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ORIGINAL ARTICLE

Nectar-inhabiting bacteria differently affect the longevity of co-occurring egg parasitoid species by modifying nectar chemistry

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Abstract

Flowering plants can be introduced in modern agroecosystems to support resident natural enemies in the context of Conservation Biological Control (CBC). Buckwheat (*Fagopyrum esculentum*) (Polygonales: Polygonaceae) has been shown to enhance the longevity of several parasitoids through the provision of high quality and easily accessible floral nectar. Yet floral nectar is ubiquitously colonized by microbes which can change nectar chemistry with consequences for parasitoids. Nonetheless, how bacteria associated with buckwheat floral nectar affect parasitoid performance is not known. In this study, adult females of *Trissolcus basalis* (Hymenoptera: Scelionidae) and *Ooencyrtus telenomicida* (Hymenoptera: Encyrtidae), two parasitoids of *Nezara viridula* (Hemiptera: Pentatomidae), were provided with synthetic nectar fermented by 14 bacterial isolates originating from buckwheat nectar. We recorded the effect of bacterial fermentation on female longevity and nectar chemistry. In the case of *T. basalis*, females consuming nectar fermented by *Bacillus* sp., *Brevibacillus* sp., *Brevibacterium frigoritolerans*, *Saccharibacillus endophyticus*, and *Terribacillus saccharophilus* significantly enhanced their longevity compared with females fed with non-fermented nectar. For *O. telenomicida*, enhanced longevity was recorded only in the case of *B. frigoritolerans* and *Pantoea dispersa*. For both parasitoids, no negative effects due to bacterial fermentation of nectar were recorded. Chemical investigations of bacteria-fermented nectars revealed an increased diversity in the composition of sugars and sugar alcohols, whereas non-fermented nectar only contained sucrose. Our findings show that nectar-inhabiting bacteria are important "hidden players" in the interactions between flowers and parasitoids, an indication that a better understanding of plant–microbe–insect interactions could improve CBC programmes.

KEYWORDS
Bacillaceae, floral nectar bacteria, *Nezara viridula*, *Ooencyrtus telenomicida*, Paenibacillaceae, *Trissolcus basalis*

1 | INTRODUCTION

Due to agricultural intensification, modern agricultural landscapes are generally characterized by a paucity of plant species diversity often resulting in the reduction of ecosystem services to agriculture, such as pollination and natural pest control (Cadotte et al., 2011; Wood et al., 2015). To support resident beneficial arthropods, Conservation Biological Control (CBC) offers a suite of agronomic practices such as the diversification of plant species within agroecosystems, aimed to preserve and improve the efficacy of these beneficial organisms (Hunt et al., 2021; Mele et al., 2022; Pollier et al., 2019; van Rijn & Wäckers, 2010; van Rijn & Wäckers, 2016). In fact, resident populations of predators and parasitoids that attack economically important insect pests often require additional resources to maintain their populations within and around agricultural fields. One common strategy of CBC is the establishment of flowering plants, which provide shelter (Griffiths et al., 2008; Wyss, 1995) and food resources such as pollen, floral and extrafloral nectar (Lu et al., 2014; Wang et al., 2022) and plant guttation (Urbaneja-Bernat et al., 2020) to a wide range of predators and parasitoids. Interestingly, nectar and pollen have recently been identified as the main drivers of CBC success (Gurr et al., 2024). Nectar in particular is a high-quality and easily accessible sugar-rich solution indispensable for covering the energetic and nutritional requirements of natural enemies such as adult parasitoids (Russell, 2015).

Although floral nectar is initially sterile, it rapidly becomes colonized by microorganisms, which are dispersed by flower-visiting insects, air or rain (Colazza et al., 2023; Lenaerts et al., 2016). Commonly, nectar is inhabited by a limited number of phylogenetically related species (Herrera et al., 2010; Pozo et al., 2015), including ascomycetous yeasts such as *Metschnikowia*, as well as bacteria from the phyla Actinomycetota, Bacillota, and Pseudomonadota (Álvarez-Pérez et al., 2012; Pozo et al., 2015). Microbial fermentation can strongly affect the chemical properties of nectar, such as the composition and concentration of sugars and amino acids (Herrera et al., 2008; Lenaerts et al., 2017), as well as the pH (Lenaerts et al., 2017; Vannette et al., 2013). At the same time, microbes can change the concentrations of secondary metabolites (Vannette et al., 2013; Vannette & Fukami, 2016), hydrogen peroxide (Martin et al., 2022) and ethanol (Ehlers & Olesen, 1997; Rering et al., 2018).

These microbe-induced changes in nectar chemical properties can alter its nutritional value, and in turn modify the nectar reward of a given nectariferous flowering plant to foraging parasitoids. Little is known about the effects of nectar-associated microbes on parasitoids, but it is becoming increasingly evident that microbes can impact the performance of insect parasitoids (Cusumano & Lievens, 2023). Consequently, the role of microbes as "hidden players" in the interactions between flowering plants and parasitoids should not be ignored when implementing CBC programmes.

A few species among the vast majority of flowering plant taxa have dominated the literature with respect to their effects on parasitoid performance (Russell, 2015). One important flowering resource widely used in CBC is buckwheat *Fagopyrum esculentum* Moench due to its demonstrated benefits to parasitoids (Araj & Wratten, 2015; Foti et al., 2017, 2019; McIntosh et al., 2020; Rahat et al., 2005). In the floral nectar of buckwheat, several bacteria have been found that can affect nectar odours and, consequently, enhance the attractiveness of the nectar to parasitoid species such as *Trissolcus basalis* (Wollaston) (Cusumano et al., 2023). This species is an egg parasitoid that has been successfully used for biological control of the southern green stink bug, *Nezara viridula* L. (Caltagirone, 1981; Conti et al., 2021), a serious polyphagous pest of many annual crops. The eggs of this stink bug species can also be parasitized by other egg parasitoids, such as *Ooencyrtus telenomicida* (Vassiliev), which co-occurs with *T. basalis* in Sicilian agroecosystems (Peri et al., 2011).

These two parasitoid species have distinct nutritional requirements given that *O. telenomicida* performs host-feeding while *T. basalis* does not (Cusumano et al., 2012, 2022); thus, it is anticipated that nectar provisioning will exert varying effects on their life-history traits, such as longevity (Jervis et al., 2008). Parasitoid efficacy can often be limited by adult female longevity and it is known that extending the lifespan of adult parasitoids not only affects the timing and frequency of encounters between males and females, but also increases the probability of encountering appropriate host stages (Rosenheim, 2011). Lifespan extension is particularly important for egg parasitoids since host eggs are often inconspicuous and suitable for parasitism for a limited amount of time (Conti & Colazza, 2012; Vinson, 1998).

