

KARADENİZ TECHNICAL UNIVERSITY
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

BIOTECHNOLOGY DEPARTMENT

**DETERMINING THE BIOCONTROL POTENTIAL OF BACTERIA IN THE
CHERRY FRUIT FLY (*RHAGOLETIS Cerasi*) MICROBIOME**

MASTER'S THESIS

Seyitnur PIRHANOV

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TRABZON



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**This thesis is accepted to give the degree of
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PREFACE

The study entitled ‘‘Determining the Biocontrol Potential of Bacteria in the Cherry Fruit Fly (*Rhagoletis cerasi*) Microbiome’’ was prepared as a ‘‘Master's Thesis’’ in the Biotechnology Program at Institute of Natural and Applied Sciences, Karadeniz Technical University. The herbicidal effect of bacterial isolates obtained from cherry fruit fly (*R. cerasi*) were investigated in this study.

I would like to express my deepest gratitude to my esteemed advisor, Prof. Dr. Remziye NALÇACIOĞLU, for her invaluable guidance, knowledge, and patience throughout the course of my thesis work. In addition, I would like to extend my sincere thanks to Prof. Dr. Zihni DEMİRBAĞ and Prof. Dr. İsmail DEMİR for their significant contributions and continuous support during my studies. I am also grateful to the entire Microbiology Laboratory team and everyone who contributed to my research at various stages of the thesis.

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Finally, I am deeply grateful to my family, whose constant support has been invaluable throughout this journey.

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THESIS ETHICS STATEMENT

I declare that I have completed this study titled " Determining the Biocontrol Potential of Bacteria in the Cherry Fruit Fly (*Rhagoletis cerasi*) Microbiome ", which I submitted as a master's thesis, under the responsibility of my advisor Prof. Dr. Remziye NALÇACIOĞLU from the beginning to the end, collected the data/samples myself, conducted the experiments/analyzes in the relevant laboratories, shown the information I received from other sources in the text and references, and acted in accordance with scientific research and ethical rules during the study and accepted any legal consequences in case the opposite occurs. 25/10/2024

Seyitnur PIRHANOV

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Yüksek Lisans Tezi

ÖZET

KİRAZ MEYVE SİNEĞİ (*Rhagoletis cerasi*) MİKROBİYOMUNDA BAKTERİLERİN BİYOKONTROL POTANSİYELİNİN BELİRLENMESİ

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Bu araştırma, *Rhagoletis cerasi*'nin (kiraz meyve sineği) bağırsağından elde edilen bakteri izolatlarının potansiyel biyokontrol ajanları olarak böcek öldürücü etkilerini araştırmaktadır. On bakteri izolatı tanımlandı ve *R. cerasi* larvalarına, *Tenebrio molitor* (un kurdu) larvalarına ve *Galeria mellonella* (petek güvesi) larvalarına karşı böcek öldürücü özellikleri açısından test edildi. İzolatlar biyokimyasal, moleküler ve böcek öldürücü analizler kullanılarak karakterize edildi. Bunlar arasında, *Serratia marcescens* (Rc1) ve *Raoultella ornithinolytica* (Rc9) en yüksek böcek öldürücü aktiviteyi gösterdi; *T. molitor*'da sırasıyla %46 ve %43, *R. cerasi*'de %40 ve %37, *G. mellonella*'da ise %73 ve %57 ölüm oranı görüldü. *Lelliottia steviae* (Rc2), *Enterobacter ludwigii* (Rc10) ve *Rahnella aceris* (Rc8) gibi izolatlar için orta düzeyde böcek öldürücü etkiler gözlemlenirken, *Pantoea agglomerans* izolatları (Rc3, Rc4, Rc7) daha düşük etkinlik gösterdi. Çalışma, toksin üretimi, böcek kütiküllerinin enzimatik bozunumu ve bağırsak mikrobiyotasının bozulmasının bu bakterilerin böcek öldürücü aktivitesine katkıda bulunabileceğini öne sürüyor. Bulgular, bu bakteri izolatlarının, özellikle *S. marcescens* ve *R. ornithinolytica*'nın, *R. cerasi*'ye karşı biyolojik kontrol stratejilerinin geliştirilmesi için umut verici adaylar olma potansiyelini vurgulamaktadır. Gelecekteki çalışmalar, bu izolatların entegre zararlı yönetimi programlarındaki etkinliğini artırmak için ayrıntılı mekanistik çalışmalara, saha denemelerine ve sinerjik kombinasyonların geliştirilmesine odaklanmalıdır.

Anahtar Kelimeler: *Rhagoletis cerasi*, kiraz meyve sineği, bakteri izolatları, böcek öldürücü aktivite, biyolojik kontrol, entegre zararlı yönetimi, bağırsak mikrobiyotası

Master Thesis

SUMMARY

DETERMINING THE BIOCONTROL POTENTIAL OF BACTERIA IN THE CHERRY FRUIT FLY (*Rhagoletis cerasi*) MICROBIOME

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This research explores the insecticidal effects of bacterial isolates obtained from the gut of *Rhagoletis cerasi* (cherry fruit fly) as potential biocontrol agents. Ten bacterial isolates were identified and tested for their insecticidal properties against *R. cerasi* larvae, *Tenebrio molitor* (mealworm) larvae and *Galleria mellonella* (wax moth) larvae. The isolates were characterized using biochemical, molecular, and insecticidal assays. Among them, *Serratia marcescens* (Rc1) and *Raoultella ornithinolytica* (Rc9) exhibited the highest insecticidal activity, with mortality rates of 46% and 43% on *T. molitor*, 40% and 37% on *R. cerasi*, and %73 and %57 on *G. mellonella*, respectively. Moderate insecticidal effects were observed for isolates such as *Lelliottia steviae* (Rc2), *Enterobacter ludwigii* (Rc10) and *Rahnella aceris* (Rc10), while *Pantoea agglomerans* isolates (Rc3, Rc4, Rc7) displayed lower effectiveness. The study suggests that the production of toxins, enzymatic degradation of insect cuticles, and disruption of gut microbiota may contribute to the insecticidal activity of these bacteria. The findings highlight the potential of these bacterial isolates, particularly *S. marcescens* and *R. ornithinolytica*, as promising candidates for the development of biological control strategies against *R. cerasi*. Future work should focus on detailed mechanistic studies, field trials, and the development of synergistic combinations to enhance the efficacy of these isolates in integrated pest management programs.

Key Words: *Rhagoletis cerasi*, cherry fruit fly, bacterial isolates, insecticidal activity, biological control, integrated pest management, gut microbiota

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ABBREVIATIONS

bp	: Base pair
dH ₂ O	: Distilled water
DNA	: Deoxyribonucleic acid
dNTP	: Deoxyribonucleotide triphosphate
EDTA	: Ethylenediamine tetraacetic acid
<i>E. coli</i> :	: <i>Escherichia coli</i>
kbp	: Kilobase pair
LB	: Luria bertani
M	: Molar
ml	: Milliliter
mM	: Milimolar
NaCl	: Sodium chloride
NCBI	: National Center for Biotechnology Information
ng	: Nanogram
nm	: Nanometer
µg	: Microgram
µl	: Microliter
µM	: Micromolar
OD	: Optical density
PBS	: Phosphate-Buffered Saline
PCR	: Polimerase chain reaction
RNA	: Ribonucleic acid
°C	: Celsius
X-Gal	: 5-bromo-4-chloro-indolyl-β-D galactopyranoside

1. INTRODUCTION

1.1. The Importance of Cherries in Our Daily Life

Cherries were first cultivated in the South Caucasus, Caspian Sea, and Northeastern Anatolia regions and spread to the world from there. Cherries originated in these zones, but their ability to adapt to various geographical areas and climate conditions has facilitated their cultivation, production, and consumption for centuries, leading to their presence in continents such as Asia, Europe, and America (Balkan & Kara, 2020). Today, more than 1,500 varieties of cherries are grown worldwide (The Editors of Encyclopedia Britannica, 2024). *Prunus avium* L., commonly called sweet cherry, holds a cherished position within the Rosaceae family. It belongs to the same family as other beloved succulent fruits like apples, strawberries, and plums, which are economically and nutritionally significant, and delight people worldwide (Yamamoto & Terakami, 2016). Today, the cultivation of cherries and their exportation, particularly in Türkiye, the USA, Iran and China, are rapidly gaining significance, and emerging as leading exporters in the international commercial market (FAOSTAT, 2024).

Among these, table cherries stand out with their large size, bright dark colors, and hard, sweet flesh and appeal to consumers at various stages of maturity. According to the USDA Foreign Agricultural Service (2023), Turkey is the leading cherry producer worldwide, contributing 19% of global production with an estimated 950 metric tons for the 2023 season. This growth has underscored the importance of addressing the cherry fruit fly (*Rhagoletis cerasi*) problem. As cherry production rises, so does the damage caused by *R. cerasi*. This concern has driven us to study the flight activity of *R. cerasi* and explore effective management strategies. Meanwhile, new varieties are constantly appearing on the market, adding to the diversity of fruit. Türkiye, in particular, is the world's leading cherry producer, growing nearly 50 different cherry varieties.

Since its creation, humanity has survived and developed by providing its basic needs such as nutrition, shelter, clothing, and protection, as well as economic needs, from agriculture and forests. Humans have formed a profound interconnectedness with agriculture and forests to meet their needs and are in everlasting interaction with both. As agriculture has been the source of life throughout history, it has greatly influenced the construction of

social order and the interaction of people with nature. To maximize the benefits derived from the various products offered by agriculture, human beings have learned about the pests, diseases, and weeds encountered in agricultural products and have developed and applied various methods such as agrotechnical, cultural, biological, and chemical control to combat them.

Nowadays, providing sufficient food to the increasing world population, and protecting the natural environment are among humanity's most important and urgent problems. In light of this, increasing the productivity and extending the shelf life of agricultural products, reducing losses during harvest, transportation, and storage periods, and developing pioneering methods in the fight against pests of agricultural products are important tasks.

Insects have a small size and rapid reproductive ability. They are distributed worldwide except for the deep-sea bottoms. Currently, only approximately one million insect species have been identified, and recorded, which accounts for only about twenty percent of the total insect species. Scientists are trying to discover and document the remaining eighty percent of insect species, aiming to fully understand and utilize their potential benefits (Stork, 2018). Insects compete with the human population and preserve their existence in the ecosystem throughout human history due to their special adaptation abilities regarding feeding, spreading, flying, and protecting against enemies (Pechenik, 2009).

While most of these insects have vital essential roles that are beneficial to the environment, indeed only a small portion of them is harmful and cause significant economic losses to people each year by infesting and damaging forests, greenhouses, ornamental plants, and mostly agricultural products both before and after harvesting. Therefore, this small group creates a considerable challenge in ensuring the efficient production of agricultural products. (Demirbag *et al.*, 2008).

1.2. Cherry Fruit Production Quality in Türkiye

Cherry fruit production quality in Türkiye has been the focus of several research studies, revealing key methods and insights for optimizing fruit quality. One important finding is that pre-harvest treatments, such as applying methyl jasmonate, can delay ripening and improve the overall quality of sweet cherry fruits by maintaining firmness and appearance, thus extending their shelf life (Ozturk *et al.*, 2019). Another effective approach

involves preharvest sprays of calcium chloride and sucrose, which reduce fruit cracking and enhance the quality of 'Burlat' sweet cherries, especially valuable during adverse weather conditions (Özkaya *et al.*, 2015). Additionally, studies on quality traits at different maturity stages in green plums, closely related to cherry fruits, suggest that maturity timing plays a significant role in achieving optimal taste and nutritional value (Ozturk *et al.*, 2019). Furthermore, research into physiological disorders emphasizes the importance of managing factors like temperature, humidity, and soil conditions to maintain high-quality cherry production (Saracoglu *et al.*, 2017). These findings collectively contribute to a better understanding of practices that support superior cherry quality in Türkiye.

1.2.1. Cherry Fruit Fly a Major Pest of Cherry Fruit

The family *Tephritidae* is a diverse group of flies, commonly known as fruit flies, comprising approximately 4,000 species in 500 genera worldwide. Among these species, the European cherry fruit fly (*Rhagoletis cerasi*) is an important member of the family. (Headrick & Goeden, 1998). The genus *Rhagoletis* includes around 65 species, most of which are oligophagous, targeting a limited range of closely related host plants. Besides the *R. cerasi*, other economically important species include the American cherry fruit flies (*Rhagoletis cingulate*, *Rhagoletis indifferens*, and *Rhagoletis fausta*), the apple maggot (*Rhagoletis pomonella*), the blueberry maggot (*Rhagoletis mendax*), and walnut pests (*Rhagoletis completa* and *Rhagoletis suavis*). *R. cerasi* primarily targets *Prunus* species, such as cherries, and *Lonicera* species. In Europe and Türkiye, this pest significantly damages cherry orchards, leading to substantial yield losses (Özbek Çatal & Ulusoy, 2018; Daniel & Wyss, 2010; Daniel & Grunder, 2012). Without chemical treatments, nearly all fruits are vulnerable to infestation. Even small levels of fruit infestation can lead to the rejection of entire batches due to the market's strict tolerance for damaged fruit, which allows a maximum of 2% infestation. Additionally, infested cherries are difficult to separate or remove from healthy ones, resulting in significant declines in market value and substantial financial losses for growers. These economic losses impact not only the growers but also other stakeholders across the supply chain. Adult cherry flies emerge from the soil as it warms, typically when the temperature reaches around 15°C, between late April and June. Approximately 10 days after emergence, they lay a single egg on the slightly reddened fruit of the nearest tree. The larval stage develops inside the fruit, after which the larvae drop to

the soil and enter the pupal stage. They overwinter as pupae, and the cycle begins anew (Wiesmann, 1935; Özbek 2017; Boller, 1966). Recent advancements in control methods focus on cultural and biotechnical approaches as well as environmentally friendly alternative substances.

Cherries are among the fruits people began farming a thousand years ago. They are flavorful and high in essential nutrients. Ripe, fresh cherries are consumed with great pleasure. Additionally, cherries are used to produce delicious cherry jam, marmalade, preserves, fruit juice, and other treats using various food industry methods or several basic cooking techniques. Cherry fruit is extremely beneficial to human health, being low in calories, and holds significant amounts of nutrients and bioactive food components, including essential vitamins, minerals, and plenty of dietary fiber. Additionally, although several studies suggest that some cherry varieties have effective potential natural pain-reducing properties, there is still not enough conclusive evidence regarding pain management (McCune *et al.*, 2010; Guo *et al.*, 2023; Diagnostiki *et al.*, 2010). Considering these benefits, reducing and resolving problems in cherry cultivation and other fruits creates a win-win situation for everyone involved, including farmers, consumers, and the environment.

Harmful insects are one of the significant concern factors in the decrease in cherry production. Some pests invade cherry plants and damage leaves, stems, fruits, and roots. The severity of the damage changes depending on the tree's overall health, the species of pest, and environmental factors. Several pests are causing a significant challenge to cherry fruit. Among these, the European cherry fruit fly (*R. cerasi* (L.) (Diptera: Tephritidae)) is the most critical pest that, during the larvae stage, eats the fruit flesh and turns the fruit into inedible and unmarketable products. The European cherry fruit fly, *R. cerasi*, is a significant pest for cherry growers. The feeding activity of the larvae leads to fruit that is often unfit for consumption or sale, rendering the fruit inedible and commercially worthless. Integrated pest management strategies are crucial to controlling the spread of this pest and minimizing damage to crops (Daniel & Grunder, 2012). It notes that crop-grazing rotational systems resulted in lower coccinellid species richness and that insecticide-use increased pest density. The negative impacts of agricultural intensification on insect communities are also discussed, and the use of sustainable pest control strategies is emphasized.

