. Samples were prepared by pipetting 100 µl of either fungus-free fermented synthetic nectar or control/non-fermented synthetic nectar in a filter paper disk Whatman No. 1 (3.8 cm diameter) placed inside a 40-ml amber vial. The vial was also equipped with a poly(tetrafluoroethylene) silicon septum-lined screw cap. Volatile organic compounds were then extracted from the headspace of the glass vial by inserting the SPME needle through the septum and exposing the fiber for 1 h. The fiber was subsequently thermally desorbed in the GC inlet for 1 min, and analyses were performed using the set parameters described above.

MSD ChemStation was utilized to quantify the peak areas of the chromatogram, while spectral libraries such as NIST 2011 and Wiley 17 and the online database, The Pherobase (www.pherobase.com) were utilized for compound annotation otherwise, available commercial standards (Sigma- Aldrich, Milan, Italy) had been used. Retention indices of the identified compounds were calculated based on the linear alkane standards that were injected immediately prior to the experiment. The unforeseen contamination was eliminated from the analysis by subtracting the background signals arising from the blank headspace of the empty vials used for volatile extraction. Five biological replicates were produced and analyzed for VOCs for each fungus-fermented synthetic nectar and the control.

3.2.7. Statistical analysis

Olfactometer data were analyzed with the R software, version R 4.4.0 (R Core Team, 2024). The residence time data were not normally distributed (Shapiro–Wilk normality test) and thus analyzed with a non-parametric test using the Wilcoxon signed rank exact test.

To analyze the peak areas of the volatile organic compounds, multivariate data analysis (projection to latent structures discriminant analysis – PSL-DA) was carried out using the software MetaboAnalyst. Before the analysis, data were log-transformed and mean-centered (Xia et al., 2009). Score and loading plots were generated to project the results of the analysis. The volatile compounds that contribute the most in explaining statistical differences were ranked according to the scores of the variable importance in the projection (VIP) (Wold et al., 2001).

3.3. Results

3.3.1. Isolation and identification of filamentous fungi from buckwheat nectar

Based on the molecular analysis of the internal transcribed spacer regions of the fungal ribosomal DNA, 6 strains of filamentous fungi belonging to 3 families (Stachybotryaceae, Cladosporiaceae, and Aspergillaceae) of the Phylum Ascomycota were found in the buckwheat nectar samples (Table 2). In the family of Stachybotryaceae, we found 1 strain of *Stachybotrys* sp. SAAF 22.1.15. On the other hand, 3 strains under Cladosporiaceae were found, namely the *Cladosporium* spp. SAAF 22.2.11, SAAF 22.2.12, and SAAF 22.3.29. Lastly, two *Aspergillus* spp. (SAAF 22.3.26 and SAAF 22.4.40) belonging to the family Aspergillaceae were also isolated and identified in the buckwheat nectar (Table 2). No yeasts have been detected according to our morphological and molecular analysis.

Phylogenetic analysis based on 27 nucleotide sequences, including sequences from reference strains retrieved from the GenBank database, showed that fungal isolates from the same species clustered together into the same clade, supporting molecular identification (Figure 6).

Table 2. Filamentous fungal strains isolated from the nectar samples of buckwheat floral nectar and their corresponding GenBank accession numbers. Identification was based on internal transcribed spacer (ITS) region sequences of the ribosomal DNA, which were compared with the already available sequences based on the type material or strain at the Genbank standard database with the help of BLASTN. Fungi were assigned to the closest homologous strain according to the highest sequence identity percentage results.

Strain ID	Accession Number[1]	Phylum	Family	Closest match in the GenBank	Sequence identity (%)	Accession no.
SAAF 22.2.11	PP990346	Ascomycota	Cladosporiaceae	*Cladosporium* sp. UTHSC DI-13 235;	99.81	LN834425.1
SAAF 22.2.12	PP990347	Ascomycota	Cladosporiaceae	*C. queenslandicum* BRIP 72447a	99.81	OL307928.1
SAAF 22.3.29	PP990348	Ascomycota	Cladosporiaceae	*C. puyae* CBS 274.80A	100	NR_152298.1
SAAF 22.3.26	PP990349	Ascomycota	Aspergillaceae	*Aspergillus amoenus* CBS 111.32; *A. versicolor* ATCC 9577	100	OL772676.1; KU729039.1
SAAF 22.4.40	PP990350	Ascomycota	Aspergillaceae	*A. uvarum* CBS 121591 *A. japonicus* CBS 114.5; 1 *A. floridensis* DTO_198-A8	100	OL711726.1; OL711724.1; MN431366.1
SAAF 22.2.15	PP990345	Ascomycota	Stachybotryaceae	*Stachybotrys phaeophialis* KAS 525; *S. subreniformis* voucher HGUP 1051	99	KU846738.1; KC305348.1

[1]GenBank accession numbers of the partial sequences of the ITS ribosomal DNA gene of filamentous fungal stains isolated from buckwheat floral nectar in this study

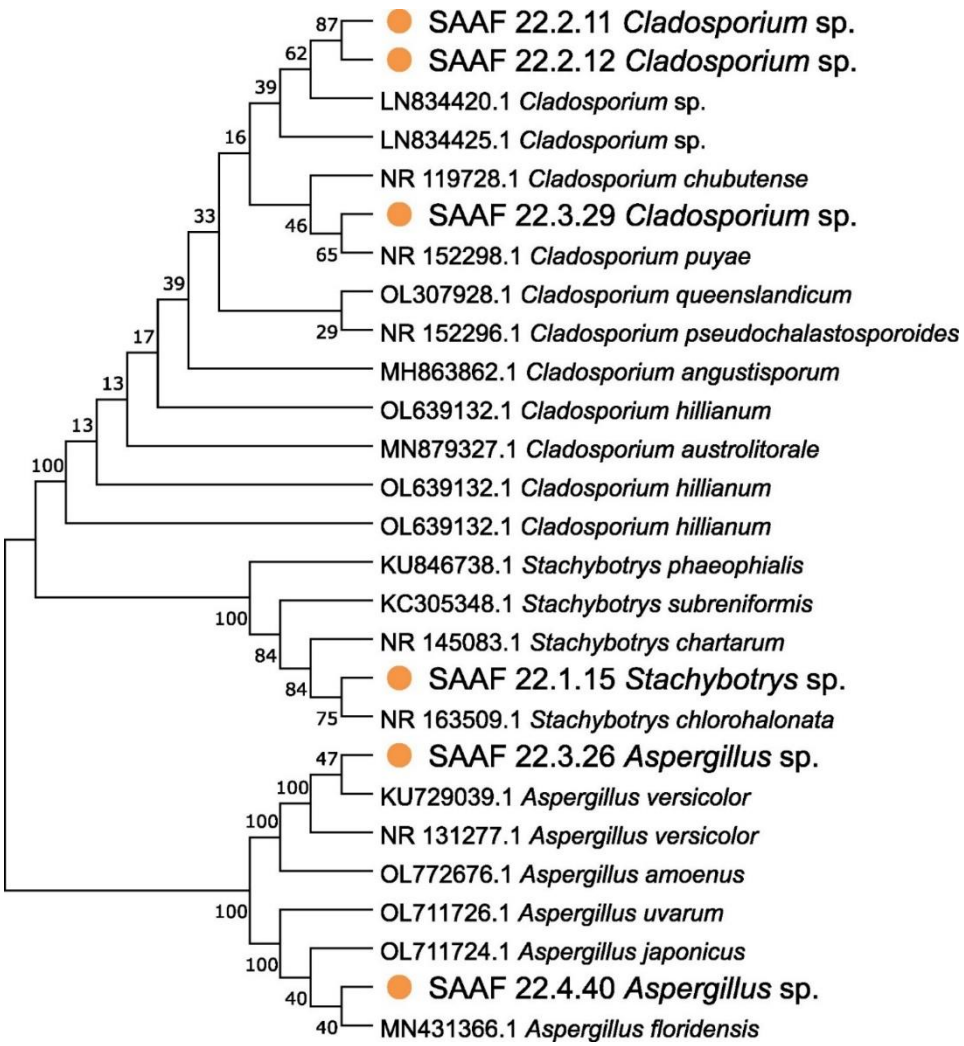

Figure 6. A phylogenetic tree of the filamentous fungal strains isolated from buckwheat floral nectar samples and a number of reference fungal strains downloaded from GenBank (Accession numbers provided). The evolutionary history was inferred using the Neighbour-Joining method (Saitou & Nei, 1987). The evolutionary distances were computed using the Jukes-Cantor method (Jukes & Cantor, 1969) and are in the units of the number of base substitutions per site. There was a total of about 668 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Fungal strains isolated in this study are marked with orange circles.

3.3.2. Olfactory response of egg parasitoids toward fungus-fermented synthetic nectar

3.3.2.1. *Trissolcus basalis*

Females of *T. basalis* showed significant attraction toward the odors emitted by synthetic nectar fermented by *Cladosporium* sp. SAAF 22.2.11 in contrast with the non-fermented synthetic nectar ($Z = 2.218$, df = 39, $p =$

0.026). On the other hand, no preferences were observed when wasps were exposed simultaneously to the odors from the non-fermented synthetic nectar and those fermented by *Cladosporium* sp. SAAF 22.2.12 (Z = 0.659, df = 39, p = 0.518), *Cladosporium* sp. SAAF 22.3.29 (Z = 0.820, df = 39, p = 0.420), *Aspergillus* sp. SAAF 22.3.26 (Z = 0.551, df = 39, p = 0.590), *Aspergillus* sp. SAAF 22.4.40 (Z = 1.102, df = 39, p = 0.276) and *Stachybotrys* sp. SAAF 22.1.15 (Z = 0.054, df = 39, p = 0.962) (Figure 7a).

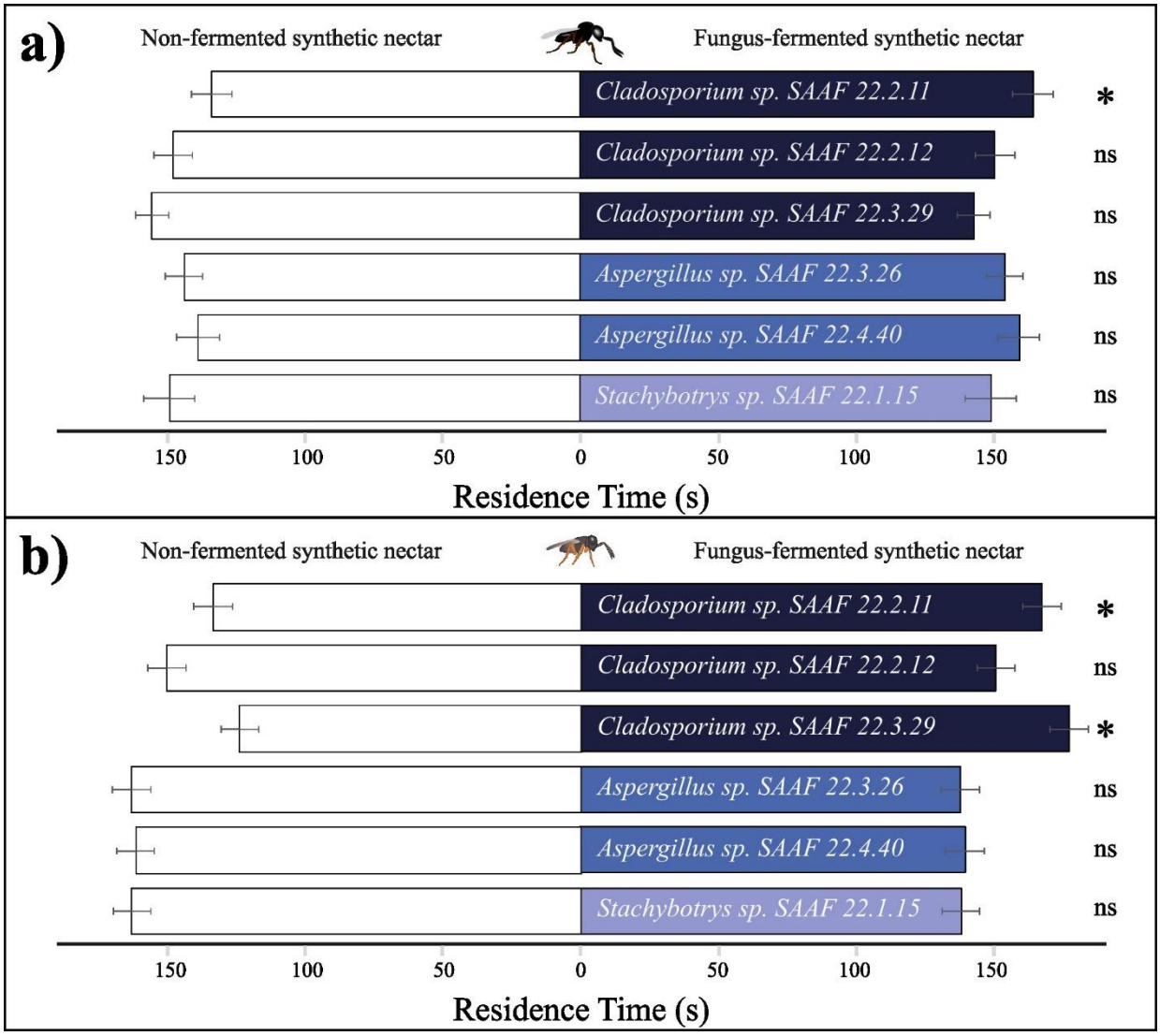

Figure 7. Olfactory responses of the adult female egg parasitoids **a**) *Trissolcus basalis* and **b**) *Ooencyrtus telenomicida* evaluated in terms of residence time (s) in two-choice bioassays over an observation time of 300 s. Test treatments represent synthetic nectars fermented by filamentous fungi *Cladosporium* sp. SAAF 22.2.11, *Cladosporium* sp. SAAF 22.2.12, *Cladosporium* sp. SAAF 22.3.29, *Aspergillus* sp. SAAF 22.3.26, *Aspergillus* sp. SAAF 22.4.40 and *Stachybotrys* sp. SAAF 22.1.15 (all blue-shaded bars). Non-fermented synthetic nectar was used in all the pairwise comparisons as a control (white bars). Each treatment was replicated 40 times (nonparametric, Wilcoxon signed rank exact test, * = $p < 0.05$; ns = not significant). Error bars represent SE (standard error).

