KARADENIZ TECHNICAL UNIVERSITY
INSTITUTE OF SCIENCE AND TECHNOLOGY

DEPARTMENT OF BIOTECHNOLOGY

IDENTIFICATION OF THE CHERRY FLY MICROBIOME AND DETERMINATION OF POTENTIAL MICROBIAL CONTROL AGENTS

MASTER THESIS

MAHDI NEAMAH SAHIB AL-SHAMMAA

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TRABZON

KARADENIZ TECHNICAL UNIVERSITY

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DEPARTMENT OF BIOTECHNOLOGY

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PREFACE

Focusing on a critical agricultural pest, the European cherry fruit fly, this thesis leverages advanced sequencing and bioinformatics technologies to explore its associated microbial communities. This research delves beyond mere identification, aiming to unlock the potential for sustainable cherry fly control, by pinpointing entomopathogenic members within the microbiome.

My fascination with the intricate relationships between insects and their microbial communities fueled this research. I am deeply grateful to my advisors, Prof. Dr. Zihni DEMIRBAG and Prof. Dr. Remziye NALCACIOGLU of Karadeniz Technical University, for their invaluable guidance and support. I sincerely thank Prof. Dr. Ingo EBERSBERGER for his expertise and mentorship during my training program in Germany and Dr. Saber DELPASAND KHABBAZI for his kind contribution regarding sample collection and RNA extraction. I would also like to thank Karadeniz Technical University Scientific Research Projects Coordination Unit (Project number: FBA-2023-10522) for financial support and the Department of Biology for allowing using infrastructure and laboratory facilities.

This journey would not have been possible without the contributions and collaboration of my colleagues. Their support has enriched this work immensely. With profound love and gratitude, I extend my heartfelt appreciation to my family my father, my mother, my brother, and my sisters for their unwavering support and belief in me. Their constant presence has been a beacon of inspiration throughout my academic journey. And I would like to express my sincere gratitude to all my friends for their unwavering support and encouragement. In particular, I want to thank and remember my dear friend, Hassan, whose absence I deeply feel.

My fervent hope is that this thesis paves the way for the development of environmentally friendly biological control strategies against the cherry fruit fly. The potential of manipulating insect microbiomes holds immense promise for sustainable pest management practices.

MAHDI NEAMAH SAHIB AL-SHAMMAA

Trabzon 2024

THESIS ETHICS STATEMENT

This study titled "Identification of the Cherry Fly Microbiome and Determination of Potential Microbial Control Agents" and presented as a Master's Thesis is written under my advisor's supervision. I hereby declare that I have completed this study under the responsibility of Prof. Dr. Zihni DEMİRBAĞ, that I have collected the data/samples myself, that I have performed the experiments/analyses in the relevant laboratories, that I have fully cited the information I have obtained from other sources in the text and bibliography, that I have acted in accordance with scientific research and ethical rules during the study process and that I accept all legal consequences in case of any contrary results. 03/05/2024

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ABSTRACT

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Cherry is a sweet and nutritious fruit grown all over the world. Türkiye has an important place in world cherry production and export. However, many factors negatively affect the yield and quality of cherry production. The cherry fruit fly (*Rhagoletis cerasi*) is a serious pest that causes big damage to cherry production and quality in Türkiye, as in almost every part of the world.

Besides the cultural measures and the use of sticky traps containing ammonium salt, chemicals are preferred for the control of this pest. However, the harm of chemicals to non-target organisms, the formation of chemical residues on the fruits, the resistance gained by insect pests against chemicals, and a problem in the viewpoint of organic cherry production led to a reconsideration of the chemicals. Biological control agents are an ideal alternative for controlling insect pests. Among biological pesticides, the place of entomopathogenic organisms (bacteria, fungi, nematodes, viruses, and protozoans) is undoubtedly the most important.

The current study aims to enlighten the microbiome of the cherry fruit fly for the elimination or reduction of the damage caused by this pest on cherries. In line with this aim, a total of 317 cherry flies from certain stations were collected, total RNA was extracted from larvae and adults of the insect, and high-efficiency sequencing (HTS) technology and bioinformatics tools were used for the detection of the microbiome of cherry fruit fly. Data analysis indicated that the most abundant entomopathogens in cherry fruit flies are

Pseudomonas fluorescens, Serratia entomophila, Serratia proteamaculans, Enterococcus gallinarum, Lysinibacillus sphaericus, and *Pseudomonas aeruginosa* as bacterial entomopathogens and also indicate other potential viral and fungal entomopathogens with less abundance. Classification of the cherry fruit fly microbiome regarding commensal, symbiotic, or entomopathogenic microorganisms was done by comparing it with information in the literature.

Keywords: Bioinformatics, cherry fruit fly, metatranscriptome, microbiome, *Rhagoletis cerasi*.

Yüksek lisans Tezi

ÖZET

KİRAZ SİNEĞİ MİKROBİYOMUNUN BELİRLENMESİ VE POTANSİYEL MİKROBİYAL KONTROL AJANLARININ TESPİTİ

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Kiraz, dünyanın her yerinde yetişen tatlı ve besleyici bir meyvedir. Türkiye, dünya kiraz üretiminde ve ihracatında önemli bir yere sahiptir. Ancak birçok faktör kiraz üretiminde verim ve kaliteyi olumsuz etkilemektedir. Kiraz meyve sineği (*Rhagoletis cerasi*) dünyanın hemen her yerinde olduğu gibi Türkiye'de de kiraz üretimine ve kalitesine büyük zarar veren ciddi bir zararlıdır.

Bu zararlı ile mücadelede kültürel önlemler ve amonyum tuzu içeren yapışkan tuzakların kullanımının yanı sıra kimyasallar da tercih edilmektedir. Ancak kimyasalların hedef dışı organizmalara zarar vermesi, meyvelerde kimyasal kalıntı oluşturması, zararlı böceklerin kimyasallara karşı direnç kazanması ve organik kiraz üretimi açısından sorun teşkil etmesi kimyasalların yeniden gözden geçirilmesine neden olmuştur. Biyolojik kontrol ajanları, böcek zararlılarının kontrolü için ideal bir alternatiftir. Biyolojik pestisitler arasında entomopatojenik organizmaların (bakteriler, mantarlar, nematodlar, virüsler ve protozoanlar) yeri şüphesiz en önemlisidir.

Bu çalışma, kirazlarda bu zararlının neden olduğu zararın ortadan kaldırılması veya azaltılması için kiraz meyve sineğinin mikrobiyomunu aydınlatmayı amaçlamaktadır. Bu amaç doğrultusunda, belirli istasyonlardan 317 kiraz sineği toplanmış, böceğin larva ve erginlerinden total RNA ekstrakte edilmiş ve kiraz sineğinin mikrobiyomunun tespiti için yüksek verimli dizileme (HTS) teknolojisi ve biyoinformatik araçlar kullanılmıştır. Veri analizi, kiraz meyve sineklerinde en bol bulunan entomopatojenlerin bakteriyel entomopatojen olarak *Pseudomonas fluorescens, Serratia entomophila, Serratia proteamaculans, Enterococcus gallinarum, Lysinibacillus sphaericus ve Pseudomonas*

aeruginosa ve aynı zamanda daha az bolluğa sahip diğer potansiyel viral ve fungal entomopatojenlerin olduğunu göstermiştir. Kiraz meyve sineği mikrobiyomunun kommensal, simbiyotik veya entomopatojen mikroorganizmalar açısından sınıflandırılması literatürdeki bilgilerle karşılaştırılarak yapılmıştır.

Anahtar Kelimeler: Biyoinformatik, kiraz meyve sineği, metatranskripttom, mikrobiyom, *Rhagoletis cerasi*.

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INDEX OF SYMBOLS AND ABBREVIATIONS

bp	: Base pair
BLAST	: Basic Local Alignment Search Tool
cDNA	: Complementary DNA
°C	: Degrees celsius
DNA	: Deoxyribonucleic acid
ddH_2O	: Double-distilled water
dNTPs	: Deoxyribonucleotide triphosphates
E-value	: Expect value
FASTQ	: File format for storing sequencing data
g	: Gram
µg	: Microgram
mg	: Milligram
ml	: Milliliter
mRNA	: Messenger RNA
NGS	: Next-generation sequencing
OD	: Optical density
PCR	: Polymerase chain reaction
q-PCR	: Quantitative PCR
RNA	: Ribonucleic acid
S_QC	: Sample QC results
STAR	: Spliced Transcripts Alignment to a Reference
TRI	: Trizol
Val.	: Integrity value
Vol.(elec.)	: Sample volume for electrophoresis

1. GENERAL INFORMATION

1.1. Introduction

Insects are important components of many ecosystems, where they perform many important functions. They aerate the soil, pollinate blossoms, and control other insects and plant pests. However, there are some groups of insects that consume forests, crops, ornamental plants, or stored products. Although less than 0.5% of identified insect species fall into this category, their serious harm leads to annual losses amounting to billions of dollars, posing a substantial threat to food security (Jankielsohn, 2018; Oliveira et al., 2014; Mereghetti et al., 2017).