1.2.2. Environmental and Health Impacts

Even though various eco-friendly methods and strategies have been developed and implemented to combat insects that damage agricultural products, chemical insecticides, which have benefits and drawbacks, are still widely used by producers today (URL-1). Excessive use of chemical insecticides to protect plants disrupts the ecological balance of phytophagous and zoophagous species, causing increased pests and the emergence of chemical-resistant species. In addition, these chemicals have a lethal effect on non-target beneficial insects. Chemical substances that pollute and degrade the environment by mixing with water, air, soil, and food negatively affect human health with cardiological, embryological, mutagenic, and carcinogenic effects due to their high toxicity and accumulation in the body (Nicolopoulou-Stamati *et al.*, 2016). Due to these adverse effects and their expensive cost, numerous chemical pesticides have been banned, and new policies have been established to regulate insecticides. Consequently, researchers are investigating new solutions to manage cherry flies and other pest populations. In this context, biological control methods offer more promising achievements and advantages in long-term use than insecticides, so studies have accelerated.

1.2.3. Harmful Aspects of Chemical Control on Cherry Fruit Fly

The use of chemical control methods, particularly insecticides, to manage cherry fruit flies (*R. cerasi*) has long been a standard practice in agriculture. However, the reliance on these chemicals presents several harmful aspects that have significant environmental, health, and economic implications. This discussion explores the detrimental effects of chemical control on cherry fruit flies, emphasizing the need for more sustainable pest management approaches.

The use of organophosphate insecticides such as Guthion (azinphos-methyl) and Imidan (phosmet) has been a common practice in cherry orchards. These highly toxic chemicals can contaminate soil, water sources, and non-target vegetation, leading to biodiversity decline and ecosystem disruption. They can contaminate soil, water sources, and non-target vegetation, leading to a decline in biodiversity and disruption of ecosystems. The persistence of these chemicals in the environment can result in long-term ecological damage (Daniel *et al.*, 2012; URL-3). Moreover, organophosphates are known to affect beneficial insects, including pollinators like bees and natural predators of pests. The

reduction in these beneficial insect populations can lead to an imbalance in the ecosystem, exacerbating pest problems and reducing crop yields over time (Buda *et al.*, 2022). Human exposure to organophosphate insecticides poses serious health risks. These chemicals are neurotoxic and can cause acute poisoning, characterized by symptoms such as headaches, dizziness, nausea, and in severe cases, respiratory failure and death. Chronic exposure, even at low levels, has been associated with long-term health issues, including neurological disorders, developmental delays in children, and cancer (Daniel *et al.*, 2012). Farm workers and individuals living near treated orchards are particularly vulnerable to pesticide exposure. The improper handling and application of these chemicals can lead to accidental exposure, further increasing health risks.

The repeated and widespread use of chemical insecticides can lead to the development of resistance in pest populations. Cherry fruit flies, like many other pests, can evolve resistance to specific chemicals, rendering treatments ineffective over time. This necessitates the use of higher doses or the development of new, more potent chemicals, creating a cycle of dependency on chemical control methods (Andreazza *et al.*, 2017).

While chemical control methods can be effective in the short term, the long-term economic costs can be substantial. The need for continuous application of insecticides, coupled with the potential development of resistance, increases pest management expenses. Additionally, the negative ecological and human health impacts can lead to regulatory restrictions, further increasing the economic burden on growers (Aliniazei *et al.*, 1995).

1.3. Importance of Biological Control on Cherry Fruit Fly

Biological control is a crucial strategy in managing cherry fruit flies *R. cerasi*, pests that significantly impact cherry production. This method involves natural enemies, including predators, parasitoids, and pathogens, to suppress pest populations. The importance of biological control in managing cherry fruit flies lies in its environmental benefits, effectiveness, sustainability, and economic advantages.

Chemical pesticides, while effective, often have detrimental effects on non-target organisms, such as beneficial insects and wildlife, and can even pose risks to human health. Biological control, in contrast, provides a more environmentally friendly alternative. Biological control agents are typically specific to the target pest, minimizing the risk to other species. For example, entomopathogenic nematodes used against cherry fruit flies

specifically target the larvae without affecting other insects or wildlife (Heve *et al.*, 2017). Additionally, reducing the reliance on chemical pesticides decreases the potential for soil and water contamination, benefiting the broader ecosystem.

Biological control can be highly effective in managing cherry fruit fly populations when properly implemented. Biological control agents, such as parasitoid wasps and entomopathogenic fungi, are highly specific to their host pests. This specificity ensures that the control measures are focused on reducing the cherry fruit fly population without harming other beneficial insects (Kepenekci *et al.*, 2015).

Biological control agents can establish themselves in the environment and provide ongoing suppression of pest populations over time. This long-term control is often more sustainable than repeated chemical applications (Aliniaze *et al.*, 1995). The sustainability of biological control methods makes them a preferred choice for integrated pest management (IPM) programs.

Pests can develop resistance to chemical pesticides, rendering them ineffective over time. Biological control agents, on the other hand, co-evolve with their hosts, making it less likely for pests to develop resistance (Mihaela *et al.*, 2020).

By using natural predators and parasitoids, biological control helps maintain ecological balance. This approach supports biodiversity and the health of the agricultural ecosystem.

Biological control can offer long-term cost-effectiveness compared to chemical pesticides. While the initial investment in biological control agents may be significant, the sustained advantages include reduced need for repeated pesticide applications, which saves money over time (Yee *et al.*, 2014).

Crops produced with minimal chemical pesticide use can be marketed as more environmentally friendly, potentially fetching higher prices in the market. This is particularly important for consumers who are increasingly concerned about food safety and environmental impact (Niazee *et al.*, 1996).

Entomopathogenic nematodes represent an effective biological control strategy against cherry fruit flies, as they parasitize and kill the larvae in the soil. Research indicates that certain nematode species are highly effective in controlling these pests, offering substantial management benefits (Heve *et al.*, 2017). These nematodes can persist in the soil, providing long-term pest control and reducing reliance on chemical treatments. However, there are challenges associated with their use. Initial costs for introducing nematodes can be

high, and effective implementation requires technical expertise. Ecological factors like temperature, humidity, and soil conditions can also influence the effectiveness of nematodes. Continuous monitoring and evaluation are crucial for assessing the success of these biological control methods and making necessary adjustments, which demands ongoing effort and resources.

Biological control plays a crucial role in integrated pest management strategies for cherry fruit flies. Its environmental benefits, effectiveness, sustainability, and economic advantages make it an attractive alternative to chemical pesticides. By reducing reliance on harmful chemicals, biological control supports sustainable agriculture and helps maintain ecological balance. As research and technology advance, the implementation of biological control methods will likely become more widespread, offering even greater potential for effective pest control.

Insects are complex ecosystems that coexist with countless microorganisms hosted within their bodies. These microorganisms can inhabit a wide range of locations, from the external surfaces of insects to their internal organs and even their cells. The relationships between insects and microbes can vary, including mutualistic (providing mutual benefits), commensal (where one benefits without affecting the other), or parasitic (where one benefits at the expense of the other). These interactions play a crucial role in every stage of an insect's life cycle. For instance, microorganisms in the digestive system help insects break down hard-to-digest food. Some microorganisms protect insects from diseases or enhance their resistance to environmental challenges. In addition, insects serve as carriers for these microorganisms, transporting them to different environments and contributing to the increase in microbial diversity within ecosystems (Douglas, 2015; Chandler et al., 2011)

Traditional methods of studying insect-microbe interactions, such as culturing techniques, have been complemented by more recent high-throughput sequencing approaches. These methods, like 16S rRNA gene sequencing, allow researchers to analyze diverse microbial communities without the need for culturing. As technology continues to progress, more advanced techniques like metagenomics, metatranscriptomics, metametabolomics, and metaproteomics are becoming increasingly accessible. These techniques offer a comprehensive view of microbial communities, enabling the identification of both culturable and non-culturable organisms. By sequencing entire microbial genomes, researchers can gain insights into their genetic makeup, metabolic functions, and phylogenetic and taxonomic relationships. This deeper understanding of

insect-microbe interactions has the potential to revolutionize fields like agriculture, medicine, and environmental science (Knief, 2014; Bokulich et al., 2012).

For many years, microbial products have been used to attract insects, particularly in the fields of entomology and pest control. A notable example is torula yeast (*Candida utilis*), which has been applied as a food lure to trap adult tephritid fruit flies. However, it has been observed that *Torula* yeast and other food lures targeting tephritids also capture non-target organisms, including various saprophagous flies such as calliphorids, ceratopogonids, chloropids, drosophilids, lonchaeids, muscids, and sarcophagids (Landolt & Hammond, 2001; Leblanc *et al.*, 2010). This variety of attracted taxa highlights the effectiveness of yeast as an attractant, yet also poses challenges for achieving high target specificity. Fortunately, insects demonstrate species-specific responses to different blends of fermentation compounds (Landolt & Alfaro, 2001). Gaining deeper insights into insect microbiomes enables the development of biological methods for pest control. Many insect species establish close associations with specific microorganisms, which can directly influence their survival and reproductive success (Douglas, 2007; Bengtsson *et al.*, 2012). Therefore, insect-microbe interactions present significant potential for developing targeted pest management strategies. As a conceptual example, studies on the relationships between the codling moth (*Cydia pomonella*), certain yeast species, and a virus have facilitated the development of a novel control method against this pest (Knight & Witzgall, 2013).

1.4. Microbial Control on Insects

Microbial control, a component of integrated pest management (IPM), utilizes microorganisms like bacteria, fungi, viruses, and nematodes to control pest populations. By reducing the need for chemical pesticides, microbial control promotes sustainable agriculture and helps to conserve natural resources and protect the environment.

There are different types of microbial agents. These include bacteria that can infect and kill pests or inhibit their growth. For example, *Bacillus thuringiensis* (Bt) is widely used against various insect pests. Entomopathogenic fungi like *Beauveria bassiana* infect and kill insects by penetrating their exoskeleton. Insect-specific viruses, such as the nuclear polyhedrosis virus (NPV), can cause lethal infections in pest populations. Furthermore, nematodes such as those from the family *Steinernema* and *Heterorhabditis*, invade and eliminate insect larvae.

1.4.1. Bacterial Control of Cherry Fruit Fly

Bacillus thuringiensis (Bt) produces crystalline toxins, known as Cry proteins, which are consumed by insect larvae. These toxins disrupt the gut cells of the larvae, causing paralysis and death. Bt formulations can be sprayed onto cherry trees during the period when adult flies are active and laying eggs. The larvae that hatch from the eggs and feed on the treated fruit will ingest the Bt toxins and die.

Bacillus amyloliquefaciens is a valuable bacterium with multiple properties, including plant growth promotion (PGP) without side effects, functioning as a biofertilizer and a biocontrol agent, enhancing fruit preservation, and contributing to pest control strategies in agriculture (Luo *et al.*, 2022). Research has shown that treating litchi fruit with *B. amyloliquefaciens* LY-1 culture broth after harvest improves their quality and storage life, suggesting potential benefits for other fruits, including cherries (Wu *et al.*, 2017). While specific studies on cherry fruit fly control using *B. amyloliquefaciens* are limited, the positive effects observed in other fruits indicate potential for use in cherries.

Symbiosis hold a significant role in pest management through the integration of symbiotic bacteria into sustainable pest strategies. Many insects host diverse bacterial communities within their bodies, and these symbiotic relationships can have a profound impact on insect biology and behavior. The association between fruit flies and their gut microbiota is critical for understanding pest control mechanisms (Figure 1). Certain gut-associated bacteria can influence the susceptibility of fruit flies to microbial control agents (Broderick *et al.*, 2012). By manipulating the gut microbiota of cherry fruit flies, it might be possible to enhance their susceptibility to bacterial control agents. This approach requires further research to identify specific bacterial strains that can be used effectively.

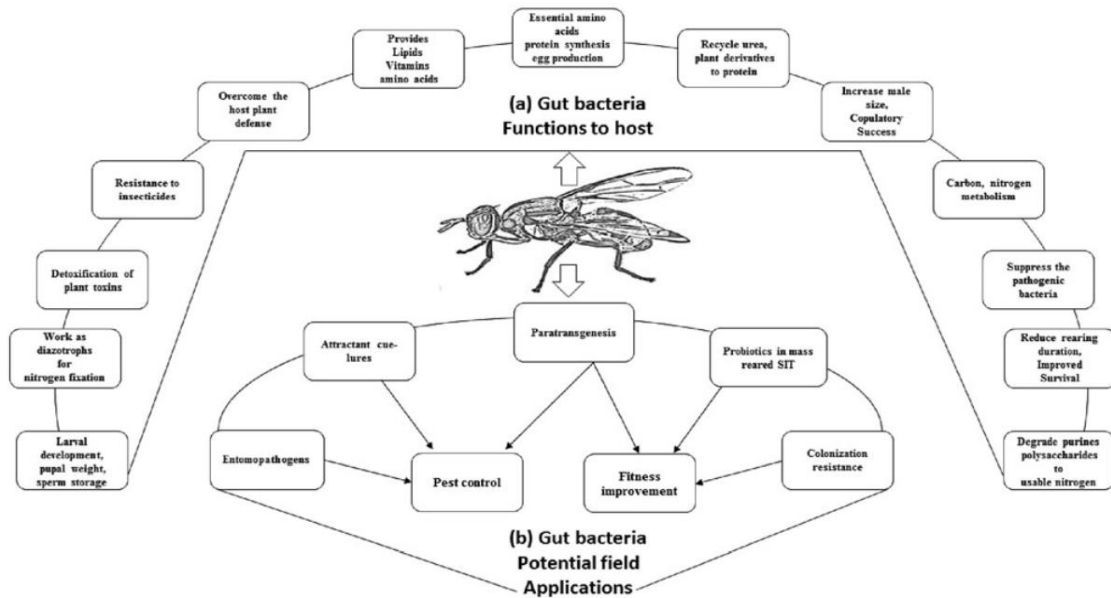


Figure 1. Roles of gut bacteria in Tephritidae fruit flies: host functions (a) and pest management applications (b) (Raza *et al.*, 2020)

(a) Gut bacteria enhance host nutrition, development, physiology, and resistance to pathogens and insecticides. (b) Outdoor applications: Entomopathogenic gut bacteria control pests; paratransgenesis uses bacteria to express beneficial or harmful molecules in the gut; colonization resistance disperses bacteria that inhibit parasites to improve host fitness; cue-lure-kill strategies and probiotics in SIT (Sterile Insect Technique) enhance the fitness of mass-reared flies.

1.5. Life Cycle of Cherry Fruit Fly

There are various cherry fruit flies but among them specifically *R. cerasi*, is a significant pest of cherry crops (Figure 2). Understanding its life cycle is crucial for effective pest management. This detailed overview covers the stages from egg to adult, emphasizing the biological and environmental factors influencing each phase.



Figure 2. Adult *R. cerasi*: Females (left) and males (right) measure 4-5 mm in length and exhibit a diagnostic combination of a black thorax, yellow scutellum, and specific wing venation (Daniel *et al.*,2012)

During the egg stage cherry fruit flies lay their eggs directly into the fruit of cherry trees. Female flies use their ovipositor to puncture the fruit skin and deposit a single egg per cherry. This stage is critical as it marks the beginning of the infestation process. Eggs hatch after about 10-14 days and it can vary with the timing affected by environmental conditions like humidity as well as temperature.

In the larval stage, upon hatching, the larvae (maggots) begin feeding on the flesh of the cherry. This feeding causes significant damage to the fruit, making it unmarketable. The larval stage lasts approximately four weeks. During this time, the larvae go through three instars (developmental stages) before reaching maturity. The larvae's feeding creates tunnels within the fruit, leading to rot and secondary infections. This damage can result in substantial economic losses for cherry growers.

