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Research Article

Culturable gut bacteria associated with the carob moth, *Ectomyelois ceratoniae* (Zeller, 1839) (Lep.: Pyralidae)

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ABSTRACT- The carob moth, *Ectomyelois ceratoniae*, is a pest of considerable global concern. The microbial communities associated with this species remain largely unexplored. In this study, we report the first isolation and comprehensive characterization of culturable bacterial symbionts from the larval gut of *E. ceratoniae*. Bacterial isolates were recovered from different larval instars and characterized using a polyphasic taxonomic approach that included morphological, biochemical, and physiological analyses, supported by 16S rRNA gene sequencing. Our results revealed a diverse gut bacterial community, revealing several isolates identified as members of the genera *Pseudomonas*, *Achromobacter*, *Exiguobacterium*, *Peribacillus*, *Pantoea*, and *Erwinia*. These isolates displayed distinct phenotypic profiles, including differences in Gram reaction, oxygen requirements, and tolerance to a range of temperatures, pH levels, and salinity conditions. This characterization of the culturable gut bacterial community provides an essential foundation for understanding the functional roles of these symbionts in host nutrition, development, and detoxification. Moreover, the findings open new avenues for research aimed at exploiting these bacterial symbionts to develop novel, eco-friendly biocontrol strategies within integrated pest management (IPM) programs targeting *E. ceratoniae*.

INTRODUCTION

The carob moth, *Ectomyelois ceratoniae* Zeller (Lep.: Pyralidae), is an economically important fruit pest with a nearly cosmopolitan distribution and a broad host range. This polyphagous species infests a wide variety of fruit-bearing plants and is particularly notorious for damaging crops such as citrus, almond, date, pistachio, and fig (Kishani-Farahani et al., 2012; Abedi et al., 2019). In Iran, pomegranate, *Punica granatum* L. (Myrtales: Punicaceae), is the principal host of *E. ceratoniae*. This insect is considered a major pest of pomegranate and is capable of causing yield losses of up to 80% during both the growing season and post-harvest storage (Shakeri, 2004). Female *E. ceratoniae* typically lay their eggs within the pomegranate calyx or in existing cracks on the fruit peel. Although larvae are capable of boring into the fruit through the calyx region, their primary mode of infestation involves exploiting pre-existing fissures or microcracks in the fruit epidermis as entry points (Hosseini et al., 2017). Once inside, the larvae feed on the internal tissues of the pomegranate, thereby facilitating colonization by saprophytic fungi and leading to deterioration in fruit quality (Abedi et al., 2021). Conventional insecticides have long been used as the main strategy for managing this pest and protecting the yield of high-quality, damage-free fruits; however, their use has been accompanied by adverse environmental effects. In addition, the extensive application of chemical control agents has contributed to the development of pest populations resistant to a range of insecticidal compounds (Zougari et al., 2023). The life cycles of many insect species are closely associated with symbiotic microorganisms, and a growing body of research indicates that these microbial partners can profoundly influence insect physiology, metabolism, and ecology (Mondal et al., 2023). Nevertheless, some insect lineages maintain only facultative associations with their microbiota rather than strict dependence on them (Douglas, 2014). Among these microbial symbionts, bacteria have attracted particular attention as promising tools for the biological control of insect pests. Through advanced biotechnological approaches, these microorganisms can be genetically engineered to produce insecticidal compounds or effector proteins, thereby offering sustainable alternatives to conventional pest management strategies (Almeida et al., 2017; Alfano et al., 2019). Moreover, manipulating the composition and functional dynamics of gut-associated bacterial communities represents a promising avenue for the

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development of novel pest management approaches. Accordingly, the gut microbiota of numerous insect species has been extensively characterized to better understand its ecological roles and potential applications in pest regulation (Sevim et al., 2012; Demirci et al., 2013; Huerta-García and Álvarez-Cervantes, 2024).