Although buckwheat has been widely demonstrated to increase parasitoid longevity, the effect of bacteria associated with buckwheat on nectar chemistry and parasitoid performance remains unexplored. To this end, synthetic nectar solutions were prepared and then undergone fermentation, by nectar-inhabiting bacteria isolated from buckwheat flowers. Subsequently, we conducted bioassays and chemical investigations with the aim to shed light on the effects of bacterial fermentation of the nectar on the performance of *T. basalis* and *O. telenomicida*. In particular, a no-choice feeding bioassay was carried out to examine the microbe-mediated effects on the longevity of adult parasitoid species. Also, a chemical analysis was performed to investigate changes in sugar/sugar-alcohols resulting from microbial metabolic activity in the attempt to link parasitoid performance with nectar chemistry.

2 | MATERIALS AND METHODS

2.1 | Parasitoid rearing

The colonies of *T. basalis* and *O. telenomicida* originated from parasitized sentinel *N. viridula* egg masses that had previously been placed in tomato fields in Palermo, Italy, where infestations of *N. viridula* had been recorded. Both parasitoids were kept in 16-mL glass tubes (70–100 wasps/tube) in a climate chamber (24 ± 2°C, 80 ± 5% RH, 16 h:8 h L:D) of the Department of Agricultural, Food and Forest Sciences (SAAF), University of Palermo. The colonies were provided with 50/50% honey–water solutions and, for their reproduction, mated females were exposed to *N. viridula* eggs, obtained from the stink bug

rearing in the department. Parasitoid females provided with only water were used in the experiments when they were about 24 h old.

2.2 | Synthetic nectar solutions

In this study, the following bacteria were used which were originally isolated from buckwheat nectar by Cusumano et al. (2023): phylum Bacillota – *Bacillus* sp. (*Bacillus* sp1) SAAF 22.2.6, *Bacillus* sp. (*Bacillus* sp2) SAAF 22.2.27, *Brevibacillus* sp. SAAF 22.4.13, *Brevibacterium frigoritolerans* SAAF 22.2.4, *Saccharibacillus* sp. SAAF 22.4.25, *Staphylococcus epidermidis* SAAF 22.3.11 and *Staphylococcus hominis* SAAF 22.3.10, *Terribacillus saccharophilus* SAAF 22.2.3; phylum Pseudomonadota—*Pantoea agglomerans* SAAF 22.4.2, *Pantoea dispersa* SAAF 22.3.3, *Pantoea* sp. (*Pantoea* sp1) SAAF 22.4.5, *Pantoea* sp. (*Pantoea* sp2) SAAF 22.4.17; phylum Actinomycetota—*Cellulosimicrobium* sp. SAAF 22.3.25, *Curtobacterium* sp. SAAF 22.4.18. The bacterial isolates were plated on trypticase soy agar (TSA) (Oxoid; Basingstoke, UK) and incubated for 2–3 days at 25°C. Then, 10 mL aliquots of trypticase soy broth (TSB) (Oxoid; Basingstoke, UK) were inoculated with a single bacterial colony from the TSA plates and kept on a rotary shaker of 150 rpm at 25°C for 1 day.

We prepared the synthetic nectar by mixing a filter-sterilized sucrose solution at a concentration of 50% w/v (Carlo Erba Reagents, Val-de-Reuil, France) with 3.16 mM casamino acid (OmniPur, Merck KGaA, Darmstadt, Germany), as described by Vannette and Fukami (2014). Next, the synthetic nectar was inoculated with individual bacterial isolates following the protocol described by Lenaerts et al. (2017).

To inoculate the nectar, 2 ml from the TSB cultures were centrifuged at 10,000 rpm for 5 min. Next, bacterial pellets were washed with sterile saline solution (0.9% NaCl) and resuspended in sterile saline solution to adjust the cell concentration to an optical density of 1 (OD 600) (about 10^8 cfu/ml). From the bacterial suspensions obtained, 100 µl was used to inoculate 10 ml of synthetic nectar in 20 ml Falcon tubes. In addition, synthetic nectar which was not inoculated with any bacterial isolate was used as control. Nectar fermentation took place for 5 days, during which both inoculated and non-inoculated synthetic nectar were incubated in static conditions at 25°C. The 5-day incubation period was selected since it represents the anthesis duration for several nectariferous flowering species (Lenaerts et al., 2016; Peay et al., 2012). At that time, bacterial densities ranged between 1×10^4 and 5.6×10^8 cfu/ml which is comparable with those observed in the field for other plant species (Fridman et al., 2012). After the fermentation, all nectar solutions were filtered (pore size 0.2 µm, Exacta + Optech Labcenter SpA, Italy) to prepare cell-free solutions. The absence of microbial contamination was confirmed by plating a subsample of each nectar solution on TSA. Lastly, aliquots of the nectar were prepared in glass amber vials of 2 ml and subsequently stored at −80°C (Peay et al., 2012) until use for bioassays and chemical analyses.

2.3 | Bacterial effects on parasitoid longevity

To assess the effect of bacterial fermentation on parasitoid longevity, a survival bioassay was carried out following the methodology described by Ermio et al. (2024). Briefly, females of *T. basalis* and *O. telenomicida* were isolated individually in a glass vial (volume 5 ml) which was closed with a cotton lid. At the side of the glass vial, for each treatment, a droplet of the test nectar was ad libitum deposited on a piece of parafilm© and replaced every 2 days using a sterile pin. All vials were kept in a climate chamber at 24 ± 2°C, 80 ± 5% RH, 16 h:8 h L:D. Parasitoids were inspected daily to record the total number of days each female stayed alive (longevity days). For both *T. basalis* and *O. telenomicida*, 15 adult females were used per each treatment, and 15 treatments (i.e., 14 bacterial isolates and the non-fermented synthetic nectar as control) were carried out, leading to a total of 225 tested females for each parasitoid species. Natural nectar was not included in the treatments mainly because our aim was to investigate the effects of *individual* bacterial isolates on nectar chemistry and parasitoid performance whereas natural nectar samples likely harbour microbial *communities*—which may consist of mixtures of bacteria and fungi—and which may differ in space, climatic conditions and time (Russell & McFrederick, 2022).