In Türkiye, the sweet and sour cherry (*Prunus avium* and *Prunus cerasus*, respectively) harvests suffer substantial damage from the cherry fruit fly (*Rhagoletis cerasi*). Cherry fruit fly larvae feed on the growing fruit, resulting in a decline in fruit quality and financial losses. Cherry fruit fly infestations can significantly impact cherry orchards in Türkiye, with damage rates reaching as high as 80% during epidemic years, leading to substantial yield losses (Alaserhat, 2022). A recent study has demonstrated that cherry fruit flies may cause damage that reduces the size and weight of the fruit as well as causing discoloration and deterioration (Canbay & Alaserhat, 2024). This affects cherry production and the resulting material impacts on cherry growers and traders in Turkey, in addition to its impact on the cherry product and sales market due to the impact it causes on a decrease in the availability of the product. Many techniques are used to combat cherry fruit flies and limit their impact on crops. In Turkey, there are various strategies such as the use of pesticides, biological control, and cultural management methods (Özbek Çatal et al., 2023). However, the damage resulting from the use of pesticides on the environment and their impact on other species must be taken into consideration (Ankit et al., 2020). Finding sustainable and environmentally friendly methods to combat this species is a top priority because of its impact on cherry crops. The most environmentally acceptable methods for combating pests is biological control, which uses the natural enemies of pests to control them (Demirbag et al., 2008). This is considered a sustainable alternative to traditional pest control techniques and one of the most common alternatives. Recently, the pathogens of pests (such as viruses, bacteria, fungi, protozoa, and nematodes) are being studied. To know the possibility of using

it to control insect pests, the main benefit of this technology is its high effectiveness and the high specificity of not affecting other species.

Insect-associated microbiota is composed of complex microbial communities (bacteria, fungi, parasites, viruses, and nematodes) with various biological functions that deserve further exploration. Manipulation of insect microbiomes has recently emerged as a promising subject for research into innovative, ecologically friendly insect-control strategies (van Lenteren et al., 2018). In this thesis, we aimed to identify the microbiome composition of the cherry fruit fly (*R. cerasi*) to detect a potential control agent against the important target pest.

1.2. Importance of Cherry Crops and Their Products

Turkish and European economies both benefit significantly from the cherry industry. In 2018, Türkiye ranked first globally in cherry production, harvesting 639,564 tons, and fourth in sour cherry production with 184,167 tons. Türkiye exported 60,121 tons of cherries and 87 tons of sour cherries, generating approximately 160 million dollars in revenue (Alaserhat, 2022), making cherries one of the top fruits produced and exported from the nation. Cherries make up a significant portion of the fruit industry in Europe (Bujdosó, 2017), where they are produced and consumed mostly in United States, Italy, and Chile. The direct contributions of cherry production as well as the industry's indirect effects on other sectors account for the cherry industry's economic significance. For instance, cherry production helps associated sectors like processing and packaging while earning money for farmers. Additionally, the export of cherry products helps Türkiye and other European countries' overall trade balance.

The effects of insect pests and illnesses on cherry orchards are only two examples of the economic difficulties that the cherry industry faces. Cherry fruit flies, or *R. cerasi*, are a big problem in Türkiye since they can seriously harm cherry harvests and lower fruit production. As a result, there may be direct financial repercussions on cherry producers as well as indirect implications on the whole economy, such as decreased cherry product exports and higher cherry production costs.

Overall, the cherry business makes a significant contribution to the economies of Türkiye and other European countries, but it also has economic difficulties that need to be resolved if the sector is to continue making a positive impact on these economies.

1.3. Cherry Fruit Fly (*Rhagoletis cerasi*) and Its Importance.

In Europe and Asia, *R. cerasi* is regarded as a serious pest of cherry harvests. It causes damage to sweet and sour cherries. In adult *R. cerasi*, the body length can range from 3.5 to 5 mm. These little fruit flies have glossy, nearly black, dark brown bodies. The wings have four transverse black lines and are translucent as shown in Figure 1. (Wakie et al., 2018)

Figure 1. The adult stage of *R. cerasi*. showcases a female (left) and male (right) with distinct features. These include a bright black thorax, a yellow scutellum, and a characteristic wing pattern. The size of the males is approximately 4 mm, while females measure around 5 mm.

The fruit fly, *R. cerasi* is also referred to as the European cherry fruit fly. It is a member of the order *Diptera's Tephritidae* family. The Integrated Taxonomic Information System (ITIS) states that it has a widespread distribution range over Europe, North Africa, and certain regions of Asia (URL-1).

The species has been brought to North America, where it is a nuisance that has become invasive (Wakie et al., 2018). The complicated life cycle of *R. cerasi* comprises the development of the larvae inside the fruit of numerous hosts. In the fruit shortly before it ripens, adult flies lay their eggs. The larvae can seriously harm cherry harvests because they eat the fruits as it is shown in Figure 2. (Daniel & Grunder, 2012).

Figure 2. The larval stage of *R. cerasi* inside the cherry fruit.

Between late May and early July, adult cherry fruit flies can be found. They feed on insects or the sweet secretions produced by cherries themselves. Females lay 50–80 eggs individually in the fruit pulp after a period of 10–15 days. After 6–12 days, white, legless larvae emerge from the eggs, measuring 4-6 mm in length, and feed on the fruit pulp. When the fruit ripens, the larvae exit and pupate in the soil. The puparium is straw yellow, cylindrical, up to 4 mm long, and 2 mm in diameter as shown in Figure 3. Pupae of *R. cerasi*, where they overwinter. This species typically has one generation every one to two years (Daniel & Grunder, 2012).

Figure 3. The pupal of *R. cerasi*.

R. cerasi, which may seriously harm cherry orchards and lower fruit production, is a big problem for cherry producers in Türkiye and other European countries. Both the direct losses to cherry growers and the indirect effects on associated businesses and the entire economy can have major economic repercussions for the cherry industry.

Economic studies have been conducted to increase the impact of the cherry fruit fly pest in Türkiye and attempt to control it and the expenses related to it. These studies indicate that the direct losses of this species are represented by the impact of crops and making them unmarketable and unsellable, in addition to high annual expenses for trying to control the pest. This has an indirect impact on the economy in a way, such as a decrease in exports of cherry goods, an increase in manufacturing costs, its impact on workers and markets, and the impact of other species due to unsafe control methods and others (Alaserhat, 2022). In terms of the global impact of this pest, production economics assessment reports have indicated Cherry crop and products in United States and Globally, indicating the significant impact of this pest on the countries destined for cherries (Wakie et al., 2018), and therefore all these studies and reports indicate that the European cherry fruit fly is classified as one of the pests that have a clear and significant impact on the economics of cherry production. The world and Türkiye make research into developing effective strategies to combat this scourge a priority, such as biological control using entomopathogens (entomopathogens are insect's pathogenic microorganisms such as bacteria, viruses, fungi, and protozoans) to reduce this economic damage and maintain sustainable cultivation of the cherry crop in Türkiye and Globally.

1.4. Investigation of Management Strategies for Cherry Fruit Fly

Due to the European cherry fruit fly *R. cerasi* having an impact and harm on cherry production, it requires effective and appropriate control methods. Methods of controlling and repelling insect pests include methods such as chemical insecticides, cultural control, and biological control. The use of chemical materials represents a danger to other species due to their low specificity and negative impact on the environment. Its cost is high, but there is a possibility that the insect pest will develop resistance to these materials. As for cultural control, it is limited to changing the crop environment, removing rotten fruits, and other traditional manual methods that consume time and effort. On the other hand, biological control represents the use of natural hostility to insect pests. Entomopathogens such as The use of *Beauveria bassiana* and *Metarhizium anisopliae* (Būda et al., 2022; Daniel & Wyss, 2009) against European cherry fruit fly *R. cerasi*, have shown positive results in laboratory experiments, which give promising results for finding ways to target European cherry fruit flies in a highly effective, specific without harming non-target organisms, and environmentally friendly way, and confirms the advantages of using control chips. The biological use of entomopathogens against *R. cerasi* makes it one of the most effective methods in terms of integrated management of this pest.

1.5. Biological Control of Noxious Insects

In agriculture, biological control is defined as a method of using living organisms or their products to control a specific pest or plant disease (Van Driesche, R., & Bellows Jr, T. S. 2012). This strategy shows great success in controlling plant pests and diseases, and it is also an alternative with high positives compared to chemicals and other control methods. Without harm to the environment or to non-target organisms, in addition to its effectiveness in reducing insect pests acceptably and clearly, biological control represents the use of natural enemies, parasites, or entomopathogens in particular and their products are with significant advantages compared to other methods of controlling insect pests (Lacey et al., 2015).

1.6. Detection of Entomopathogens of The Target Insect

The detection of entomopathogens in target insects, such as the cherry fruit fly (*R. cerasi*), is critical for the development of effective biological control strategies. Various methods are utilized for this purpose, including conventional techniques like culturing, microscopic examination, and polymerase chain reaction (PCR), which provide valuable but time-consuming results requiring specialized skills. Culturing involves isolating and growing pathogens from the insect sample on specific media, allowing for their identification based on characteristic growth patterns and morphology (Thiery & Frachon, 1997). Microscopic examination allows for the visualization of pathogens using microscopy techniques, providing insights into their morphology and structure (Bich et al., 2021). PCR amplifies specific regions of the pathogen's DNA, enabling its detection through the amplification of characteristic genetic markers (Khashaba et al., 2020). These methods have been fundamental in the initial identification and characterization of entomopathogens but may have limitations in terms of sensitivity and specificity.

In contrast, modern molecular techniques like metagenomics offer a more comprehensive and high-throughput approach to detecting entomopathogens' nucleic acid sequences with high sensitivity, even in low concentrations (Crandall et al., 2020). Metagenomics involves the direct sequencing of genetic material from environmental samples, bypassing the need for culturing or isolation (Hiebert et al., 2020). This approach allows for the detection of a wide range of microorganisms present in the sample, including both known and novel species, providing a more holistic view of the microbial community. Metagenomic data can be analyzed using bioinformatics tools to identify and characterize entomopathogens based on their genetic signatures, offering insights into their diversity, abundance, and potential interactions with the host and other microorganisms (Picciotti et al., 2023).