This study aimed to isolate and identify bacterial species associated with *E. ceratoniae* in Iran, thereby providing insight into the complex symbiotic relationships that influence the biology of this economically important pest. Elucidating the diversity and functional roles of these symbiotic bacteria may help pave the way for the development of environmentally sustainable and biologically informed management strategies for this agriculturally threatening species.

MATERIALS AND METHODS

Collection of larvae

Infested pomegranate fruits containing carob moth larvae were collected during the autumn of 2023 from an orchard in Khalil-Abad, Khorasan-e-Razavi Province, Iran (35.2531° N, 58.2890° E), and transported to the laboratory. The infested fruits were carefully opened, and the larvae were collected and classified according to their developmental instars for subsequent bacterial isolation and characterization.

Isolation of bacteria from larvae

The collected larvae were divided into two developmental groups based on their instar stage: early instars, comprising the first and second larval stages, and late instars, comprising the fourth and fifth larval stages. Neonate larvae were also examined as a separate group. To obtain neonate larvae, approximately 50 infested pomegranate fruits containing carob moth larvae were placed in wooden cages (60 × 60 × 60 cm^3) under controlled environmental conditions of 28 ± 3 °C, 60 ± 5% relative humidity, and a photoperiod of 16 h light and 8 h dark. After adult emergence, moths were collected using an oral aspirator and transferred to containers measuring 10 × 30 × 40 cm^3 and lined with rough tissue paper to facilitate oviposition. For nutritional supplementation, adults were provided with a cotton wick moistened with 10% honey solution. Egg hatching was monitored at 12-h intervals, and freshly hatched larvae were collected. Bacterial isolation was carried out independently for each experimental group. For this purpose, ten larvae from each group were selected as sources of bacterial isolates. Before isolation, the larvae were starved for 24 h, surface-sterilized in 70% ethanol for 2-3 min, and then rinsed three times with sterile distilled water to remove residual contaminants (Sevim et al., 2018). After removal of the larval head and terminal abdominal segment, the dissected gut tissues from each experimental group were transferred to 1.5-mL microcentrifuge tubes containing 100 µL Phosphate Buffered Saline (PBS) buffer (NaCl 0.137 M, KCl 0.0027 M, Na_2HPO_4 0.01 M, and KH_2PO_4 0.0018 M; pH 7.4) and thoroughly homogenized using a sterile plastic pestle (Sevim et al., 2018). The homogenates were then centrifuged, and the resulting supernatants were aseptically spread onto nutrient agar plates and incubated at 30 °C for 48 h. Distinct bacterial colony morphotypes were subsequently restreaked on nutrient agar to obtain pure cultures. The purified bacterial strains were then preserved in 20% glycerol at - 20 °C for long-term storage.

Phenotypic characterization of the isolated strains

The bacterial isolates were morphologically characterized based on their colony features, as presented in Table 1. Several important phenotypic characteristics, including Gram reaction (using KOH 3%), oxidative/fermentative metabolism, oxidase and catalase activities, levan production, and motility, were evaluated using standard bacteriological tests (Schaad and Chun, 2001).

Physiological attributes of the isolated strains

The physiological characteristics of the bacterial isolates were evaluated by examining their growth under different temperature conditions, NaCl concentrations, and pH levels (Sevim et al., 2018). Each isolate was cultured in 3 mL of nutrient broth and incubated at 4, 30, 37, 45, and 55 °C to assess thermal tolerance. In parallel, growth was evaluated in media supplemented with sodium chloride concentrations ranging from 3% to 10% (w/v) to determine salinity tolerance. Finally, all isolates were cultured in 3 mL of nutrient broth adjusted to pH values from 5 to 12. Bacterial growth was assessed qualitatively by visual inspection of culture turbidity. This qualitative screening approach was used to determine the physiological tolerance range of each isolate, consistent with standard bacterial characterization protocols (Sevim et al., 2012).