2.4 | Bacterial effects on nectar chemistry

To carry out sugar analyses, 200 µl of nectar samples from both non-fermented and fermented treatments, were aliquoted into 1.5 ml Eppendorf tubes, lyophilized, and subsequently weighed. For methoxyamination, 10 µl of methoxyamination reagent (prepared by dissolving 20 mg/ml of methoxyamine hydrochloride in pure pyridine) was added per milligram of nectar sample. The mixtures were vortexed until fully dissolved, subjected to 3 min of sonication, and then placed on a shaker for 90 min. For internal standardization, 20 µl of adonitol (20 ng/µl in methanol) was added to gas chromatography screw-capped glass vials containing a glass insert (200 µl). The internal standard was dried using a speed vac (RVC 2–25 CD plus, Germany) at 30°C for 300 min. Subsequently, an aliquot of 20 µl of the sample with methoxyamination reagent was transferred to a glass vial containing adonitol. Finally, 20 µl of N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) was added and the mixture was vortexed for 10 s. Samples were analysed using a gas chromatograph–mass spectrometer (Agilent Technologies, GC: 7890B, MSD: 5977B) fitted with a Restek Rtx-5 w/Integra-Guard column (30 m × 0.25 mm ID × 0.25 µm). An autosampler (Agilent Technologies, PAL RSI 85) was used to inject 1 µl of the sample with a split ratio of 0.1:1. The inlet temperature was 205°C and the pressure was 17.2 psi. The oven temperature was held at 70°C for 2 min and then increased gradually to a final temperature of 325°C, held for 10 min. Helium (1 ml min^{-1}) was used as the carrier gas. Due to the very high concentration of sucrose in the samples, the mass spectrometer was switched off at the retention time where sucrose appeared to avoid damage to the instrument. *n*-Alkane standards (C8–C40) were analysed for determining retention indices. Metabolite derivatives (sugars and

sugar alcohols) were identified by comparing their mass spectra and retention indices with data bank entries in the Golm Metabolome Database (GMD), and Fein BinBase database with the help of MS-DIAL (ver. 4.8) software. Metabolites were quantified relative to the peak area of the internal standard. Furthermore, the pH in the different nectars was determined with a pH electrode (WTW Inolab, Weilheim, Germany).

2.5 | Statistical analysis

Longevity data were analysed using Generalized Linear Models (GLMs), fitting a gamma error distribution and a reciprocal link function (Crawley, 2007). For each parasitoid species we fitted two different GLMs: in a first GLM we tested whether parasitoid longevity was significantly different when wasps were fed with nectars fermented by the three different phyla of bacterial isolates. To do so, we pooled together the results of all isolates within a given phylum (eight Bacillota isolates, four Pseudomonadota isolates and two ,Actinomycetota isolates). We hypothesized that isolates belonging to the same phylum would elicit similar effects in terms of parasitoid longevity given the phylogenetic relatedness of the strains. Thus, we expected to observe differences among the three phyla (Bacillota, Pseudomonadota and Actinomycetota). In a second GLM, we tested whether parasitoid longevity was significantly different between the 14 fermented nectars and the control, non-fermented synthetic nectar. For both GLMs, Fisher's LSD test was used for post hoc comparisons of means. Significance of the fixed terms in the model was determined directly with *F*-tests (Crawley, 2007). Model fit was assessed with diagnostic plots. All statistical analyses of longevity data were performed with R software version R 4.1.3 (R Core Team, 2022).

Projection to latent structures discriminant analysis (PLS-DA) was used to analyse peak areas of the sugar/sugar-alcohols detected after bacterial fermentation of the nectar media. In these analyses we excluded the control as it only contained sucrose. We carried out three separated PLS-DA, one where we compared all Bacillota isolates, one for the Pseudomonadota isolates and one for the Actinomycetota isolates. Peak areas were first log-transformed, mean-centred 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. The variable importance in the projection (VIP values), which indicates the relative ranking of the compounds contributing the most in explaining statistical differences in the PLS-DA, was also calculated (Wold, 2001).

3 | RESULTS

3.1 | Bacterial effects on parasitoid longevity

In the case of *T. basalis*, the effect of nectar fermentation among the three phyla (Bacillota, Pseudomonadota and Actinomycetota) was statistically significant (GLM: $F = 17.634$, df $= 2$, p <.001) (Figure 1a). Post-hoc comparisons revealed that females provided with nectar fermented by Bacillota and Actinomycetota lived significantly longer than those provided with nectar fermented by Pseudomonadota (p <.001), whereas no significant difference was found between Bacillota and Actinomycetota (Figure 1a). The effect of individual isolates on the longevity of the parasitoids was statistically significant (GLM: $F = 3.515$, df $= 14$, p <.001) (Figures 1b and S1). When compared with the control nectar, differences were observed (p <.05), with positive effects on the longevity of the parasitoids for the following isolates: *Bacillus* sp. SAAF 22.2.6 increased the longevity by 42.30%, *Brevibacillus* sp. SAAF 22.4.13 by 39.61%, *B. frigoritolerans* SAAF 22.2.4 by 37.5%, *S. endophyticus* SAAF 22.4.25 by 42.08% and *T. saccharophilus* SAAF 22.2.3 by 40.17%. Regardless of the bacteria species involved, longevity of *T. basalis* was never significantly decreased compared with *T. basalis* fed with control nectar.

In the case of *O. telenomicida*, there was no statistically significant effect of nectar fermentation among the three phyla (GLM: $F = 0.1735$, df $= 2$, $p = .8408$) (Figure 2a). However, the effect of individual isolates on the longevity of females was statistically significant (GLM: $F = 4.554$, df $= 14$, p <.001). When compared with the control nectar, differences were observed (p <.05) for *B. frigoritolerans* SAAF 22.2.4 and *P. dispersa* SAAF 22.3.3 (Figures 2b and S2), which increased female longevity by 26.88% and 24.88%, respectively. As for *T. basalis*, longevity of *O. telenomicida* was never significantly decreased by any of the bacteria species involved, when compared with females fed with control nectar.

3.2 | Bacterial effects on nectar chemistry

Analysis of sugars and sugar alcohols revealed that bacterial fermentation resulted in the de novo production of 14 metabolites compared with the non-fermented synthetic nectar, which consisted only of sucrose. Overall, all these compounds were detected in all nectars inoculated with the bacterial isolates, but in different proportions (Table 1). A comparison by PLS-DA among the eight isolates of bacteria within the Bacillota phylum resulted in a significant model (permutation test, p <.001) (Figure 3a). The first five compounds, ranked in descending order of VIP values, are: erythrose, sophorose, galactinol, fructose and 1-kestose. Within the Pseudomonadota phylum, a comparison by PLS-DA among the four bacterial isolates also resulted in a significant model (permutation test, p <.001) and the following compounds have the highest VIP values: fructose, tagatose, L-iditol, glucose and galactose (Figure 3b). Within the Actinomycetota phylum, a comparison by PLS-DA among the two isolates of bacteria resulted in a significant model (permutation test, p <.001) where the following five compounds have the highest VIP values: tagatose, raffinose, trehalose, galactose and fructose (Figure 3c). The pH of the bacteria-fermented nectar ranged from 5.21 (±0.03) in the case of *Bacillus* sp2 SAAF 22.2.27 to 6.62 (±0.03) in the case of *Brevibacillus* sp. SAAF 22.4.13 (Table 1).