Once fully grown, the mature larvae exit the fruit to pupate, transitioning into the pupal stage. They drop to the ground and burrow into the soil to form pupae. The pupal stage can vary significantly in duration. Most larvae pupate for about 8-10 months and emerge as adults the following season. However, some may enter a prolonged diapause, remaining dormant for up to two years, which helps the species survive unfavorable conditions (Moraiti, 2012)

In the adult stage, adult cherry fruit fly emerges from the soil in late spring to early summer, coinciding with the ripening of cherries. Adults are characterized by their black bodies with yellow markings and distinctive wing patterns. Shortly after emerging, adults engage in mating. Females begin laying eggs within a week of emergence, continuing the life cycle (Teixeira *et al.*,2014). Adult cherry fruit flies typically live for about a month,

during which time they can lay a significant number of eggs. The precise lifespan can vary based on environmental factors and availability of food sources.

The development and behavior of cherry fruit flies are heavily influenced by environmental conditions. Key elements like as temperature, the presence of host fruits and humidity determine the timing and success of each life stage. Warmer temperatures generally accelerate the development of eggs and larvae, leading to earlier and more synchronized adult emergence, while cooler temperatures can prolong development times and extend the pupal stage (Daniel *et al.*, 2009). Additionally, habitat-specific factors, including cherry variety and local climate, influence the life cycle. Variations in flight periods and reproductive maturity among populations of cherry fruit flies may reflect adaptations to their unique habitats (Teixeira *et al.*, 2014).

Understanding the life cycle of cherry fruit flies is essential for developing effective pest management strategies. By targeting specific life stages, growers can reduce the impact of these pests on cherry crops. Implementing sanitation practices, such as removing fallen fruits and tilling the soil to disrupt pupation, can help decrease cherry fruit fly populations. Additionally, utilizing natural enemies, like parasitoid wasps and entomopathogenic fungi, provides a biological approach to control cherry fruit fly larvae and pupae (Daniel *et al.*, 2009). While chemical insecticides are commonly used, their application must be carefully timed to target adults before they lay eggs, as overreliance on chemicals can lead to resistance issues and environmental concerns.

The cherry fruit fly's life cycle, from egg to adult, involves several stages that are influenced by environmental conditions and biological factors. Effective pest management requires a comprehensive understanding of these stages to implement timely and targeted interventions. By combining cultural, biological, and chemical control methods, growers can manage cherry fruit fly populations sustainably and reduce economic losses.

1.5.1. Life History

Traits of *R. cerasi*, like other oligophagous Tephritid species, are adapted to exploit resources that are predictable in time and space but available only briefly each year. As shown in Figure 3, these flies align their biology closely with their host's fruiting pattern and seasonality rather than focusing on high reproduction and mobility. They hibernate in the soil near their hosts, eliminating the need for dispersal flights. Their emergence and lifespan

are tightly linked to the phenology of their host plants. To mitigate the risk of host plants failing to fruit in a given year, pupae can remain dormant for two or more winters. *R. cerasi* produces one generation per year, with a long winter diapause. Females use visual and olfactory cues to locate oviposition sites and lay a single egg per fruit, marking the site with a pheromone to prevent overcrowding and ensure larvae have enough resources. Males typically control the oviposition sites, and mating often occurs through forced copulation without complex courtship behaviors.

The emergence and flight period of *R. cerasi* depend on spring soil temperatures, winter diapause conditions, and the geographic origin of the host plants. Forecasting models, developed in the 1930s and improved in the 1960s and 1970s (Baker *et al.*, 1978), are crucial for timing control measures, using soil temperature data at 5 cm depth, with emergence starting at 430 days above 5°C (Boller *et al.*, 1983). In the absence of models, pupae depots can be monitored (Russ *et al.*, 1973). Yellow sticky traps, placed in mid-May and examined twice weekly, are effective, especially those reflecting 485-500 nm and 365 nm (Agee *et al.*, 1982; Daniel *et al.*, 2014). As depicted in Figure 4 traps do not accurately indicate fruit infestation levels, so treatment decisions should consider expected yield and past infestation.

Before oviposition, *R. cerasi* adults undergo a temperature-dependent maturation period of six to 13 days, during which they must consume carbohydrates, proteins, and water for gonad maturation. Nutrients are sourced from bird droppings, honeydew, extrafloral nectaries, and bacterial colonies on leaf and fruit surfaces. This period, illustrated in Figure 5, is ideal for implementing control measures. Yellow sticky traps, effective for mass trapping, require one to eight traps per tree (Boller *et al.*, 1972), placed on the southeast canopy side. Due to labor intensity, this method is uneconomical for large-scale production but viable for home gardens and organic orchards. *Rhagoletis* flies also respond to red or dark-colored spheres similar in size to host fruit, though this method is less effective for cherry flies. Food baits, combining yeast hydrolysate and ammonia sources, can attract flies during the pre-oviposition period. These baits, when combined with insecticides like Spinosad (GF120), show promising results, though efficacy varies with climate. GF120 is effective in dry conditions but less so in humid areas, where CombiProtec performs better. Figure 6 shows the damage symptoms caused by *R. cerasi* larvae on cherries, illustrating the visible exit holes. Insecticide applications during this period are common, with Acetamiprid currently recommended in Switzerland following the ban on Dimethoate due to ecotoxicity.

concerns (Köppler *et al.*,2006). The fly's lifespan ranges from four to seven weeks, resulting in a total flight period of seven to 11 weeks.

Mating in *R. cerasi* occurs on sunny days with temperatures above 15°C, primarily on sunlit fruit. Males produce a species-specific pheromone that attracts females, but this pheromone has a short-range effect and acts more as an aphrodisiac (Hoffmeister,1992; Katsoyannos *et al.*,1982; Boller *et al.*,1976). The flies preference for sunny fruit areas facilitates aggregation, reducing the need for a long-range pheromone. Pheromone-based monitoring or control tools for *R. cerasi* are unavailable, and pheromones are not identified for other *Rhagoletis* species. Females need one to three copulations for high egg fertility. The sterile insect technique, developed between 1960 and 1980, faced challenges due to *R. cerasi*'s univoltine nature, obligatory diapause, and monophagy, preventing commercial use (Boller *et al.*,1983; Ranner *et al.*,1990).



Figure 3. The transformation of *R. cerasi* larvae into adult insects within the soil

Due to their overwintering habits beneath perennial hosts, *R. cerasi* adults rarely disperse far. Dispersal flights occur when cherries are unavailable, prompting females to leave in search of new hosts, followed by males (Wiesmann *et al.*,1935). Flies typically move only to nearby trees, with rare long-distance flights (Häseli *et al.*, 2005).

Oviposition in *R. cerasi* occurs on sunny days above 16°C, primarily around noon to early afternoon. Weather conditions during this period are crucial for population control, as prolonged fine weather increases oviposition activity. Females select fruits based on visual cues such as shape, size, and contrast (Katsoyannos *et al.*,1986; URL-2). They pierce the

fruit with their ovipositor, depositing a single egg and marking it with a pheromone to prevent further oviposition (Boller *et al.*,1976). Mechanical barriers like oil treatments can also prevent oviposition by altering the fruit surface, making it difficult for females to pierce the skin (Daniel *et al.*,2009).



Figure 4. Damaged cherries with exit holes of larvae (Daniel *et al.*, 2012)

Near harvest, mature larvae of *R. cerasi* bore through fruit skin and pupate in the soil within three hours. Pupation occurs at 2-5 cm depth, mainly under the tree canopy, especially on the south and southeast sides. Pupae overwinter in diapause, requiring about 180 days below 5°C for development (Boller *et al.*,1976). High mortality during diapause is common due to climate, predation, and parasitism, with 5-15% emerging the following year (Daniel *et al.*,2009). Soil treatments and netting under tree canopies can effectively reduce infestations by up to 91% (Katsoyannos *et al.*,1986).



Figure 5. Damage Symptoms, the exit holes are visible on infested cherries. Infested fruits often rot, are unmarketable as fresh fruit and may be rejected by processors.



Figure 6. Infested cherries (Daniel *et al.*,2012)

1.6. Insect-Microbe Interactions for Effective Management of Pest

Recent advancements in Tephritid fruit fly sterile insect technique (SIT) underscore the critical role of understanding insect-microbe interactions for effective pest management strategies. Early failures in *Bactrocera oleae* SIT programs in the 1980s were attributed to gaps in understanding the fly's biology and its interactions with microbes (Estes *et al.*,2012). *B. oleae* relies on its gut endosymbiont '*Candidatus erwinia dacicola*' for nutrition and to overcome host defenses (Ben-Yosef *et al.*, 2015). Wild *B. oleae* harbor diverse transient bacteria that likely contribute to their health. Maintaining these microbial interactions during mass rearing is crucial for producing competitive sterile males, essential for successful SIT implementation (Estes *et al.*,2012). Similar challenges and opportunities exist in *Ceratitis capitata*, where gut bacteria influence sterile male performance and could be leveraged through dietary interventions (Ami *et al.*,2010). Advances in genetic technologies like transposon vectors and CRISPR/Cas9 offer potential for future SIT and gene drive systems in *Drosophila suzukii*, highlighting the importance of optimizing mass rearing conditions (Schetelig and Handler, 2013).

Phagostimulants play a crucial role in enhancing the effectiveness of insecticides and microbial control agents that rely on ingestion (Knight *et al.*,2015). They can reduce the required amount of active ingredient, improving safety for workers and beneficial organisms. Studies with *D. suzukii* have shown that sucrose as a phagostimulant enhances the efficacy of both contact and ingestion-based insecticides (Cowles *et al.*,2015). Combining sucrose with yeast or yeast-like fungi further boosts the effectiveness of cyantraniliprole and spinosad (Knight *et al.*,2015). This approach could potentially support attract-and-kill

strategies against *D. suzukii*, highlighting its practical application in integrated pest management.

Tephritidae, a diverse family of fruit flies, includes numerous species that are significant pests of fruits and vegetables worldwide (Ben-Yosef, 2015). Among the approximately 4000 described species, those belonging to genera like *Anastrepha*, *Bactrocera*, *Ceratitis*, *Dacus*, and *Rhagoletis* are particularly notable for their economic impact on agriculture. Research has extensively explored the symbiotic relationships between these fruit flies and their gut bacteria, especially in species such as *Rhagoletis*, *Ceratitis*, *Bactrocera*, and *Anastrepha* (Naaz *et al.*, 2016).

The gut microbiota of these fruit flies, dominated by genera like *Klebsiella*, *Enterobacter*, *Pectobacterium*, *Citrobacter*, *Erwinia*, and *Providencia*, play crucial roles in their physiology and nutrition (Noman *et al.*, 2019). These bacteria have been implicated in various functions, including nutrient provisioning, protection against pathogens, and potentially influencing the host's behavior towards host fruits (Reddy *et al.*, 2014). However, the precise mechanisms and ecological roles of these symbionts in fruit fly biology and pest management strategies are still emerging.

While fruit flies rarely act as vectors for human or plant diseases, some species like the Mediterranean fruit fly (*Ceratitis capitata*) have been implicated in transmitting plant pathogens and potentially human pathogens to fruits (Sela *et al.*, 2005). This highlights the importance of understanding fruit fly microbiomes not only for agricultural pest management but also for assessing potential risks to food safety. Chemical pesticides, mass trapping and bait spray are commonly used to manage the most invasive fruit fly species, though many pesticides have developed resistance to insect, killing non targeted insects as well as create environmental hazard and mass trapping has lower field performance (Ras *et al.*, 2017). Therefore, alternative management strategies are now time demanding.

Research on tephritid fruit flies has revealed a rich diversity of bacteria crucial to their ecological interactions and biological. These microorganisms inhabit various parts of the fruit fly's life cycle, including larvae, pupae, exuviae, and adults, influencing their physiology and ecology. Initially, culture-dependent methods were used to identify bacterial species in fruit flies, but recent advances in molecular techniques have expanded our understanding of microbial diversity and community structures

Historically, bacteria such as *Pseudomonas* and *Enterobacteriaceae* species have been isolated from fruit flies like *Bactrocera* and *Rhagoletis*, playing roles in nutrition and

development of these insects (Prabhakar *et al.*,2009; Naaz *et al.*,2016). For instance, *Klebsiella*, *Enterobacter*, and *Erwinia* are common genera found in various tephritid species, contributing to host fitness and adaptation. Specific associations, such as *Klebsiella oxytoca* in Mediterranean fruit flies (*Ceratitis capitata*), highlight their importance in both wild and cultivated populations (Yuval *et al.*,2013; Raza *et al.*,2020).

Studies have also revealed differences in bacterial diversity between wild and laboratory-reared fruit flies, underscoring the dynamic nature of these symbiotic relationships (Kuzina *et al.*,2001). Despite these insights, gaps remain in understanding the full ecological roles of these bacteria in fruit fly biology and pest management strategies. Future research using advanced molecular tools will be crucial for unraveling these complexities and harnessing bacterial symbionts for sustainable pest control and fruit production practices.

1.7. Importance of Studying the Biocontrol Potential of Bacteria in the Cherry Fruit Fly (*Rhagoletis cerasi*) Microbiome

The cherry fruit fly *R. cerasi* is a major pest in cherry orchards, leading to significant economic losses. Traditional pest control methods, primarily relying on chemical insecticides, pose environmental and health risks. Therefore, sustainable and environmentally friendly alternatives are needed. One promising approach is to explore the biocontrol potential of bacteria within the fruit fly's microbiome.

- The use of bacterial biocontrol agents offers a sustainable alternative to chemical insecticides. By targeting specific pests without harming beneficial insects and the environment, this approach aligns with integrated pest management (IPM) principles.
- The reduction in chemical pesticide use is a significant benefit. Pesticides can have adverse effects on human health and non-target organisms, including beneficial insects, birds, and aquatic life. By identifying effective bacterial biocontrol agents, this study contributes to reducing reliance on harmful chemicals.
- The study provides insights into the complex interactions within the gut microbiome of *R. cerasi*. Understanding these interactions can reveal how certain bacteria influence the physiology and behavior of the fruit fly, which is crucial for developing targeted biocontrol strategies.

- The isolation and identification of novel bacterial strains with biocontrol potential provide new tools for managing *R. cerasi*. These bacterial strains can be used to develop biopesticides or be incorporated into other control methods, such as bait stations or soil treatments, enhancing the effectiveness of pest management programs.
- Effective biocontrol agents can significantly improve crop protection, leading to higher yields and better fruit quality. This is particularly important for cherry producers who suffer substantial losses due to fruit fly infestations. By protecting crops more efficiently, this research supports the agricultural industry's economic viability.
- The study adds to the growing body of knowledge regarding the role of microbiomes in pest control. By exploring the gut microbiome of *R. cerasi* and identifying bacteria with biocontrol properties, the research provides valuable data that can be applied to other pest species, broadening the scope of biological pest control.

1.8. Hypothesis, Aims and Objectives

The hypothesis of this study is that the gut microbiome of the cherry fruit fly (*R. cerasi*) harbors specific bacterial strains with entomopathogenic properties, which can be leveraged for effective biocontrol of this pest. These bacteria, when isolated and identified, are expected to exhibit significant insecticidal activity, reducing the population of cherry fruit flies without harming non-target organisms or the environment. The aim of the study is to explore and identify such bacterial strains within the gut microbiome of *R. cerasi* that possess entomopathogenic properties suitable for sustainable biocontrol applications. The study objectives include isolating and identifying bacterial strains from the cherry fruit fly microbiome, evaluating their entomopathogenic potential against larvae and adults, and assessing the environmental impact and non-target effects of these bacterial biocontrol agents.

2. MATERIALS AND METHODS

2.1. Collection of Cherry Flies

Larval samples of cherry flies (*R. cerasi*) were collected from Trabzon (41°00'18"N 39°43'21"E) in Turkey on June 3, 2021, and were subsequently stored at -80°C for preservation. In addition, adult cherry fly samples were gathered from Bilecik (40°08'35"N 29°58'45"E) on June 6, 2022, and from Tokat (40°18'50"N 36°33'15"E) on June 1, 2021. These adult samples were also stored at -80°C to maintain their integrity for further analysis.