3.3.2.2. *Ooencyrtus telenomicida*

Females of *O. telenomicida* were significantly attracted toward the odors emitted by the synthetic nectar fermented by *Cladosporium* sp. SAAF 22.2.11 ($Z = 2.191$, df = 39, $p = 0.027$) and *Cladosporium* sp. SAAF 22.3.29 ($Z = 2.137$, df = 39, $p = 0.032$) when compared to the non-fermented synthetic nectar. However, no preferences were observed when *O. telenomicida* females were exposed simultaneously to the odors from the non-fermented synthetic nectar and those fermented by other filamentous fungi, such as the *Cladosporium* sp. SAAF 22.2.12 ($Z = 0.013$, df = 39, $p = 0.994$), *Aspergillus* sp. SAAF 22.3.26 ($Z = 1.095$, df = 39, $p = 0.276$), *Aspergillus* sp. SAAF 22.4.40 ($Z = 1.048$, df = 39, $p = 0.301$) and *Stachybotrys* sp. SAAF 22.1.15 ($Z = 1.035$, df = 39, $p = 0.307$) (Figure 7b).

3.3.3. Volatile analysis of fungus-fermented synthetic nectar

Headspace analyses of the synthetic nectar fermented by the six filamentous fungi isolated from buckwheat nectar revealed a total of 36 different VOCs, 35 of which were found in *Aspergillus* sp. SAAF 22.3.26, 34 compounds found in both *Aspergillus* sp. SAAF 22.4.40 and *Cladosporium* sp. SAAF 22.2.11 and 33 compounds were detected in *Cladosporium* sp. SAAF 22.2.12, *Cladosporium* sp. 22.3.29 and *Stachybotrys* sp. SAAF 22.1.15. However, only 29 VOCs were detected from the headspace of the non-fermented synthetic nectar. It is also worth noting that seven compounds may be considered microbial VOCs as these compounds were only detected from the headspace of at least one fungus-fermented synthetic nectar and never found in the non-fermented synthetic nectar. These compounds were methyl benzoate (ID 14), hydroxyacetophenone (ID 18), α-terpineol (ID 21), 2,4-dimethyl glutarate (ID 22), 2,6-di-tert-butyl-p-benzoquinone (ID 29), heptadecane (ID 33) and hexadecanoic acid (ID 36) (Table 3).

Based on the PLS-DA, a significant separation of the volatile blends was found across the fungus-fermented synthetic nectar group as compared with the non-fermented synthetic nectar (permutation test $p > 0.005$) (Figure 8). Moreover, the model indicated that seven VOCs had a VIP score > 1.5, suggesting that these compounds strongly contributed to explaining the differences among treatments. These compounds were phenol, 3,4-dimethyl (ID 19), 2,4-dimethyl glutarate (ID 22), α-terpineol (ID 21), hydroxyacetophenone (ID 18), 2,6-di-tert-butyl-p-benzoquinone (ID 29), methyl benzoate (ID 14) and 2-methyl benzaldehyde (ID 12), (Table 3).

Table 3. Volatile organic compound (VOC) composition of synthetic nectars fermented by the filamentous fungi *Aspergillus* sp. SAAF 22.3.26, *Aspergillus* sp. SAAF 22.4.40, *Cladosporium* sp. SAAF 22.2.11, *Cladosporium* sp. SAAF 22.2.12, *Cladosporium* sp. SAAF 22.3.29, *Stachybotrys* sp. SAAF 22.1.15 and the non-fermented control.

ID	**Volatile Organic Compounds**	**RT** (min)	**RI**	**Synthetic nectar***						
				Cont	**ASP26**	**ASP40**	**CLAD11**	**CLAD12**	**CLAD29**	**STACH15**
1	Urea	2.47		7.73±0.29	10.00±1.15	9.97±1.96	10.30±1.56	9.67±1.23	11.52±1.13	10.25±2.51
2	Oxime methoxy phenyl	9.96	903	19.63±10.99	13.03±1.05	14.20±5.66	13.18±1.81	7.92±1.60	10.11±1.57	13.49±4.54
3	2(5h)furanone	10.14	908	4.17±1.93	4.22±1.13	4.50±1.78	3.42±1.09	4.91±1.69	2.74±0.69	5.44±0.73
4	Benzaldehyde	11.79	953	18.70±8.14	29.52±1.95	34.72±10.60	31.72±4.84	28.67±11.39	19.23±3.47	20.07±3.43
5	Benzonitrile	12.64	976	13.84±6.03	22.73±3.09	33.63±6.78	27.50±4.84	17.06±4.72	22.08±5.68	12.73±6.02
6	5-hepten-2-one	12.76	979	4.74±2.03	8.55±2.15	9.76±1.90	6.02±0.65	8.60±1.74	5.78±0.46	5.52±1.84
7	Octanal	13.44	998	0.80±0.41	1.53±0.36	1.79±0.61	1.41±0.55	1.36±0.13	1.02±0.40	1.07±0.26
8	1-hexanol-ethyl	14.37	1025	72.50±21.75	72.52±11.79	120.80±29.57	77.86±17.58	74.80±15.09	120.98±5.95	74.32±19.2
9	2-hydroxybenzaldehyde	14.83	1039	5.53±2.38	7.87±1.85	8.86±3.23	8.56±1.74	5.30±0.84	4.06±0.73	4.65±0.82
10	Acetophenone	15.58	1061	14.61±5.31	22.09±2.39	22.80±5.37	21.34±2.54	12.06±1.44	17.01±0.81	20.00±1.88
11	7-octen-2-ol, 2,6-dimethyl	15.88	1070	9.85±3.09	16.59±11.10	11.71±3.60	7.14±0.51	6.69±1.42	6.34±0.79	9.80±3.80
12	2-methylbenzaldehyde	16.19	1079	641.37±107.52	66.04±1.29	48.21±9.29	66.47±3.88	79.94±6.42	58.53±2.07	103.64±12.55
13	Benzenediol, 4-ethyl	16.38	1085	2.31±0.70	1.79±0.51	2.12±0.72	3.05±0.34	3.53±0.44	1.92±0.22	2.73±0.30
14	Methyl benzoate	16.56	1090	ND	3.26±0.75	2.28±1.36	2.85±0.91	0.89±0.27	1.05±0.48	ND
15	Undecane	16.79	1097	2.00±1.07	1.16±0.87	1.83±0.98	1.82±1.02	1.59±0.45	0.97±0.47	2.95±1.64
16	Nonanal	16.97	1103	11.07±1.78	12.18±1.27	13.15±4.58	9.02±0.61	11.98±1.68	9.51±1.69	11.24±1.88
17	2,4,6-cycloheptatrien-1-one	18.36	1148	3.86±0.70	3.54±0.36	3.52±0.58	3.62±0.69	3.42±0.45	3.09±0.22	3.33±0.29
18	Hydroxyacetophenone	18.65	1157	ND	2.52±1.18	ND	3.61±0.82	ND	ND	ND
19	Phenol, 3,4-dimethyl	18.94	1166	2.50±0.70	ND	1.78±0.77	2.11±0.65	2.49±0.42	2.61±0.29	3.18±0.53
20	Unk ID_20	19.44	1183	1.33±0.65	3.25±0.82	3.57±0.64	3.02±0.98	3.15±0.44	4.01±0.18	4.21±0.26
21	α terpineol	19.79	1194	ND	2.35±0.40	2.54±0.13	2.61±0.68	2.26±0.30	2.96±0.36	3.19±0.58
22	2,4-dimethylglutarate	19.94	1199	ND	3.21±0.56	2.50±0.72	2.97±0.94	3.02±0.49	2.35±0.64	3.93±1.12
23	Decanal	20.13	1205	15.68±2.99	18.18±1.04	18.74±4.55	11.07±1.34	15.81±1.36	13.82±3.06	14.25±2.25
24	2,5-dimethylbenzaldehyde	20.37	1214	31.25±8.85	17.24±0.88	28.73±7.44	17.09±0.98	29.27±7.79	29.31±8.57	27.75±5.08
25	Benzothiazole	20.65	1224	2.10±1.08	1.99±0.85	2.60±1.03	3.01±0.90	3.22±1.26	4.69±1.13	2.36±1.27

26	Nonanoic acid	21.82	1264	2.95±0.39	3.53±0.46	4.02±0.51	3.36±0.60	3.34±0.72	2.96±0.31	2.81±0.71
27	Dodecanal	25.76	1409	1.40±0.59	2.69±0.77	2.97±0.84	2.94±0.34	3.61±0.60	4.44±0.37	8.43±5.05
28	(E)-geranyl acetone	26.71	1447	26.35±6.57	31.49±5.48	30.00±4.35	16.71±1.95	30.01±5.28	40.99±8.94	18.04±1.50
29	2,6-di-tert-butyl-p-benzoquinone	27.06	1461	ND	5.31±0.75	6.85±0.82	4.65±0.61	4.74±1.42	6.47±0.75	6.23±1.75
30	Unk ID_30	28.25	1508	6.44±2.47	3.49±0.28	8.24±1.59	7.15±2.14	6.21±2.27	6.08±1.52	5.24±2.59
31	Hexadecane	30.44	1600	1.56±1.27	1.57±0.70	1.41±0.74	1.70±0.88	2.42±1.20	4.93±0.60	2.09±1.11
32	Benzophenone	31.10	1629	8.99±3.89	8.61±1.49	12.40±2.42	10.84±2.11	11.22±4.15	16.37±1.48	7.76±2.22
33	Heptadecane	32.71	1700	ND	2.09±0.92	2.63±1.12	3.77±0.98	3.19±1.53	6.43±0.71	1.82±0.98
34	Tetradecanoic acid	33.92	1753	1.00±0.43	1.23±0.54	ND	ND	ND	ND	ND
35	Unk ID_35	37.94	1952	4.01±1.08	2.59±0.26	3.45±0.88	3.36±0.60	3.31±0.64	3.30±0.95	3.57±1.63
36	Hexadecanoic acid	38.03	1957	ND	7.10±3.43	3.27±2.41	ND	ND	ND	1.82±0.85

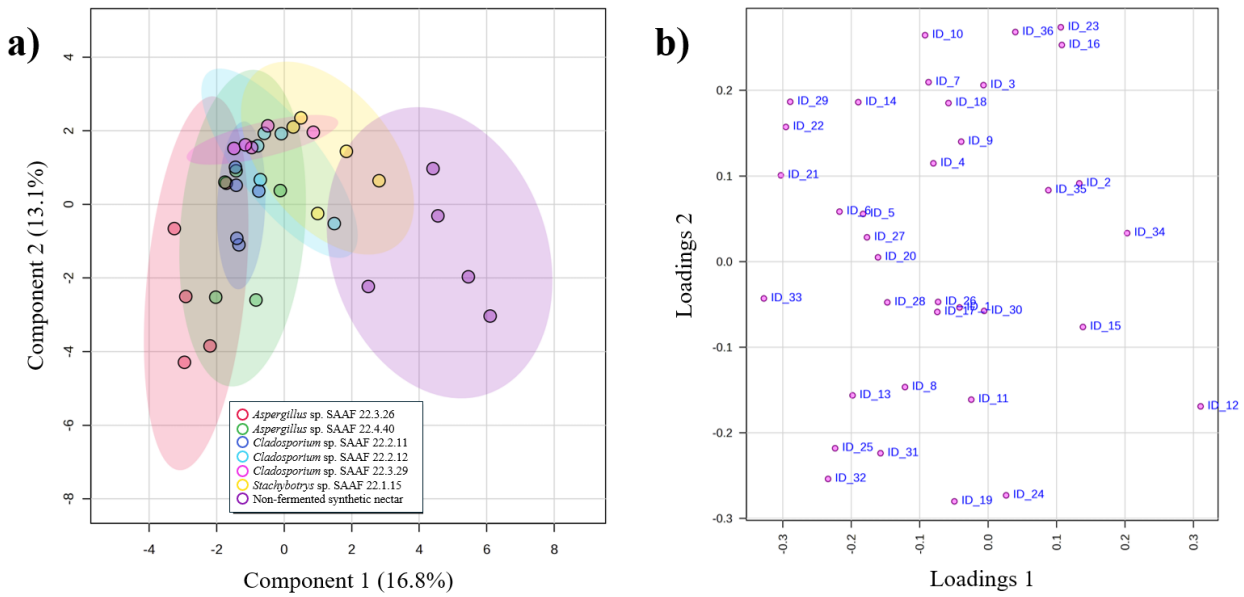

Figure 8. Projection to latent structures discriminant analysis (PLS-DA) of synthetic nectar fermented by filamentous fungi *Cladosporium* sp. SAAF 22.2.11, *Cladosporium* sp. SAAF 22.2.12, *Cladosporium* sp. SAAF 22.3.29, *Aspergillus* sp. SAAF 22.3.26, *Aspergillus* sp. SAAF 22.4.40 and *Stachybotrys* sp. SAAF 22.1.15 and negative control (non-fermented synthetic nectar). All biological replicates (n = 5) indicate fungus-free synthetic nectars. **a**) The score plot visualizes the grouping pattern of the samples according to the first two principal components (PCs) with the explained variance in parenthesis. Ellipses indicate a 95% confidence interval. **b**) Loading plot of the first two PCs showing the contribution of each compound to the two PLS-DA components. Compounds with VIP (variable important to the projection) scores greater than 1.5 are 2-methyl benzaldehyde (ID 12), benzoic acid methyl ester (ID 14), hydroxyacetophenone (ID 18), phenol, 3,4-dimethyl (ID 19), α-terpineol (ID 21), 2,4-dimethyl glutarate (ID 22), and 2,6-di-tert-butyl-p-benzoquinone (ID 29). See Table 1 for the complete list of compound IDs.