Detecting and diagnosing entomopathogens and diagnosing the activity of the pathogen in the target insect pest, such as (*R. cerasi*) is considered the cornerstone for the application of biological control techniques. Entomopathogens are discovered and studied using various techniques, as well as traditional manual laboratory techniques, which give good results, but require specialized skills, effort, and a relatively long time, these techniques include laboratory cultivation, microscopic detection, and polymerase chain reaction. Laboratory cultivation involves isolating and growing the target microorganisms in selected

culture media and studying their shape, patterns, and distinctive phenotypic properties (Thiery & Frachon, 1997). Microscopic examination includes isolating the microorganisms and studying them under the microscope. Their properties are also distinguished based on their shape and apparent properties in terms of shape and structure (Bich et al., 2021), while the polymerase chain reaction is considered a relatively contemporary technique, as the target microorganisms are detected by amplifying specific genetic regions and specific genetic markers in the targeted microorganisms (Khashaba et al., 2020), Although these techniques are effective in many cases, it should also be taken into account that they have limitations in terms of sensitivity and specificity during the detection process.

The latest developments in molecular technologies in recent times are represented by Omics techniques and methods, such as metagenomics, metatranscriptomics, and other omics techniques, these techniques offer significant advantages, such as high sensitivity and the ability to work with small samples. They also minimize the risks associated with traditional culturing methods, ensuring more accurate detection of microorganisms. (Crandall et al., 2020). For example, studying the metagenomics of environmental samples provides a flexible and relatively fast technique with high specificity and sensitivity. The microbial community of a sample containing thousands or millions of microbes is detected through One Workflow, which gives it an additional advantage because it comprehensively detects all microorganisms, including newly discovered ones if databases with the latest updates are used (Crandall et al., 2020). In addition, these technologies provide detailed information about these microorganisms and study the characteristics of the target microorganisms and their interactions and activities with the rest of the microbial community and with the host (Picciotti et al., 2023).

1.7. Next-generation Sequencing for Detection and Profiling of Microorganisms of Insects

Next Generation Sequencing (NGS) term refers to advanced technologies that provide the possibility for scientists to obtain huge DNA or RNA sequence data in a relatively short time. NGS includes different technologies and systems, the most popular NGS systems are Illumina, Ion Torrent, PacBio, and Oxford Nanopore, but it is considered the most widely used and common technology in the world is Illumina, as 90% of the sequence data was produced by Illumina platforms (Hu et al., 2021).

Illumina sequencing relies on the principle of sequencing using synthesis. This technique sequences DNA fragments in parallel on a flow cell using reverse terminators marked with fluorescent markers. A nucleotide is added in each sequencing cycle, and the final base is detected based on the fluorescent signals. It is considered this is part of a group of other techniques, such as amplification, partitioning, and evaluation of fragments, and the creation of libraries through this process is carried out with Illumina sequencing which provides accurate, high-throughput, and economical sequencing of large sequences of DNA or RNA, which is ultimately represented in the form of transcriptomes or genomes data ready to be studied using bioinformatics techniques and tools, which has revolutionized various fields of science, including medical, forensic, and agricultural sciences and research (Modi et al., 2021).

In terms of research and development in the field of biological control, represented by the filtration and detection of microorganisms, especially entomopathogens, NGS is considered one of the most effective or most efficient techniques in this field for detecting the microbial composition of insects (Greay et al., 2018), identifying factors for biological control, and simplifying their study and detection, especially in samples that It is difficult to work with traditional laboratory techniques, and this does not deny that NGS does not have difficulties, such as its need for high-quality DNA or RNA samples. It is also considered a relatively expensive process, with the need for experience and high skills in the field of bioinformatics to analyze and study the data produced by these techniques. NGS, as well as the need to confirm discoveries and results using the laboratory in subsequent steps (Paula, 2021), and among the data provided from NGS techniques, the complete RNA data represented by Metatranscriptomic is considered one of the most important and most useful methods with the ability to detect active agents and detect all types of microorganisms, including both DNA and RNA-based pathogens that are candidates for biological control, in addition to providing an insight into its activity, effectiveness, and interaction with other microbes and the host environment (Douglas, 2018).

1.8. Metatranscriptome

The term metatranscriptome refers to the study of the complete set of mRNA molecules within a population of organisms at a specific stage and time using advanced sequencing techniques such as NGS. The field of metatranscriptome is considered one of the most important fields for studying the factors (mRNA) that are most important and influential for gene expression, and functional protein synthesis, which makes the field of metatranscriptome of high importance in biological studies and research (Mason et al., 2012; Di Bella et al., 2013).

Metatranscriptome sequencing includes many steps and processes to produce high-throughput data and accurate, detailed data. These processes include reverse transcription-polymerase chain reaction (RT-PCR), microarrays, and NGS such as RNA-seq and single-cell (Chung et al., 2020). This approach generates metatranscriptome sequence data, which is then analyzed using advanced bioinformatics techniques, including machine learning algorithms and statistical analyses, to identify and characterize the microbial communities. (Y. Zhang et al., 2021). The study of the metatranscriptome frequently includes network analysis, pathway analysis, and differential gene expression analysis, where the study of comparative gene expression provides information about expression levels. Gene analysis in various samples, such as healthy tissues and diseased tissues, helps to understand and identify the genes involved in biological processes, while pathway analysis provides detailed insight into the metabolic pathways of gene expression, which gives in-depth information about biological processes. Network analysis also provides insight into genes and Interacting proteins, their potential functions, and complex interactions analysis (Wang et al., 2020; Liu et al., 2021).

In addition, the metatranscriptome is a powerful tool for studying microbial communities, providing comprehensive detection of active microbes by analyzing mRNA, which represents gene expression and protein functions. Unlike metagenomics, which only detects DNA-based organisms, the metatranscriptome can identify both DNA and RNA-based pathogens, offering a more complete picture of microbial activity. Moreover, the metatranscriptome also provides information about RNA of the host and the microbial community within it, which gives an in-depth insight into the interactions and effective molecular functions between the host and the microbial community.

1.9. Metatranscriptomics Studies with Insects

Recently, it has been noted with the increased development in the field of meta-omics, and in particular, the metatranscriptome (Kumawat et al., 2022), that its use has increased in the field of studying biological control strategies and identifying potential pathogens for biological control of insect pests that pose risks to food security and crops, as the metatranscriptome is considered a powerful tool for studying and discovering the microbial community (Rozadilla et al., 2020). The discovery of active entomopathogens, their interactions, genetic productivity, their interaction with each other, and their interaction with the host, which gives a deep insight into their behavior and response to environmental conditions such as heat and humidity, and their reproduction and evolution, which provides valuable information to detect the natural enemies of the insect pest to be used in biological control (Picciotti et al., 2023).

For instance, Abbà and colleagues (2022) demonstrated the effectiveness of the metatranscriptome in discovering and characterizing the microbial community associated with insect vectors.

In another study, Batson and colleagues (2021) used the metatranscriptome to reveal the sources of blood in mosquitoes caught in the wild and identify associated pathogens.

Additionally, Kariuki and colleagues (2023) analyzed the intestinal microbiome of black soldier fly larvae, providing an in-depth look at its effectiveness and productivity using metatranscriptome techniques.

These studies and developments indicate the important role of the metatranscriptome in developing areas of research in the field of biological control and providing a deep understanding of insects, their microbial communities, their interactions, and their effectiveness.

1.10. Aim of Study

Cherry crops are susceptible to significant damage from the *R. cerasi* insect, which can diminish yields and create financial losses for farmers. Entomopathogens can infect and kill insect pests, minimizing the need for chemical pesticides and supporting environmentally friendly pest control approaches in cherry production.

Insights into the associated microbiota of the cherry fry *R. cerasi* insect its can be discovered by metatranscriptomic sequencing, by identifying important microbial species

and their roles, researchers can create specialized biological control techniques to manage this pest in cherry production.

Our study aims to leverage metatranscriptome analysis to identify entomopathogens in the cherry fruit fly, offering a comprehensive view of the microbial composition of adults and larvae. By employing this powerful technique, we aim to contribute to the advancement of microbial identification in complex ecological systems, furthering our understanding of microbial dynamics and their implications for biological control strategies.

2. MATERIALS & METHODS

2.1. Insect Collection

Chery fruit flies (*R. cerasi*) were collected from three different locations in Türkiye as shown in Figure 4. Both larvae and adult stages were collected. Adult insects were collected during the period of cherry ripening in June 2022 from Bilecik (40°08′35″N 29°58′45″E) and Tokat (40°18′50″N 36°33′15″E) cities. Larvae samples were collected from cherries after they began to appear in markets in Trabzon (41°00′18″N 39°43′21″E) in July 2022.

Figure 4. Locations where the Chery fruit flies (*R. cerasi*)were collected.

Sticky yellow traps (Rebell®amarillo, Andermatt Biocontrol) were used to collect adult *R. cerasi*. To attract the insects a pheromone is specifically designed to attract *R. cerasi* (Cerasiwit ®, biowet). was attached to these traps (Yan et al., 2021). The traps and the pheromone together hanged to the cherry trees as shown in Figure 5.

Figure 5. Traps hung on cherry trees to catch insects.