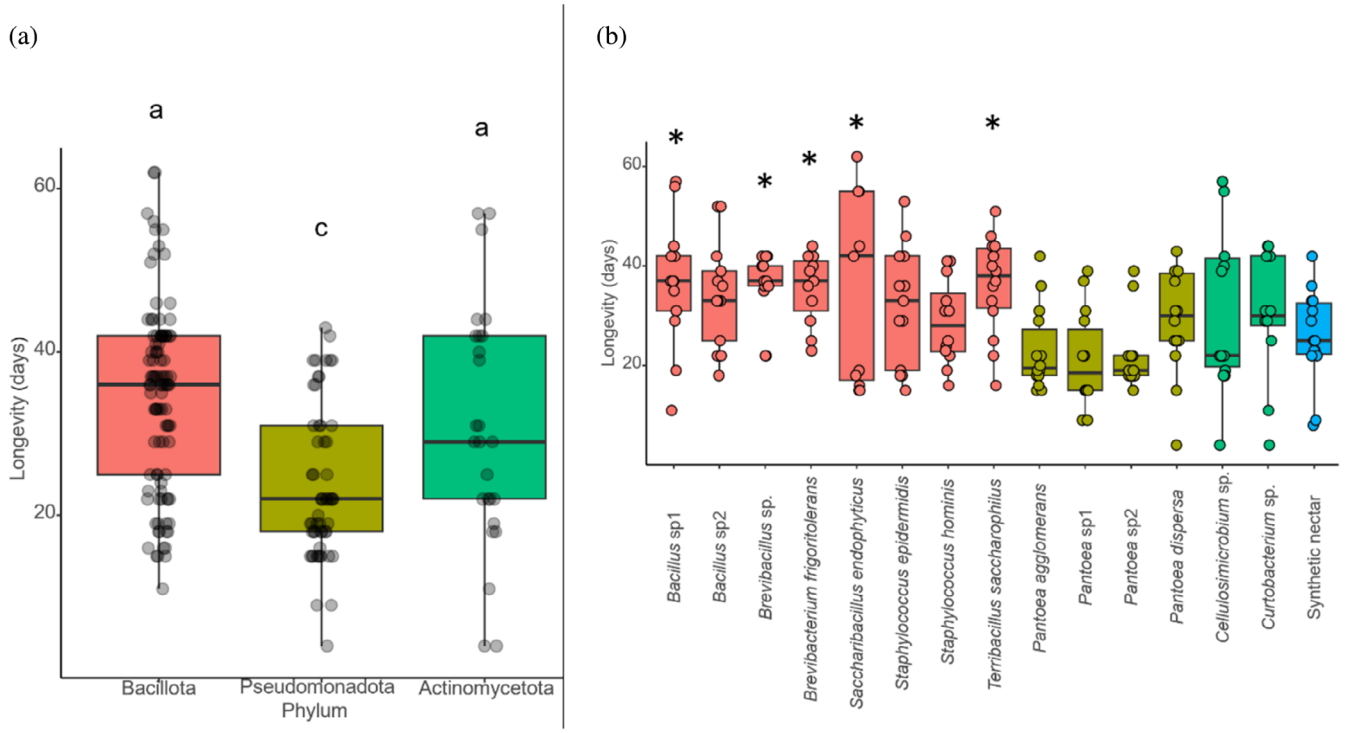

FIGURE 1 Boxplot illustrating the distribution of longevity (days) for the adult female egg parasitoid *Trissolcus basalis* fed on synthetic nectar fermented by the different bacterial isolates. The synthetic nectar was fermented by: for Bacillota—*Bacillus* sp1 SAAF 22.2.6, *Bacillus* sp2 SAAF 22.2.27, *Brevibacillus* sp. SAAF 22.4.13, *Brevibacterium frigoritolerans* SAAF 22.2.4, *Saccharibacillus endophyticus* SAAF 22.4.25, *Staphylococcus epidermidis* SAAF 22.3.11, *Staphylococcus hominis* SAAF 22.3.10, and *Terribacillus saccharophilus* SAAF 22.2.3; for Pseudomonadota—*Pantoea agglomerans* SAAF 22.4.2, *Pantoea dispersa* SAAF 22.3.3, *Pantoea* sp1 SAAF 22.4.5, and *Pantoea* sp2 SAAF 22.4.17; for Actinomycetota—*Cellulosimicrobium* sp. SAAF 22.3.25 and *Curtobacterium* sp. SAAF 22.4.18. (a) Comparison among the 3 phyla (different letters indicate significant differences, GLM, $p \leq .05$). (b) Comparison among the 14 bacterial isolates and the non-fermented synthetic nectar (asterisks indicate significant differences, GLM, $p \leq .05$). Pink = Bacillota, Olive green = Pseudomonadota, Green = Actinomycetota; Blue = Non-fermented synthetic nectar. All biological replicates ($n = 15$) indicate cell-free nectars.

4 | DISCUSSION

This study demonstrates that bacterial fermentation of nectar affects the longevity of two egg parasitoids of stink bugs. To our knowledge, this is the first study that assessed how parasitoid longevity can be affected by nectar-inhabiting bacteria in buckwheat, a flowering plant which is widely used in Conservation Biological Control (CBC) programmes.

We found that the longevity of *T. basalis* females feeding on certain fermented nectars was significantly prolonged by approximately 40% compared to non-fermented synthetic nectar. In particular, nectar fermented by the bacterial isolates *Bacillus* sp. SAAF 22.2.6, *Brevibacillus* sp. SAAF 22.4.13, *B. frigoritolerans* SAAF 22.2.4, *S. endophyticus* SAAF 22.4.25 and *T. saccharophilus* SAAF 22.2.3 had positive effects. This observed longevity is also higher compared to the mean longevity of females exposed to excised inflorescences of buckwheat (Rahat et al., 2005). Furthermore, we did not record any negative effect due to fermentation by nectar-inhabiting bacteria on the longevity of *T. basalis*. It is noteworthy that all bacteria that enhanced longevity of *T. basalis* belong to the phylum Bacillota, suggesting a specific effect elicited by these bacterial isolates on the positive fitness-related effects of *T. basalis*.

Positive effects due to bacterial fermentation of the nectar media were also found for *O. telenomicida* females, although enhanced longevity was recorded only in the case of *B. frigoritolerans* SAAF 22.2.4 and *P. dispersa* SAAF 22.3.3—by 26.88% and 24.88% respectively—when compared with non-fermented synthetic nectar. These isolates belong to different phyla (Bacillota and Pseudomonadota, respectively), indicating that non-related bacteria can enhance longevity in this parasitoid species.