3.4. Discussion

In this study, we found that insect parasitoids can respond to changes in nectar odors caused by the fermentation of nectar-inhabiting fungi isolated from buckwheat nectar. We thus contribute to unraveling the interactions between flower-visiting insects and flower-associated microbes beyond yeasts and bacteria, which are the most common microbial taxa reported to colonize nectar. From the floral nectar of buckwheat, we were able to culture 6 different strains of filamentous fungi belonging to 3 families (Stachybotryaceae, Cladosporiaceae, and Aspergillaceae) of the Phylum Ascomycota; yet only 2 strains within the Cladosporiaceae (*Cladosporium* sp. SAAF 22.2.11 and *Cladosporium* sp. 22.3.29) were found attractive for the co-occurring parasitoids *T. basalis* and *O. telenomicida* suggesting a specificity of parasitoid olfactory responses.

Although not much is known about the role played by nectar-inhabiting filamentous fungi on the foraging behavior of flower-associated insects, an increasing body of evidence is accumulating rapidly for yeasts. In olfactometer studies, nectar-fermentation by specialist yeasts *Metschnikowia* spp. has been shown to elicit attraction in the aphid parasitoid *A. ervi* (Sobhy et al., 2018, 2019) and the egg parasitoid *T. basalis* and *O. telenomicida* (Ermio et al., 2024) which is in agreement with our results on filamentous fungi. Taking together, these results indicate that insect parasitoids display broad olfactory responses to nectar fermented by fungi from the Ascomycota phylum. In the case of specialist yeasts, evidence accumulated so far indicate that parasitoid attraction to *Metschnikowia*-fermented nectar is not negatively associated with a decrease in nectar quality, which suggests that parasitoid attraction to *Metschnikowia* spp.-fermented nectar may increase parasitoid efficiency in localizing profitable food resources (Sobhy et al., 2018, 2019). Yet, to understand if parasitoid olfactory responses towards nectar fermented by filamentous fungi are adaptive, further studies should be conducted to explore whether feeding on *Cladosporium* sp.-contaminated nectar confers a fitness benefit to the parasitoids, for example, in terms of extended longevity or increased fecundity. A possible reason for the absence of olfactory responses by the egg parasitoids towards *Aspergillus* sp.- and *Stachybotrys* sp.-fermented nectars could indeed be explained if parasitoids experience a fitness cost when consuming such fermented nectar. Further studies should also explore if there is a benefit for the filamentous fungi to attract parasitoids, as it is currently not known whether parasitoids may act as effective dispersal agents of nectar-inhabiting microbes.

In our study, we found that fungal fermentation affected the composition of floral odors, both quantitatively by modifying the constitutive VOC emission of nectar and qualitatively by producing de novo microbial VOCs. According to our multivariate statistical analyses, the compounds that possess the highest VIP values and are thus likely correlated with parasitoid attraction are both constitutive VOCs (phenol, 3,4-dimethyl and 2-methyl benzaldehyde) as well as mVOCs which are missing in the non-fermented synthetic nectar (2,4-dimethyl glutarate, α-terpineol, hydroxyacetophenone, 2,6-di-tert-butyl-p-benzoquinone, methyl benzoate). It is thus possible that both qualitative and quantitative differences in the odor blend due to fungal fermentation may be important for explaining the olfactory responses of *T. basalis* and *O. telenomicida*. For example, 2-methyl benzaldehyde was a compound present in higher abundance in non-fermented synthetic nectar, and in

a recent study, it was reported to be repellent toward other hymenopteran species, ants in particular (Kay et al., 2023). The compounds acetophenone and hydroxyacetophenone, previously reported as signal nectar sources to parasitoids, were found to enhance plant attractiveness and promote natural pest control (Rohrig et al., 2008). Among other chemicals, α-terpineol was shown to have electroantennographic activity toward the bark beetle parasitoid species *Roptrocerus xylophagorum* (Ratzeburg) (Hymenoptera: Pteromalidae) (Pettersson et al., 2000). Among the microbial volatiles, methyl benzoate and 2,6-di-tert-butyl-p-benzoquinone have been reported to have antimicrobial properties (Salvatore et al., 2021) and may thus confer competitive advantages when other microbes colonize the same buckwheat flower. In addition, methyl benzoate is reported to be a plant defensive chemical with eco-friendly botanical-insecticide properties while safe to non-target organisms such as the natural predators coccinellids *Coccinella septempunctata* L. and *Harmonia axyridis* Pallas. Methyl benzoate was reported as the main volatile emitted in the scent of snapdragon flowers (Dudareva et al., 2000), whereas p-benzoquinone was found in the headspace of buckwheat flowers (Foti et al., 2017), suggesting a microbial origin of the latter compounds. Although it is reasonable to suspect that the above-mentioned compounds could be responsible for the attraction of *T. basalis* and *O. telenomicida* towards nectar fermented by filamentous fungi, this hypothesis should be validated with the aid of synthetic chemical compounds.

Given the widespread occurrence of yeasts in floral nectar, the lack of taxa such as *Metschnikowia* in our study was surprising, as we were able to detect only filamentous fungi, and a wide array of bacteria (Cusumano et al., 2022). A possible explanation for the absence of nectar-inhabiting yeasts could lie, at least partially, in the community composition of insect pollinators associated with buckwheat: flowers are commonly visited by honeybees but not by bumblebees (Liu et al., 2020), which are considered the main dispersal agents of specialist yeasts such as *Metschnikowia* spp. In fact, nectar-inhabiting yeasts have been shown to increase flower visitation rates, particularly in the case of bumblebees (Herrera et al., 2013; Schaeffer et al., 2014, 2017), whereas honeybees often displayed neutral or even negative foraging responses towards nectar-inhabiting yeasts (Crowley-Gall et al., 2022; Rering et al., 2020). It is also possible to argue that, in the absence of yeasts, floral nectar represents an empty niche available for colonization by less specialized fungal taxa such as molds in the genera *Cladosporium* and *Aspergillus*. These filamentous fungi are more commonly associated with pollen (Rutkowski et al., 2023), but they can grow in sugar-rich resources and were also reported to occur in floral nectar (Ehlers & Olesen, 1997; von Arx et al., 2019). As we are not aware of any other studies that have investigated the microbial composition of buckwheat floral nectar, further research is needed to better understand which microbial taxa dominate and whether the absence of yeasts is common in the nectar of buckwheat flowers.

In this work, we contribute to generating awareness that interactions between parasitoids and flowering plants should not be viewed as a simple “bipartite” interaction; instead, a “tripartite” plant-insect-microbe approach is needed, keeping into account that microbes can act as “hidden players” in plant-insect interactions (Colazza et al., 2023; Dicke et al., 2020; Frago et al., 2012). Among nectar-inhabiting microbes, bacteria and yeasts

have been increasingly studied in the ecology of pollinators and parasitoids (Cusumano & Lievens, 2023; Vannette, 2020). By showing that fermentation by filamentous fungi can also affect the foraging behavior of insect parasitoids, we expand the microbial taxa known to play a role in the interaction between flower-visiting insects and flowering plants. Finally, our results are also relevant for sustainable agriculture, as flowering resources such as buckwheat are implemented in agroecosystems to enhance the efficacy of natural enemies as biological control agents of insect pests (Gurr et al., 2017; Heimpel & Mills, 2017). Since flower visitation is crucial for insect parasitoids to exploit floral resources, it is important to consider the microbial component, as parasitoid visitation rates may be enhanced by attractive microbial volatiles emitted from nectar-inhabiting filamentous fungi.

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Chapter 4

The direct consequence of nectar-inhabiting filamentous fungi on the olfaction of stink bug egg parasitoid *Trissolcus basalis* through nectar fermentation

Abstract

Floral nectar is a conducive medium for a variety of microbial taxa, which are found to alter nectar traits crucial for insect attraction. A multitude of nectar-inhabiting microbes have already been documented, but none has looked into how filamentous fungi mediate interactions between flowering plants and parasitoids. Moreover, most laboratory studies have only shown the indirect effects that microbial fermentation may have on parasitoids, but not so far the direct role played by the microbial bodies themselves on the insect foraging behavior. Here, we investigated the direct fungus-mediated effects of fermented nectar on the foraging behavior of the egg parasitoid *Trissolcus basalis*, the major antagonist of the vegetable pest *Nezara viridula*. Culturable microbes from buckwheat *Fagopyrum esculentum* were used to ferment synthetic nectars, and their direct impact on parasitoid olfaction was studied. Out of 6 fungal strains, *T. basalis* only preferred the odors emitted by fermented nectar with fungal bodies of *Cladosporium* sp. SAAF 22.2.11. In terms of the comparative responses of wasps when exposed simultaneously to fermented nectar with and without fungal bodies, *T. basalis* generally preferred odors emitted by fungus-free nectar over its counterpart, especially the ones fermented by *Aspergillus* sp. SAAF 22.3.26 and *Stachybotrys* sp. SAAF 22.1.15. Our results demonstrate that direct effects due to microbial bodies in nectar should be highly considered to generalize the potential role that nectar-inhabiting microbes play in flowering plant-insect interaction in laboratory assays instead of just mere indirect effects due to microbial fermentation.

Keywords: *Trissolcus basalis* • Microbial bodies • *Cladosporium* • *Fagopyrum esculentum* • *Nezara viridula* • Filamentous fungi

4.1. Introduction

One of the approaches in sustainable cropping systems is the establishment of biodiversity patches around the crop fields that would sustain basic ecosystem services, like pest regulation (Gurr et al., 2017; Landis et al., 2000). A pest regulation process by natural enemies that encompasses several strategies of habitat modification is actually the key principle of conservation biological control (CBC) programs (Gurr et al., 2017; Landis et al., 2000). Planting of non-crop flowering plants in strips or hedges has been one of the tactics being practiced since time immemorial that falls under such an approach (Kowalska et al., 2022; Pollier et al., 2019). Flowering plants serve as the foundation of important resources like shelter and food for a multitude of insect species that play indispensable roles in the ecosystem (Gurr et al., 2017; Landis et al., 2000). Floral nectar is among the important resources that flowering plants offer to nectarivorous insects and has been recognized to mediate the interactions of both organisms (Foti et al., 2017; Luizzi et al., 2024). Aside from pollinators and nectar robbers, hymenopteran parasitoids rely heavily on floral nectar (Brito Vera & Pérez, 2024; Jervis et al., 1993; Luizzi et al., 2024). In fact, nectar is a highly sought-after food by many insects, including parasitoids, because of its nutrient contents, such as sugar, amino acids, essential minerals, vitamins, and lipids, among others, essential for their survival and immunity, thereby maximizing their full potential (Jervis et al., 1993; Lievens et al., 2015). However, in order for CBC programs to be effective, flowers should not only offer high-quality nectar rewards but also must be highly attractive to the parasitoids for efficient location (Foti et al., 2017). Thus, volatile plumes produced by flowering plants are exploited by nectarivorous insects, including parasitoids, which serve as signals that orient themselves toward a nectar resource (Bianchi & Wäckers, 2008; Colazza et al., 2023; Raguso, 2008; Rusman et al., 2024; Schiestl, 2015).

Floral nectar, similar to other ecosystems, is an appealing habitat for certain living organisms (Lievens et al., 2015). Despite its harsh nature and limited quantity, floral nectar is colonized by microorganisms of different identities and varying densities (Christensen et al., 2021; de Vega et al., 2009; Fridman et al., 2012; Herrera et al., 2009). It has been reported in many previous works that bacteria and yeasts are among the most prevalent groups of microbes to inhabit floral nectar, and more often than not, they require insect vectors for dispersal among nectar environments (Brysch-Herzberg, 2004; Good et al., 2014; Herrera et al., 2008; Rering et al., 2018; Vannette, 2020). Their presence actually makes the interaction between flowering plants and insects even more complex than previously thought. In fact, nectar-inhabiting microbes contribute to the entirety of volatile emissions of flowering plants that, consequently, influence the foraging behavior of the community of flower visitors (Cusumano et al., 2022; Golonka et al., 2014; Rering et al., 2018; Sobhy et al., 2018). On top of that, the metabolic activities of microbes could also enhance or degrade the quality

of floral nectar, thereby affecting the reward offered by flowers to nectarivorous insects (Lenaerts et al., 2017; Schaeffer et al., 2014). Among the nectar traits that nectar-inhabiting microbes can modify are the proportions and compositions of sugars and amino acids, the pH level, nectar temperature, the presence of primary metabolites, and even the nectar volume (De Vega & Herrera, 2013; Herrera et al., 2008; Herrera & Pozo, 2010; Vannette et al., 2013; Vannette & Fukami, 2016, 2018). One or combinations of these alterations in nectar may be considered significant factors for floral visitors in selecting excellent nectar resources (Vannette, 2020).