16S rRNA gene sequencing

The taxonomic identity and phylogenetic affiliation of the bacterial isolates were elucidated through partial 16S rRNA gene sequencing. Genomic DNAs were extracted by Tissue Genomic DNA Extraction Mini Kit (FAVORGEN, Iran) and preserved at -20 °C until subsequent PCR amplification. The amplified fragment corresponded to an approximately 1,500-base pair region of the 16S ribosomal RNA gene. The primer pairs of 27F (5′-AGAGTTTGATCMTGGCTCAG-3′ as forward) and then beside the adjacent pair 1492R (5′-GGYTACCTTGTTACGACTT-3′ as reverse) (Sevim et al., 2018) were utilized for amplification. The total volume of PCR was 25 µL, containing 11.9 µL sterile distilled water, 10 pmol of each primer, 12.5 µL PCR Mastermix (CinnaClone, Iran) and 0.2 µL of DNA. PCR amplification was initiated with an initial denaturation step at 95 °C for 1 min, followed by 35 cycles of denaturation at 94 °C for 45 s, primer annealing at 55 °C for 30 s, and DNA strand extension at 72 °C for 1.5 min. The protocol concluded with a final extension step at 72 °C for 5 min. Subsequently, a 5 µL aliquot of each amplicon was subjected to electrophoresis on a 1% (w/v) agarose gel, stained with Green Viewer (Genet Bio, Korea), and visualized under UV light. The PCR

products were then sequenced by MACROGEN (Seoul, South Korea) using conventional Sanger sequencing technology. The resulting nucleotide sequences were analyzed using the BLAST algorithm against the NCBI GenBank database to identify the closest phylogenetic relatives and determine the percentage similarity of the bacterial isolates to reference species.

Phylogenetic analysis

The bacterial isolates were subjected to phylogenetic reconstruction together with their closest relatives to infer molecular relationships and determine their taxonomic placement. Sequence editing was performed using BioEdit version 7.2.5 (Hall, 1999). The nucleotide sequences were aligned using Clustal X 1.83 (Thompson et al., 1997). A phylogenetic tree was then constructed based on the neighbor-joining algorithm and visualized using MEGA 11 (Kumar et al., 2018), with bootstrap analysis performed using 1000 replicates. The 16S rRNA gene sequences obtained from the bacterial isolates were deposited in GenBank, and their corresponding accession numbers are listed in Table 2.

RESULTS

A total of eight bacterial isolates were recovered from treated larvae of *E. ceratoniae*. Among these, one isolate was obtained from neonate larvae (1N), four from the early larval instars (1E, 2E, 3E, and 4E), and three from the late instars (1L, 2L, and 3L). Comprehensive characterization of the isolates was performed based on their morphological, biochemical, physiological, and molecular attributes.

Phenotypic characterization of the isolated bacterial strains

Isolates 1N and 2E produced orange and pale-yellow colonies, respectively. Two isolates (2L and 3L) formed yellow colonies, while four isolates (1E, 3E, 4E, and 1L) produced creamy-colored colonies. The colony surfaces of all tested isolates, except 1E, were smooth. Three isolates, namely 4E, 2L, and 3L, produced mucoid colonies in the presence of 5% sucrose in the solid medium. The results of key biochemical assays (Table 1) showed that six isolates (2E, 3E, 4E, 1L, 2L, and 3L) were Gram-negative, whereas the remaining two isolates (1N and 1E) exhibited Gram-positive characteristics. All examined isolates were catalase positive. Only one isolate (1L) was oxidase positive, while the other seven isolates (1N, 1E, 2E, 3E, 4E, 2L, and 3L) showed a negative reaction. In the oxidative-fermentative test, isolates 1N, 4E, 2L, and 3L were able to grow and utilize the carbon source in both the presence and absence of oxygen, indicating that they were facultative anaerobes. In contrast, the remaining isolates (1L, 1E, 2E, and 3E) showed activity only in the presence of oxygen, classifying them as obligate aerobes. All eight isolates were also able to move in motility medium and were therefore recorded as motile (Table 1).