The egg parasitoid species studied in this study have distinct nutritional requirements given that *O. telenomicida* performs host-feeding while *T. basalis* does not; thus, it is not surprising to observe divergent effects from different fermented nectars, although the fermentation by *B. frigoritolerans* SAAF 22.2.4 enhanced longevity in both stink bug egg parasitoids. To the best of our knowledge, the effects of nectar-inhabiting bacteria on parasitoids have only been studied in the aphid parasitoid *Aphidius ervi* (Haliday) where positive, negative or neutral effects have been reported (Lenaerts et al., 2017). Fermentation by *Lactococcus* sp. increased the longevity of *A. ervi*, whereas *Asaia* had a detrimental effect on the longevity of the aphid parasitoid (Lenaerts et al., 2017). The negative effects of the latter bacterium, which belongs to the group of acetic acid bacteria, could

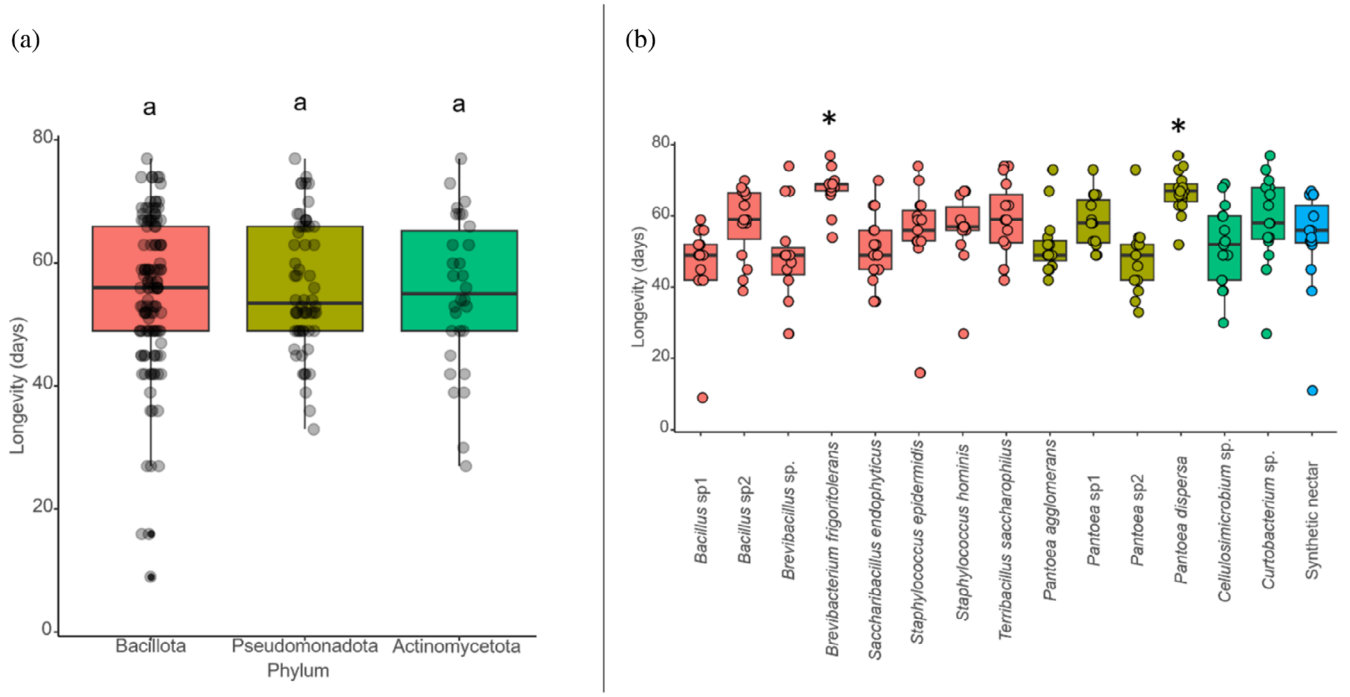

FIGURE 2 Boxplot illustrating the distribution of longevity (days) for the adult female egg parasitoid *Ooencyrtus telenomicida* fed on synthetic nectar fermented by the different bacterial isolates. The synthetic nectar was fermented by: for Bacillota—*Bacillus* sp1 SAAF 22.2.6, *Bacillus* sp2 SAAF 22.2.27, *Brevibacillus* sp. SAAF 22.4.13, *Brevibacterium frigoritolerans* SAAF 22.2.4, *Saccharibacillus endophyticus* SAAF 22.4.25, *Staphylococcus epidermidis* SAAF 22.3.11, *Staphylococcus hominis* SAAF 22.3.10, and *Terribacillus saccharophilus* SAAF 22.2.3; for Pseudomonadota—*Pantoea agglomerans* SAAF 22.4.2, *Pantoea dispersa* SAAF 22.3.3, *Pantoea* sp1 SAAF 22.4.5, and *Pantoea* sp2 SAAF 22.4.17; for Actinomycetota—*Cellulosimicrobium* sp. SAAF 22.3.25 and *Curtobacterium* sp. SAAF 22.4.18. (a) Comparison among the 3 phyla (different letters indicate significant differences, GLM, $p \leq .05$). (b) Comparison among the 14 bacterial isolates and the non-fermented synthetic nectar (asterisks indicate significant differences, GLM, $p \leq .05$). Pink = Bacillota, Olive green = Pseudomonadota, Green = Actinomycetota; Blue = Non-fermented synthetic nectar. Each treatment comprised of 15 replicates.

be due to the dramatic decrease in the pH which reduced the quality of the nectar for the parasitoid. In contrast, bacteria belonging to Bacillota are not known to cause such drastic reductions of pH in the fermented media (Schleifer, 2009), and our results confirm this finding. Other nectar-inhabiting microbes, such as yeasts, have been reported to affect attraction and performance of parasitoids, such as the aphid parasitoid *A. ervi* (Sobhy et al., 2018) and the egg parasitoids *T. basalis* and *O. telenomicida* (Ermio et al., 2024).

From a chemical perspective, we found that bacterial fermentation greatly influenced the sugar profile of the nectar. The non-fermented synthetic nectar contained only sucrose, whereas a total of 14 sugars and sugar alcohols were identified in the fermented products of all bacteria, although in different proportions. While previous studies focusing on nectar fermentation by microbes have mainly examined the dynamics between sucrose, glucose, and fructose (Lenaerts et al., 2017; Sobhy et al., 2018), we show here that bacterial fermentation of nectar results in a huge variety of sugars and sugar alcohols, providing a more complete picture of the effects of bacterial fermentation from a chemical perspective. It is reasonable to assume that the positive effects of the six bacterial isolates (five Bacillota and one Pseudomonadota) on the longevity of *T. basalis* and *O. telenomicida* are due to the diversity and abundance of these sugars and sugar alcohols, which may complement the nutritional needs of the parasitoids. Nevertheless, only a single strain of each bacterial species was tested in this study, so the effect of intra-specific microbial diversity on parasitoids' longevity should be explored in future studies. Also, the role of bacterial cells themselves on parasitoid performance should be investigated given that nectar, in nature, is ingested together with microbial cells (Cusumano & Lievens, 2023). In fact, microorganisms known to inhabit floral nectar, such as *Metschnikowia* yeasts, have been identified in internal organs of insect parasitoids under field conditions (Srinatha et al., 2015).