Several nectar microbes have already been studied for their impact on floral nectar physicochemical properties, which also resulted in differential microbial-mediated effects on pollinators, as well as parasitoids of insect pests (Cusumano et al., 2022; Ermio et al., 2024; Herrera et al., 2009; Lievens et al., 2015; Sobhy et al., 2018). For example, honeybees (Rering et al., 2018; Vannette & Fukami, 2016) and bumblebees were found to be attracted to the volatiles emitted by the nectar specialist yeasts, *Metschnikowia* spp. (Herrera et al., 2013; Schaeffer et al., 2014, 2017). Also, studies have indicated that *M. reukaufii* produced more attractive volatiles to honeybees in contrast with the generalist nectar yeast and bacteria (Rering et al., 2018). However, one report has claimed that *M. reukaufii*-inoculated nectar was less preferred by honeybees than uncolonized nectar in a field experiment (Rering et al., 2020). On the other hand, the behavioral responses of pollinators toward nectar-inhabiting bacteria could also be variable among the different isolates tested (Good et al., 2014; Junker et al., 2014; Rering et al., 2018, 2020). In the case of other insect guilds, adult parasitoids of aphids (*Aphidius ervi*) and egg parasitoids of southern green stink bugs (*Trissolcus basalis* and *Ooencyrtus telenomicida*) were found to be attracted to volatile emitted by *Metschnikowia* spp. (Ermio et al., 2024; Sobhy et al., 2018). Moreover, strains of bacteria *Staphylococcus epidermidis*, *Terrabacillus saccharophilus*, *Pantoea* sp., and *Curtobacterium* sp. isolated from buckwheat nectar exhibited favorable behavioral responses in *T. basalis* via nectar fermentation (Cusumano et al., 2022).

Although numerous reports have focused on yeasts and bacteria in studying nectar microbial ecology, no information has been published yet showing how other microbial taxa in nectar, like filamentous fungi, mediate flowering plant-insect interactions. In fact, filamentous fungi were found to inhabit flowers (Lupo et al., 2001; Tadych et al., 2012) and have been reported to colonize sugar-rich environments such as floral nectars, like in the case of *Cladosporium* and *Aspergillus* (Ehlers & Olesen, 1997; von Arx et al., 2019). The previous chapter of this manuscript, however, could be the first to investigate the nectar-inhabiting filamentous fungi and their impact on insect olfaction via nectar fermentation. Nonetheless, all bioassays conducted with parasitoids only tested cell- or fungus-free fermented nectar without taking into account the effect of microbial cells or bodies themselves

on the wasps' behavior. Microbial bodies are thought to provide crucial benefits for the general well-being of insects (Gibson & Hunter, 2010; Stefanini, 2018). For instance, microbes could provide an additional nutritional source for the insects and may be involved in their detoxification, growth and development (Gibson & Hunter, 2010; Noda & Koizumi, 2003; Shen & Dowd, 1992). Also, it was found that bumblebees may exhibit a preference behavior toward nectar with live yeast over treatment with inactivated yeast cells (Pozo et al., 2020). Moreover, some experiments have also tested the direct effects of microbes in fermented nectar, but those studies were unfortunately only limited to bumblebees and honeybees (Rering et al., 2018, 2020; Schaeffer et al., 2014, 2017). Thus, further research is highly needed to generalize previous results, taking into account the direct microbial-mediated effects on the response behavior of parasitic wasps in floral nectar.

Here, we studied the direct effect of nectar-inhabiting filamentous fungi on the foraging behavior of egg parasitoid *Trissolcus basalis*, the major antagonist of southern green stink bug *Nezara viridula*, a highly destructive sucking insect pest for a multitude of vegetable crops globally (Esquivel et al., 2018). This parasitoid is well known to feed on floral nectar, and thus, it is ideal to consider this insect species in establishing a model system for investigating nectar microbial ecology. We utilized the previously isolated filamentous fungi from buckwheat nectar (see Chapter 3). Buckwheat (*Fagopyrum esculentum* Moench) is actually a flowering plant widely used in CBC programs to enhance the parasitism rates by parasitoids of insect pests. Briefly, we fermented synthetic nectar through inoculation of filamentous fungi. Aside from producing fungus-free fermented nectar, we also produce fermented nectar with fungal bodies. Finally, we investigated how these microbes would directly impact the olfactory responses of egg parasitoids. To do that, we conducted two olfaction bioassays: 1) the response of wasps on the fermented nectar with fungal bodies and uninoculated synthetic nectar (control), and 2) the comparative response of the wasps on the fermented nectar with fungal bodies and fungus-free fermented nectar.

4.2. Materials and Methods

4.2.1. Experimental organisms

Nectar filamentous fungi. The following nectar filamentous fungi used in this study were initially isolated from the floral nectar of buckwheat *Fagopyrum esculentum*: *Stachybotrys* sp. SAAF 22.1.15 (Ascomycota: Stachybotryaceae), *Cladosporium* spp. SAAF 22.2.11, SAAF 22.2.12, SAAF 22.3.29 (Ascomycota: Cladosporiaceae), *Aspergillus* spp. SAAF 22.3.26 and SAAF 22.4.40 (Ascomycota: Aspergillaceae). All these fungal isolates underwent a thorough confirmation as to their true association with floral nectar by conducting a series of growth trials using synthetic nectar with varying sucrose concentrations (15%, 30%, and 50%). Observations after 5-day fermentation revealed that the isolates had grown and thrived in the synthetic nectar irrespective of the sucrose concentrations, which was later confirmed by their viability in potato dextrose agar (PDA) after plating of the individual fungus-fermented synthetic nectar.

Trissolcus basalis. The colony of egg parasitoid *T. basalis* (Hymenoptera: Scelionidae) was initially established after the wasps that emerged from the egg masses of southern green stinkbug *Nezara viridula* (Hemiptera: Pentatomidae) found in the cultivated fields in Palermo, Italy (38°03'57"N, 13°28'10"E) that were mainly planted to tomatoes. Diluted organic honey in water (80:20 v/v) was used to sustain the parasitoid colonies inside the 55-ml glass tubes (25 × 150 mm) closed with cotton plugs and kept in a climate chamber (24±1°C; 70±5% RH and 14L:10D photoperiod). The egg parasitoid was maintained using the freshly collected egg masses of stinkbugs from the laboratory mass rearing. Basically, egg masses were exposed to 2-3 gravid female wasps for two days and incubated in glass tubes until the adult wasp emergence. Both male and female wasps were kept in the same tube subsequent to adult emergence to allow mating. A day before the experiment, the egg parasitoids were deprived of food to induce starvation, and only young mated female wasps (2-5 days old) without prior exposure to the odors of synthetic nectars were used in the olfaction bioassays.

The laboratory populations of stinkbugs were initially established from field-collected adults around the cultivated and uncultivated areas in Palermo, Italy. Regular introduction of new field-collected bugs was done to cope with the possible inbreeding within the colonies. The populations were maintained in insect cages (47.5 × 47.5 × 47.5 cm, BugDorm-44545 MegaView Science Co. Ltd, Taichung, Taiwan) inside a climate chamber (24 ± 2 °C, 70 ± 5% RH, and a L:D 16:8 photoperiod). Different instars and adult bugs were reared separately to manage the populations easily. Fresh vegetables (carrots, cabbages, tomatoes, and green beans) and sunflower/soybean seeds were supplied at all times and replenished every 2–3 days. Water dispensers with cotton pads were also provided in Petri dishes, and crumpled tissue papers were made available inside the adult cages to

serve as the substrate for oviposition. Egg masses were checked every other day and collected for the maintenance of both the stink bug and egg parasitoid colonies.

4.2.2. Inoculation and fermentation of synthetic nectar

To prepare the different filamentous fungus-fermented synthetic nectars, the mother culture of individual fungal strains was plated in PDA, followed by incubation of 7 days at 25°C. If the fungal growth was sufficient enough following a visual assessment, the plates were flooded with sterile distilled water to harvest the conidia. Then, the conidial suspensions were prepared, counted using a Thoma hemocytometer, and adjusted to the desired concentration (10^6 per ml). Afterward, 100 µl of fresh conidial suspension of the individual filamentous fungi was inoculated to every 20 ml of filter-sterilized synthetic nectar in sterile 50-ml falcon tubes. A mixture of 50% w/v sucrose (Carlo Erba Reagents S.A.S., Val-de-Reuil, France) added with 3.16 mM digested casamino acid (OmniPur, Merck KGaA, Darmstadt, Germany) was followed to produced synthetic nectar solutions (Vanette & Fukami, 2014), imitating the blend of natural sugar concentration found in buckwheat and several floral nectar (Cawoy et al., 2006). Control treatment was also prepared with synthetic nectar only without any fungal inoculation. Subsequently, all falcon tubes with synthetic nectar underwent fermentation in a rotary shaker at 25°C for five days – a duration that conforms to the approximate lifespan of several flower species (Lenaerts et al., 2016, 2017; Peay et al., 2012; Vannette & Fukami, 2014). In this study, two sets of fermented nectar products were prepared: one that has undergone filtration or removal of fungal bodies after the fermentation period to produce fungus-free fermented nectar and another set in which fungal bodies were retained in the nectar.

All fungus-fermented synthetic nectars from the latter set were vortexed to dislodge the fungal bodies and suspend evenly in the nectar solution. Then, these fermented nectars were used to investigate how the presence of fungal bodies in the nectar may directly affect the olfaction response of the egg parasitoid *T. basalis*. Also, a comparative study was conducted to establish whether the response behavior of the parasitoid was being affected by the presence of fungal bodies per se or just the indirect effect of the microbial fermentation alone (fungus-free nectar). Finally, all fungus-fermented synthetic nectars were aliquoted to 1-ml amber vials and stored at -80°C until further use. Such temperature is regarded as non-detrimental to the filamentous fungi (Kitamoto et al., 2002; Ito & Nakagiri, 1996), as confirmed by the fungal viability test. In addition, the freezing of samples is believed to have no adverse impact that might be incurred on the quality of the fermented nectar (Peay et al., 2012).

4.2.3. Olfactory response of *T. basalis* toward fungus-fermented synthetic nectar

To assess the behavior response of *T. basalis* to the volatiles emitted by the different fungus-fermented synthetic nectar, two olfaction bioassays were conducted: 1) the response of wasps on the fermented nectar with fungal bodies and uninoculated, unfermented synthetic nectar (control), and 2) the comparative response of the wasps on the fermented nectar with fungal bodies and fungus-free fermented nectar. These experiments

were done using a four-chamber static olfactometer (Steidle & Schoeller, 1997). Basically, the olfactometer device has a cylindrical body (4.5 × 20 cm) with four divisions called chambers by the two intersecting vertical plates, all made of acrylic glass. A walking arena made of plastic gauze (0.5 mm mesh) was also situated on the top of the four chambers, and a gauze cover (~0.5 mm mesh) supported by a glass rim (1.5 cm high) to confine the insects when released inside the arena. The chambers served as the enclosure to separate the odor sources, but two diagonally positioned chambers always had similar odor sources. For example, two chambers were assigned to the test odors (fermented nectar with fungal bodies), while the other two were either for the control odors (uninoculated, unfermented) or fungus-free fermented nectars. Two hundred µl of either nectar was pipetted to a sterile filter paper disc (3.8 cm diameter, Whatman No. 1) laid on the Petri plates and placed in their respective chambers. The individual starved female parasitoid that had been acclimatized in the room was then released in the middle of the walking arena and was given a minute to get accustomed to the olfactometer. Thereafter, the olfactory response of the wasps was observed and evaluated for five minutes using a video recording camera (Logitech C920 HD Pro Webcam), and the residence time spent by the wasp above each chamber was scored using an event recording program JWatcher V0.9 (https://www.jwatcher.ucla.edu/). Forty female parasitoids were used as independent replicates in each of the bioassays (total N = 480). Each parasitoid was used only once in the experiment, and the filter paper discs were replaced after one run. To lessen the probabilities of positional bias in the setup, regular rotation of the olfactometer was done after one replication. Bioassays were also carried out in a dark room on a rack covered with a blackout curtain to eliminate possible visual biases from the lights other than the one coming from the white fluorescent lamp (Philips, TLD 58 W/640) attached just above the static four-chamber olfactometer. Regular cleaning of the olfactometer was done with 90% ethyl alcohol and tap water every day. Afterward, the device was left overnight in the room to dry up. The experiments were carried out during the day between 09h00 and 17h00 at 21 ± 5 °C and 50 ± 10% RH.