Physiological attributes of the isolated bacterial sStrains

Based on the results in Table 2, all isolates grew at NaCl concentrations of 3% and 5%. Growth at 7% NaCl was variable, whereas no growth was observed at 10% NaCl. All isolates also exhibited growth at pH 5, 7, and 9, while growth at pH 10 and 12 was variable. Specifically, isolates 2E, 3E, 4E, 2L, and 3L showed no growth at pH 10 and similarly failed to grow at pH 12. In contrast, isolates 1N, 1E, and 1L demonstrated robust growth at both pH 10 and 12 (Table 2). In addition, none of the isolates showed growth at 4 °C. All bacterial symbionts grew at 30 and 37 °C, whereas growth at 45 °C was variable and no growth was observed at 55 °C.

16S rRNA sequencing

To validate the conventional characterization of the bacterial isolates, molecular identification was performed through 16S rRNA gene sequencing. Phylogenetic and molecular analyses revealed that the isolates belonged to distinct taxa, i.e., *Exiguobacterium* sp. (1N), *Peribacillus acanthi* (1E), *Pseudomonas* sp. (2E, 3E), *Erwinia* sp. (4E), *Achromobacter* sp. (1L), and *Pantoea* sp. (2L, 3L) (Table 3). The phylogenetic relationships inferred from sequence analysis further confirmed these taxonomic groups (Fig. 1).

Table 1. The morphological and biochemical features of the bacterial isolates obtained from *Ectomyelois ceratoniae* larvae

Isolate	Instar	Colony surface	Colony color	Gram reaction	Motility	O/F*	Levan production	Oxidase	Catalase
1N	Neonate larvae	smooth	orange	+	+	O/F	-	-	+
1E	1st & 2nd instars	rough	cream	+	+	O	-	-	+
2E		smooth	pale yellow	-	+	O	-	+	+
3E		smooth	cream	-	+	O	-	+	+
4E		smooth	cream	-	+	O/F	+	-	+
1L	4th & fifth instars	smooth	cream	-	+	O	-	+	+
2L		smooth	yellow	-	+	O/F	+	-	+
3L		smooth	yellow	-	+	O/F	+	-	+

*O/F: The oxidative/fermentative

Table 2. The physiological attributes of the bacterial isolates obtained from *Ectomyelois ceratoniae* larvae

Isolate	Growth													
	NaCl (w/v)				pH					Temperature (°C)				
	3%	5%	7%	10%	5	7	9	10	12	4	30	37	45	55
1N	+	+	+	-	+	+	+	+	+	-	+	+	+	-
1E	+	+	+	-	+	+	+	+	+	-	+	+	+	-
2E	+	+	-	-	+	+	+	-	-	-	+	+	-	-
3E	+	+	-	-	+	+	+	-	-	-	+	+	-	-
4E	+	+	-	-	+	+	+	-	-	-	+	+	+	-
1L	+	+	+	-	+	+	+	+	+	-	+	+	+	-
2L	+	+	+	-	+	+	+	-	-	-	+	+	+	-
3L	+	+	+	-	+	+	+	-	-	-	+	+	+	-

Table 3. Similarity percentages of the 16S rRNA gene sequences of bacterial strains isolated from *Ectomyelois ceratoniae* larvae to their closest genus/species relatives, based on BLASTn searches in the NCBI GenBank database

Isolate	Bacterial genus/species	GenBank accession number	Query cover (%)	Similarity (%)
1N	*Exiguobacterium* sp.	PP447393	100	99.58
1E	*Peribacillus acanthi* (syn. *Bacillus acanthi*)	PP439710	100	100
2E	*Pseudomonas* sp.	PP454076	100	99.78
3E	*Pseudomonas* sp.	PP447318	99	100
4E	*Erwinia* sp.	PP439827	99	100
1L	*Achromobacter* sp.	PP464079	100	99.57
2L	*Pantoea* sp.	PP447256	100	99.86
3L	*Pantoea* sp.	PP447254	100	99.79