According to our PLS-DA model comparing the nectars fermented by Bacillota, the compounds most likely to correlate with parasitoid performance (based on the highest VIP values) were erythrose, sophorose and galactinol, fructose and 1-kestose. In addition, a PLS-DA model among the four nectars fermented by Pseudomonadota showed that the highest VIP values were found for fructose, tagatose, L-iditol, glucose and galactose. Hexoses (glucose and fructose) are usually encountered in high concentrations in the floral nectar of exposed nectaries (McIntosh et al., 2020). For instance, fructose accounts for 50% of the total sugars in buckwheat nectar (Lee & Heimpel, 2003). Although parasitoid species may vary in their ability to utilize different nectar sugars, glucose and fructose are known to

TABLE 1 Concentration (mean ± SE, $N = 4$) of sugar metabolites, expressed in ng/mg of dry weight of synthetic nectar fermented by the 14 distinct bacterial isolates.

RT (RI)	Chemical compounds	Phylum													
		Bacillota								Pseudomonadota				Actinomycetota	
		Ba.sp1	Ba.sp2	Br.sp	Br.fr	Sa.en	St.ep	St.ho	Te.sa	Pa.ag	Pa.di	Pa.sp1	Pa.sp2	Ce.sp	Cu.sp
21.62 (1430)	Erythrose	0.28 (±0.17)	1.11 (±0.95)	2.53 (±0.66)	1.36 (±0.44)	17.61 (±1.85)	3.37 (±0.71)	15.20 (±4.06)	6.21 (±2.65)	18.23 (±1.05)	22.38 (±6.54)	7.90 (±2.51)	34.68 (±6.37)	5.50 (±2.49)	5.05 (±2.19)
27.77 (1688)	Xylitol	2.44 (±1.24)	40.57 (±7.85)	5.17 (±2.59)	22.50 (±5.49)	6.34 (±1.15)	4.57 (±1.85)	10.18 (±2.83)	1.50 (±0.40)	2.46 (±0.74)	2.10 (±0.89)	13.09 (±8.03)	4.76 (±1.41)	5.41 (±1.87)	6.31 (±1.49)
30.22 (1800)	Tagatose	6.97 (±1.63)	10.69 (±3.24)	1.93 (±1.06)	9.85 (±2.66)	2.03 (±0.54)	2.05 (±0.78)	32.99 (±10.20)	4.02 (±1.83)	2.96 (±2.30)	0.23 (±0.08)	9.20 (±2.40)	194.20 (±39.85)	10.66 (±1.39)	144.32 (±7.67)
30.33 (1806)	Fructose	2.57 (±1.77)	80.34 (±9.75)	3.86 (±1.07)	1.08 (±1.01)	4.78 (±1.36)	6.22 (±3.30)	135.79 (±13.95)	4.42 (±1.50)	3.45 (±1.43)	2.01 (±0.56)	36.59 (±7.97)	555.87 (±68.96)	19.65 (±1.35)	59.94 (±10.91)
30.64 (1820)	Galactose	1.98 (±0.66)	128.04 (±44.90)	7.72 (±1.13)	3.30 (±1.48)	14.24 (±3.60)	43.26 (±10.33)	145.47 (±17.26)	3.06 (±0.81)	3.48 (±0.43)	4.92 (±2.22)	231.92 (±25.83)	333.12 (±110.96)	26.17 (±7.22)	194.46 (±32.60)
30.66 (1822)	Glucose	4.54 (±1.85)	9.65 (±2.67)	1.54 (±0.95)	5.99 (±2.60)	0.78 (±0.16)	3.31 (±2.06)	31.65 (±8.89)	1.50 (±0.92)	1.01 (±0.42)	0.29 (±0.12)	7.61 (±3.11)	28.68 (±4.62)	10.10 (±5.70)	8.98 (±1.41)
32.27 (1899)	L-Iditol	3.16 (±0.51)	26.47 (±7.87)	19.59 (±6.41)	28.22 (±14.96)	20.72 (±1.83)	49.26 (±8.11)	20.31 (±4.74)	5.68 (±1.84)	7.64 (±1.33)	7.20 (±1.83)	12.12 (±3.25)	43.01 (±11.12)	9.88 (±3.12)	20.79 (±1.62)
43.32 (2524)	Lactose	0.95 (±0.65)	1.50 (±1.50)	1.28 (±1.21)	2.04 (±2.04)	2.05 (±2.05)	3.26 (±3.26)	2.69 (±1.98)	2.18 (±2.18)	1.00 (±0.77)	2.73 (±1.58)	19.34 (±8.05)	6.03 (±3.05)	2.03 (±1.20)	5.76 (±5.71)
43.82 (2556)	Maltose	0.68 (±0.49)	3.23 (±2.69)	125.60 (±46.61)	2.58 (±1.52)	1.40 (±1.02)	1.48 (±0.94)	24.94 (±9.65)	19.66 (±16.36)	2.57 (±) 2.00	1.83 (±1.11)	49.92 (±17.51)	207.32 (±23.68)	39.97 (±24.18)	225.20 (±50.44)
44.65 (2610)	Trehalose	1.19 (±0.46)	1.25 (±0.86)	19.57 (±7.22)	1.11 (±0.64)	4.81 (±1.92)	5.27 (±0.95)	13.81 (±5.63)	3.56 (±1.67)	3.36 (±1.34)	6.94 (±5.44)	63.55 (±8.83)	386.95 (±20.64)	45.85 (±10.53)	209.06 (±20.67)
47.03 (2772)	Sophorose	4.42 (±0.55)	21.56 (±6.11)	45.27 (±14.14)	42.86 (±37.79)	364.76 (±68.52)	61.48 (±9.85)	23.46 (±9.68)	172.58 (±44.39)	25.28 (±4.40)	257.12 (±78.07)	14.48 (±3.38)	60.67 (±10.18)	56.70 (±20.19)	35.31 (±8.67)
48.50 (2877)	Galactinol	0.28 (±0.22)	1.06 (±0.89)	2.85 (±1.49)	0.41 (±0.15)	0.78 (±0.15)	0.93 (±0.28)	3.17 (±1.22)	0.41 (±0.10)	1.35 (±0.52)	0.74 (±0.33)	1.86 (±0.83)	7.32 (±0.93)	1.90 (±0.93)	2.85 (±0.91)
53.49 (3257)	Raffinose	0.63 (±0.20)	0.70 (±0.23)	3.72 (±1.85)	42.42 (±7.59)	0.84 (±0.44)	2.09 (±0.68)	5.61 (±1.52)	1.06 (±0.49)	2.75 (±1.05)	2.17 (±0.96)	3.83 (±1.46)	43.98 (±13.97)	0.29 (±0.05)	4.52 (±1.25)
55.48 (3374)	1-Kestose	3.73 (±1.68)	0.55 (±0.40)	0.96 (±0.51)	8.24 (±0.40)	0.46 (±0.12)	3.96 (±1.87)	0.96 (±0.32)	0.14 (±0.04)	0.54 (±0.20)	0.25 (±0.11)	0.41 (±0.09)	1.63 (±0.18)	1.02 (±0.57)	1.94 (±0.59)
pH		5.73 (±0.03)	5.69 (±0.01)	6.62 (±0.01)	6.57 (±0.02)	6.60 (±0.03)	5.89 (±0.01)	6.58 (±0.05)	5.64 (±0.04)	5.60 (±0.01)	5.21 (±0.03)	5.94 (±0.01)	5.88 (±0.01)	6.59 (±0.02)	6.33 (±0.02)