Traps were strategically deployed in cherry farms to collect insects over a period of three weeks. With daily controlling and collecting, the insects were carefully transported promptly to the laboratory under controlled conditions for storage. In the laboratory, the insects, including all body parts, were meticulously collected from the traps and frozen at −80 °C until further processing (Cooper et al., 2020). *R. cerasi* larvae were collected from inside the infected cherry fruits and processed similarly to the adult insects.

2.2. RNA Extraction from Adult and Larvae of Chery Fruit Flies

Adults and larvae of *R. cerasi* were homogenized and ground separately using a sterilized mortar and pestle. During the grinding and homogenization, liquid nitrogen was used to maintain a temperature at which the RNA would not disintegrate in the tissues. Insects were flash-frozen in liquid nitrogen and homogenized in mortar. A total of 100 mg of homogenized tissue was then placed into a pre-frozen microcentrifuge tube. This process was applied to both adult and larvae samples before RNA extraction (Whitworth & Watson, 2023).

For RNA extraction, the TRI Reagent® RNA isolation kit (T9424, Sigma-Aldrich, Burlington, MA, USA) was employed, following the manufacturer's guidelines (Ahmad et al., 2023; Hou et al., 2021). Initially, 1 mL of TRI Reagent was added to homogenized insect tissue in a microcentrifuge tube, and cell lysis was achieved by repetitive pipetting. After cell lysis, the samples were incubated at room temperature for 5 minutes. Subsequently, 0.2 ml of chloroform was added, and the tubes were vigorously shaken for 15 seconds before being allowed to stand for 2–15 minutes at room temperature. Upon centrifugation at 12,000 ×g for 15 minutes at 2–8 °C, the mixture separated into three phases: a red organic phase containing proteins, an interphase containing DNA, and a colorless upper aqueous phase containing RNA. The upper aqueous phase was transferred to a new sterile tube, and 0.5 ml of 2-propanol was added. After incubating at room temperature for 5–10 minutes, the samples were centrifuged at 12,000 × g for 10 minutes at 2–8 °C, resulting in RNA precipitation forming a pellet at the tube's bottom. The supernatant was carefully removed, and the RNA pellet was washed by adding at least 1 ml of 75% ethanol per 1 ml of TRI Reagent used in the initial sample preparation step. After vortexing and centrifugation at 7,500 × g for 5 minutes at 2–8 °C, the RNA pellet was air-dried for 5–10 minutes and then dissolved in ddH_2O.

RNA integrity was analyzed using 1.0% agarose gel electrophoresis, and the purity, and concentration of the RNA were tested using a Nanodrop spectrophotometer (Thermo Scientific™ - NanoDrop™ 2000 Spectrophotometers) at a wavelength of 260/280 (Iwanicki et al., 2020).

2.3. Next-Generation Sequencing (NGS)

The insect RNA samples were carefully transferred under controlled conditions and temperature and sent to Novogene's BM Labosis company branch in Ankara. Upon arrival, the samples underwent a rigorous four-step quality check process. This process included agarose gel analysis to detect contamination or RNA degradation, RNA Purity Testing (OD260/OD280) using a Nanodrop, RNA quantification using a Qubit fluorometer, and RNA integrity assessment using an Agilent 2100 instrument. Only RNA samples that passed these quality checks were further processed. The subsequent steps involved sequencing, library construction, and quality assessment according to the company’s standard procedure. The raw data, which consists of sequence information (reads) produced by Illumina

machines (Novaseq 6000 platform), A FASTQ file containing sequence information (reads) and associated sequence quality information is used to store raw data.

2.4. Data Analysis

The data analysis for this study was conducted in collaboration with the Applied Bioinformatics group at Goethe Frankfurt am Main University's Institute of Cell Biology & Neuroscience. We utilized the university's servers and Linux system to install and use the required data analysis tools. This collaboration facilitated a comprehensive analysis using advanced bioinformatics resources, ensuring the accuracy and reliability of our results.

2.4.1. Quality Control and Filtering

The evaluation of raw metatranscriptome read data for larval and adult samples commenced with the implementation of FastQC (version 0.12.1) (URL - 2). Following the initial assessment, a refinement process was executed using Trimmomatic (version 0.39) (URL - 2). This step involved the removal of adapter sequences and the culling of low-quality reads, employing Trimmomatic with a sliding window size of 4, a quality score of 15, and a minimum read length of 36. After this, a reassessment of read quality post-trimming was conducted to validate the efficacy of the filtering process (Williams et al., 2016).

To eliminate the influence of host insect reads, the Spliced Transcripts Alignment to a Reference (STAR) (version 2.7.11a) (URL - 4) a mapping tool was employed (Zakrzewski et al., 2018). Raw reads for both larval and adult samples were individually mapped to the NCBI reference genome of *Rhagoletis cerasi* (NCBI GenBank accession number GCA_029783565.1). The "–outReadsUnmapped" option in the STAR mapping tool facilitated the removal of host insect reads, ensuring a focused analysis of the target transcriptome.

2.4.2.Assembly of The *de novo* Transcript

Following quality control, trimming, and filtering, *de novo* assembly was undertaken for larval and adult specimens separately. Trinity (version 2.15.1) (URL - 5), a tool known for its proficiency in *de novo* transcriptome assembly, was employed with default parameters

(Grabherr et al., 2011). This crucial step aimed at reconstructing comprehensive transcriptomes from the quality-controlled and filtered reads, laying the groundwork for subsequent analyses and the visualization of the length distribution of transcripts done by Python matplotlib (version 3.8.2) (URL - 6).

Each stage in this methodological framework was executed with meticulous attention to detail, reinforcing the integrity of the data. This comprehensive approach not only underscores the methodological rigor but also contributes to the robustness and reliability of the subsequent analyses in the unfolding investigation.

2.4.3. Microbiota Detection

Following the assembly of filtered and quality-checked reads into transcripts using Trinity, a meticulous taxonomic classification of these transcripts was separately conducted for larvae and adults. Kraken 2 (version 2.1.3) (URL - 7), a sophisticated tool relying on *k*-mers (sequence fragments) mapping, was employed for this purpose, utilizing a k=35 as the default setting (Wood et al., 2019).

The taxonomic classification was executed through Kraken 2, utilizing a custom database created using "kraken2-build," which encapsulated comprehensive data from the complete NCBI RefSeq, spanning bacteria, viruses, fungi, protozoa, and archaea (URL - 12), accessed on November 2023). To enhance the precision and reliability of the classified microorganisms, the parameters "--minimum-hit-groups" were set to 5, and a cutoff threshold of 0.03 was applied (Lu et al., 2022). These parameters were selected to reduce false positives and increase the confidence of sequences assigned to specific taxonomic groups. Additionally, the results were visualized using KronaTools (version 2.8.1) (URL - 8) and the Pavian R package (version 1.0) (URL - 9) (Goloshchapov et al., 2023; Lu et al., 2022).

Before applying the Kraken 2 tool, host readings from the samples were judiciously filtered out, aligning with recommendations in the natural Protocols for pathogen classification (Lu et al., 2022). Utilizing a database closely aligned with sample characteristics is preferable, and the custom Kraken database was tailored to maintain logical and expectation-driven taxonomic classifications specific to the nature of the studied samples.

This comprehensive methodology ensures a robust taxonomic classification process that aligns with established best practices in the field. By incorporating Kraken 2 with a custom database and setting stringent parameters, the study enhances the credibility and reliability of subsequent analyses, especially in the context of identifying and classifying potential entomopathogens.

2.4.4. Determination of Entomopathogens among Microbiota

In terms of selecting entomopathogens from the microbiota of *R. cerasi*, we leveraged established literature to categorize these microorganisms into distinct classes based on their known entomopathogenic properties (Deka et al., 2021). The identified classes encompass a wide range of microorganisms, each with documented pathogenicity towards various insect species. Table 1 presents the selected entomopathogens, along with their references.

Table 1. Identified entomopathogenic microorganisms in insects.

Class	Entomopathogen	Reference
Bacteria	*Bacillus subtilis*	(Torres et al., 2022)
	Bacillus thuringiensis	(Aronson et al., 1986)
	Lysinibacillus sphaericus	(Berry, 2012)
	Paenibacillus	(Grady et al., 2016)
	Photorhabdus	(Nielsen-LeRoux et al., 2012)
	Xenorhabdus	(Nielsen-LeRoux et al., 2012)
	Serratia entomophila	(Vaughan et al., 2022)
	Serratia proteamaculans	(Vaughan et al., 2022)
	Yersinia entomophaga	(Hurst et al., 2019)
	Pseudomonas entomophila	(Lemaitre, 2015)
	Pseudomonas fluorescens	(Otsu et al., 2004)
	Pseudomonas aeruginosa	(Chin et al., 2021)
	Enterococcus gallinarum	(Gouli et al., 2021)
	Raoultella terrigena	(Gouli et al., 2021)
Viruses	*Baculoviridae*	(Valicente, 2019)
	Iflaviridae	(Carvalho & Long, 2021)
	Reoviridae: CPV	(Valicente, 2019)
	Entomopoxvirinae: EPV	(Valicente, 2019)
	Iridoviridae: IV	(Valicente, 2019)
	Ascoviridae	(Valicente, 2019)
	Polydnaviridae	(Valicente, 2019)
	Parvoviridae: DNV	(Carvalho & Long, 2021)
	Birnaviridae	(Carvalho & Long, 2021)
	Caliciviridae	(Carvalho & Long, 2021)
	Nodaviridae	(Carvalho & Long, 2021)
	Picornaviridae	(Carvalho & Long, 2021)

Table 1: Continued.