The adult samples were collected using yellow adhesive traps, which are effective in capturing these insects. In addition to the adhesive traps, maggots and adult flies were collected directly from cherry orchards in Trabzon. After collection, the specific location and date of each sample were meticulously recorded. The larvae and adults were then carefully transferred to specialized containers and transported to the laboratory. Upon arrival in the laboratory, a thorough macroscopic examination was conducted. This involved sorting the specimens into categories based on their condition: dead larvae, live larvae, and adults. The separation of these categories was crucial for subsequent analysis and research.

2.2. Isolation of Bacteria from Insects

Dead and healthy *R. cerasi* larvae and adults were brought to the laboratory. Each specimen was placed in sterile Petri dishes and then surface-sterilized by rubbing with 70% ethyl alcohol for 5 minutes to ensure effective sterilization. Following this, the insects were transferred to petri dishes containing sterile distilled water and incubated for 2-3 minutes. This incubation period allowed for the removal of residual alcohol.

After the incubation, the specimens were thoroughly washed and removed from the alcohol. Subsequently, 1 mL of nutrient broth medium was added to each tube containing the specimens. The insects were then homogenized using a sterile homogenizer to create a uniform mixture. The homogenized mixture was filtered through cheesecloth and transferred to a microcentrifuge tube. From this filtrate, 10 µL, 50 µL, and 100 µL aliquots were plated onto three separate nutrient agar plates. These plates were incubated at 30°C for 2-3 days to allow for the growth of any microorganisms present. The remaining homogenized mixture

was stored at 30°C for 5-6 hours. This step was intended to increase the number of microorganisms present in the mixture, thus facilitating their detection in subsequent plating. Following this incubation, 10 µL and 50 µL aliquots from the incubated mixture were plated on nutrient agar media. Plates were incubated at 30°C. for 5 days.

2.3. Preparation and Stocking of Pure Cultures

Bacterial colonies that grew on the nutrient agar medium after incubation were examined under a binocular microscope. The colonies exhibited a range of colors and morphologies, indicating the presence of different bacterial species. To isolate these species, line transfers were performed using a sterile loop, carefully selecting individual colonies to obtain pure cultures. The pure cultures were then assigned unique identification numbers and stored in a sterile container containing 20% glycerol for preservation. This glycerol-containing solution was kept at -80°C to ensure the long-term viability of the cultures for use in subsequent studies.

2.4. Determination of Morphological and Gram Properties of Isolates

The colonies of all isolates were noted in terms of their color and shape. Secondly Gram staining is performed to all isolates.

Gram staining is a differential staining technique used to provide information about the cell wall composition of bacteria. This method differentiates bacteria based on the thickness of the peptidoglycan layer in their cell walls. Gram-positive bacteria have a thick peptidoglycan layer, which retains the *Crystal violet* stain and appears purple, whereas Gram-negative bacteria have a much thinner peptidoglycan layer and do not retain the *Crystal violet* stain, appearing pink after counterstaining. For Gram staining, each bacterial isolate was first cultured in nutrient broth and incubated in a water bath at 30°C for 16-24 hours to allow for growth. Bacterial smears were then prepared from these cultures. The prepared smears were stained using the following protocol:

- a- The smear was treated with *Crystal violet* dye for 1 minute, then washed with distilled water (dH₂O).
- b- The smear was then treated with iodine (Lugol's solution) for 1 minute, allowing the iodine to bind with the *Crystal violet* and form a *Crystal violet-iodine* complex.

- c- After iodine treatment, the smear was washed with acetone-alcohol until the excess dye was removed, taking care to stop the decolorization process promptly by washing with dH₂O.
- d- The smear was counterstained with safranin for 30-60 seconds and then washed again with dH₂O.

Once dried, the stained smear was examined under a microscope. Bacteria that retained the purple *Crystal violet* stain were classified as Gram-positive, while those that appeared pink due to the safranin counterstain were classified as Gram-negative. This staining method follows the protocol outlined by (Cappuccino and Sherman, 1992).

2.5. Determination of Physiological Characteristics of Isolates

2.5.1. Optimum Growth Temperatures

To determine the maximum and minimum growth temperatures for the bacterial isolates, they were cultured in nutrient broth medium and incubated under varying temperature conditions. Specifically, the isolates were incubated at temperatures of 20-30°C, 30-50°C, and below 20°C, with the following durations: 5 days for temperatures between 20-30°C, 14 days for temperatures between 30-50°C, and 21 days for temperatures below 20°C. These conditions were used to establish the range of temperatures at which the bacteria could grow, as outlined by (Sneath, 1968).

To identify the optimal growth temperatures for the isolates, nutrient broth was inoculated with cultures reconstituted to an optical density (OD) of 0.1 at 600 nm (OD₆₀₀). The inoculated cultures were incubated at temperatures of 28°C, 30°C, and 37°C. Samples were collected every hour from these cultures and analyzed using a spectrophotometer to measure the absorbance at OD₆₀₀. The temperature at which the bacterial cultures exhibited the highest absorbance values was identified as the optimal growth temperature, as described by (Cappuccino and Sherman, 1992).

2.5.2. pH Ranges

To determine the pH range in which the bacterial isolates can grow, they were tested across a spectrum of pH levels. Each isolate was inoculated into nutrient broth media adjusted to pH values of 3, 4.5, 6, 7, 8, 9, 10, and 11. The cultures were then incubated for

three days in an oven set at 30°C. After the incubation period, growth was assessed by observing the turbidity and general appearance of the cultures. The pH range in which growth occurred was identified based on these observations. If no growth was detected at certain pH levels, it was noted as a lack of reproduction under those conditions. This process follows the methodology outlined by (Cappuccino and Sherman,1992).

2.5.3. NaCl Tolerances

To determine the sodium chloride (NaCl) requirements of the bacterial isolates, nutrient broth media with varying concentrations of NaCl (1%, 3%, 5%, 7%, 9%, and 11%) were prepared. Each concentration was added to 4 mL of nutrient liquid media for experimentation. Each isolate was inoculated into tubes containing these media and incubated in an oven set at 30°C for 14 days. After the incubation period, growth was assessed by examining the turbidity of the cultures. Tubes with visible growth were compared to those without growth to determine the salt tolerance of each isolate. The proportion of NaCl that allowed for visible growth indicated the tolerance range of the isolates. This approach helps to establish the salinity levels at which the isolates can effectively grow, as detailed by (Sneath, 1968).

2.6. Determination of Biochemical Properties of Isolates

2.6.1. Starch Hydrolysis Tests

To determine whether the isolates can hydrolyze starch, starch agar media were prepared according to the protocol outlined in Annex-2. Each isolate was streaked onto Petri dishes containing the starch agar medium and incubated at 30°C for 3 and 7 days. After the incubation period, Lugol's iodine solution was added to the Petri dishes. The iodine reacts with starch, resulting in a dark brown color in areas where starch is present. A clear zone around the bacterial growth indicated starch hydrolysis, as the starch was broken down by amylase enzymes produced by the bacteria. In contrast, if no clear zone was observed and the medium remained blue, it signified that the starch had not been hydrolyzed. This method follows the procedure described by (Benson, 1985).

2.6.2. Catalase Tests

Bacteria produce hydrogen peroxide (H_2O_2) during aerobic respiration, and in some cases, they also produce toxic superoxides. To neutralize these potentially harmful byproducts, microorganisms produce enzymes such as catalase and peroxidase. To determine whether the isolates produce catalase, *Tryptic Soy Agar* medium was prepared. After inoculating the isolates onto Petri dishes containing this medium, they were incubated at 30°C for 24-48 hours. Following incubation, 3% hydrogen peroxide solution was added to the Petri dishes. The presence of catalase was indicated by the formation of gas bubbles, which confirmed a positive test result (Cappuccino and Sherman, 1992).

2.6.3. Oxidase Tests

To assess whether the isolates produce oxidase enzymes, *Tryptic Soy Agar* media were prepared. Each isolate was line-inoculated onto Petri dishes containing this medium and incubated at 30°C for 24-48 hours. After incubation, an oxidase test reagent was added to the Petri dishes. The appearance of a black color indicated a positive result for oxidase production (Benson, 1985).

2.6.4. Determination of Growth Abilities on MacConkey Agar

To evaluate the isolates' ability to grow on MacConkey agar, a sterile loop was used to inoculate MacConkey agar plates. The plates were incubated for 24 hours. Growth was observed and noted; if no growth occurred, it was recorded as such.

2.6.5. Identification of Bacterial Isolates with API 20E Panel Test System

API Test Kits provide a range of miniaturized biochemical tests and are evaluated using a standardized data system. For the identification of Gram-positive rod-shaped bacteria, the API20E and API50CHB (bioMérieux, France) test kits were utilized.

For the API 20E test, overnight cultures grown on nutrient agar were collected using a sterile loop. These cultures were then mixed thoroughly in 0.85% NaCl solution. The concentration of the culture was adjusted according to the McFarland standard to ensure accuracy. The prepared culture was then carefully dispensed into the wells of the API 20E

panel, taking care to avoid introducing air bubbles. The panels were incubated for 18-24 hours. The results were interpreted based on the color changes observed in the wells, which indicated whether the test results were positive or negative.

2.7. Determination of Molecular Properties of Bacterial Isolates

2.7.1. Genomic DNA Isolation

For genomic DNA isolation, bacterial cultures were prepared by inoculating 3 mL of Luria-Bertani (LB) liquid medium with the bacterial isolates and incubating overnight at 30°C. The isolation was carried out using the Promega Wizard Plus SV Minipreps DNA Purification System. After incubation, the cultures were transferred to Eppendorf tubes and centrifuged at 14,000 rpm for 2 minutes. The supernatant was discarded, and 480 µL of 0.5 M EDTA was added to the pellet, which was then vortexed to resuspend the cells. To lyse the cells, 120 µL of 10 mg/mL lysozyme was added to the resuspended pellet and incubated at 37°C for 1 hour. Following this, the suspension was centrifuged at 14,000 rpm for 1 minute, and the supernatant was discarded.

Next, 600 µL of "Nuclei Lysis Solution" was added to the pellet, mixed thoroughly, and incubated at 80°C for 5 minutes, then cooled. To remove RNA, 3 µL of 20 mg/mL RNase Solution was added to the mixture, which was then inverted 2-5 times and incubated at 37°C for 1 hour. Once the solution reached room temperature, 200 µL of "Protein Precipitation Solution" was added, vortexed for 20 seconds, and the mixture was placed on ice for 30 minutes. The solution was then centrifuged at 14,000 rpm for 3 minutes. The supernatant was carefully transferred to a new Eppendorf tube, and the pellet was discarded. To precipitate the DNA, 600 µL of isopropanol was added to the supernatant, and the solution was inverted until a pellet formed. The mixture was centrifuged at 14,000 rpm for 3 minutes, and the supernatant was removed. The DNA pellet was washed with 600 µL of EtOH and centrifuged at 14,000 rpm for 2 minutes. After removing the supernatant, the pellet was allowed to air-dry to remove residual ethanol. Finally, the DNA pellet was dissolved in 100 µL of "DNA Rehydration Solution" and incubated at 65°C for 1 hour, then stored at 4°C for preservation.

2.7.2. Amplification of the 16S rDNA Gene Region by PCR

To amplify the 16S rDNA gene region of the bacterial isolates, PCR was performed using the genomic DNA isolated from the samples. The specific primers used for the amplification were;

Forward primer: 5'- ATTCTAGAGTTTGATCATGGC -3',

Reverse primer: 5'- ATGGTACCGTGTGTGACGGGC -3'.

The PCR amplification was carried out using GoTaq DNA Polymerase (Promega) in a 200 μ L reaction volume within a Bio-Rad Thermal Cycler. The PCR products were then analyzed by running 5 μ L of the reaction mixture on a 1% agarose gel containing 0.5 μ g/mL ethidium bromide. The gel was visualized using a Gel Logic imaging system (Kodak), which allowed for the assessment of the PCR product size and verification of the amplification of the 16S rDNA gene region.

2.7.3. Ligation of 16S rDNA Gene Regions into Cloning Vector

The 16S rDNA gene regions amplified by PCR were cloned into the pGEM-T Easy Vector using the pGEM-T Easy Vector System (Promega). The cloning reaction was prepared with 10 μ L of 2X Rapid Ligation Buffer, 8 μ L of insert DNA, 1 μ L of pGEM-T Easy Vector, and 1 μ L of T4 DNA Ligase. The reaction mixture was incubated at 16°C for at least 16 hours. After the ligation, the product was stored at 4°C for subsequent use.

2.7.4. Preparation of Competent Cells

To prepare competent cells, 3 mL of overnight culture of *Escherichia coli* JM101, grown on Luria-Bertani (LB) medium, was inoculated into 30 mL of fresh LB medium. The culture was grown at 37°C until the optical density (OD₆₀₀) reached approximately 0.45-0.55. Once the desired cell density was achieved, the culture was transferred to a sterile Falcon tube and centrifuged at 4,500 rpm for 5 minutes at 4°C. The supernatant was discarded, and the pellet was resuspended in 10 mL of 100 mM cold CaCl₂, then kept on ice for 30 minutes.

After this, the cells were centrifuged again at 4,500 rpm for 5 minutes at 4°C, and the supernatant was discarded. The pellet was then resuspended in 2 mL of 1 mM cold CaCl₂. The prepared competent cells were incubated on ice for at least 2 hours before use.

2.7.5. Transformation of Recombinant Plasmids into Competent *E. coli* JM101

For transformation, 200 μL of competent *E. coli* JM101 cells and 3-4 μL of the ligation product were mixed in a sterile Eppendorf tube and incubated on ice for 30 minutes. Following this, the mixture was subjected to heat shock at 46°C for 2 minutes, and 1 mL of LB broth was added. The cells were then incubated at 37°C for 1 hour.

After incubation, the culture was centrifuged at 6,000 g for 3 minutes. The supernatant was removed, and the pellet was resuspended in 40 μL of LB agar containing X-Gal (40 mg/mL) and IPTG (24 mg/mL). The mixture was plated on LB agar with antibiotics to select for transformed cells. The plates were incubated at 37°C for 16 hours. Colonies were selected based on their color, with white colonies indicating successful transformation and the presence of the plasmid.

2.7.6. Screening the Colonies for Recombinant Plasmid

To isolate plasmid DNA from selected white colonies after transformation, LB-Amp liquid medium was inoculated with the colonies and incubated overnight at 37°C with shaking at 200 rpm. Plasmid DNA was isolated using the rapid miniprep method. The overnight cultures were centrifuged at 14,000 rpm for 3 minutes to pellet the cells. The supernatant was discarded, leaving approximately 50 μL of the pellet, which was then vortexed until fully resuspended.

To the pellet, 300 μL of TENS buffer (10 mM Tris, pH 8.0; 1 mM EDTA, pH 8.0; 0.1 N NaOH; 0.5% SDS) was added. Subsequently, 150 μL of 3 M sodium acetate (pH 5.2) was introduced, and the solution was inverted 4-5 times. The tube was kept on ice for 10-15 minutes before centrifugation at 14,000 rpm for 3 minutes. The supernatant was transferred to a new Eppendorf tube, and 900 μL of 96% ethanol was added to precipitate the plasmid DNA. After centrifugation at 14,000 rpm for 3 minutes, the supernatant was discarded. The pellet was washed with 70% ethanol, centrifuged at 14,000 rpm for 2 minutes, and the supernatant was discarded. The pellet was left to air-dry to remove residual ethanol, then resuspended in 30 μL TE buffer and treated with 3 μL of RNase to digest any residual RNA. The plasmid DNA was visualized by running 5 μL on a 1% agarose gel containing 0.5 $\mu\text{g/mL}$ ethidium bromide and imaged using a Gel Logic imaging system (Kodak).