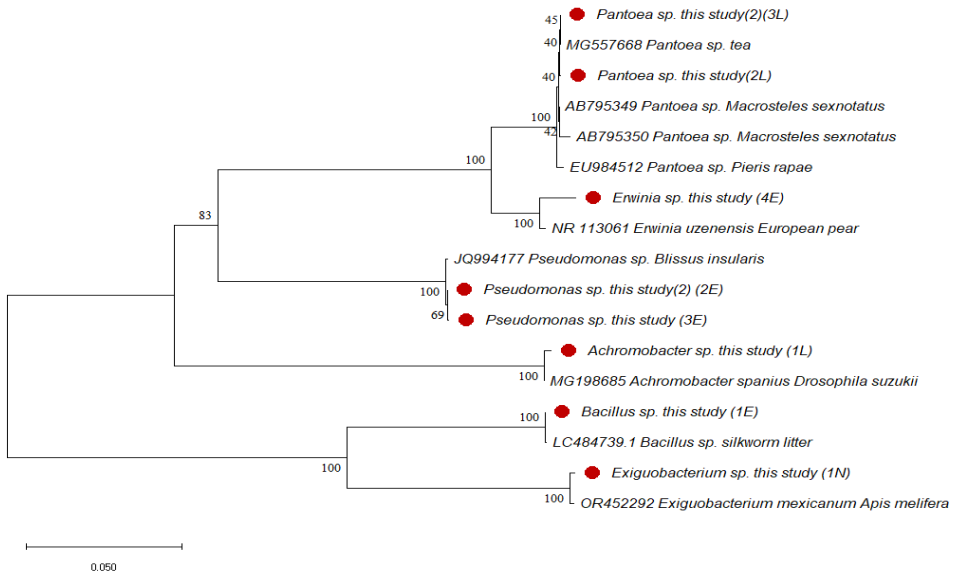

Fig. 1. Phylogenetic tree derived from neighbor-joining analysis of 16S rRNA sequences related to bacterial symbionts associated with *Ectomyelois ceratoniae* larvae and their closely related genera/species. Bootstrap values based on 1000 replicates were indicated above nodes.

DISCUSSION

Lepidoptera are one of the largest and most ecologically significant members of the class Insecta, encompassing lineages that include both major agricultural pests and species of considerable ecological or economic concern. The remarkable adaptive radiation of this order is closely associated with the essential roles played by their microbial communities. The diverse microbiota of Lepidoptera contributes substantially to nutrition, reproductive regulation, and defense against pathogens (Zhang et al., 2022). In this study, eight morphologically distinct bacterial isolates were recovered from carob moth larvae representing different instar stages. The isolates obtained from older larvae, designated 1L, 2L, and 3L, belonged to the families Alcaligenaceae and Erwiniaceae. Four strains recovered from younger larvae, namely 1E, 2E, 3E, and 4E, were assigned to the families Bacillaceae, Pseudomonadaceae, and Erwiniaceae. In addition, one strain (1N) belonging to the family Bacillaceae was identified from neonate larvae. Dietary composition, host plant specificity, seasonal variation, and geographical distribution are among the key factors influencing the diversity and composition of insect-associated bacterial communities, including those of Lepidoptera (Priya et al., 2012; Hu et al., 2022).

González-Serrano et al. (2020) demonstrated that the bacterial composition of the digestive system in *Brithys crini* larvae varies according to the developmental stage. Similarly, in the present study, bacterial diversity was observed among different larval instars, with distinct bacterial compositions detected in neonate, younger, and older larvae. Exiguobacterium was identified in neonate larvae, whereas *Peribacillus* sp., *Erwinia* sp., and Pseudomonas sp. were detected in younger larvae. Moreover, two genera, *Achromobacter* sp. and *Pantoea* sp., were identified in older larvae.