	Rhabdoviridae	(Carvalho & Long, 2021)
Fungi	*Aspergillus fumigatus*	(Ebani & Mancianti, 2021)
	Fusarium	(Ebani & Mancianti, 2021)
	Verticillium lecanii	(Ebani & Mancianti, 2021)
	Alternaria cassia	(Ebani & Mancianti, 2021)
	Phytophthora palmivora	(Ebani & Mancianti, 2021)
	Beauveria bassiana	(Ebani & Mancianti, 2021)
	Metarhizium anisopliae	(Ebani & Mancianti, 2021)
	Hirsutella thompsonii.	(Ebani & Mancianti, 2021)
	Verticillium lecanii	(Ebani & Mancianti, 2021)
	Paecilomyces	(Ebani & Mancianti, 2021)
	Isaria spp.	(Ebani & Mancianti, 2021)
	Miscellaneous	(Ebani & Mancianti, 2021)
	Neozygites fresenii	(Ebani & Mancianti, 2021)
	Nomuraea rileyi	(Ebani & Mancianti, 2021)
	Entomophthora muscae	(Ebani & Mancianti, 2021)
	Metarhizium brunneum	(Ebani & Mancianti, 2021)
	Lecanicillium longiosporun	(Ebani & Mancianti, 2021)
	Poecilomyces lilacinus	(Ebani & Mancianti, 2021)
	Isaria fumosorosea	(Ebani & Mancianti, 2021)
Protozoa	*Haemogregarina*	(Kwenti, 2017)
	Nosema	(Kwenti, 2017)
	Babesia	(Kwenti, 2017)
	Theileria	(Kwenti, 2017)

The identified microorganisms present a diverse and promising selection of potential entomopathogens that hold significant promise for the biological control of the *R. cerasi* pest. Our subsequent objective is to verify the presence of transcripts associated with these selected entomopathogens using Basic Local Alignment Search Tool (BLASTx) (URL - 10) analysis. This verification process will provide crucial insights into their potential roles in regulating *R. cerasi* populations, thereby contributing to the development of an effective biological control strategy.

2.4.5. Conformations of Entomopathogens Transcripts

To confirm the classified entomopathogens, we searched for the Kraken report to identify and extract the transcripts associated with entomopathogens. Using Kraken Tools

(version 1.2) (URL - 11), we performed a search based on microorganisms characterized as entomopathogens in the literature. The extracted entomopathogen transcripts were then confirmed using a local BLASTx (version 2.14.0) analysis (Johansson et al., 2013). For each target entomopathogen, we downloaded the protein sequences from NCBI and ran BLASTx with the classified entomopathogen transcripts against the NCBI data, using an E-value threshold of 1e-5 was applied for the BLASTx analysis. Subsequently, the results were filtered to remove hits with low identity and high E-values, retaining only the best BLASTx hits for each transcript query identifier. This process aimed to improve the accuracy of the findings.

3. RESULTS

3.1. Insects Used for RNA Extraction

Rhagolettis cerasi were meticulously collected from Bilecik, Tokat, and Trabzon, encompassing both larvae and adult stages (Table 2).

A total of 203 adult insects were collected from Bilecik and Tokat during the cherry ripening period in June 2022. The collection process involved strategically deploying sticky yellow traps equipped with pheromones on cherry trees in both cities. Traps were controlled daily, the insects were separated from the traps carefully, transported to the laboratory, and frozen at −80 °C, until being processed.

In July 2022, 114 larvae were diligently collected from cherries in the vicinity of Trabzon. The larvae extraction process involved careful handling to preserve all body parts, and the collected specimens were promptly frozen at -80 °C until further processing.

Table 2. Summary of insect collection.

Location	Insect Stage	Collected Insect
Bilecik	Adult	110
Tokat	Adult	93
Trabzon	Larvae	114

3.2. RNA Extraction

In order to prepare high-quality RNA samples for downstream analysis, we carried out RNA extraction from *R. cerasi* adults and larvae. This involved homogenizing and grinding the flash-frozen insects using liquid nitrogen for temperature control. Subsequently, 100 mg of tissue was processed for RNA extraction using the TRI Reagent® RNA isolation kit. The extraction process also included chloroform addition, centrifugation, and RNA precipitation with 2-propanol. Finally, Nanodrop spectrophotometry was utilized to assess the quality and concentration of the extracted RNA. This step is crucial as it ensures the integrity and purity of the RNA samples, which are essential for downstream applications such as sequencing and analysis.

The results of the RNA extraction are summarized in Table 3. The extracted RNA samples from adult flies in Bilecik and Tokat regions showed RNA amounts of 30 µl and 20

µl, with RNA quality (measured by the 260/280 ratio) of 1.80 and 1.90, and concentrations of 5.993 µg/µl and 2.119 µg/µl, respectively. The larvae sample from Trabzon region exhibited an RNA amount of 90 µl, a quality ratio of 2.20, and a concentration of 28.607 µg/µl.

Table 3. The quality and quantity of extracted RNA samples from adult and larvae of *R. cerasi*.

RNA Sample location	**RNA Amount** (µl)	**RNA Quality 260/280**	**RNA Concentration** (µg/µl)
Adult / Bilecik	30	1.80	5.993
Adult / Tokat	20	1.90	2.119
Larvae / Trabzon	90	2.2	28.607

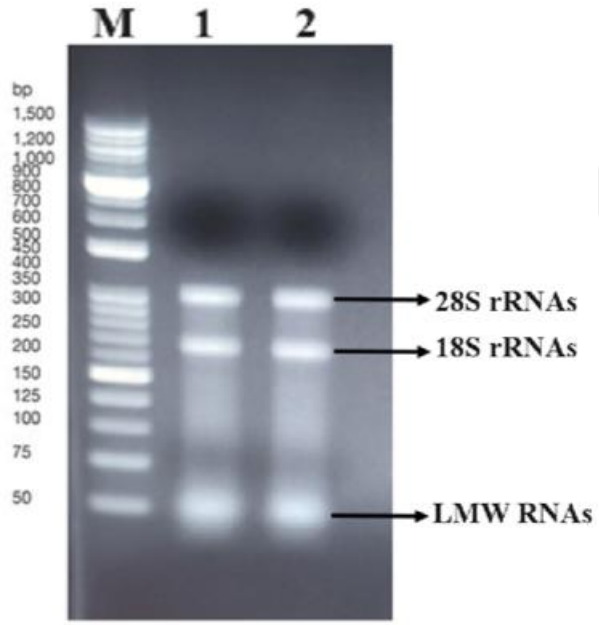


Figure 6. Agarose gel electrophoresis of extracted RNA samples from *R. cerasi*. Lane M contains a molecular weight marker (Invitrogen™ - E-Gel™ Sizing DNA Ladder - Size Range:50 to 1500 bp), facilitating the estimation of RNA sizes in other lanes. Lane 1 displays RNA from adult samples, with visible 28S and 18S bands representing ribosomal RNA (rRNA) along with low molecular weight RNAs (LMW RNAs) and indicating RNA integrity. Lane 2, containing RNA from larvae, exhibits similar bands, offering insights into RNA composition. The gel serves as a visual assessment of RNA quality.

3.3. Next-Generation Sequencing: Metatranscriptome Sequencing

Next-generation sequencing (NGS) is crucial for identifying potential targets for biological control in *R. cerasi* adults and larvae. Following rigorous quality control at Novogene's BM Labosis company branch (Ankara, TR) to ensure the integrity and purity of RNA samples during transportation and processing of the RNA samples from *R. cerasi* adults and larvae were assessed in detail, including multiple steps to ensure sample integrity and quality, such as agarose gel analysis, Nanodrop RNA Purity Testing, Qubit fluorometer quantification, and RNA integrity testing.

After passing these tests, the samples underwent ribosomal RNA depletion, followed by library construction and quality assessment. The library concentration was measured using a Qubit 2.0 fluorometer before sequencing on the Illumina (Novaseq 6000 platform) machine.

Table 4 provides a detailed overview of the quality and quantity of the extracted RNA samples before sequencing. For the adult samples, the RNA concentration was 399.000 ng/µl, with a volume of 16.00 µl, resulting in a total amount of 6.38400 µg. The integrity value was 5.90, indicating a high-quality RNA sample. The sample passed the quality control (QC) test and required 0.33 µl for electrophoresis.

Similarly, for the larvae samples, the RNA concentration was 2380.000 ng/µl, with a volume of 62.00 µl, resulting in a total amount of 147.56000 µg. The integrity value was 6.00, indicating an even higher quality RNA sample than the adult sample. The sample also passed the QC test and required 0.05 µl for electrophoresis.

Table 4. Quality control of extracted RNA samples.

R_Samp	Conc. (ng/µl)	Vol.(µl)	Tot.Amt.(µg)	Int. Val.	S_QC	Vol. (elec.)
Adults	399.000	16.00	6.38400	5.90	Pass	0.33
Larvae	2380.000	62.00	147.56000	6.00	Pass	0.05

Raw sequencing reads were obtained as FASTQ files with 142,815,914 sequences totaling 49 GB of data for the adults sample and 154,923,122 sequences totaling 53.2 GB of data for the larvae sample.

3.4. Analysis of Raw Data for Microbiota Composition Associated with *Rhagoletis cerasi* and Detection of Entomopathogen-Specific Transcripts

3.4.1. Quality Control Before Trimming

The initial quality assessment using FastQC (URL - 2) before trimming the metatranscriptome data was performed to ensure the high quality of the row data for both larval and adult samples, as demonstrated in Table 5.