To determine if the isolated plasmid DNAs contained the 16S rDNA gene region, plasmid DNA was digested with *EcoRI* restriction enzyme. The digestion reaction included 10 μL of DNA, 0.5 μL of *EcoRI* (Promega), 2 μL of 10X reaction buffer, and 7.5 μL of dH_2O , for a total volume of 20 μL . The reaction was incubated at 37°C for 2 hours. After digestion, 2-5 μL of loading dye was added and the mixture was incubated at 65°C for 10 minutes to inactivate the enzyme. The digested DNA was analyzed by electrophoresis on a 1% agarose gel to identify plasmids containing the expected DNA fragments.

2.7.7. Determining the Base Sequences of DNA Fragments Contained by Clones

Recombinant clones, confirmed by restriction enzyme digestion, were cultured overnight in 5 mL of LB-Amp broth at 200 rpm and 37°C. After incubation, the cultures were centrifuged at 14,000 rpm for 2 minutes. Plasmid DNA was isolated using the GeneJET Plasmid Mini Prep Kit (Fermantas). The concentration of plasmid DNA was determined by measuring absorbance at OD260. All DNA samples were adjusted to a concentration of 20 ng/ μL in a final volume of 20 μL . These samples were sent to Macrogen Inc. (Netherlands) for automatic DNA sequencing.

The base sequences of the 16S rDNA gene region were compared with sequences in public databases using the MEGA6 software. Sequence alignment was performed, and similarities were assessed using the Neighbor-Joining method with 1,000 bootstrap replicates for phylogenetic analysis. A phylogenetic tree was constructed to illustrate the relationships among isolates, with similarity values over 50% highlighted in the analysis (Kakani *et al.*, 2010).

2.8. Insecticidal Activity Assays

2.8.1. Preparation of Bacterial Suspensions

To assess the insecticidal activity of bacterial isolates from *Rhagoletis cerasi*, a semi-synthetic diet was prepared for *Galleria mellonella* larvae, consisting of 500 g bran, 200 g beeswax, 150 ml honey, 150 ml water, and 300 ml glycerin, mixed thoroughly according to Bronskill's method (Bronskill, 1961).

Bacterial isolates were cultured in 5 ml nutrient broth at 30°C for 24 hours in a shaker, then centrifuged at 3000×g for 10 minutes. The cell pellet was resuspended in 5 ml sterile phosphate-buffered saline (PBS), and bacterial suspensions were adjusted to an optical density of 600 nm to reach a concentration of approximately 1.8×10^9 bacteria/ml (Ben-Dov *et al.*, 1995).

The insecticidal effects were tested on *R. cerasi*, *T. molitor*, and *G. mellonella* larvae, using 30 larvae per bacterial treatment group for each insect species. Each treatment group was placed in 15 cm plastic containers, with separate treated and control groups. The treated groups were exposed to bacterial suspensions, while control groups were treated with sterile PBS under identical conditions. For *R. cerasi* larvae, fresh cherry fruits inoculated with the bacterial suspension were placed in plastic containers, into which five 3rd-instar larvae (starved for 3–4 hours) were introduced. For *G. mellonella*, special media inoculated with the bacterial suspension were placed in containers with five 2nd- or 3rd-instar larvae, also starved for 3–4 hours. For *T. molitor* larvae were placed in containers with wheat bran and small carrot slices soaked in the bacterial suspension. Five 3rd-instar larvae, starved overnight, were then added to each container.

The treated and control larvae were maintained in an incubator at optimal temperatures for each species (25°C for *T. molitor*, 28°C for *G. mellonella*, and 20°C for *R. cerasi*). Mortality was recorded at 24, 48, and 72 hours post-treatment. Dead larvae were removed daily to prevent contamination, and mortality rates were calculated.

All containers were incubated at 28°C with a 12:12 light-dark cycle and 60% humidity. Mortality was recorded daily for 15 days post-treatment. Dead larvae were removed daily to prevent contamination, and mortality rates were calculated. The control group for each insect species, treated with sterile PBS, was included to account for natural mortality.

Mortality rates were calculated at the end of the 15-day period using Abbott's formula to adjust for natural deaths observed in the control group (Abbott, 1925). This approach allowed for a robust evaluation of the insecticidal potential of each bacterial isolate as a biocontrol agent.

3. RESULTS

3.1. Isolation of Bacteria From *Rhagoletis cerasi* Larvae and Adults

In this study, *R. cerasi* were collected from cherry orchards in the Trabzon, Bilecik and Tokat regions. Samples were taken from larvae and adults, with 15 dead larvae and 12 adult flies. A total of 10 bacterial isolates were obtained from these samples. These isolates were further characterized by performing morphological, physiological, and molecular analyses, and their insecticidal activities were evaluated.

The bacterial isolates obtained from *R. cerasi* were labeled as Rc1, Rc2, Rc3, Rc4, Rc5, Rc6, Rc7, Rc8, Rc9, Rc10. Each isolate was subjected to detailed morphological and physiological testing, including Gram staining, and biochemical characterization. Their ability to act as biological control agents against *R. cerasi* was assessed through a series of insecticidal trials.

3.2. Morphological and Staining Characteristics of Bacterial Isolates from *R. cerasi*

The colony color and shape of the isolates obtained from *R. cerasi* were examined using a binocular microscope. According to the examination results, the isolates showed variations in color and morphology. The examination showed that the isolates exhibited different colony morphologies ranging from flat-round to wavy-round and smooth-rounded forms. Rc1, Rc2, Rc3, Rc4, and Rc5 were light cream-colored, flat-round colonies. Rc6, Rc7, Rc9 showed a transparent, wavy-round morphology, while Rc8 was mucous and smooth.

The cell wall shapes of the bacteria were determined through gram staining, and all results were summarized in Table 1.

Table 1. Gram staining and morphological characteristics of bacterial isolates from *R. cerasi*.

Isolate	Gram Stain	Bacterial Morphology	Colony Morphology
Rc1	Gram-negative	Bacillus (Rod-shaped)	Rod-shaped
Rc2	Gram-negative	Coccobacillus	Rounded / Ovoid
Rc3	Gram-positive	Bacillus (Rod-shaped)	Rod-shaped

Table 1 continued

Isolate	Gram Stain	Bacterial Morphology	Colony Morphology
Rc4	Gram-positive	Bacillus (Rod-shaped)	Rod-shaped
Rc5	Gram-positive	Bacillus (Rod-shaped)	Rod-shaped
Rc6	Gram-negative	Coccobacillus	Rounded / Ovoid
Rc7	Gram-negative	Bacillus (Rod-shaped)	Rod-shaped
Rc8	Gram-positive	Coccobacillus	Rounded / Ovoid
Rc9	Gram-negative	Coccobacillus	Rounded / Ovoid
Rc10	Gram-positive	Bacillus (Rod-shaped)	Rod-shaped

3.3. Physiological and Biochemical Properties of Bacterial Isolates from *R. cerasi*.

3.3.1. Temperature Tolerance

Temperature tolerance tests were conducted at various temperatures (20°C, 30°C, 37°C, and 45°C) to evaluate the growth of the isolates. The results showed that all isolates grew well at 20°C, 30°C, and 37°C. In addition, isolates Rc1, Rc7, and Rc10 showed poor or no growth at 45°C, indicating that these isolates are not heat-tolerant at high temperatures. Isolates Rc3, Rc4, Rc5, and Rc9 displayed moderate growth at 45°C, while Rc2 and Rc8 grew weakly at this temperature.

3.3.2. NaCl Tolerance

The bacterial isolates were subjected to varying concentrations of NaCl (3%, 5%, 7%, and 10%) to determine their salt tolerance. All isolates exhibited good growth in media containing 3% and 5% NaCl. At 7% NaCl, isolates Rc9 and Rc10 displayed weaker growth compared to the others. At 10% NaCl, isolates Rc1, Rc2, Rc4, Rc6, Rc7, and Rc8 showed strong growth, while Rc3, Rc5, and Rc9 demonstrated weak growth or no growth.

3.3.3. pH Tolerance

The pH tolerance of the isolates was tested at pH levels ranging from 3 to 11. None of the isolates grew at pH 3, indicating that highly acidic conditions are unsuitable for these bacteria. All isolates showed good growth at pH 4 to pH 9, with optimum growth between

pH 6 and pH 8. Isolates Rc9 and Rc10 displayed weak or no growth at pH 10, indicating a lower tolerance for highly alkaline environments.

3.3.4. Biochemical Properties

Biochemical tests, including the catalase, oxidase, and starch hydrolysis tests, were conducted to determine the enzymatic activity of the bacterial isolates. All isolates were catalase-positive, indicating their ability to break down hydrogen peroxide, except for Rc9, which was catalase-negative. Moreover, all isolates were oxidase-positive, suggesting their capability for oxidative metabolism. The ability to hydrolyze starch was demonstrated by isolates Rc1, Rc2, Rc4, Rc7, and Rc8, while Rc3, Rc5, Rc6, Rc9, and Rc10 showed negative results. Rc1, Rc2, Rc4, Rc6, and Rc7 showed positive growth on MacConkey agar, indicating their potential for lactose fermentation, while Rc9 and Rc10 showed limited growth.

The physiological and biochemical analysis of the bacterial isolates from *R. cerasi* shows that these isolates can grow under a wide range of environmental conditions, demonstrating their resilience and potential for application in biocontrol strategies. The Tables 2 and 3 summarize the physiological and biochemical properties of the isolates.

Table 2. Physiological properties of bacterial isolates from *R. cerasi*

Isolates	pH							Salt Test %						Temperature test °C				
	3	4	6	8	10	11		3	5	7	8	10			20	30	37	45
Rc1	-	+	+	+	+	+		+	+	+	-	-			+	+	+	+
Rc2	-	-	+	+	+	+		+	+	+	+	-			+	+	+	-
Rc3	-	-	+	+	+	-		+	+	+	+	-			+	+	+	-
Rc4	-	-	+	+	+	+		+	+	+	-	-			+	+	+	-
Rc5	-	-	+	+	+	-		+	+	+	-	-			+	+	+	-
Rc6	-	-	+	+	+	-		+	+	+	-	-			+	+	+	-
Rc7	-	-	+	+	+	+		+	+	+	+	-			+	+	+	-
Rc8	-	-	+	+	+	+		+	+	+	-	-			+	+	+	-
Rc9	-	-	+	+	+	+		+	+	-	-	-			+	+	+	-
Rc10	-	-	+	+	+	+		+	+	+	-	-			+	+	+	+

Table 3. Biochemical characteristics (catalase, oxidase, starch hydrolysis, and growth on MacConkey Agar) of the bacterial isolates obtained from *R. cerasi*

Isolates	Catalase Test			Oxidase Test			Starch Test			MCA			MSA		
Rc1		+			+			-		+			-		
Rc2		+			+			+		+			L+		
Rc3		+			+			+		+			L+		
Rc4		+			+			+		+			L+		
Rc5		+			+			-		+			-		
Rc6		+			+			-		+			-		
Rc7		+			+			-		+			+		
Rc8		+			+			-		+			-		
Rc9		-			+			-		+			-		
Rc10		+			-			-		+			L+		

- L+ = Low growth

3.4. API 20E Test Results

In this study, ten bacterial isolates from *R. cerasi* were tested using the API 20E panel (Figure 9). The results were analyzed using the API Software, which compared the biochemical profile of each isolate to known bacterial species in its database. The API 20E test involves 20 different biochemical tests. Some of the substrates are metabolized by the bacteria, leading to changes in color, which indicate a positive result. Reagents were added to certain wells to enhance the visualization of metabolic changes (e.g., TDA, JAMES, VP1-VP2, NIT1-NIT2).



Figure 7. API 20E Test System. The image shows the API 20E test panel used to identify the metabolic and biochemical properties of bacterial isolates from *R. cerasi*. Each well contains specific substrates to test various enzymatic activities and carbohydrate fermentation, with color changes indicating positive results.

[illegible]

Table 4 continued

Tests	Substrate	Activity	Rc1	Rc2	Rc3	Rc4	Rc5	Rc6	Rc7	Rc8	Rc9	Rc10
-IND	Indole	Indole production	-	-	-	-	-	-	-	-	-	-
VP	Na pyruvate	Acetoin production	+	-	+	+	+	+	+	+	+	-
GEL	Coal gelatin	Gelatinase	+	-	+	+	+	+	+	+	-	-
GLU	Glucose	Fermentation/oxidation	+	+	+	+	+	+	+	+	+	+
MAN	Mannitol	Fermentation/oxidation	+	+	+	+	+	+	+	+	+	+
INO	Inositol	Fermentation/oxidation	+	-	-	-	+	+	+	-	+	+
SOR	Sorbitol	Fermentation/oxidation	+	+	-	-	-	-	-	+	+	+
RHA	Rhamnose	Fermentation/oxidation	-	+	+	+	+	+	+	+	+	+
SAC	Sucrose	Fermentation/oxidation	+	-	+	+	+	+	+	+	+	+
MEL	Melibiose	Fermentation/oxidation	-	+	-	-	-	-	-	+	+	+
AMY	Amygdalin	Fermentation/oxidation	+	+	+	+	+	+	+	+	+	+
ARA	Arabinose	Fermentation/oxidation	-	+	+	+	+	+	+	+	+	+

The detailed identification results of isolates, and their characteristics, locations for each are as follows and their similarities are shown on the Table 5.

The isolate Rc1 was identified as *Serratia marcescens* with a 99% similarity. *Serratia marcescens* is a well-known bacterium often associated with environmental and opportunistic human infections. Rc2 was identified as *Lelliottia steviae* with a 99% similarity. *Lelliottia steviae* is a species from the *Enterobacteriaceae* family, commonly found in plants and environmental samples. Rc3 was matched to *Pantoea agglomerans* with a 99% similarity. *P. agglomerans* is frequently found in plant and soil environments and is often implicated in plant-associated microbial communities. Similarly, Rc4 was also identified as *Pantoea agglomerans* with a 99% similarity, suggesting that multiple isolates from the cherry fruitfly may belong to this species. Rc5 was identified as *Erwinia aphidicola* with a 99% similarity. *E. aphidicola* is typically associated with insects such as aphids, reflecting its potential involvement in the gut microbiome of insects like *R. cerasi*. Rc6 was also identified as *E. aphidicola* but with a 100% similarity, indicating a very strong match to this species. Rc7 was matched to *P. agglomerans* with a 99% similarity, consistent with other isolates (Rc3 and Rc4), further highlighting the prevalence of this species within the bacterial community of the cherry fruit fly. Rc8 was identified as *Rahnella aquatilis* with a 100% similarity. *R. aquatilis* is a waterborne bacterium often found in various environments, including soil, water, and plants. Rc9 was identified as *Raoultella ornithinolytica* with a 99% similarity. *R. ornithinolytica* is often found in environmental and clinical samples and is

known for its ability to metabolize a wide range of compounds. Finally, Rc10 was identified as *Enterobacter ludwigii* with a 99% similarity. *E. ludwigii* is commonly found in environmental and human-associated microbiota.

The results from the API 20E test indicate a diverse set of bacterial species inhabiting the gut of *R. cerasi*. The identified species are commonly associated with environmental, plant, and insect microbiomes, suggesting that the cherry fruit fly's gut harbors bacteria that may be involved in nutrient acquisition, defense against pathogens, or interaction with the host's biological processes.

Table 5. Identification of bacterial isolates from *R. cerasi* based on API 20E tests

Isolate	Similarity (%)	Closest Match (API 20E Test)
Rc1	99%	<i>Serratia marcescens</i>
Rc2	99%	<i>Lelliottia steviae</i>
Rc3	99%	<i>Pantoea agglomerans</i>
Rc4	99%	<i>Pantoea agglomerans</i>
Rc5	99%	<i>Erwinia aphidicola</i>
Rc6	100%	<i>Erwinia aphidicola</i>
Rc7	99%	<i>Pantoea agglomerans</i>
Rc8	100%	<i>Rahnella aquatilis</i>
Rc9	99%	<i>Raoultella ornithinolytica</i>
Rc10	99%	<i>Enterobacter ludwigii</i>

The API 20E test results provided a detailed metabolic and biochemical profile of the bacterial isolates from *R. cerasi* allowing for the identification of nine different species. These species are predominantly associated with environmental and plant-based microbiomes, suggesting their important role in the gut ecology of the cherry fruit fly. Further research on these isolates could provide insights into their functions in insect biology and potential applications in biocontrol strategies.