One of the bacterial isolates identified in this study belonged to the genus Pseudomonas, within the class Gammaproteobacteria and also Pseudomonadaceae. The genus Pseudomonas currently comprises 313 species and is characterized by remarkable metabolic diversity, which enables its members to inhabit a wide range of ecological niches (Girard et al., 2021). Although many species within this genus are pathogenic to various insects, beneficial associations between Pseudomonas and insects have also been reported in certain taxa (Teoh et al., 2021). Endosymbiotic Pseudomonas species can provide their insect hosts with essential nutrients, including amino acids, cofactors, and vitamins, while also offering protection against predators, pathogens, and plant defensive compounds. For example, *Pseudomonas trivialis*, a symbiont of *Ips typographus* larvae, supplies nutrients to its host by degrading plant cell wall components and also protects it against pathogens (Peral-Aranega et al., 2020). In addition, Rogowska-van et al. (2022) reported that leguminous plants naturally produce the toxic compound nitropropionic acid, which does not affect green peach aphids. Further investigation identified a Pseudomonas strain associated with this pest that is resistant to nitropropionic acid and capable of converting it into mineral nitrogen and carbon dioxide.

The genus *Achromobacter* was among the bacteria identified in the larvae of *E. ceratoniae*. This genus belongs to the class Betaproteobacteria and the family Alcaligenaceae. Members of *Achromobacter* have been isolated from a wide range of environments, including laboratory samples, water, soil, plant tissues, and insect digestive systems (Bing et al., 2021; Sun et al., 2022). Isolates of this genus have also been reported as cellulose-degrading bacteria from the larvae of Lepidoptera and Coleoptera, as well as from adult flies (Handique et al., 2017; Msango et al., 2024). In addition, *Achromobacter* has been isolated from the digestive system of the American cockroach (*Periplaneta americana*), where it exhibited anti-Klebsiella pneumoniae activity (Ma et al., 2023). In a study by Bing et al. (2021), 25 cohabiting bacterial species were isolated from the pest *Drosophila suzukii*. Among them, *Alcaligenes faecalis*, *Achromobacter spanius*, and *Serratia marcescens* were pathogenic when injected into adult insects. These bacterial species also caused disease in pest larvae through feeding. The genera *Pantoea* and *Erwinia*, which were also identified in the current study, belong to the class Gammaproteobacteria and the family Erwiniaceae. The genus *Pantoea* comprises more than 20 identified species (Walterson and Stavrinides, 2015). The relationship between *Pantoea* and *Lepidoptera* appears to vary considerably depending on the species involved, and current understanding of these associations remains incomplete. Symbiotic *Pantoea* species can produce antimicrobial compounds that help insects defend themselves against harmful microorganisms. In some cases, they also produce substances that assist in the breakdown of essential food materials that insects are otherwise unable to digest (Li et al., 2017). *Pantoea stewartii* subsp. stewartii, the causal agent of Stewart's wilt in corn, is transmitted by beetle vectors (Coleoptera). Unspecified *Pantoea* species, as well as the species *stewartii*, *ananatis*, and *agglomerans*, have been isolated from various insects (Walterson and Stavrinides, 2015). Moreover, the beneficial effects of *Pantoea* species as endosymbionts on the growth of three different thrips species have been reported in the literature (Jin and Kim, 2023).

Various species of the genus Erwinia have been identified in a range of insects, including Lepidoptera. These bacteria may establish either symbiotic or pathogenic relationships with their insect hosts (de Vries et al., 2004; Paniagua Voirol et al., 2018; Ge et al., 2025). Erwinia can enhance insect adaptation to host plants by influencing dietary utilization (de Vries et al., 2004). In addition, members of this genus contribute to the digestion of food materials and may promote insect growth and development because of their strong metabolic capabilities (Xue et al., 2021; Cambronero-Heinrichs et al., 2023). In contrast, some *Erwinia* species are pathogenic to Lepidoptera. Certain species can also induce wilting, yellowing, and death in host plants. When such infected plants are consumed by Lepidopteran larvae, the bacteria may accumulate in the digestive tract and cause disease symptoms in the insects (Grenier et al., 2006). Among the Gram-positive bacteria identified from carob moth larvae, two genera belonging to the phylum Firmicutes, i.e., *Exiguobacterium* and *Peribacillus*, were detected. Members of *Exiguobacterium* are capable of growing under diverse conditions of temperature, acidity, salinity, and heavy metal concentrations (White et al., 2019). Strain 1N examined in the present study exhibited morphological and physiological characteristics similar to those reported for strains isolated from microbialite sources.