Table 5, provides a summary of the key metrics obtained from the FastQC report. For the adult sample, the total number of sequences was 142,815,914, with a sequence length of 150 base pairs and a %GC content of 45%. Similarly, for the larvae sample, the total number of sequences was 154,923,122, with a sequence length of 150 base pairs and a %GC content of 41%. These results indicate that the initial metatranscriptome data exhibited high-quality characteristics, setting a solid foundation for subsequent analyses.

Table 5. FastQC analysis of the row reads before trimming.

Measure	Adults Sample	Larvae Sample
Total Sequences	142,815,914	154,923,122
Sequence Length (bp)	150	150
%GC	45	41

To assess the quality of the metatranscriptome data before trimming, we conducted a per-base sequence quality analysis using FastQC (Figures 7 A and B). This analysis helps us to understand the distribution of sequence quality along the length of the reads. In the figures, The graph displays quality scores across all bases in your sequence data. The x-axis represents the position in the read (bp), ranging from 1 to 150. The y-axis shows quality scores (encoded in Sanger/Illumina 1.9 format) ranging from 0 to 36. Color Coding, Green Zone (Quality Scores: 28-36). The initial positions (approximately 1-120) are predominantly green, indicating high-quality base calls. Bases in this zone have lower error rates and are

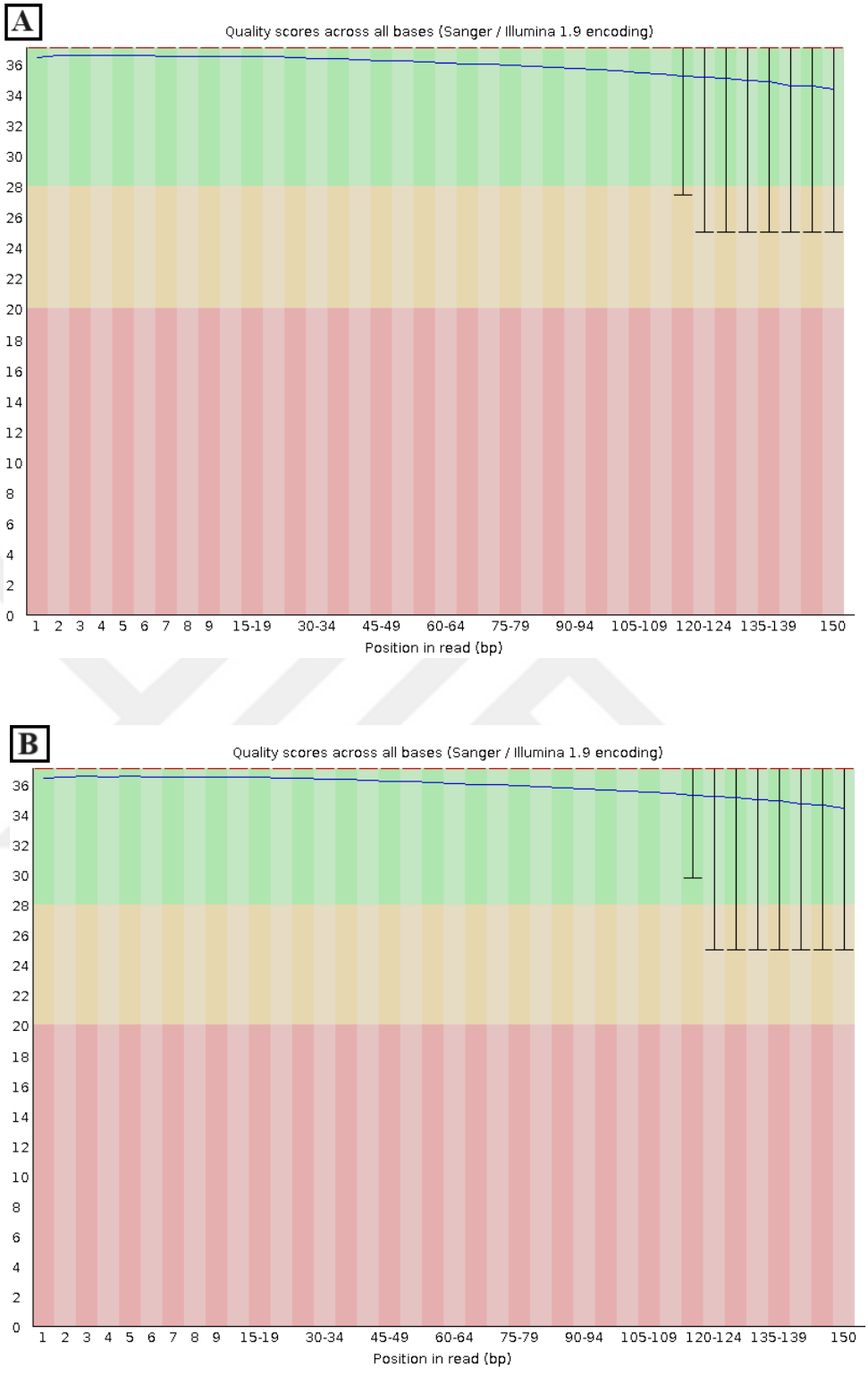


Figure 7. The Quality control report on per base row sequence quality before trimming. The figure illustrates the Per Base Sequence Quality for both adult (labeled as A) and larval (labeled as B) samples before any trimming procedures. The analysis provides a comprehensive view of the sequencing data quality, allowing for an assessment of base-wise sequence quality across the entire length of the reads for both adult and larval datasets.

reliable for downstream analyses. Yellow Zone (Quality Scores: 20-28) Around positions 121-135, there's a small section of yellow. These bases exhibit moderate quality, with slightly higher error rates. They can often be used in analyses with caution. Red Zone (Quality Scores: <20), these bases have low-quality scores and high error rates.

Most of the bases exhibit good quality for adults and larvae as showen in (Figures 7 A and B), as indicated by the yellow and green coloration, as for row metatranscriptome data, Quality Scores are 24-36 for reads (bp), ranging from 110 to 150.

. These figures illustrate the overall high quality of the metatranscriptome data for both adult and larval row reads.

3.4.2. Data Trimming

To ensure high-quality data for subsequent analysis, the trimming process was carried out using Trimmomatic (version 0.39) (URL - 3), employing a sliding window size of 4, a quality score of 15, and a minimum read length of 36. This process effectively removed low-quality reads, very short reads and Illumina universal adapter reads from the raw metatranscriptome reads. The results of the trimming process are illustrated in Figure 8, and summarized in Table 6. Despite the removal of these reads, a high percentage of surviving reads was retained for both larval and adult samples.