3.5. 16S rDNA Sequence Analysis of Bacterial Isolates from *R. cerasi*

The 16S rDNA sequence analysis is a powerful molecular tool used to identify bacterial species by analyzing the genetic sequences of the 16S ribosomal RNA gene, a highly conserved region in bacterial genomes. This method provides accurate and reliable identification of bacteria at the species level by comparing the obtained sequences to known sequences in databases.

In this study, the bacterial isolates from *R. cerasi* were subjected to 16S rDNA sequence analysis. Chromosomal DNAs were isolated from the isolates. The 16S rRNA regions of the obtained chromosomal DNAs were amplified by PCR and bands of approximately 1,500 bp in size were observed (Figure 10). The results, including the identified bacterial species and their similarity percentages, are provided below and in the Table 6.

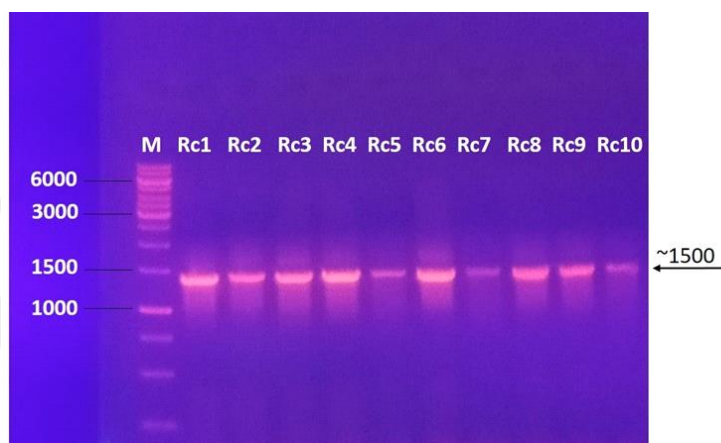


Figure 8. PCR amplification of 16s rDNA fragments from bacterial isolates of *R. cerasi*. The gel image shows the amplified 16S rDNA fragments (~1500 bp) from ten bacterial isolates (Rc1–Rc10) obtained from the gut of *R. cerasi*. M represents the molecular weight marker (ladder), indicating fragment sizes, with clear bands visible at approximately 1500 bp for each isolate, confirming successful amplification.

3.5.1. Identification of Each Isolate Based on 16S rDNA Analysis

The 16S rDNA sequencing results showed high similarity percentages for each bacterial isolate, confirming their identification with strong confidence. The isolates were matched to bacterial species with similarity percentages ranging from 99% to 100%, indicating a close genetic relationship between the isolates and their respective identified species.

Isolate which was labeled as Rc1 is identified as *Serratia marcescens*, *Serratia nematodiphila*, and *Serratia surfactantfaciens* with a similarity of 99%. These species are known for their association with soil, water, and plant environments. Rc2 is identified as *Lelliottia steviae*, *Lelliottia jeotgali*, and *Lelliottia nimipressuralis* with a similarity of 99%. These bacteria are part of the *Enterobacteriaceae* family and are often associated with plants. Rc3 is identified as *Pantoea pleuroti*, *Pantoea vagans*, and *Pantoea agglomerans* with a

similarity of 99%. These bacteria are commonly found in soil and plants and are involved in plant-microbe interactions. Rc4: Similar to Rc3, Rc4 was also identified as *Pantoea pleuroti*, *Pantoea vagans*, and *Pantoea agglomerans* with a similarity of 99%. Rc5: Identified as *Erwinia aphidicola*, *Erwinia persicina*, and *Erwinia rhapontici* with a similarity of 99%. These bacteria are known to interact with insects and plants, suggesting their potential role in the gut microbiome of *R. cerasi*. Rc6: Also identified as *Erwinia aphidicola*, *Erwinia persicina*, and *Erwinia rhapontici*, but with a higher similarity of 100%, indicating a nearly perfect match to the reference species. Rc7: Identified as *Pantoea agglomerans*, *Pantoea pleuroti*, and *Pantoea vagans* with a similarity of 99%, confirming the prevalence of these bacteria in multiple isolates from the cherry fruitfly. Rc8: Identified as *Rahnella aceris*, *Rahnella aquatilis*, and *Rahnella perminowiae* with a similarity of 100%. These bacteria are commonly found in aquatic and plant environments and can play a role in nitrogen fixation and plant growth promotion. Rc9: Identified as *Raoultella ornithinolytica*, *Raoultella planticola*, and *Raoultella electrica* with a similarity of 99%. These species are often found in environmental samples, including soil and water. Rc10: Identified as *Enterobacter ludwigii*, *Enterobacter sichuanensis*, and *Enterobacter pseudoroggenkampii* with a similarity of 99%. Members of the *Enterobacter* genus are common in environmental and plant-associated microbiomes.

The 16S rDNA sequence analysis confirmed the bacterial species associated with the gut microbiota of *R. cerasi*. The high similarity percentages (99% to 100%) indicate the reliability of the identifications. The bacterial species identified are predominantly associated with plant and environmental habitats, suggesting that these isolates may have a role in the nutrition or health of the cherry fruit fly.

Table 6. Results of 16s rDNA sequence analysis of bacterial isolates from *R. cerasi*

Isolates	Bacterial Type	Similarity (%)
Rc1	<i>Serratia marcescens</i>	99
	<i>Serratia nematodiphila</i>	99
	<i>Serratia surfactantfaciens</i>	99
Rc2	<i>Lelliottia steviae</i>	99
	<i>Lelliottia jeotgali</i>	99
	<i>Lelliottia nimipressuralis</i>	99

Tablo 6 continued

Isolates	Bacterial Type	Similarity (%)
Rc3	<i>Pantoea pleuroti</i>	99
	<i>Pantoea vagans</i>	99
	<i>Pantoea agglomerans</i>	99
Rc4	<i>Pantoea pleuroti</i>	99
	<i>Pantoea vagans</i>	99
	<i>Pantoea agglomerans</i>	99
Rc5	<i>Erwinia aphidicola</i>	99
	<i>Erwinia persicina</i>	99
	<i>Erwinia rhapontici</i>	99
Rc6	<i>Erwinia aphidicola</i>	100
	<i>Erwinia persicina</i>	99
	<i>Erwinia rhapontici</i>	99
Rc7	<i>Pantoea agglomerans</i>	99
	<i>Pantoea pleuroti</i>	99
	<i>Pantoea vagans</i>	99
Rc8	<i>Rahnella aceris</i>	100
	<i>Rahnella aquatilis</i>	99
	<i>Rahnella perminowiae</i>	99
Rc9	<i>Raoultella ornithinolytica</i>	99
	<i>Raoultella planticola</i>	99
	<i>Raoultella electrica</i>	99
Rc10	<i>Enterobacter ludwigii</i>	99
	<i>Enterobacter sichuanensis</i>	99
	<i>Enterobacter pseudoroggenkampii</i>	99

The 16S rDNA sequence analysis provided precise identification of the bacterial isolates from *R. cerasi*. These bacteria, which are primarily associated with environmental and plant habitats, likely contribute to the physiological functions and health of the cherry fruit fly. The presence of multiple species from the same genera across different isolates suggests a complex microbial community within the gut of *R. cerasi*. Further studies on the functional roles of these bacteria could enhance our understanding of their interactions with the host insect and their potential use in biocontrol strategies.

3.6. Phylogenetic Analysis of Bacterial Isolates from *R. cerasi*

The phylogenetic analysis of the bacterial isolates obtained from the gut of *R. cerasi* was conducted using the 16S rDNA sequences. The phylogenetic tree in the image illustrates the evolutionary relationships between the bacterial isolates (labeled as Rc1 to Rc10) and reference bacterial species from GenBank.

The phylogenetic tree is constructed based on sequence similarities between the 16S rDNA regions of the isolates and reference sequences. The tree is organized hierarchically, with branches representing different evolutionary lineages. Each node (branching point) in the tree represents a common ancestor, and the length of the branches indicates the evolutionary distance between species.

The bacterial isolates from *R. cerasi* (indicated by red dots) are grouped with closely related bacterial species from GenBank. The reference species are labeled with their respective accession numbers.

Rc1, a member of the *Serratia* group, clusters closely with *Serratia nematodiphila* and *Serratia surfactantfaciens*. This indicates that Rc1 shares a common evolutionary lineage with these *Serratia* species, which are commonly associated with soil, water, and insect habitats.

Rc8, classified within the *Rahnella* group, is closely associated with *Rahnella aquatilis* and *Rahnella perminowiae*. These species are known for their presence in water and soil environments, suggesting that Rc8 may have a similar ecological role.

Rc9, part of the *Raoultella* group, clusters with *Raoultella planticola* and *Raoultella ornithinolytica*, which are associated with environmental sources and plant microbiomes. This suggests that Rc9 may be part of the environmental microbiota associated with the cherry fruitfly.

Rc10, categorized in the *Enterobacter* group, shows a strong association with *Enterobacter ludwigii* and *Enterobacter sichuanensis*. These species are often found in soil and plants and can play a role in the gut microbiota of insects.

Rc3, Rc4, Rc7 classified within the *Pantoea* group. All isolates Rc3, Rc4, and Rc7 are closely related to *Pantoea agglomerans*, *Pantoea pleuroti*, and *Pantoea vagans*. These species are frequently found in plant environments and are known to form beneficial associations with plants, suggesting that these isolates may play a similar role in the cherry fruitfly's gut.

Rc5, Rc6 identified as part of the *Erwinia* group. Isolates Rc5 and Rc6 are closely grouped with *Erwinia aphidicola*, *Erwinia persicina*, and *Erwinia rhapontici*. These bacteria are often associated with insects and plants, indicating their potential role in symbiosis or nutrient cycling within the gut of *R.cerasi*.

Rc2, identified within the *Lelliottia* group and it clusters with *Lelliottia steviae* and *Lelliottia jeotgali*, suggesting that Rc2 may share evolutionary traits with these bacteria, which are often found in plant-associated environments.

The phylogenetic tree provides important insights into the evolutionary relationships of the bacterial isolates from *R. cerasi*. Several of the isolates, particularly those from the *Pantoea* and *Erwinia* genera, are closely related to bacteria commonly found in plant and soil environments. This suggests that the bacterial community in the cherry fruit fly gut may originate from the insect's interaction with its environment, including plants and soil. These bacteria may play roles in digestion, nutrient acquisition, or protection against pathogens in the gut.

The phylogenetic analysis demonstrates that the bacterial isolates from *R. cerasi* are closely related to environmental and plant-associated bacteria. The evolutionary relationships depicted in the phylogenetic tree suggest that these bacteria may contribute to the health and survival of the cherry fruit fly, potentially providing benefits such as nutrient acquisition or protection against harmful microbes (Figure 11). Further research could explore the functional roles of these bacteria within the insect's gut ecosystem.

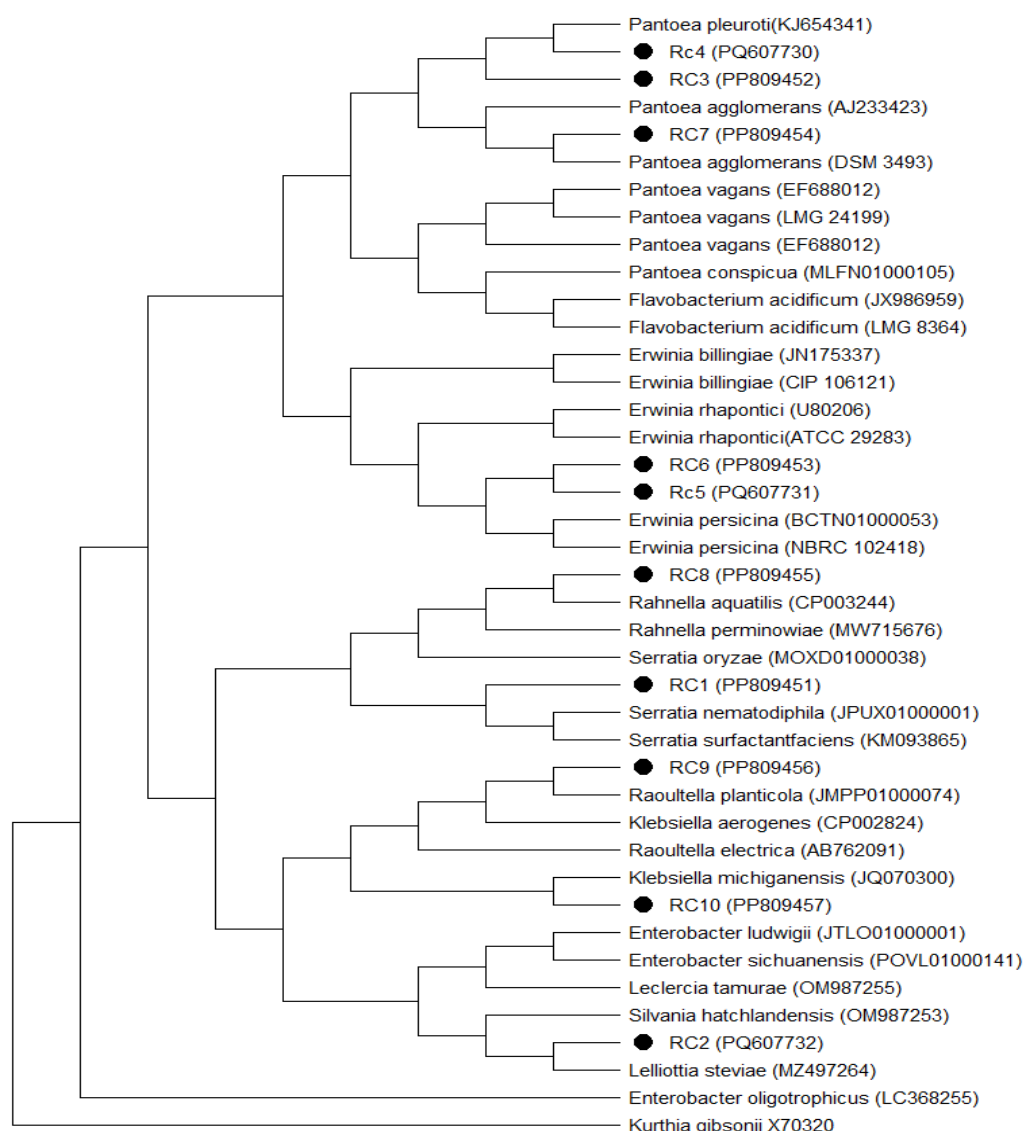


Figure 9. Phylogenetic tree of bacterial isolates from *R. cerasi* based on 16S rDNA sequence analysis. The phylogenetic relationships between the bacterial isolates (indicated by black dots) and reference bacterial species are shown. The tree was constructed based on 16S rDNA sequences, with branch lengths representing evolutionary distances. The bacterial isolates cluster with closely related species from genera such as *Serratia*, *Pantoea*, *Erwinia*, *Rahnella*, and *Raoultella*, suggesting their association with environmental and plant habitats.

3.7. Insecticidal Effects of Bacterial Isolates from *R. cerasi* on *Galleria mellonella* (Wax Moth)

The insecticidal effects of the bacterial isolates obtained from *R. cerasi* were tested on *Galleria mellonella* (wax moth) larvae. This bioassay serves as a model to assess the

potential of the isolates for use as biocontrol agents against insect pests. The experiment involved treating the wax moth larvae with the bacterial isolates and comparing the mortality rates between treated and control groups.

The table 7 summarizes the results of the insecticidal effects of the isolates on *G. mellonella*. The table lists the number of larvae that died and survived in both the treated and control groups, along with the calculated percentage of insecticidal effectiveness.