Another identified Gram-positive genus in digestive systems of carob moth larvae was *Peribacillus*. The name of this genus signifies its close relation to the genus *Bacillus* from the family Bacillaceae. While most representatives of this genus have been isolated from soil or the gastrointestinal tract of humans and animals (Rodríguez et al., 2022), *Peribacillus* spp. have also been documented in insect gut microbiomes, including honeybees (*Apis mellifera jemenitica*) exposed to pesticide stress (Ghramh et al., 2025). A total of 17 species within the genus *Peribacillus* were classified in a monophyletic group based on 16S rRNA sequencing (Manetsberger et al., 2023, Sato et al., 2023). Certain members of this genus, including *P. simplex*, have demonstrated antifungal activity (Manetsberger et al., 2023, Baranova et al. 2024).

CONCLUSION

By integrating a polyphasic taxonomic framework with 16S rRNA phylogenetic analysis, this study provides the first in-depth characterization of the culturable gut bacteriome of the carob moth, *E. ceratoniae*, thereby addressing a key knowledge gap at the intersection of insect microbiology, chemical ecology, and pest management. The documented bacterial portfolio herein, including taxa such as *Pseudomonas*, *Pantoea*, *Achromobacter*, *Exiguobacterium*, *Peribacillus*, and *Erwinia*, reveals a metabolically and ecologically versatile symbiotic consortium with recognized potential for nutrient provisioning, detoxifications of allelo chemicals and pesticides, and modulation of plant-insect interactions. Nevertheless, species-level identification remains provisional, as the molecular analyses were based solely on 16S rRNA gene sequences combined with basic phenotypic profiling. A more robust taxonomic resolution would require sequencing of additional housekeeping genes, such as gyrB, rpoB, and atpD, together with comprehensive biochemical and physiological characterization using standardized identification schemes. Viewing these bacteria as manipulable components of the pest holobiont offers a promising avenue for microbiome-informed agricultural innovation, in which insect-derived strains and engineered or selectively disrupted symbioses may be incorporated into sustainable and environmentally compatible biological control strategies within integrated pest management (IPM) programs. Future studies combining improved taxonomic resolution with genome-resolved functional analyses, in vivo manipulation of symbiont communities, and field-level validation will be essential for translating this preliminary symbiotic inventory into robust microbiome-based tools capable of reducing chemical inputs while maintaining effective crop protection.

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Conceptualization: Lida Fekrat, Sareh Baghaee Ravari, and Mohammad ZakiAghl; Methodology: Lida Fekrat, Sareh Baghaee Ravari, and Mohammad ZakiAghl; Software: Lida Fekrat; Validation: Lida Fekrat and Sareh Baghaee Ravari; Formal analysis: Lida Fekrat and Zohreh Izadi; Investigation: Zohreh Izadi; Resources: Lida Fekrat and Sareh Baghaee Ravari; Data curation: Zohreh Izadi; Writing, original draft preparation: Lida Fekrat; Writing, review and editing: Sareh Baghaee Ravari and Mohammad ZakiAghl; Visualization: Lida Fekrat; Supervision: Lida Fekrat and Sareh baghaee Ravari; Project administration: Lida Fekrat; Funding acquisition: Lida Fekrat.

DECLARATION OF COMPETING INTEREST

The authors declare no conflicts of interest.

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