4.2.4. Statistical analysis

The data on the olfaction bioassays were analyzed using R software version 4.4.0 (R Core Team 2024). Preceding the hypothesis testing, the residence time spent by the parasitoids above the chambers of the olfactometer underwent a test of the assumption of normality (Shapiro–Wilk normality test). Analyses revealed a p-value > 0.05, indicative of no violation of the assumption of normality, which was thus analyzed with a parametric test using the t-test for the independent samples.

4.3. Results

4.3.1. Olfactory response of *T. basalis* toward fungus-fermented synthetic nectar

4.3.1.1. Response of wasps on the fermented nectar with fungal bodies and unfermented synthetic nectar

T. basalis females only preferred the odors emitted by fermented synthetic nectar with fungal bodies of *Cladosporium* sp. SAAF 22.2.11 to unfermented synthetic nectar (t = − 2.462, df = 39, p = 0.0183). However, no differences in terms of residence time were found when wasps were given a choice between the unfermented synthetic nectar (control) and fermented synthetic nectar with fungal bodies of either *Cladosporium* sp. SAAF 22.2.12 (t = −1.573, df = 39, p = 0.124), *Cladosporium* sp. SAAF 22.3.29 (t = −1.205, df = 39, p = 0.236), *Aspergillus* sp. SAAF 22.3.26 (t = 0.261, df = 39, p = 0.795), *Aspergillus* sp. SAAF 22.4.40 (t = 0.174, df = 39, p = 0.863) or *Stachybotrys* sp. SAAF 22.1.15 (t = − 0.655, df = 39, p = 0.516) (Figure 9).

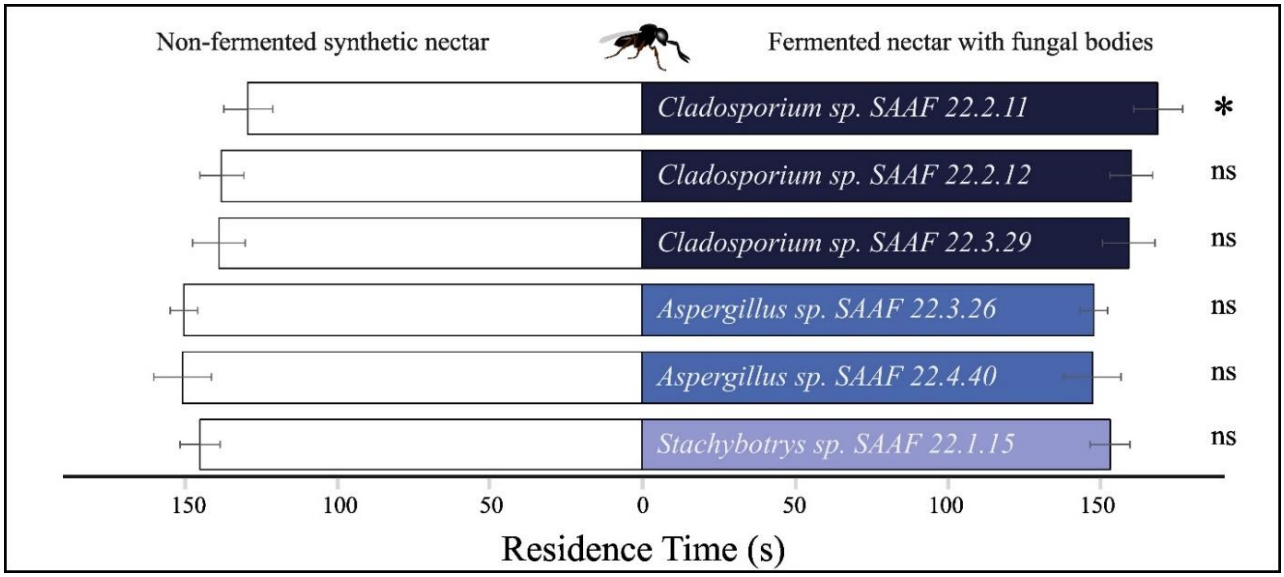

Figure 9. Olfactory responses of the female egg parasitoid *Trissolcus basalis* in terms of residence time in two-choice assays over an observation time of 300 s. Test treatments represent fermented synthetic nectars with fungal bodies of the filamentous fungi *Cladosporium* spp. SAAF 22.2.11, SAAF 22.2.12, SAAF 22.3.29, *Aspergillus* spp. SAAF 22.3.26 and SAAF 22.4.40 and *Stachybotrys* sp. SAAF 22.1.15 (colored bars). Non-fermented, uninoculated synthetic nectar was used in all the pairwise comparisons as a control (white bars). Each treatment was replicated 40 times (paired t-tests for dependent samples, *p < 0.05; ns = not significant). Error bars represent SE.

4.3.1.2. Comparative response of the wasps on the fermented nectar with and without fungal bodies

T. basalis females showed a preference for odors emitted by two fungus-free fermented synthetic nectar over its counterpart fermented synthetic nectar with fungal bodies, especially the ones fermented by *Aspergillus* sp. SAAF 22.3.26 (t = 2.680, df = 39, p = 0.011) and *Stachybotrys* sp. SAAF 22.1.15 (t = 2.029, df = 39, p = 0.049). On the other hand, no preferences were displayed between fungus-free fermented nectar and its

counterpart fermented nectar with fungal bodies of either *Cladosporium* sp. SAAF 22.2.11 ($t = 0.71971$, df = 39, $p = 0.476$), *Cladosporium* sp. SAAF 22.2.12 ($t = -0.662$, df = 39, $p = 0.512$), *Cladosporium* sp. SAAF 22.3.29 ($t = -1.384$, df = 39, $p = 0.174$) or *Aspergillus* sp. SAAF 22.4.40 ($t = 0.591$, df = 39, $p = 0.558$) (Figure 10).

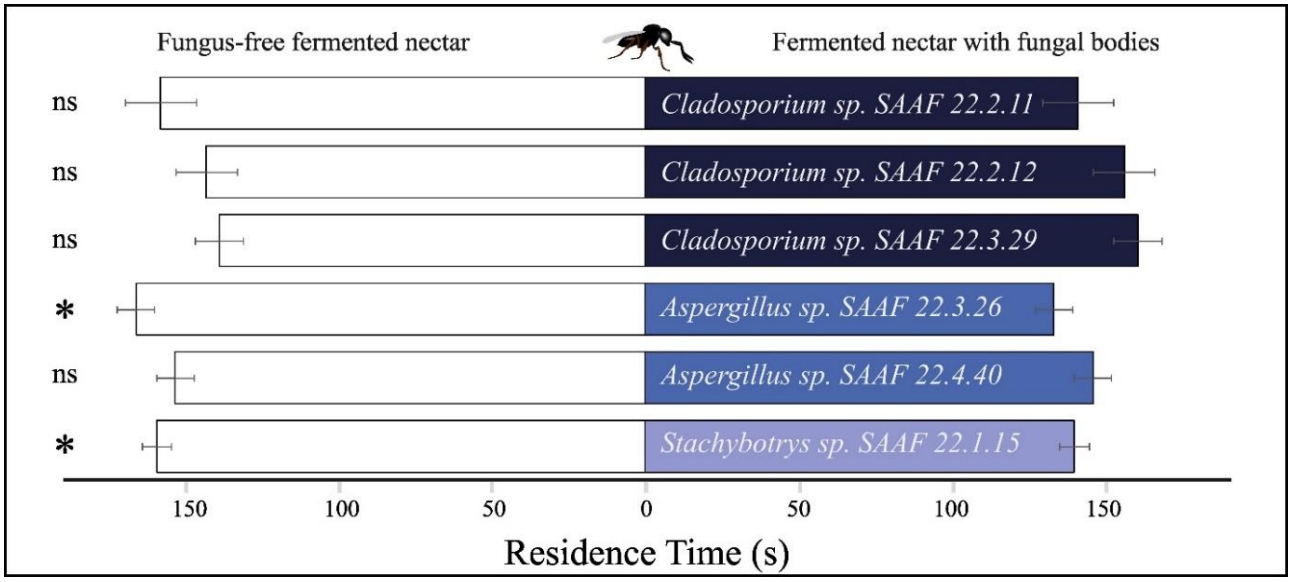

Figure 10. Olfactory responses of the female egg parasitoid *Trissolcus basalis* in terms of residence time in two-choice assays over an observation time of 300 s. Test treatments represent fermented synthetic nectars with fungal bodies of the filamentous fungi *Cladosporium* spp. SAAF 22.2.11, SAAF 22.2.12, SAAF 22.3.29, *Aspergillus* spp. SAAF 22.3.26 and SAAF 22.4.40 and *Stachybotrys* sp. SAAF 22.1.15 (colored bars). The counterpart fungus-free fermented synthetic nectar of each test treatment was used in its respective pairwise comparisons as a control (white bars). Each treatment was replicated 40 times (paired t-tests for dependent samples, $*p < 0.05$; ns = not significant). Error bars represent SE.

4.4. Discussion

In this study, we investigated the direct effect of nectar-inhabiting filamentous fungi isolated from buckwheat nectar on the olfaction behavior of a stink bug egg parasitoid, *Trissolcus basalis*. The direct effect is described as the direct role played by the fungal bodies themselves, in addition to the fermentation, on the insect foraging behavior. Results showed that out of the six filamentous fungi, *T. basalis* displayed preference behavior only toward *Cladosporium* sp. SAAF 22.2.11 in contrast with the non-inoculated control nectar in two-choice olfactometer bioassay. Interestingly, when wasps were exposed simultaneously on both fermented nectars with and without filamentous fungi, fungus-free nectar fermented by *Aspergillus* sp. SAAF 22.3.26 and *Stachybotrys* SAAF 22.1.15 were preferred by wasps over their counterpart nectars with fungal bodies. So far, this has not been investigated yet elsewhere and our study is the first to demonstrate the direct effect of a highly neglected microbial guild in the nectar environment, the filamentous fungi, on egg parasitoids.

Our results corroborate with the other findings that, indeed, nectar-inhabiting microbes influence the foraging behavior of floral visitors, including the parasitoids of insect pests. While no particular studies have been done yet about nectar microbial ecology pertaining to filamentous fungi, few reports have already shown that yeast and bacteria influence parasitoid foraging behaviors via nectar fermentation. For example, nectars fermented by *Metschnikowia gruessii* and *M. reukaufii* were significantly attractive to the adult parasitoid of aphids, *Aphidius ervi* (Sobhy et al., 2018). In another experiment, this aphid parasitoid could significantly recall the volatiles previously perceived from *M. reukaufii* even after 24 hours and may learn to associate them with the different yeast odors (Sobhy et al., 2019). Also, it was found that *M. gruessii* produced nectar highly attractive to stink bug egg parasitoids, *T. basalis* and *Ooencyrtus telenomicida* (Ermio et al., 2024). On the other hand, bacterial isolates from buckwheat nectar were also found to alter nectar traits and shape the foraging behavior of egg parasitoid *T. basalis* (Cusumano et al., 2022).

However, in previous studies, tests were carried out by using only cell-free fermented nectar without taking into account the possible direct role the microbial bodies themselves may play in the foraging behavior of insects. Microbial bodies, in general, could influence several fitness-related traits of insects (Gibson & Hunter, 2010; Stefanini, 2018), and thus, we can safely assume that it has a crucial impact on the preference behavior of insects, especially when the latter encounter microbes in nature. For example, it was found that the presence of live yeast *Candida bombiphila* in nectar was highly preferred by bumblebees in contrast with the treatment containing the inactivated yeast cells, which was reflected in the frequent insect visitations of live yeast-containing flowers (Pozo et al., 2020). A similar trend was also observed between nectars with or without inactivated yeast cells, where bumblebees showed a significant preference for the former (Pozo et al., 2020). Apparently, it was not the case in *Cladosporium* sp. SAAF 22.2.11 since it was consistently attractive to the egg parasitoids irrespective of the presence or absence of microbial bodies in its fermented nectar (see also Chapter 3). Nevertheless, in addition to the volatile by-products of microbes in nectar, insects may also exploit the presence of microbes themselves as honest signals in searching for available nectar sources. With that being said, it is necessary to consider the microbial bodies in investigating the flowering plant-insect interactions as

mediated by nectar microbes in future studies. Anyhow, some have also demonstrated the direct effects of nectar microbes on the foraging behavior of insects; however, those studies were unfortunately only limited to the general pollinators (Rering et al., 2018, 2020; Schaeffer et al., 2014, 2017). Considering that parasitoids are among the community of flower visitors, our findings are additional to the emerging consensus that microbial bodies of nectar-inhabiting microorganisms influence insect behavior.

In the second experiment, where we assessed wasp response when exposed to both fermented nectars with or without filamentous fungi, it was observed that wasps generally preferred the latter. This can be seen in the response of insects to the *Aspergillus* sp. SAAF 22.3.26 and *Stachybotrys* sp. SAAF 22.1.15, by which the presence of microbial bodies repelled them. This particular scenario is contrary to our previous expectations. However, we can speculate that the presence and abundance of microbial bodies in the nectar might influence the response of wasps (Pozo et al., 2020). In a study conducted with bumblebees, Junker et al. (2014) found insects were repelled by the different nectar-inhabiting bacterial strains, where a dose-dependent response was observed to at least one strain. This suggests that *T. basalis* could probably detect a certain abundance of microbial bodies and avoid nectar that is heavily colonized. However, this hypothesis is fairly speculative and has to be tested in the future. Apparently, we did not observe in our previous studies (*i.e.*, Chapter 3 and the first experiment of this chapter) any attraction nor repellence of wasps toward nectar fermented by these fungal strains, regardless of the presence of microbial bodies when compared to the control. However, in this particular experiment, wasps were exposed to totally different choices in the olfactometer. Thus, we cannot expect that the wasps would exhibit responses parallel to the previous studies. At any rate, our results suggest that parasitic wasps, similar to other insects, exhibit divergent species-specific responses toward nectar microbes and it is highly dependent on the choices of stimuli being offered at the same time.