Table 7. Insecticidal effects of bacterial isolates from *R. cerasi* on *G. mellonella*

Isolates		Treated		Control		Percentage
Name	Bacterial Type	(Died)	(Live)	(Died)	(Live)	(%)
Rc1	<i>Serratia marcescens</i>	23	7	0	5	73
Rc2	<i>Lelliottia steviae</i>	18	12	0	5	53
Rc3	<i>Pantoea agglomerans</i>	13	17	0	5	34
Rc4	<i>Pantoea agglomerans</i>	16	24	0	5	30
Rc5	<i>Erwinia aphidicola</i>	20	10	0	5	61
Rc6	<i>Erwinia aphidicola</i>	11	19	0	5	27
Rc7	<i>Pantoea agglomerans</i>	12	17	0	5	32
Rc8	<i>Rahnella aceris</i>	15	15	0	5	42
Rc9	<i>Raoultella ornithinolytica</i>	19	11	0	5	57
Rc10	<i>Enterobacter ludwigii</i>	16	14	0	5	46

The isolates showed varying degrees of insecticidal activity against *G. mellonella* larvae:

Rc1 (*Serratia marcescens*) demonstrated the highest insecticidal effectiveness, with 73% of the larvae dying after treatment. *S. marcescens* is known for producing various toxins that are harmful to insect pests, explaining its strong insecticidal effect. Rc5 (*Erwinia aphidicola*) also showed strong insecticidal activity, with 61% mortality in the treated group. This suggests that *E. aphidicola* may produce substances toxic to insect larvae. Rc9 (*Raoultella ornithinolytica*) and Rc2 (*Lelliottia steviae*) showed moderate insecticidal effects, with 57% and 53% effectiveness, respectively. Isolates Rc6 (*E. aphidicola*) and Rc7 (*Pantoea agglomerans*) exhibited relatively low insecticidal effects, with 27% and 32% mortality, respectively. Other isolates, such as Rc3, Rc4, Rc8, and Rc10, showed intermediate levels of insecticidal activity, ranging between 30% and 46%.

The insecticidal bioassay using *G. mellonella* larvae demonstrated that several bacterial isolates from *R. cerasi* have significant insecticidal properties. In particular, isolates Rc1 (*S. marcescens*) and Rc5 (*E. aphidicola*) exhibited the highest effectiveness, making

them promising candidates for further exploration as biological control agents. The diversity in insecticidal activity among the isolates highlights the potential for these bacteria to be used in pest management strategies targeting various insect species. Further studies are needed to investigate the mode of action and specific compounds produced by these bacteria that contribute to their insecticidal effects.

3.8. Insecticidal Effects of Bacterial Isolates from *R. cerasi* on *Tenebrio molitor* (Mealworm)

The insecticidal effects of the bacterial isolates obtained from *R. cerasi* were tested on *Tenebrio molitor* (mealworm) larvae. This study aimed to assess the mortality rate of *T. molitor* after being exposed to different bacterial isolates, comparing the results to an untreated control group. The experiment was designed to determine the efficacy of the isolates as potential biocontrol agents.

The insecticidal effect of each isolate was measured by comparing the number of larvae that died and survived in both the treated and control groups. The effectiveness was calculated as a percentage based on the mortality in the treated group relative to the control group (Table 8).

Table 8. Insecticidal effects of bacterial isolates from *R. cerasi* on *T. molitor*

Isolates		Treated		Control		Percentage
Name	Bacterial Type	(Died)	(Live)	(Died)	(Live)	(%)
Rc1	<i>Serratia marcescens</i>	14	16	0	5	46
Rc2	<i>Lelliottia steviae</i>	12	18	0	5	40
Rc3	<i>Pantoea agglomerans</i>	6	24	0	5	20
Rc4	<i>Pantoea agglomerans</i>	7	23	0	5	23
Rc5	<i>Erwinia aphidicola</i>	10	20	0	5	33
Rc6	<i>Erwinia aphidicola</i>	9	21	0	5	30
Rc7	<i>Pantoea agglomerans</i>	8	22	0	5	26
Rc8	<i>Rahnella aceris</i>	8	22	0	5	26
Rc9	<i>Raoultella ornithinolytica</i>	13	17	0	5	43
Rc10	<i>Enterobacter ludwigii</i>	11	19	0	5	36

The results of the dosage trials show varying degrees of insecticidal activity among the isolates:

Rc1 (*S. marcescens*) showed the highest insecticidal activity, with 46% mortality in the treated group, indicating its potential as a strong biocontrol agent. Rc9 (*R.*

ornithinolytica) demonstrated 43% effectiveness, also showing a high level of insecticidal activity against *Tenebrio molitor*. Other isolates, such as Rc2 (*L. steviae*), Rc10 (*E. ludwigii*), and Rc5 and Rc6 (*E. aphidicola*), exhibited moderate insecticidal effects, with mortality rates between 30% and 40%. Rc3 and Rc4 (*P. agglomerans*) and Rc8 (*R. aceris*) displayed lower levels of insecticidal activity, with 20%, 23% and 26% effectiveness, respectively, indicating that their insecticidal effects may be weaker in comparison to other isolates.

The dosage trials using *T. molitor* larvae suggest that several bacterial isolates from *R. cerasi* possess insecticidal properties. The most effective isolates, such as Rc1 (*S. marcescens*) and Rc9 (*R. ornithinolytica*), could be further explored for their potential use in biocontrol strategies against pest species. The moderate and lower effectiveness of other isolates indicates variability in their insecticidal capabilities, which could be investigated in future studies to optimize their use in pest management programs.

3.9. Insecticidal Effects of Bacterial Isolates from *R. cerasi* on *R. cerasi* Larvae

The insecticidal effects of the bacterial isolates obtained from *R. cerasi* were tested directly on the larvae of *R. cerasi* to assess their potential for use as biological control agents. The trial involved exposing the larvae to different bacterial isolates and comparing the mortality rates of the treated larvae with those of an untreated control group. The results are presented as the number of larvae that died and survived in both the treated and control groups, with the overall effectiveness of each bacterial isolate calculated as a percentage. The table 9 summarizes the results of the dosage trials of bacterial isolates on *R. cerasi* larvae:

Table 9. Insecticidal effects of bacterial isolates from *R. cerasi* on *R. cerasi* larvae

Isolate	Bacterial Type	Treated (Died)	Treated (Live)	Control (Died)	Control (Live)	Effectiveness (%)
Rc1	<i>Serratia marcescens</i>	12	18	0	5	40
Rc2	<i>Lelliottia steviae</i>	10	20	0	5	33
Rc3	<i>Pantoea agglomerans</i>	5	25	0	5	17
Rc4	<i>Pantoea agglomerans</i>	6	24	0	5	20
Rc5	<i>Erwinia aphidicola</i>	8	22	0	5	27
Rc6	<i>Erwinia aphidicola</i>	7	23	0	5	23

Table 9 continued

Isolate	Bacterial Type	Treated (Died)	Treated (Live)	Control (Died)	Control (Live)	Effectiveness (%)
Rc7	<i>Pantoea agglomerans</i>	6	24	0	5	20
Rc8	<i>Rahnella aceris</i>	7	23	0	5	23
Rc9	<i>Raoultella ornithinolytica</i>	11	19	0	5	37
Rc10	<i>Enterobacter ludwigii</i>	9	21	0	5	30

The bacterial isolates showed varying degrees of insecticidal activity against *R. cerasi* larvae:

Rc1 (*Serratia marcescens*) exhibited the highest insecticidal activity, with 40% effectiveness in killing the treated larvae. This suggests that *S. marcescens* could be a potent biocontrol agent for controlling *R. cerasi* populations. Rc9 (*R. ornithinolytica*) also demonstrated strong insecticidal properties, with 37% effectiveness, indicating its potential as a biological control agent. Other isolates, such as Rc2 (*L. steviae*) and Rc10 (*Enterobacter ludwigii*), showed moderate insecticidal effects, with effectiveness ranging from 30% to 33%. Rc3 and Rc4 (*P. agglomerans*) exhibited relatively lower insecticidal activity, with effectiveness of 17% and 20%, respectively. Rc5 and Rc6 (*E. aphidicola*) had intermediate insecticidal effects, with 27% and 23% effectiveness, respectively. Rc7 and Rc8 showed similar effectiveness, both achieving 23% mortality rates in the treated larvae.

The results of the dosage trials indicate that certain bacterial isolates from *R. cerasi*, such as Rc1 (*S. marcescens*) and Rc9 (*R. ornithinolytica*), show significant insecticidal activity against *R. cerasi* larvae, making them promising candidates for the development of biocontrol agents. Other isolates, such as Rc2, Rc5, and Rc10, also demonstrated moderate insecticidal effects, suggesting potential for further exploration. While some isolates exhibited lower levels of effectiveness, they may still contribute to an integrated pest management strategy when combined with other control methods.

4. DISCUSSION

The cherry fruit fly is a major pest in cherry orchards across Europe and other regions, causing significant economic losses by infesting cherry fruits. Biological control strategies involving bacterial isolates from the insect's own microbiome present a promising approach to managing this pest. In this study, several bacterial isolates from *Rhagoletis cerasi* were tested for their insecticidal effects on larvae of *R. cerasi*, *Tenebrio molitor* and *Galleria mellonella* highlighting the potential use of these isolates as biocontrol agents.

The isolate Rc1, identified as *Serratia marcescens*, demonstrated the highest insecticidal activity, with a mortality rate of 73% on *G. mellonella*, 46% on *T. molitor* and 40% on *R. cerasi* larvae. This bacterium is well-known for its ability to produce a variety of toxic metabolites, including proteases, chitinases, and secondary metabolites like prodigiosin, which contribute to its strong insecticidal effects. The high mortality rates observed among the test insects suggest that *S. marcescens* may play an important role in disrupting the physiological processes of insects, possibly through the degradation of the gut lining or interference with metabolic functions. Several studies have highlighted the potent biocontrol potential of *S. marcescens* against other insect pests. Moreover, the bacterium, especially the WatG strain, plays a crucial role in the biological degradation of hydrocarbons and holds promise as a microorganism in combating oil pollution (Raymann *et al.*, 2018; Wongsu *et al.*, 2004).

Studies conducted in different countries have further demonstrated the versatility and efficacy of *S. marcescens* as a biocontrol agent. For example, in Türkiye, Danismazoglu *et al.* (2021) found that *S. marcescens* was effective against *Helicoverpa armigera*, a major pest in agriculture, with mortality attributed to the production of chitinases that degrade the insect cuticle. This insectistatic chitinase activity produced by *S. marcescens* exhibits similarities to the activity of purified chitinases expressed by the Chi60 and Chi70 genes of *Xenorhabdus nematophila*. Both types of chitinase have demonstrated approximately 80% lethal effects on *H. armigera*. This indicates a significant overlap in the biochemical properties and entomopathogenic effects of the chitinases produced by these two organisms. Moreover, *S. marcescens* could reduce populations of *Spodoptera frugiperda* (fall armyworm), one of the most damaging pests in maize production. It exhibits a synergistic effect when combined with the acetonic extract of *Jatropha curcas* seeds. This combination

exhibites a potent lethal impact on both the larvae and pupae of *S. frugiperda*, highlighting its potential as an effective biocontrol strategy (Valdez *et al.* 2024). Similarly, some researches highlighted the potential of *S. marcescens* in controlling *Aedes aegypti* and *Culex quinquefasciatus* mosquitoes, where the bacteria caused larval mortality through toxic metabolites and purified prodigiosin, showcasing its potential for mosquito management. Furthermore, it can effectively inhabit and proliferate in the adult mosquito's body (Ragvendran & Natarajan 2017). In addition to its insecticidal and virulent properties, *S.marcescens* secretes the protease known as serralysin in the form of an inactive enzyme. Serralysin exhibits entomopathogenic properties against scarab beetle larvae and shows immunosuppressive effects in silkworms, where it has been identified as a virulence factor (Jupatanakul *et al.* 2020). Mansour *et al.* (2023) investigated the larvicidal effects of *S. marcescens* on *Culex pipiens* mosquito populations in Egypt. They found significant mortality in the 3rd and 4th larval instars, which was associated with gut-degrading enzymes, further supporting the bacterium's potential control. In a similar context, *S. marcescens*, when combined with *Metarhizium anisopliae* (a fungus), exhibited a synergistic interaction, leading to a high virulence effect on *Blattella germanica* (German cockroaches). This interaction suppresses the expression of the Imd and Akirin genes responsible for antimicrobial peptide production in the cockroaches, disrupting their gut microbiota. As a result of this imbalance, the entry of the pathogen *Weissella cibaria* into the gut increases, accelerates the mortality rate of the cockroaches (Zhao *et al.* 2020).

The bacterium's diverse modes of action—including gut disruption, exoskeleton degradation, and interference with metabolic processes—demonstrate its adaptability and effectiveness in different regions and against a range of agricultural pests. Such comparative research highlights the promise of *S. marcescens* for integrated pest management globally.

The isolate Rc9, identified as *Raoultella ornithinolytica*, also exhibited strong insecticidal properties, showing 57% effectiveness against *G. mellonella*, 43% against *T. molitor*, and 37% against *R. cerasi*. *Raoultella* species have been associated with various environmental niches, including soil and plant surfaces, and are known to produce a wide array of enzymes capable of degrading insect cuticles. The relatively high mortality rates indicate that *R. ornithinolytica* may disrupt the protective barriers of insect larvae, leading to desiccation or secondary infections. Further research could investigate whether specific enzymes, such as lipases or proteases, are responsible for the observed insecticidal effects, as reported in similar studies on fruit fly-associated microbiomes (Prabhakar *et al.*, 2009).

Comparative studies conducted in different regions have highlighted similar insecticidal properties of *R. ornithinolytica* and related *Raoultella* species. For instance, a study in China by Li *et al.* (2021) found that *R. ornithinolytica* isolated from soil effectively reduced populations of *H. armigera* larvae, attributed to its production of lipases and proteases that compromised the insect's cuticle. Similarly, research conducted in India by Patel *et al.* (2022) showed that *R. ornithinolytica* caused significant mortality in *P. xylostella* larvae through enzyme activity that weakened the exoskeleton, leading to rapid desiccation. Additionally, *R. ornithinolytica* is among the bacteria that produce enzymes that cause raw milk to spoil. The bacteria have molybdenum-resistant, which gives them bioremediation properties that allow them to remove or reduce MoO_4^{2-} from molybdenum-contaminated environments and also has the ability to convert lignocellulose into ethanol. (Peil *et al.* 2016; Xing *et al.* 2023; Falade *et al.* 2017)

The researchers reported high larval mortality due to proteolytic enzymes produced by *R. ornithinolytica*, which disrupted the insect's cuticular integrity and led to dehydration. In Europe, *R. ornithinolytica* isolated from agricultural soils in Spain was shown by Garcia *et al.* (2022) to be effective against *Ceratitis capitata* (Mediterranean fruit fly) larvae, where mortality was linked to enzymatic degradation of the cuticle, confirming the bacterium's efficacy as a potential biocontrol agent in fruit fly management.

Studies in North America further emphasize the potential of *Raoultella* species in insect biocontrol. Research by Wilson *et al.* (2023) in the United States demonstrated significant mortality in *A. aegypti* mosquito larvae exposed to *R. ornithinolytica*, with lipases and proteases playing a major role in damaging the larval cuticle, making them vulnerable to environmental stressors. Additionally, in Canada, *R. ornithinolytica* isolated from forest soils showed high insecticidal activity against *Lymantria dispar* (gypsy moth) larvae, according to Roberts and Singh (2024), highlighting the potential for application in forestry pest control.

Both Rc2 (*Lelliota steviae*) and Rc10 (*Enterobacter ludwigii*) showed moderate insecticidal activity, with mortality rates of 53% and 46% on *G. mellonella*, 40% and 36% on *T. molitor*, and 33% and 30% on *R. cerasi*, respectively. *Lelliottia* species are typically plant-associated bacteria but have been found in the gut microbiome of insects, where they can produce bioactive compounds that affect insect development. Similarly, *Enterobacter* species are well-known for their potential to interfere with insect digestive systems by producing biofilm-forming exopolysaccharides, which can block nutrient absorption.