Note: For Bacillota—Ba.sp1 = *Bacillus* sp1 SAAF 22.2.6, Ba.sp2 = *Bacillus* sp2 SAAF 22.2.27, Br.sp. = *Brevibacillus* sp. SAAF 22.4.13, Br.fr = *Brevibacterium frigoritolerans* SAAF 22.2.4, Sa.en = *Saccharibacillus endophyticus* SAAF 22.4.25, St.ep = *Staphylococcus epidermidis* SAAF 22.3.11, St.ho = *Staphylococcus hominis* SAAF 22.3.10 and Te.sa = *Terribacillus saccharophilus* SAAF 22.2.3; for Pseudomonadota—Pa.ag = *Pantoea agglomerans* SAAF 22.4.2, Pa.di = *Pantoea dispersa* SAAF 22.3.3, Pa.sp1 = *Pantoea* sp1 SAAF 22.4.5, and Pa.sp2 = *Pantoea* sp2 SAAF 22.4.17; for Actinomycetota— Ce.sp. = *Cellulosimicrobium* sp. SAAF 22.3.25 and Cu.sp. = *Curtobacterium* sp. SAAF 22.4.18.
Abbreviations: RI, retention index; RT, retention time.

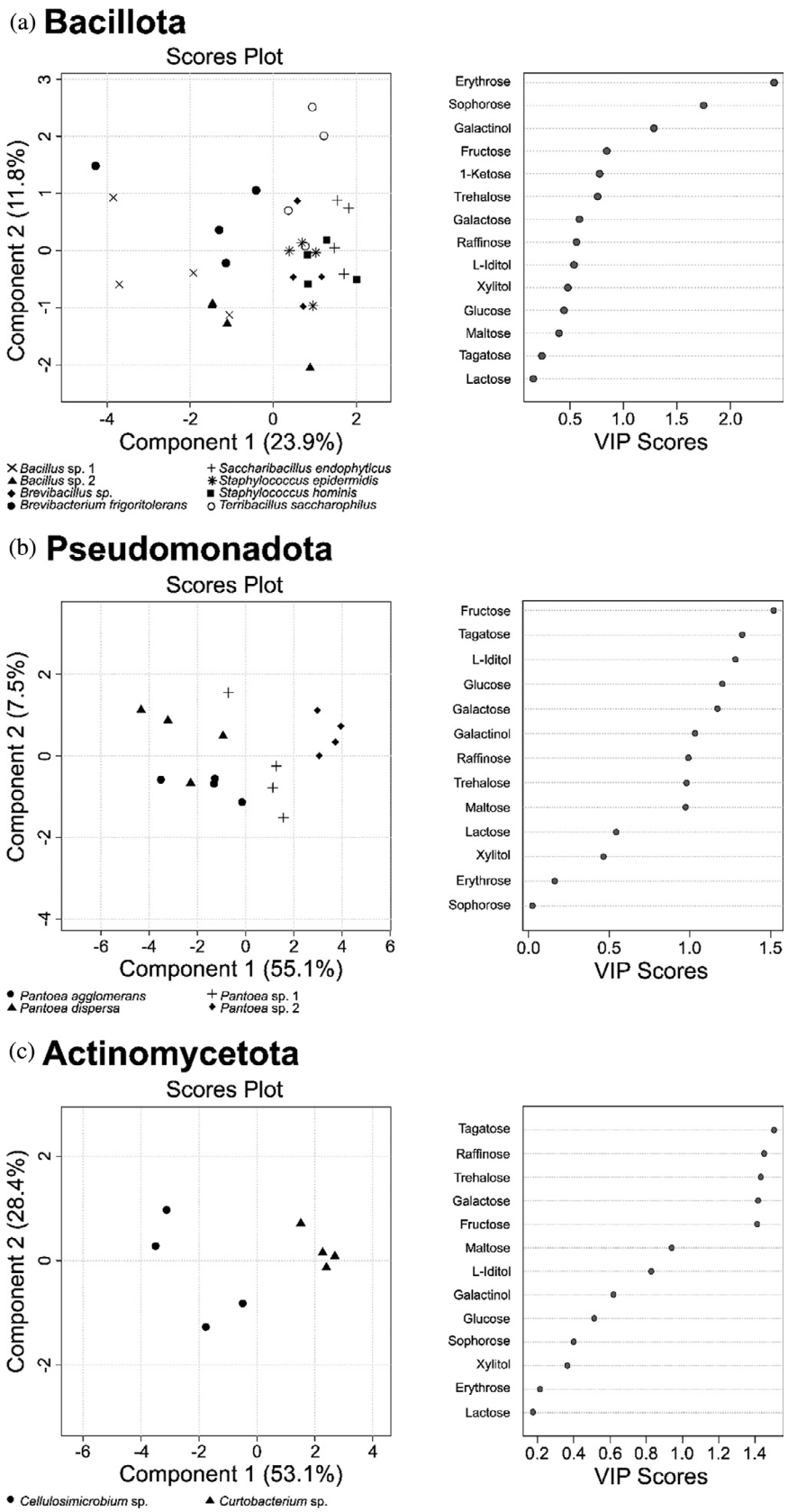

FIGURE 3 Projection to latent structures discriminant analysis (PLS-DA) of synthetic nectar fermented by the different bacterial isolates. The synthetic nectar was fermented by: for Bacillota—*Bacillus* sp1 SAAF 22.2.6, *Bacillus* sp2 SAAF 22.2.27, *Brevibacillus* sp. SAAF 22.4.13, *Brevibacterium frigoritolerans* SAAF 22.2.4, *Saccharibacillus endophyticus* SAAF 22.4.25, *Staphylococcus epidermidis* SAAF 22.3.11, *Staphylococcus hominis* SAAF 22.3.10, and *Terribacillus saccharophilus* SAAF 22.2.3; for Pseudomonadota—*Pantoea agglomerans* SAAF 22.4.2, *Pantoea dispersa* SAAF 22.3.3, *Pantoea* sp1 SAAF 22.4.5, and *Pantoea* sp2 SAAF 22.4.17; for Actinomycetota—*Cellulosimicrobium* sp. SAAF 22.3.25 and *Curtobacterium* sp. SAAF 22.4.18. Comparisons were carried out among isolates belonging to the same phylum: (a) Bacillota (8 isolates); (b) Pseudomonadota (4 isolates); (c) Actinomycetota (2 isolates). Left panels: Score plot visualizing the grouping pattern of the samples according to the first two principal components (PCs) with the explained variance in parenthesis. Right panels: Compounds ranked according to values of Variable Important for the Projection (VIP). All biological replicates ($n = 4$) indicate cell-free nectars.

be important for the longevity of a wide range of insects from this taxonomic group (Goelen et al., 2018; Hirose et al., 2009; Xia et al., 2021; Zhang et al., 2014). These sugars are immediately directed towards energy generation or are transformed into glycogen and/or trehalose within the fat body around the gut of most insects (Jervis et al., 2008; Rivero & Casas, 1999; Wyatt, 1967). There are

indications that the accumulation of sugar and glycogen reserves as a result of fructose- and glucose-rich diets correlate with parasitoid longevity enhancement (Luo et al., 2010).