The mechanism responsible for the positive behavior of egg parasitoids toward the fungus-fermented nectar is rather not well understood. However, studies have highlighted the role of volatile organic compounds (VOCs) emitted by nectar-dwelling microbes in parasitoid foraging behaviors (Cusumano et al., 2022; Ermio et al., 2024; Sobhy et al., 2018). Although it is unknown which volatiles produced by filamentous fungi themselves could influence parasitoid behavior, the previous chapter has analyzed the volatile profile of the fermented nectar but only those that underwent removal of the fungal bodies after fermentation. Based on the chemical analyses, seven VOCs namely, the phenol, 3,4-dimethyl; 2,4-dimethyl glutarate; α-terpineol; hydroxyacetophenone; 2,6-di-tert-butyl-p-benzoquinone; methyl benzoate; and 2-methyl benzaldehyde are suggested that may have contributed to explaining the differences in the olfaction of wasps observed among treatments. While these compounds could be possibly similar to the VOCs that the microbes themselves emit, we can speculate that there could be a variation of some compounds in terms of the concentrations. Nonetheless, further volatile analyses of the odor that microbes released, which may have influenced egg parasitoid attraction toward the nectar fungi and its fermented nectar, are highly warranted.

In summary, our results demonstrated that aside from the fermentation by-products of nectar-inhabiting filamentous fungi, fungal bodies themselves have influenced the olfaction behavior of the egg parasitoid. However, studies involving the filamentous fungi in nectar and their possible impact on parasitoids have not been reported elsewhere, and thus, there are a few ecological aspects related to these microbes that have to be further investigated. For example, it has to be known what impact these microbes can have on the nectar qualities and, consequently, on the life history parameters of the nectar feeders, including the parasitoids. In addition, it would be of great importance to assess how feeding live filamentous fungi may affect the performance of insects, like adult longevity, survival and even fecundity. Likewise, most research that has been done so far, including this work, only investigated the impact of microbial monoculture without taking into account the microbial assemblages that are naturally found in floral nectar. Microbes of different identities and densities may coexist in nectar that might have a collaborative impact on the nectar scent and quality, thereby shaping insect foraging behavior (Rering et al., 2020). Finally, field validation with flowering plants must be done in the future since current laboratory studies were carried out in a controlled environment, and results may not be ecologically feasible. Overall, nectar-inhabiting filamentous fungi could be an additional tool to consider in planning CBC programs targeting the nectar reward offered by the flowering plants by making it more attractive to the parasitoids of insect pests

4.5. References

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Chapter 5

Consequence of nectar-inhabiting filamentous fungi on the performance of stink bug egg parasitoids

Abstract

Flowering plants are rich in nectar, which attracts and supports parasitoids of insect pests in agroecosystems. Nectar is known to harbor microorganisms of various species, with yeasts and bacteria being the most prevalent. While reports are inconsistent, the presence of microbes in nectar has implications for the nectar quality and, thus, poses cascading effects on the community of nectarivorous insects. However, among the reported nectar-inhabiting microbes, filamentous fungi have been neglected in the tri-trophic interactions between flowering plants and flower visitors, like the parasitoids. Here, the isolated nectar filamentous fungi from buckwheat (*Fagopyrum esculentum*) were used to test the hypothesis that fungi affect the longevity and survival of the stink bug egg parasitoids *Trissolcus basalis* and *Ooencyrtus telenomicida* via nectar fermentation. Performance bioassay results showed that no significant microbe-mediated effect was found in terms of the adult longevity of two wasp species. While the survival rates of *O. telenomicida* were not affected by the nectar filamentous fungi, *T. basalis* that fed on nectar fermented by *Cladosporium* sp. SAAF 22.2.12 and *Cladosporium* sp. SAAF 22.3.29 had reduced survival rates in contrast with those that fed on non-fermented nectar. Since buckwheat has been widely used as a component of conservation biological control, our findings are thus significant contributions to the advancement of this strategy for effective pest management by taking into account the role of untapped nectar microbes in the complex flowering plant-parasitoid interactions.

Keywords: *Trissolcus basalis* • *Ooencyrtus telenomicida* • *Cladosporium* • *Fagopyrum esculentum* • Filamentous fungi • Longevity • Survival

5.1. Introduction

In conventional farming systems, vast land areas are regularly planted to a single or a couple of crop varieties in successive cropping seasons. This farming approach often led to scarcity of food resources and refuge for the beneficial insects (Sentil et al., 2022; van Rijn & Wäckers, 2016). These resources become a limiting factor that puts selective pressure on the biodiversity of beneficial insects, including the resident natural enemies, driving them away from the agricultural landscapes. Natural enemies are important components of agroecosystems because of their role in pest regulations. To counteract the possible loss of the community of natural enemies, conservation measures have to be put in place. For instance, plant biodiversity has to be structured in such a way that it preserves and promotes more natural enemies of crop pests (Landis et al., 2000). This strategy is under the principle of conservation biological control (CBC), wherein the agroecosystem is manipulated to favor natural enemies (Landis et al., 2000; Shields et al., 2019). As a practice, select flowering plants are planted around the field to act as a sink of resources like food and shelter for the resident natural enemies (Foti et al., 2017; Kowalska et al., 2022). Hymenopteran parasitoids are among those natural enemies that require abundant calories to fuel their energy-demanding tasks like foraging, flight, host location and reproduction (Araj et al., 2011; Cusumano & Lievens, 2023; Nafziger & Fadamiro, 2011; Takasu & Lewis, 1995). Floral nectar, a flower-based sugary secretion, is the primary source of nutrition for most hymenopteran adult parasitoids because it is compact with sugar and amino acids, as well as lipids, vitamins, and essential minerals, among other compounds that could support insect fitness (Jervis et al., 1993; Lievens et al., 2015). This floral resource is a key factor that mediates the mutualistic interactions between flowering plants and parasitoids (Parachnowitsch et al., 2019; Schaeffer et al., 2017).

Floral nectar qualities may vary among the flowers of conspecifics or from plant to plant within the community (Chalcoff et al., 2006; Herrera et al., 2006). The variation among floral nectar has become more apparent because studies have also shown that it is colonized by different microbial species that can tolerate the challenging nature of the nectar and increase their population density over time, thereby influencing nectar quality (Christensen et al., 2021; de Vega et al., 2009; Fridman et al., 2012; Herrera et al., 2009; Vannette, 2020). Bacteria in the Phyla Actinobacteria, Firmicutes and Proteobacteria are often found to be associated with the nectar of various flowering plant species (Álvarez-Pérez et al., 2012; Cusumano et al., 2022; Fridman et al., 2012; Vannette et al., 2021; von Arx et al., 2019). Moreover, specialist yeasts under the *Metschnikowia* clade, along with other generalist yeasts, are frequently described in several floral nectars across the globe. For example, *Metschnikowia* spp., among other yeasts, were found to be associated with the floral nectar of Mediterranean plants in Spain and various nectar environments of bee-pollinated flowers in Belgium and Germany (Álvarez-Pérez & Herrera, 2013; Bartlewicz et al., 2016; Brysch-Herzberg, 2004; Jacquemyn et al., 2013; Pozo et al., 2011). Furthermore, several microbiological surveys were conducted in other parts of the world, where they also reported the presence of different yeast species associated with floral nectar, such as in Asia (Tsuji & Fukami, 2018; Yang et al., 2019), Africa (de Vega et al., 2009) and North America (Belisle et al., 2012; Canto et al., 2017; Dhami et al., 2018; Klaps et al., 2020; Schaeffer et al., 2014, 2015; Vannette et al., 2021; Vannette & Fukami, 2017). To date, a microbiological survey of floral nectar that would look at the

presence of other microbial guilds is still unknown. However, reports have confirmed that filamentous fungi, a closely related microbial guild to yeasts, were found to inhabit flower tissues (Lupo et al., 2001; Tadych et al., 2012) as well as in nectar environments (Ehlers & Olesen, 1997; von Arx et al., 2019), suggesting that filamentous fungi, like bacteria and yeasts, should be investigated in the context of nectar microbial ecology. For instance, the previous chapter (Chapter 3) has identified a few filamentous fungi from the nectar of buckwheat flowers. Buckwheat is actually an important component of CBC programs because it recruits and supports parasitoids of insect pests in the agroecosystems (Gurr et al., 2017). Having knowledge of the microbiome of buckwheat nectar would mean that another factor should be considered in the nectar-mediated interaction between parasitoids and flowering plants, especially that microbes could alter nectar qualities. In fact, the presence of microbes in floral nectar involves catabolic activities and is linked to the alteration of nectar's physicochemical properties to a certain degree. For instance, microbes may affect the nectar volume and the component sugar or amino acid as well as their proportions in nectar (De Vega & Herrera, 2013; Herrera et al., 2008; Vannette et al., 2013; Vannette & Fukami, 2018). Moreover, because of the microbial by-products during fermentation, nectar acidity could be affected (Vannette et al., 2013) and can affect the occurrence of secondary plant metabolites (Vannette & Fukami, 2016). A study also demonstrated that dynamics in nectar temperature were directly dependent on microbial abundance in a perennial herb *Helleborus foetidus* (Herrera & Pozo, 2010). This alteration in nectar traits as affected by microbes could benefit nectar feeders since warmer nectar is characterized by low viscosity and, thus, facilitates easy ingestion (Nicolson et al., 2013). These changes in nectar quality brought about by nectar-inhabiting microbes have a greater implication on the performance of the nectar feeders, including the parasitoids. For instance, adult longevity of parasitoid *Aphidius ervi* had increased after feeding on *Lactococcus* sp.-fermented nectar, in contrast to those insects that fed on *Asaia* sp.-fermented nectar (Lenaerts et al., 2017). Another report also showed that *A. ervi* consumed less nectar fermented by generalist yeasts *Aureobasidium pullulan* and *Sporobolomyces roseus*, which had compromised adult longevity (Sobhy et al., 2018). However, other reports have also demonstrated that some nectar-inhabiting microbes did not affect insects' nectar intake and performance. For example, *M. reukaufii* and *Asaia astilbes* or even by their assemblages in nectar, did not affect the nectar intake of honeybees (Rering et al., 2020). A similar trend was also observed in bumblebees and egg parasitoids of stink bugs wherein nectar specialist yeasts *Metschnikowia* spp. did not affect pollen consumption and fecundity (Schaeffer et al., 2017) and adult longevity and survival rates (Ermio et al., 2024), respectively. However, drawing a generalization based on one or two nectar microbes or on one or a few insect species is practically impossible because impacts on a few would not always mean universally true. Thus, further research that would investigate the effect of other nectar microbes, like filamentous fungi, on other floral visitor species, like parasitoids, is highly warranted.

In this study, we tested the hypothesis that nectar-inhabiting filamentous fungi, similar to other microbes, affect the performance in terms of adult longevity and survival of nectar-feeding organisms. Here, the culturable nectar-inhabiting filamentous fungi from buckwheat nectar were used to ferment synthetic nectar and

underwent filtration after 5 days to produce fungus-free fermented synthetic nectar. Then, we conducted a no-choice feeding bioassay to investigate whether consuming fungus-fermented synthetic nectar impacts the longevity and survival of the two stink bug egg parasitoids, *Trissolcus basalis* and *Ooencyrtus telenomicida*. Both egg parasitoids are antagonists of the southern green stink bug *Nezara viridula*, which is considered an economically-important sucking insect pest of major crops all over the world. These egg parasitoids are dependent on floral nectar for their energy requirement and co-exist in areas in Southern Italy where their host has been documented to be a key pest of several crops (Colazza & Bin, 1995; Peri et al., 2014). Interestingly, they engage in intrinsic and extrinsic interspecific competition among their larvae and adults for possession of host resources (Cusumano et al., 2022; Peri et al., 2011). Moreover, *T. basalis* and *O. telenomicida* may require seemingly different nutrients because *O. telenomicida* exhibits concurrent host feeding in contrast with T. *basalis* (Cusumano et al. 2015). Moreover, *O. telenomicida* typically lays yolk-rich eggs; thus, it is predictable that it would require more nutrition in terms of protein than *T. basalis* would (Cusumano et al., 2015). Thus, parasitoids like *O. telenomicida* execute non-destructive host-feeding patterns before oviposition to augment their nutritional demands (Jervis & Kidd, 1986). Given their differences, we could hypothesize that these two stink bug egg parasitoids would have divergent responses in terms of performance toward fungus-fermented synthetic nectar.