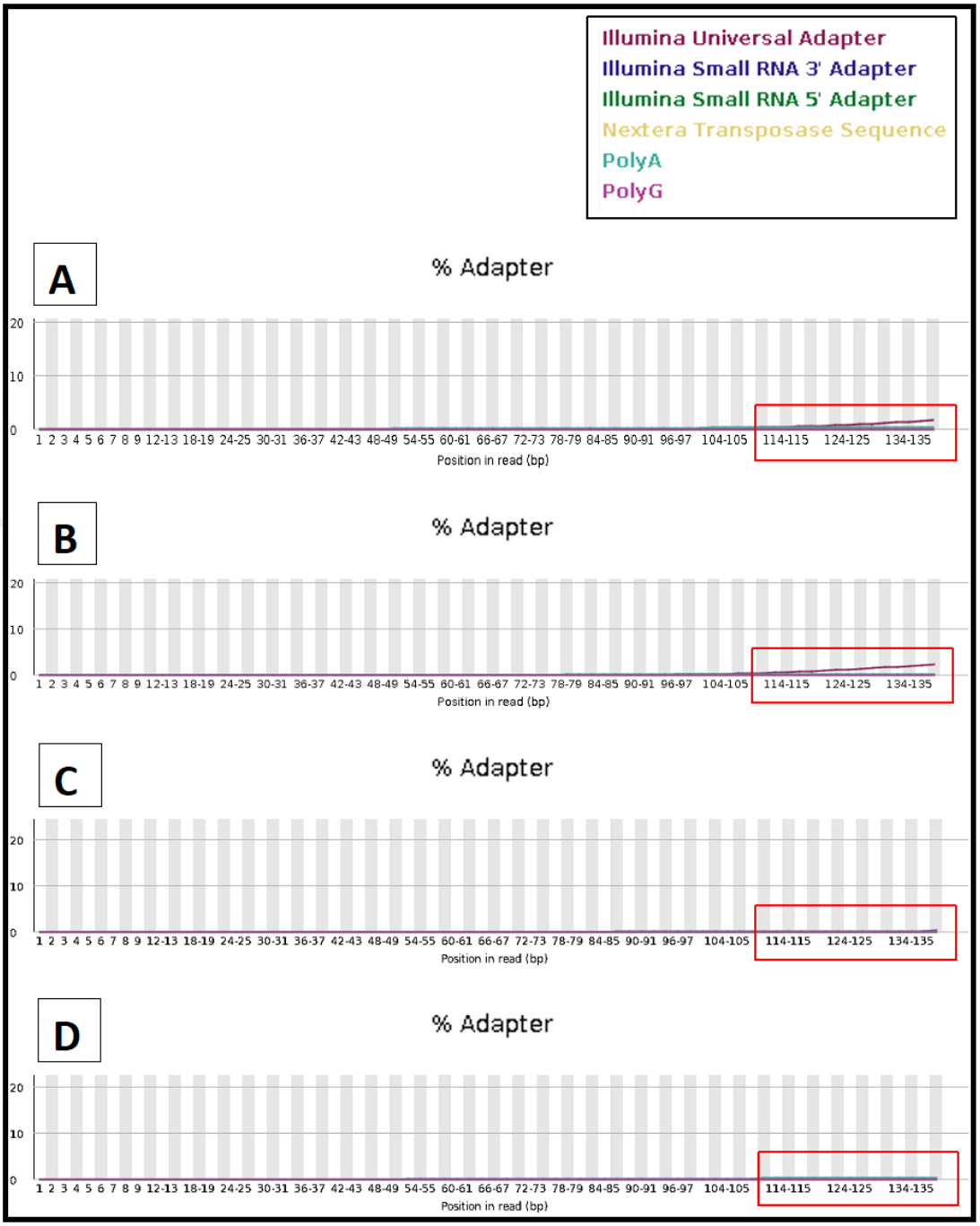


Figure 8. Presentation of the FastQC report on adapter content for adults and larvae samples before and after trimming. Panels A and B represent Adapter Content before removal of adapters for adults and larvae, respectively. Panels C and D display Adapter Content after adapter removal for adults and larvae. A red box emphasizes the region indicating the process of adapter removal, highlighting its impact on the Adapter Content curve, and a red box emphasizes the region indicating the process of adapter removal, highlighting its impact on the Adapter Content curve.

In Figure 8, the Adapter Content figure from the FastQC (URL - 2) report provides insight into the presence and distribution of adapter sequences in the raw data. The y-axis

represents the amount of adapter content, while the x-axis shows the position in read base pairs (bp). The curves on the graph represent different types of adapter content, each depicted in a different color. The Illumina universal adapter is shown in red, the Illumina small RNA 3' adapter in blue, the Illumina small RNA 5' adapter in green, the Nextera transposase sequence in yellow, polyA in light green, and polyG in violet. This analysis helps identify and quantify adapter contamination in the raw data, which is crucial for ensuring the accuracy and reliability of downstream analyses.

In Figures 8 A and 8 B, representing the raw data before trimming for adults and larvae, respectively, the presence of the Illumina universal adapter is depicted in red boxes. However, after the trimming process, as shown in Figures 8 C and 8 D for adults and larvae, respectively, the Illumina universal adapter is no longer present. This removal is indicated by the absence of the red curve in the trimmed data, visually highlighted with a red box, signifying the successful elimination of adapter contamination from the metatranscriptome data.

Table 6. Trimming of row reads

Sample	Input Reads	Surviving	Dropped
Adults	142,815,914	141,914,081	901,833 (0.63%)
Larvae	154,923,122	153,998,938	924,184 (0.60%)

Table 6, provides a detailed overview of the trimming results. For the Adults sample, the initial input reads were 142,815,914, with 141,914,081 reads surviving after trimming. This resulted in 901,833 reads being dropped, accounting for 0.63% of the initial reads. Similarly, for the Larvae sample, the initial input reads were 154,923,122, with 153,998,938 reads surviving after trimming. This led to 924,184 reads being dropped, which represented 0.60% of the initial reads.

3.4.3. Quality Control After Trimming

To ensure the quality of the data after the trimming process, we rechecked the quality of the data for adults and larvae datasets. A post-trimming FastQC (URL - 2) report was generated to evaluate the quality of the metatranscriptome read data. The report

demonstrated significant improvements in the quality metrics, indicating enhanced data quality. The summary of key metrics, including sequence length and %GC content, is presented in Table 7, provides an overview of the post-trimming FastQC results for both the adult and larvae samples. For the adult sample, the total number of sequences after trimming was 141,914,081, while for the larvae sample, it was 153,998,938. The sequence length ranged from 36 to 150 for both samples, and the %GC content was measured at 45% for the adult sample and 41% for the larvae sample. The metrics presented collectively demonstrate the enhanced quality and suitability of the trimmed metatranscriptome data for further analysis. Figure 9 provides a visual representation of the quality improvement in the trimmed data. Specifically, Figure 9 A depicts the per base sequence quality for adult samples, while Figure 9 B corresponds to larval samples. Most of the bases exhibit good quality for adults and larvae as shown in (Figures 7 A and B), as indicated by the yellow and green coloration, as for row metatranscriptome data, Quality Scores are 24-36 for reads (bp), ranging from 124 to 150. These figures illustrate the distribution of sequence quality along the length of the reads after trimming, showing the overall high quality of the trimmed metatranscriptome data for both adult and larval reads.

Table 7. FastQC analysis of the row reads after trimming.

Measure	Adults sample	Larvae sample
Total Sequences	141,914,081	153,998,938
Sequence length	36-150	36-150
%GC	45	41

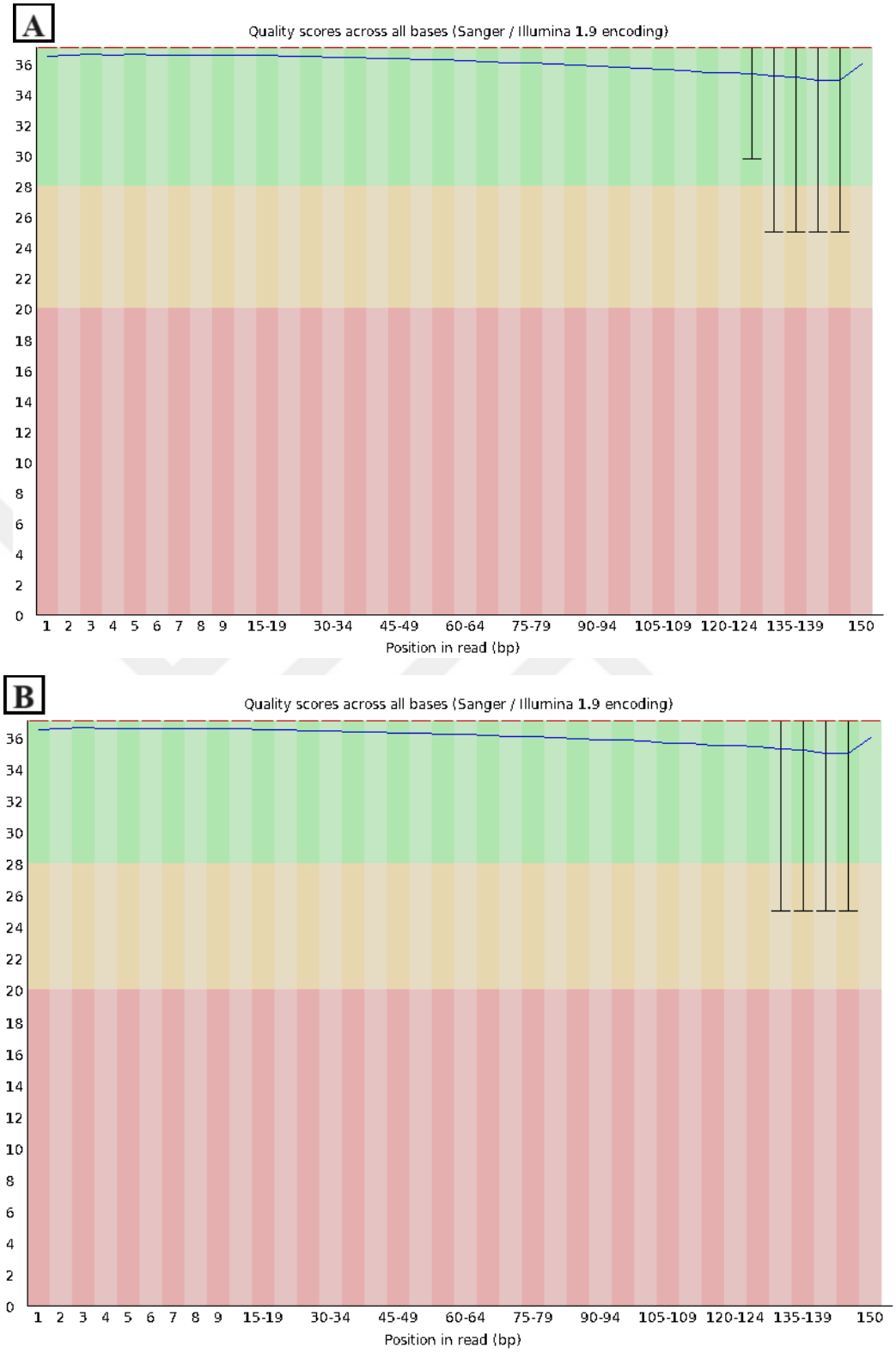


Figure 9. The Quality control report on per base row sequence quality after trimming. displays Sequence Quality Per Base after trimming for adult and larval samples, panels A and B represent the Per Base Sequence Quality for adults and larvae, respectively, post-trimming.

3.4.4. Host Reads Mapping

In the process of eliminating host insect reads, we utilized the STAR (version 2.7.11a) (URL - 4) mapping tool, and the corresponding results in Table 8 elucidate the percentage of uniquely mapped reads for both adult and larval samples, affirming the successful removal of host-related reads.

Table 8 presents the STAR mapping results for both the adult and larval samples. For the adult sample, 52,953,597 reads out of 141,914,081 input reads were uniquely mapped, accounting for 37.31% of the total input reads. Similarly, for the larval sample, 97,932,898 reads out of 153,998,938 input reads were uniquely mapped, representing 63.59% of the total input reads. These results indicate the successful removal of host-related reads from our dataset, ensuring the quality and integrity of our metatranscriptome data for further analysis.