While glucose and fructose are known to positively affect the longevity of parasitoids, it is less clear how the other sugars may affect parasitoid performance. In general, monosaccharides (glucose, fructose) do not crystallize as fast as oligosaccharides such as trehalose (Wäckers, 2000) and raffinose (Luo et al., 2010). Therefore glucose and fructose are more palatable, and parasitoids are more likely to feed on them compared to oligosaccharides such as raffinose (Wäckers, 2000). There is evidence that feeding parasitoids with glucose or fructose can increase their glycogen levels compared to galactose (Luo et al., 2010). Galactose has been shown to marginally enhance the longevity of parasitoids (Goelen et al., 2018; Wäckers, 2001). Moreover, it has been suggested that erythrose may offer limited nutritional value for insects (Choi et al., 2017), while sophorose and galactinol derivatives are associated with antimicrobial properties. Sophorose lipids are known for their antifungal properties (Kulakovskaya et al., 2014), whereas galactinol, a D-galactose-containing oligosaccharide, is known for its role in plant resistance to microbial infections (Cho et al., 2010; Kim et al., 2008). Additionally, 1-kestose, a fructan sugar that can be found in the nectar of flowers utilized in CBC programmes (van Laere & van den Ende, 2002), has been observed to be consumed by microbial communities colonizing the nectar (Lenaerts et al., 2016).

Future research efforts are needed to ascertain the impact of such sugars and sugar alcohols on the longevity of the egg parasitoid species investigated in this study. Within the Actinomycetota phylum, multivariate analysis also showed differences between strains. Nevertheless, no parasitoid species showed increased or decreased longevity when fed with nectar fermented by Actinomycetota isolates, making it difficult to discuss how the chemistry of sugars and sugar alcohols correlates with parasitoid performance.

Further investigations should explore how bacterial fermentation affects the concentration of sucrose in the nectar media which could not be quantified in this study. A higher ratio of sucrose to hexose (glucose and fructose) has been hypothesized as preferable for parasitoids, since it gradually increases the osmotic pressure in their bodies, maintaining the water balance (Vattala et al., 2006). Lenaerts et al. (2017) showed that bacterial fermentation significantly reduced sucrose, while increasing fructose and glucose concentrations, possibly pointing to competition for sugar resources between nectar-inhabiting bacteria and parasitoids. Moreover, even though nectar is a sugar-rich solution, it contains in much lower amounts amino acids and secondary metabolites, such as alkaloids, that might also affect parasitoid longevity (Lo & Hwang, 2024; Nicolson, 2022) There are indications that nectar may also contain compounds that are not directly associated with its nutritional and energetic value, such as non-protein amino acids (Nepi, 2014). How such compounds affect *T. basalis* and *O. telenomicida* should be further investigated.

To increase the efficiency of parasitoids in CBC, nectar-inhabiting microbes should not only enhance parasitoid performance, but also attract the parasitoids to nectar sources, to ensure high visitation rates of floral resources (Colazza et al., 2023). In a study focused on olfactory responses, it was found that several nectar-inhabiting bacteria attracted *T. basalis*, including *T. saccharophilus* SAAF 22.2.3 (Cusumano et al., 2023). This bacterial isolate is therefore particularly interesting for CBC as it has the potential to enhance not only the longevity of the parasitoid, but also its attraction to flowering plants in the fields, such as buckwheat. This flowering plant has received considerable attention in CBC as it provides high-quality and easily accessible nectar to several parasitoids, including species attacking stink bug eggs (Araj et al., 2019; Blassioli-Moraes et al., 2022; Campbell et al., 2016; Foti et al., 2017; McIntosh et al., 2020; Rahat et al., 2005; Russell, 2015; van Rijn & Wäckers, 2010; Winkler et al., 2006). The beneficial outcomes seen in CBC might be, at least partially, attributed to the bacterial fermentation of floral nectar.

Given the importance of nectariferous plants for sustainable crop protection (Gurr et al., 2016), further research should be undertaken to investigate how nectar-inhabiting bacteria of buckwheat and other plants used in CBC programmes affect the performance of parasitoids, a novel scenario that deserves to be further investigated. In particular, the effect of the most promising nectar-inhabiting bacteria should be confirmed in field conditions. We foresee two main strategies by which nectar-inhabiting bacteria could be taken into account in CBC programmes: (1) flowering plants could be selected based on their likelihood to host beneficial nectar-inhabiting bacteria; (2) candidate bacterial isolates could be delivered into crops with the aid of spray applications in order to support beneficial arthropods (Colda et al., 2021).

Further research efforts should be made to extend the number of case studies in order to understand under which conditions nectar-inhabiting microbes act as beneficial partners for parasitoids (Cusumano & Lievens, 2023). In addition, a topic that remains largely unexplored is whether nectar-inhabiting microbes may also affect other natural enemies, including predators such as syrphids and lacewings which are known to rely on floral nectar and other sugar-rich resources at the adult stage (Leroy et al., 2011). Undoubtedly, fungi and bacteria ubiquitously colonize the nectar in nature and therefore, the impact of their interactions should not be neglected (Álvarez-Pérez et al., 2019), as the correct choice of nectar-inhabiting microbes is likely to be contingent on the beneficial organism's identity and its relative abundance.

AUTHOR CONTRIBUTIONS

Ezio Peri: conceptualization, methodology, writing—review & editing, supervision. **Stefano Colazza:** conceptualization, methodology, writing—review & editing, supervision. **Bart Lievens:** conceptualization, methodology, writing—review & editing. **Michael Rostás:** conceptualization, methodology, writing—review & editing. **Antonino Cusumano:** conceptualization, methodology, writing—review & editing, supervision. **Patrizia Bella:** methodology, investigation, writing—review & editing. **Jay Darryl L. Ermio:** investigation, visualization. **Mirella Lo Pinto:** investigation. **Alfonso Agrò:** investigation. **Shahinoor Rahman:** investigation, formal analysis. **Evgenia Sarakatsani:** investigation, formal analysis, visualization, writing—original draft, writing—review & editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

DATA AVAILABILITY STATEMENT

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

ETHICS STATEMENT

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain studies with human participants performed by any of the authors.

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