5.2. Materials and Methods

5.2.1. Experimental organisms

Nectar filamentous fungi. The following nectar filamentous fungi used in this study were initially isolated from the floral nectar of buckwheat *Fagopyrum esculentum*: *Stachybotrys* sp. SAAF 22.1.15 (Ascomycota: Stachybotryaceae), *Cladosporium* spp. SAAF 22.2.11, SAAF 22.2.12, SAAF 22.3.29 (Ascomycota: Cladosporiaceae), *Aspergillus* spp. SAAF 22.3.26 and SAAF 22.4.40 (Ascomycota: Aspergillaceae). All these fungal isolates underwent a thorough confirmation as to their true association with floral nectar by conducting a series of growth trials using synthetic nectar with varying sucrose concentrations (15%, 30%, and 50%). Observations after 5-day fermentation revealed that the isolates had grown and thrived in the synthetic nectar irrespective of the sucrose concentrations, which was later confirmed by their viability in potato dextrose agar (PDA) after plating of the individual fungus-fermented synthetic nectar.

Egg parasitoids. The original colonies of egg parasitoids *Trissolcus basalis* Wollaston (Hymenoptera: Scelionidae) and *Ooencyrtus telenomicida* Vassiliev (Hymenoptera: Encyrtidae) were obtained from parasitized egg masses of southern green stinkbugs *Nezara viridula* L. (Hemiptera: Pentatomidae) that originated in fields with vegetation (with and without tomato) in Palermo, Italy (37°44'23'' N –13°08'27'' E). The populations of these two egg parasitoids were maintained inside a climate chamber (24 ± 2°C, 70 ± 5% RH, 16:8 L:D photoperiod) using fresh egg masses of stinkbugs in 55 ml glass tubes (25 × 150 mm) fed with drops of 80:20 (v/v) honey/water solution on parafilm. Initially, the stinkbug egg masses were exposed to five gravid females of either parasitoid for 48 h and incubated in glass tubes until emergence. Only drops of water were provided for the newly emerged parasitoids, and females that were 24 h old were used for the longevity and survival bioassays.

The stinkbug population was maintained in another climate chamber (24 ± 2°C, 70 ± 5% RH, 16:8 L:D photoperiod), reared inside net insect cages (47.5 × 47.5 × 47.5 cm, BugDorm-44545 MegaView Science Co. Ltd, Taichung, Taiwan). These stinkbugs were primarily established from field-collected adults around Palermo, Italy. Adults and nymphs were separated into different cages or boxes to facilitate the handling and managing of various generations. Inside each insect cage or box, diets like fresh vegetables (carrots, cabbages, tomatoes, and green beans) were provided all the time and supplemented with sunflower and soybean seeds. Changing the diets with fresh ones was done regularly (every 2-3 days) to avoid the establishment of molds. To maintain the humidity, water dispensers with cotton pads on Petri plates were also placed inside the cages. Moreover, crumpled paper towels were scattered or hung in the adult cages to provide additional substrate for oviposition. Egg masses were checked every 2-3 days and collected for egg parasitoid maintenance and the establishment of new stinkbug generation.

5.2.2. Inoculation and fermentation of synthetic nectar

The production of synthetic nectars began with the preparation of synthetic nectar solutions by mixing filter-sterilized 50% (w/v) sucrose (Carlo Erba Reagents S.A.S., Val-de-Reuil, France) solution and 3.16 mM casamino acid (OmniPur, Merck KGaA, Darmstadt, Germany) as outlined by Vanette and Fukami (2014). This

concentration models several floral nectars found in nature, including buckwheat nectar (Cawoy et al., 2006). Synthetic nectar solutions were individually inoculated with the six filamentous fungi to produce fungus-fermented synthetic nectars. In brief, fungal strains from the mother cultures were plated on PDA and incubated for 7 days at 25°C. Then, the fungal conidia were harvested using sterile distilled water with the aid of a disposable sterile plastic wire loop. The resulting fungal suspension was subsequently filtered using sterile cloth gauze to eliminate the other fungal bodies and retain the conidia in the suspension. The number of conidia was counted using the Thoma hemocytometer to ensure the consistency of the conidial counts across the different fungal suspensions for nectar inoculation. Fresh 100 µL of 10^6 conidia per ml fungal suspension of each strain was inoculated to 20 ml of sterile synthetic nectar solution prepared in sterile Falcon tubes. After that, all tubes were incubated at 25°C for 5 days, which is the approximate floral lifespan of many plant species (Lenaerts et al., 2016, 2017; Peay et al., 2012; Vannette & Fukami, 2014). After the fermentation/incubation period, the fungus-fermented synthetic nectars were centrifuged, and the supernatants were filtered using 0.20-micron filters. To confirm that the synthetic nectars were fungus-free, streaking in PDA with filtered fungus-fermented synthetic nectars was carried out, and possible growth of fungi was observed for another 3 to 5 days. Finally, fungus-fermented synthetic nectars were aliquoted to 1-ml amber vials and directly stored in a -80°C freezer for further use.

5.2.3. Impact of fungus-fermented synthetic nectar on parasitoid longevity and survival

To determine whether the changes in synthetic nectar due to fungal fermentation affect egg parasitoid fitness, longevity and survival bioassays were conducted. Longevity is basically considered the measure of time that the adult egg parasitoid lived from emergence until its point of death. In contrast, the survival rate is regarded as the percentage of egg parasitoids that are still alive in a given period after being exposed to or fed on different fungus-fermented synthetic nectars. In this experiment, egg parasitoids were isolated individually in sterile 5-ml glass vials equipped with cotton plugs. Drops of fungus-fermented synthetic nectar were supplied to the parasitoids on parafilm and inserted inside the glass vials. This technique was done to ensure that contamination of the vials was contained while making sure that nectar was available at all times to the parasitoids by replenishing them regularly. A control group fed on non-fermented synthetic nectar was included in the experiment as the reference control. The bioassays were conducted in a climate chamber with conditions maintained at 24 ± 2°C, 70 ± 5% RH, 16 h:8 h L:D photoperiod. For each treatment, 15 mated female parasitoids were used. Dead insects were scored daily, and the experiments were concluded after all insects had died.

5.2.4. Statistical analysis

Longevity data were analyzed using Generalized Linear Models (GLMs), fitting a gamma error distribution and a reciprocal link function(Crawley, 2007) with the R software, version R 4.4.0 (R Core Team, 2024). Tukey's test was used for post hoc comparisons of means, given that means among different treatments were significantly different. The survival of the adult egg parasitoids fed on the different nectars was analyzed using

the Kaplan-Meier estimator with the log-rank test of the Benjamini-Hochberg procedure for pairwise comparisons. Consequently, survival probability curves were generated for both egg parasitoids.

5.3. Results

5.3.1. Impact of fungus-fermented nectars on parasitoid longevity and survival

By comparing the fungus-mediated effects of the different fermented synthetic nectars on the longevity of *T. basalis*, no statistical significance was found among the treatments ($F = 1.811$, df = 6,98 $p < 0.105$). Adult wasps that fed on nectars fermented by *Cladosporium* sp. SAAF 22.2.11, *Cladosporium* sp. SAAF 22.2.12, *Cladosporium* sp. SAAF 22.3.29, *Aspergillus* sp. SAAF 22.3.26, *Aspergillus* sp. SAAF 22.4.40, *Stachybotrys* sp. SAAF 22.1.15 and non-fermented synthetic nectar lived for 118.53 ± 2.89, 92.07 ± 7.69, 97.87 ± 5.49, 83.87 ± 10.57, 97.80 ± 8.25, 99.60 ± 7.62 and 106.40 ± 8.44 days, respectively (Figure 11A). In terms of the survival rates of *T. basalis*, wasps that fed on non-fermented synthetic nectar had comparable survivability with those that fed on *Cladosporium* sp. SAAF 22.2.11, *Aspergillus* sp. SAAF 22.3.26, *Aspergillus* sp. SAAF 22.4.40, *Stachybotrys* sp. SAAF 22.1.15. However, wasps that fed on *Cladosporium* sp. SAAF 22.2.12 and *Cladosporium* sp. SAAF 22.3.29 had reduced survival rates in contrast with those that fed on non-fermented nectar and *Cladosporium* sp. SAAF 22.2.11, but comparable with the other treatments ($\chi^2 = 26.7$, df = 6, $p < 0.0002$) (Figure 11C).

No significant differences were found in the longevity of *O. telenomicida* when fed on either individual fermented nectars or non-fermented nectar ($F = 0.836$, df = 6,98 $p < 0.545$). Adult females that fed on nectars fermented by *Cladosporium* sp. SAAF 22.2.11, *Cladosporium* sp. SAAF 22.2.12, *Cladosporium* sp. SAAF 22.3.29, *Aspergillus* sp. SAAF 22.3.26, *Aspergillus* sp. SAAF 22.4.40, *Stachybotrys* sp. SAAF 22.1.15 and non-fermented synthetic nectar lived for 72.27 ± 3.27, 73.47 ± 5.39, 75.07 ± 6.15, 75.00 ± 4.30, 77.87 ± 5.34, 78.07 ± 3.50 and 65.60 ± 4.39 days, respectively (Figure 11B). A similar trend of no significant differences was found for the survival curves of *O. telenomicida* ($\chi^2 = 10$, df = 6, $p < 0.12$) (Figure 11D).

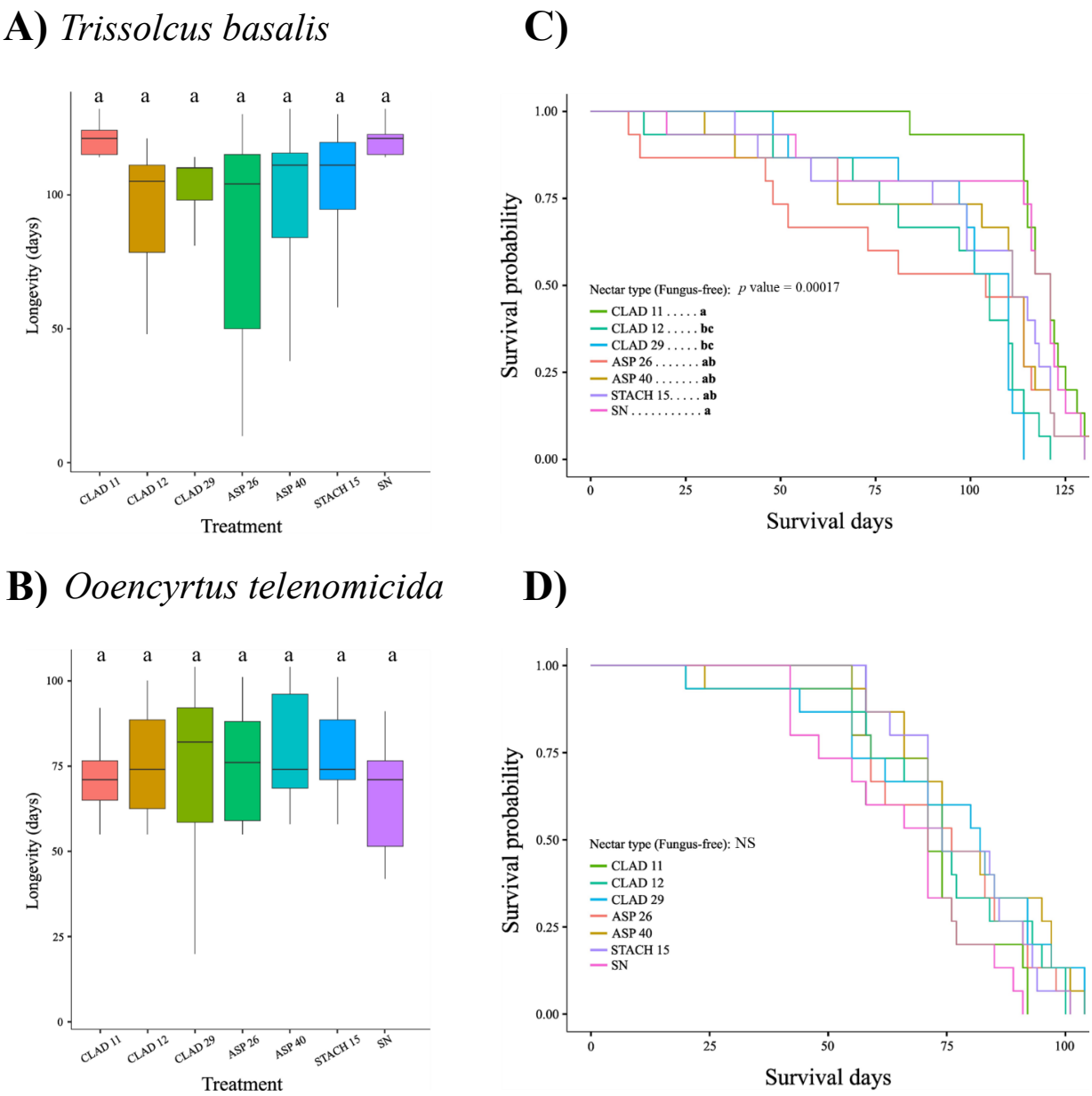

Figure 11. **A-B**) Median longevity and **C-D**) Kaplan–Meier survival curves for the adult female egg parasitoids *Trissolcus basalis* and *Ooencyrtus telenomicida* fed on: synthetic nectar fermented by CLAD11-*Cladosporium* sp. SAAF 22.2.11, CLAD12-*Cladosporium* sp. SAAF 22.2.12; CLAD29-*Cladosporium* sp. SAAF 22.3.29; ASP26-*Aspergillus* sp. SAAF 22.3.26; ASP40-*Aspergillus* sp. SAAF 22.3.29; STACH15-*Stachybotrys* sp. SAAF 22.1.15; and SN-non-fermented/uninoculated synthetic nectar (reference). Each treatment was replicated 15 times. Different letters in A-B indicate significant differences among treatments (GLM, $p < 0.05$). Bolded horizontal lines inside the box show medians, boxes contain the 25th, 50th percentiles, and whiskers that show the upper and lower quartiles. In C-D, different letters indicate significant differences among parasitoid survival curves according to the pairwise log-rank post hoc tests with the Benjamini-Hochberg correction (p <0.05); NS = not significant.