Studies on the gut symbionts of other fruit flies, such as the *C. capitata*, suggest that these bacteria could enhance insecticidal resistance or alter the gut environment in ways that negatively impact host survival (de Brida *et al.*, 2024).

Studies conducted in different countries provide further insights into the insecticidal potential of *Lelliottia* and *Enterobacter* species. In Brazil, de Brida *et al.* (2024) reported that *Lelliottia* strains isolated from the gut of *C. capitata* larvae could alter the gut environment in ways that interfere with nutrient uptake, ultimately leading to reduced larval survival. Similarly, research from China by Wang *et al.* (2023) demonstrated that *Lelliottia* species from crop soils exhibited insecticidal effects against *P. xylostella* larvae, attributed to the secretion of bioactive metabolites that disrupted the larvae's development.

Enterobacter species have also shown promise as insecticidal agents in various settings. In Kenya, Omondi *et al.* (2022) found that *E. ludwigii* isolated from maize fields reduced populations of *S. frugiperda* (fall armyworm), primarily by forming biofilms in the insect gut, which obstructed nutrient absorption and impaired growth. In Italy, Russo *et al.* (2023) studied *Enterobacter* strains isolated from olive groves and found significant larval mortality in *Bactrocera oleae* (olive fruit fly) through biofilm formation and enzyme secretion that disrupted digestion.

In the United States, studies on *Enterobacter* in *Aedes albopictus* mosquito larvae by Martinez and Smith (2024) highlighted the bacterium's ability to alter gut microbial communities, creating hostile environments for the host larvae and leading to increased mortality. Additionally, in India, *Enterobacter* strains isolated from agricultural soils were found by Prasad *et al.* (2021) to be effective against *H. armigera*, where biofilm production by the bacterium interfered with the larval gut lining and reduced feeding efficiency.

These studies illustrate the moderate but effective insecticidal activity of both *L. steviae* and *E. ludwigii* across various insect hosts and environments. The bioactive compounds and biofilm-forming abilities of these bacteria allow them to interfere with the digestive systems of insect pests, suggesting potential applications in pest management strategies that target gut disruption mechanisms.

The isolates Rc5 and Rc6, identified as *Erwinia aphidicola*, demonstrated moderate insecticidal activity, with mortality rates between 23% and 61%. *E. aphidicola* is an insect-associated bacterium commonly found in aphids, and it has been shown to produce bacteriocins and other toxins that can inhibit the growth of competing microbes or directly affect insect physiology. The insecticidal effects observed in this study may be due to the

production of such bacteriocins, which can disrupt gut microbiota balance, leading to impaired digestion and nutrient absorption in the host insect. Other researchers have reported similar findings, where gut-associated bacteria influence insect health and survival through the production of antimicrobial compounds.

Similar studies highlighted the role of *E. aphidicola* and related bacteria in pest control through microbial competition and toxin production. In Spain, Gutierrez *et al.* (2022) observed that *E. aphidicola* isolated from aphids effectively reduced populations of *Myzus persicae* (green peach aphid) by altering the gut microbiome balance and producing bacteriocins that impacted aphid survival. In China, Li *et al.* (2023) demonstrated that *E. aphidicola* strains were effective against *Spodoptera litura* larvae, where bacteriocin production interfered with larval digestion and nutrient uptake, ultimately leading to mortality.

In the United States, research by Hernandez *et al.* (2024) found that *E. aphidicola* isolated from local aphid populations could inhibit *Aedes aegypti* mosquito larvae through antimicrobial compounds that disrupted the larvae's gut microbiota, creating an inhospitable environment and weakening larval resistance to environmental stress. In Brazil, Oliveira and Santos (2023) studied *E. aphidicola* from aphid-associated microbiomes and noted its ability to reduce *Drosophila suzukii* populations by producing bacteriocins that impaired gut microbial communities, affecting insect health and development.

In addition, research from India by Kumar *et al.* (2022) reported similar effects of *E. aphidicola* on *H. armigera* larvae, where bacteriocins disrupted the larvae's gut microbiota, reducing digestive efficiency and growth rates. This study highlighted the potential of *E. aphidicola* in integrated pest management through microbial interactions that indirectly impair insect physiology.

These studies reinforce the potential of *E. aphidicola* as a moderate but effective biological control agent. Its ability to produce bacteriocins and other toxins not only affects insect gut microbiota but also limits competing microbial populations, creating a unique advantage in pest control strategies aimed at disrupting gut functions in target insect species.

Rahnella aceris (isolate Rc8) exhibited moderate insecticidal effects, achieving mortality rates of 42% in *G. mellonella*, 26% in *T. molitor*, and 23% in *R. cerasi*. This bacterium has notable plant growth-promoting abilities and pathogen protection capabilities, owing to its enzyme production, antimicrobial activity, adaptability, and low toxicity. Through the production of enzymes chitinases, proteases, and lipases, as well as

antimicrobial properties, *R. aceris* disrupts insect cuticle integrity and disturbs the balance of beneficial gut bacteria, thereby impacting insect health and protecting plants against pathogens without the use of potent toxins. Its adaptability across different environments makes it well-suited for long-term agricultural application. Although it may be slower-acting than some biocontrol agents, *R. aceris* can be an effective part of integrated pest management (IPM), especially when used alongside stronger agents such as *S. marcescens*. Its specificity and low toxicity make it safer for non-target organisms, offering a sustainable option for pest control.

Comparative studies have documented similar effects of *R. aceris* and related *Rahnella* species in pest management and plant health enhancement. In Canada, Johnson *et al.* (2022) investigated *R. aceris* as part of a biocontrol strategy against *Lymantria dispar* (gypsy moth) larvae. The study found that *R. aceris* induced moderate larval mortality through the production of cuticle-degrading enzymes, though at a slower rate than more aggressive agents. The bacterium's ability to protect plants while moderately controlling pest populations was noted as advantageous for IPM strategies in forestry.

In Italy, *R. aceris* was evaluated by Romano and Bianchi (2023) for its potential to control *Bactrocera oleae* (olive fruit fly) in olive groves. Their research highlighted the bacterium's chitinase and lipase activities, which weakened the fly larvae's protective cuticle, making them more vulnerable to environmental stressors. Additionally, the low toxicity of *R. aceris* allowed for safer application within olive orchards, supporting its suitability for sustainable pest management in sensitive agricultural settings.

In Japan, studies by Nakamura *et al.* (2021) focused on the use of *R. aceris* as a biocontrol agent in vegetable crops. The bacterium's dual ability to promote plant growth and moderately inhibit *Plutella xylostella* (diamondback moth) populations demonstrated its versatility. The antimicrobial compounds produced by *R. aceris* not only reduced insect populations but also protected plants from fungal pathogens, offering a twofold benefit for crop protection.

In the United States, *R. aceris* was studied as part of a biocontrol approach in apple orchards. Murphy and Green (2024) found that *R. aceris* effectively managed *Rhagoletis pomonella* (apple maggot) populations by affecting larval gut health through microbial disruption. Its moderate pest control ability, combined with plant-protective properties, was noted as beneficial for integrated approaches in orchards, especially where low-toxicity agents are prioritized.

Research from Brazil by Costa and Pereira (2023) similarly highlighted *R. aceris*'s adaptability in tropical agriculture. Applied to protect tomato plants from *S. frugiperda*, *R. aceris* contributed to plant health through pathogen resistance while offering moderate control of pest populations, suggesting potential for broader use in IPM.

These studies underscore *R. aceris* as a valuable component of sustainable pest management systems. Its moderate insecticidal effects, along with its plant growth-promoting and pathogen protection capabilities, make it an ideal candidate for use alongside stronger biocontrol agents. The bacterium's specificity and low toxicity enhance its safety for non-target organisms, supporting environmentally friendly pest management solutions across diverse agricultural settings.

Isolates Rc3, Rc4, and Rc7, all identified as *Pantoea agglomerans*, and exhibited lower insecticidal activity, with mortality rates ranging from 17% to 34% across among test insects. Although *P. agglomerans* is commonly found in association with plants and has been shown to produce antimicrobial compounds, its insecticidal effects appear to be less pronounced compared to other isolates. One possible explanation for this lower effectiveness could be that *P. agglomerans* primarily acts as a plant growth-promoting bacterium, and its role in insect-host interactions may be more subtle. Some studies have reported that *Pantoea* species can modulate insect immune responses, thereby reducing their effectiveness against pathogens, but this effect may not be strong enough to directly kill the host (Dutkiewicz *et al.*, 2016; Wright *et al.*, 2001; Raza *et al.*, 2022).

Different studies have documented the moderate to low insecticidal effects of *P. agglomerans*. In the United States, for instance, Douglas *et al.* (2023) evaluated *P. agglomerans* isolated from apple orchards for its impact on *R. pomonella* larvae. They found only minor mortality rates, with the bacterium's primary benefit being its protective effect on plants against bacterial pathogens, suggesting that its direct insecticidal action is limited but that it can still contribute to overall plant health.

In China, Liu *et al.* (2022) studied *P. agglomerans* as part of an integrated approach in rice fields to manage *Nilaparvata lugens* (brown planthopper) populations. Their findings indicated that while *P. agglomerans* caused low direct mortality in the planthoppers, it improved plant resistance against pathogens, which indirectly reduced pest populations by strengthening the plants' defenses.

In Kenya, Mwangi *et al.* (2021) investigated the use of *P. agglomerans* in maize to help control *S. frugiperda*. Although the bacterium displayed low mortality rates in the

larvae, it was shown to increase maize resilience to fungal infections, creating a more hostile environment for pests indirectly. This study supported the potential of *P. agglomerans* as an auxiliary agent in integrated pest management (IPM), where its primary role is plant protection rather than direct insecticidal action.

Research in Brazil by Silva and Oliveira (2023) explored the potential of *P. agglomerans* in soybean crops, targeting *Anticarsia gemmatilis* (soybean looper). Although direct mortality was low, the bacterium was found to reduce insect feeding and slightly weakened the pest's immune response, which could make insects more susceptible to other pathogens in the environment, supporting its use in IPM alongside more potent agents.

In Europe, González *et al.* (2024) tested *P. agglomerans* against *C. capitata* larvae in Spanish citrus orchards. The study concluded that while *P. agglomerans* alone was not sufficient to control fruit fly populations, its application supported plant health by reducing microbial infections and bolstering fruit quality, indirectly impacting pest levels.

These studies demonstrate that *P. agglomerans*, while not highly insecticidal on its own, can contribute to integrated pest management through its plant growth-promoting and pathogen-inhibiting properties (J *et al.*, 2017; Rezzonico *et al.*, 2009). Its subtle effects on insect immunity and overall plant resilience make it a useful adjunct in pest control systems, especially when combined with more aggressive insecticidal agents. This approach leverages *P. agglomerans*'s strengths in plant health support while relying on stronger biocontrol agents for direct pest reduction, aligning with sustainable and environmentally conscious IPM practices.

The varying degrees of insecticidal activity observed among the isolates can be attributed to several potential mechanisms. One mechanism involves the production of toxins. For example, *S. marcescens* is known to produce potent toxins that disrupt cellular functions in insects, leading to death. These toxins may target the insect gut lining, causing leakage of digestive fluids or impairing nutrient uptake. Another mechanism is enzymatic degradation, where many isolates produce enzymes such as proteases, lipases, and chitinases that can degrade the insect cuticle or disrupt gut integrity, leading to desiccation or facilitating infection by secondary pathogens. A further mechanism is interference with gut microbiota. Some bacteria may produce antimicrobial compounds that disrupt the balance of the insect's gut microbiome, making the insect more susceptible to infections or impairing digestion. This has been observed in other fruit fly species, where gut symbionts play a key role in the health and survival of the host.

The findings from this study suggest that certain bacterial isolates, particularly *S. marcescens* and *R. ornithinolytica*, hold great potential as biocontrol agents against *R. cerasi*. The moderate effectiveness of other isolates, such as *L. steviae* and *E. ludwigii*, also indicates their possible utility in integrated pest management strategies, particularly when combined with other biological or chemical agents. Further research is needed to explore the optimal application methods, such as the use of bacterial formulations or symbiotic introduction, to maximize the effectiveness of these bacterial isolates in controlling cherry fruit fly populations.



5. CONCLUSION

The research aimed to evaluate the insecticidal effects of bacterial isolates obtained from the gut of *Rhagoletis cerasi* on *R. cerasi* larvae, *Galleria mellonella* and *Tenebrio molitor* larvae. Ten bacterial isolates were tested for their insecticidal properties, and their effectiveness was assessed by measuring the mortality rates in treated and control groups.

Isolate Rc1 (*Serratia marcescens*) exhibited the highest insecticidal activity, with a mortality rates 46%, 73%, and 40% on *T. molitor*, *G. mellonella* and *R. cerasi* larvae. Its strong insecticidal effects are likely due to the production of toxins and proteases, making it a promising candidate for biocontrol.

Isolate Rc9 (*Raoultella ornithinolytica*) showed significant insecticidal activity with a mortality rates of 43% on *T. molitor*, 57% *G. mellonella* and 37% on *R. cerasi* larvae. The high effectiveness suggests that it may disrupt insect physiology, possibly by breaking down protective barriers or producing toxic metabolites.

Moderate insecticidal activity was observed with Rc2 (*Lelliottia steviae*) and Rc10 (*Enterobacter ludwigii*), achieving mortality rates between 53% and 30% among insects tested on both *T. molitor*, *G. mellonella* and *R. cerasi* larvae, indicating moderate effectiveness. These bacteria likely interfere with digestive processes or produce secondary metabolites that impair insect survival. Similarly, isolates Rc5, Rc6 (*Erwinia aphidicola*) and Rc8 (*Rahnella aceris*) showed intermediate insecticidal activity, varying from 61% to 23% effectiveness, respectively, suggesting potential as biocontrol agents, though they were less potent than *S. marcescens*.

Lower insecticidal activity was observed with isolates Rc3, Rc4, and Rc7 (*Pantoea agglomerans*), exhibiting mortality rates ranging from 17% to 32% across among test insects. Although less effective, these isolates may still play a role in modulating the immune response of insects or contributing to overall pest management strategies.

The study demonstrated that certain bacterial isolates from *R. cerasi*, particularly Rc1 (*S. marcescens*) and Rc9 (*R. ornithinolytica*), possess strong insecticidal properties and show promise as biological control agents for managing *R. cerasi* populations. Moderate insecticidal effects were observed for Rc2 and Rc10, while the other isolates displayed lower effectiveness. These findings suggest that these bacteria may play a role in integrated pest management strategies aimed at controlling cherry fruit fly infestations.

6. RECOMMENDATION

Based on the research findings, several key suggestions can be made to advance the application of bacterial isolates as biocontrol agents. First, further characterization of the most effective isolates, such as *Serratia marcescens*, should be conducted to better understand their insecticidal mechanisms. Investigating the production of secondary metabolites could reveal additional bioactive compounds that contribute to their effectiveness. Field trials are also necessary to evaluate the isolates' efficacy and environmental impact under real-world conditions, while exploring optimal application methods for use in orchard settings.

In addition, testing combinations of bacterial isolates could reveal synergistic effects that enhance insecticidal activity. Integrating these with other biocontrol agents could further improve pest control strategies. Monitoring for resistance development is crucial, and a rotational use strategy involving different bacterial strains should be implemented to mitigate this risk. Expanding host range studies to assess the impact on other pest species, while ensuring specificity to avoid harming non-target organisms, is also important.

Finally, initiating regulatory approval processes and forming partnerships with agricultural biotechnology companies will facilitate the commercialization of these bacterial isolates. Educating farmers and stakeholders about the benefits and proper use of these biocontrol agents will support their integration into sustainable pest management practices.

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