5.4. Discussion

In this study, we assessed the role played by nectar-inhabiting filamentous fungi found in buckwheat on the adult longevity and survival of two sink bug egg parasitoids, *Trissolcus basalis* and *Ooencyrtus telenomicida*. Data analyses revealed that the adult longevity of both egg parasitoid species was not affected by consuming nectars fermented by the six filamentous fungi. While there were no fungus-mediated effects in terms of the survival rates of *O. telenomicida* via nectar consumption, *T. basalis* had reduced survival rates when fed on nectar fermented by *Cladosporium* spp. SAAF 22.2.12 and SAAF 22.3.29 in contrast with those wasps fed on control nectar. This is, thus far, the first assessment of the effects of filamentous fungi found in buckwheat nectar on the performance of egg parasitoids.

So far, studies pertaining to nectar-inhabiting microbes that impact the performance of nectarivorous insects via changes in nectar qualities are entirely focused on the ascomycete yeasts and different bacterial species. While it has not been widely documented yet, we found that other than yeasts and bacteria, nectar-inhabiting filamentous fungi isolated from buckwheat flower could also shape the performance of nectarivorous insects, in this case, the egg parasitoids of stink bugs. Filamentous fungi have already been reported to colonize floral nectar (Ehlers & Olesen, 1997; von Arx et al., 2019), and thus, we can assume that these microbes may also play a role in influencing the appeal of nectar rewards that plants offer to a community of flower visitors. Generally, we did not find any significant fungal-mediated effects on the adult longevity and survival of the two wasp species, except that the survival rates of *T. basalis* fed on *Cladosporium* spp. SAAF 22.2.12 and 22.3.29 were reduced as compared with the control. Interestingly, our results corroborate with other findings that, indeed, nectar-inhabiting microbes would not totally affect the performance of insects. For instance, the bacterium *Rosenbergiella nectarea* yielded fermented nectar that did not affect the adult longevity of *Aphidius ervi* (Lenaerts et al., 2017). Likewise, *Metschnikowia gruessii* and *M. reukaufii* could produce nectar that was non-detrimental to the longevity and survival of egg parasitoids *T. basalis* and *O. telenomicida* and the adult aphid parasitoids *A. ervi* (Ermio et al., 2024; Sobhy et al., 2018). Also, while *M. reukaufii* affected the foraging behavior of *Bombus impatiens*, the performance in terms of the reproduction of bee workers was not affected (Schaeffer et al., 2017). Similarly, *B. terrestris* was not largely affected by the different yeast species tested except for the species-specific impact on the nest development (Pozo et al., 2020). On the other hand, there are also other reports that nectar microbes could negatively affect insect performance. For instance, an acetic acid bacterium, *Asaia platycodi*, was found to reduce the adult longevity of *A. ervi* (Lenaerts et al., 2017). Moreover, continuous consumption by this parasitoid on nectar fermented by yeasts like *Sporobolomyces roseus* and *Aureobasidium pullulans* resulted in a reduction in adult longevity and survival rates (Sobhy et al., 2018). However, microbes could also enhance the nectar quality, like in the case of the lactic acid bacterium *Lactococcus garvieae*, which could improve insect performance in terms of adult longevity (Lenaerts et al., 2017). This is particularly important from a biological control perspective because insects that have longer life spans would have more chances to reproduce and encounter their hosts in the field, thereby increasing their efficiency as natural enemies (Heimpel & Jervis, 2005).

Although the mechanism that drives the differentiated fungus-mediated effects on egg parasitoids of stink bugs is unknown, we can assume that alterations in nectar component sugar and amino acids and their proportions could be used to explain such results. For example, microbes have the ability to improve or degrade the nectar quality by means of increasing or decreasing the amounts of component amino acids (Lenaerts et al., 2017; Sobhy et al., 2018; Vannette & Fukami, 2018). These changes in amino acid concentrations in nectar may also play a role in how appealing this reward is to the nectarivorous insects, which consequently influences consumption (Petanidou et al., 2006). On the other hand, it could also be plausible to argue that the alterations in the components and composition of sugars in the nectar due to microbial metabolism may influence the performance of insects as microbes digest sucrose and produce varying levels of fructose and glucose (Lenaerts et al., 2017; Sobhy et al., 2018). Nevertheless, whether filamentous fungi could also cause variations in nectar chemistry is something that has to be investigated in the future by conducting appropriate metabolome analyses.

Egg parasitoids *T. basalis* and *O. telenomicida* have divergent nutrient needs since the latter require more protein to produce yolk-rich eggs (Cusumano et al., 2015), but unexpectedly, continuous consumption of fungus-fermented nectar resulted in their seemingly similar performances. These findings signify that nectar-inhabiting filamentous fungi may produce equitable rewards that could satisfy species-specific nutritional requirements among the community of egg parasitoids. However, we have to deal with our generalization with caution since several ecological aspects of these microbes must first undergo further investigation to support our assertion. One, we have only assessed the impact of filamentous fungi via nectar fermentation, without putting into consideration the microbial bodies themselves on the performance of egg parasitoids. In nature, insects may encounter or forage for microbes. These microbes, in one way or another, play an integral function in their general health, growth and development (Gibson & Hunter, 2010; Noda & Koizumi, 2003; Shen & Dowd, 1992). Thus, future studies should take a look at how feeding on microbial bodies by parasitoids would affect their performance. Second, since we only tested individual microbes on the nectar fermentation, it is, therefore, worth investigating the possible synergistic or additive effects of the assemblages of microbes on the nectar quality and performance of insects, which may mimic the natural scenario in nectar environments. Different microbes may coexist in nectar, which might have a concerted impact on the nectar odors and quality, thereby influencing insect response and performance (Rering et al., 2020). Third, aside from adult longevity and survival, it is also of great importance to assess the effect of feeding on fungus-fermented nectar on the other life history parameters of insects, like reproductive potential or fecundity. Lastly, the caveat of laboratory studies is that, more often than not, they contradict the field results. Thus, field validation with flowering plants inoculated by filamentous fungi must be carried out and evaluate how microbial inoculation affects the performance of the community of parasitoids.

Overall, we have demonstrated the impact of the nectar-inhabiting filamentous fungi on the adult longevity and survival rate of two egg parasitoids*, T. basalis* and *O. telenomicida,* through nectar fermentation. Evidence

suggests that, aside from yeasts and bacteria, filamentous fungi could also play a crucial role in the complex interactions between flowering plants and egg parasitoids. The fact that some of the filamentous fungi were attractive to both egg parasitoids (see Chapters 3 and 4) and did not cause deleterious effects on the insect performance is considerably important from a conservation biological control perspective. These microbes hold the potential to attract and facilitate parasitoids to locate excellent nectar sources through honest microbial signaling, thereby strengthening parasitoid fitness that may translate to effective pest regulations. Thus, in developing pest management programs, we should consider not only how a flowering plant attracts parasitoids into agricultural landscapes but also how likely it is that their nectar hosts beneficial microbes for better conservation biological control.

5.5. References

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General Conclusion

Taken altogether, our results support the general notion that a mere bipartite interaction between flowering plants and insects should transition into a more complex tripartite interaction mediated by nectar-inhabiting microbes (Colazza et al., 2023). We have demonstrated that nectar-inhabiting fungi, filamentous and yeasts, modify nectar traits by producing a unique bouquet of volatile organic compounds and, consequently, mediate interactions between flowering plants and flower visitors, such as the hymenopteran parasitoids. On the other hand, while there was a species-specific insect response toward the nectar fermented by filamentous fungi isolated from buckwheat, our results propose that this microbial guild, in addition to yeasts and bacteria, should not be neglected in investigating nectar microbial ecology, especially in its ecological relevance in plant-insect interactions. Apart from the fermentation products of nectar microbes, we also showed that microbial bodies themselves play a role in the attraction or repulsion of insects toward floral nectar. In that regard, our results further supported the general hypothesis that the nectar microbes that are dependent on insects as their dispersal agents enhance the volatile-mediated attraction toward the nectar. Moreover, we have shown that continuous consumption of microbe-fermented synthetic nectars, especially those that elicited positive preference behavior, did not affect insects' adult longevity and survival rates. This is highly important from a biological control perspective because we aim to augment the attraction of the parasitoids in the field through microbe-driven approaches without causing any unforeseen negative effect on their performance.

However, our results on how filamentous fungi mediated plant-insect interaction are only limited to egg parasitoids. It is, therefore, worth investigating how these microbes influence the foraging behavior of other members of flower visitors. Also, we have not yet looked at how microbe-mediated effects would have on other life history parameters of insects, like reproductive potential or fecundity, and so it merits further studies. On the other hand, in a natural nectar environment, different microbes could co-exist (Rering et al., 2020); thus, we have to examine the contributing factors of microbial assemblages on the nectar scent and quality and on the responses of insects. Microbes that live in an extreme environment are expected to show competition for resources, and this may cause total deviation of the nectar traits in contrast with only singular microbial inoculation. Finally, field validation with nectar-producing flowering plants inoculated with fungi should be considered in the near future, and assess how this scenario would affect the behavior and performance of the community of insects, both pests and parasitoids. It can be assumed that different species of insects with varying roles in ecosystem services could respond uniquely to the nectar-inhabiting microbes, and that would be an interesting area to be further investigated.

The framework of our research could have several applications in sustainable agricultural systems. First, microbial volatiles emitted by fungi hold the potential to be used as attractants in monitoring instruments for insect distribution in the field and pest control (Beck & Vannette, 2017). Although this has to be checked in future studies, this can be feasible for some reasons. For instance, semiochemical-baited traps are not uncommon in insect monitoring, and volatile signals from microbes could be used as an alternative means that

would enhance the attraction of the target species. Moreover, buckwheat has been used extensively in agriculture as a component of conservation biological control programs, which aim to attract and support parasitoids into the agricultural landscapes. Fungi inhabiting the floral nectar could be exploited as a supplementary tool in enhancing the attraction of flowering plants toward the parasitoids by targeting the floral nectar environments. This approach could provide honest microbial signaling that would facilitate parasitoids in locating nutritious nectar rewards, with positive consequences in their fitness and thereby increasing effectiveness in delivering pest regulation services. While flowering plants used in conservation biological control are generally selected in terms of attractiveness, anthesis, nectar accessibility and quality, criteria based on the probability of their nectar hosting beneficial, non-pathogenic microbes is rather uncommon. However, this strategy may provide an innovative tool for the improvement of the traditional conservation biological control programs, and thus, further studies are warranted.

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About the Author

Jay Darryl Labrador Ermio was born on the 20th of November 1994 in Bacolod City in the Province of Negros Occidental, Philippines, to Mr. Eduardo C. Ermio and Ma. Luz L. Ermio. He is the youngest among the five siblings in the family, and he grew up and lived in their humble abode in the middle of a rice field in the countryside of Purok Cabayabasan, Barangay Abuanan, Bago City, Philippines. Darryl finished his elementary at Abuanan Elementary School in Bago City in April 2007 and his high school at Cansilayan National High School (now Cansilayan Farm School) in Murcia in April 2011. After finishing high school, Darryl went to the Island Province of Leyte to pursue his bachelor's degree in Agriculture, majoring in Plant Protection with a specialization in Entomology at the Visayas State University with the support of the local scholarship (NOSP-Pagkaon), and finished his degree with flying colors in April of 2016. He worked on the entomopathogenic nematodes against the larvae of coconut rhinoceros beetle as his undergraduate thesis. During his senior year at the university, he was given the chance to study for six months at the University of Goettingen in Germany as an undergraduate exchange student under the Expert4Asia Project of the Erasmus Mundus. Subsequent to his graduation, Darryl worked as a science research assistant for a public-funded project on the management of queen pineapple pests under the supervision of Dr. Maria Juliet C. Ceniza of the National Coconut Research Center-Visayas, Visayas State University. Two years later, Darryl was accepted as one of the scholars of the Erasmus Mundus Joint Master Degree in Plant Health in Sustainable Cropping Systems and had the chance to study at the University of Goettingen, Germany, and Polytechnic University of Valencia, Spain, and went to the Crop Research Institute Prague in Czechia for his master's thesis on ecotoxicology of insecticides on bumblebees and springtails. In 2020, after finishing his master's degree, he went back to the Philippines to work as a junior lecturer at the Visayas State University, where he is currently connected. Fortunately, in November 2021, Darryl was given a chance to pursue his doctorate studies at the University of Palermo under the supervision of Dr. Stefano Colazza and Dr. Antonino Cusumano. He also spent some time in Dr. Michael Rostas's lab at the University of Goettingen as part of his research stay abroad. For his entire doctoral years, he focused on the role of nectar-inhabiting microbes, specifically yeast and filamentous fungi, for the biocon agents in the context of conservation biological control. As of this writing, Darryl is preparing to finish his doctorate by the end of the year of 2024. Finally, Darryl is looking forward to continuing his work on the chemical ecology of insects when he is back in the Philippines and building a laboratory that would cater research activities not only to the faculty members of his university but also to the students and all their stakeholders.