Table 8. STAR Mapping with host reference genome

Sample	Input Reads	Uniquely Mapped Reads	Uniquely Mapped Reads %
Adult	141,914,081	52,953,597	37.31%
Larva	153,998,938	97,932,898	63.59%

It is noteworthy that we relied on the single available genome reference for *R. cerasi* (NCBI Taxonomy ID 43399), acknowledging certain limitations such as a small genome size (1.2 Gb) and fragmented N50 values. Despite these challenges, the chosen genome reference was employed due to the absence of alternative references. Consequently, not all host transcripts were filtered out, as reflected in the moderately high percentage of uniquely mapped reads.

These findings collectively underscore the effectiveness of our quality control and filtering processes, demonstrating the enhanced quality of the metatranscriptome data for subsequent analyses, despite the inherent challenges associated with the selected genome reference.

3.4.5. Assembly of The *de novo* Transcripts

Following the meticulous steps of quality control, trimming, and filtering, de novo assembly was carried out separately for larval and adult specimens. Trinity (version 2.15.1) (URL - 5), a highly regarded tool for *de novo* transcriptome assembly, was employed with default parameters. This critical phase aimed at reconstructing comprehensive metatranscriptomes from the quality-controlled and filtered reads, providing a foundation for subsequent analyses.

The *de novo* assembly with Trinity using default parameters resulted in the generation of metatranscriptomes for both larval and adult specimens. The characteristics of the assembled transcripts are summarized in Table 3.7. The length distribution of transcripts for adults and larvae is visually represented in Figure 10, which illustrates the frequency (y-axis) of transcripts at various lengths (x-axis). Figures A and B correspond to adults and larvae, respectively, and depict that the majority of transcripts fall within the 200 to 1000 base pair length range.

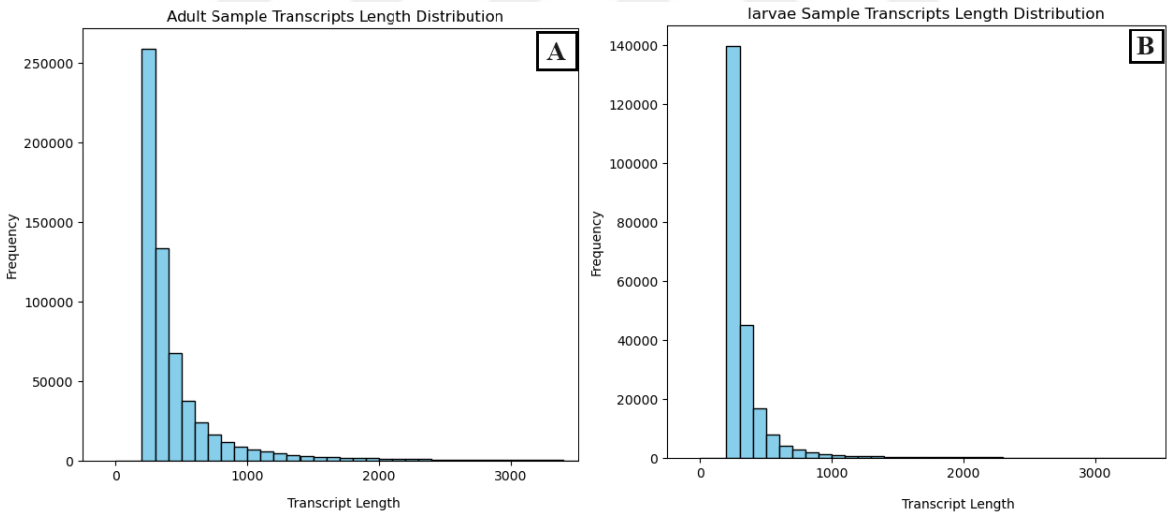


Figure 10. Assemblies length distribution, provides a visual representation of the length distribution of transcripts for both adult and larval-filtered samples. A corresponds to adults, while B pertains to larvae.

Table 9 presents the results of the *de novo* assembly for both larval and adult specimens. For the adult specimen, the assembled transcripts range from 201 to 35,818 base pairs in length, with a mean length of 498.907 base pairs and a total of 608,025 transcripts.

In comparison, the larval specimen's assembled transcripts range from 201 to 9,116 base pairs in length, with a mean length of 496.132 base pairs and a total of 226,568 transcripts. These results provide insights into the metatranscriptome characteristics of the studied specimens and serve as a foundation for further analyses.

Table 9. Statistics of *de novo* assembled transcripts.

Sample	Min Length	Max Length	Mean Length	Total Transcripts
Adults	201	35,818	498.907	608,025
Larvae	201	9,116	496.132	226,568

3.4.6. Detection of Microbiota

In the process of taxonomic classification using Kraken 2 (version 2.1.3) (URL - 7), we implemented additional measures to enhance result accuracy. In addition to the "--minimum-hit-groups 5" option, we set the confidence score threshold to 0.03. This stringent criterion, coupled with the requirement for a minimum of three distinct hit groups for confident classification, significantly improved precision, reducing the likelihood of false positives and reinforcing result reliability. The outcomes for adults and larvae are detailed in Table 10, complemented by Figure 11, providing a visual representation of the distribution of classified microorganism transcripts across major taxonomic groups and highlighting the relative abundance in adults (blue) and larvae (orange) metatranscriptomes.

Table 10 summarizes the results of taxonomic classification using Kraken 2 for both adult and larval samples. For the adult sample, out of 608,025 input sequences, 351,400 (57.8%) were microbial classified, while 256,625 (42.2%) remained microbial unclassified. In comparison, for the larval sample, out of 226,568 input sequences, 14,471 (6.39%) were microbial classified, and 212,097 (93.6%) remained microbial unclassified. These results provide insights into the taxonomic composition of the microbiota in the studied specimens and their relative abundance, serving as a foundation for further analysis and interpretation.

Figure 11 displays the distribution of classified microbial transcripts in adults and larvae. In adults, bacteria accounted for 57.5% (349,698 transcripts), fungi for 0.2% (1,194 transcripts), viruses for 0.02% (115 transcripts), and protozoa for 0.01% (64 transcripts). In larvae, bacteria were the most abundant at 5.8% (13,149 transcripts), followed by fungi at

0.44% (987 transcripts), viruses at 0.03% (71 transcripts), and protozoa at 0.04% (98 transcripts).

Although archaea were present, their count was too low to be displayed in the figure. In adults, archaea represented only 0.0007% with four classified transcripts, while in larvae, they were present at a very low level, accounting for just 0.0013% with three classified transcripts.

Table 10. Kraken 2 classification analysis of microbial composition.

Sample	Sequences Input	Sequences Classified	Sequences Unclassified
Adults	608,025	351,400 (57.8%)	256,625 (42.2%)
Larvae	226,568	14,471 (6.39%)	212,097 (93.6%)

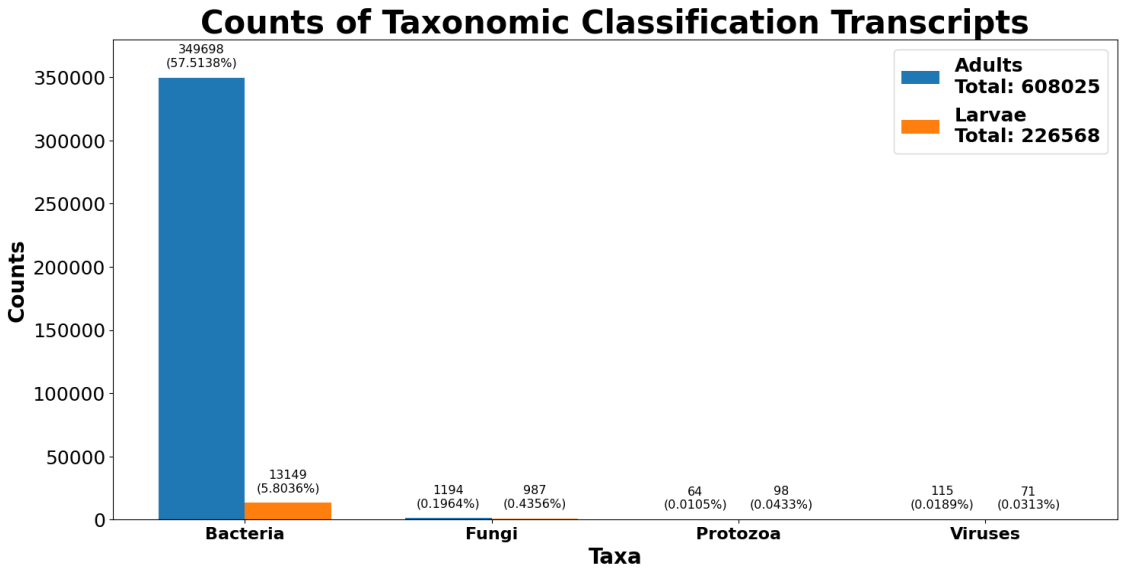


Figure 11. Classified microbial groups. It presents the distribution of classified microorganism transcripts among major taxonomic groups, including bacteria, viruses, fungi, and protozoa. The color-coded bars distinguish between adults (blue) and larvae (orange), offering a comprehensive overview of the relative abundance of these microbial categories within the metatranscriptomes of adults and larvae.

This comprehensive methodology, combining Kraken 2 with a custom database and stringent parameters, not only reflects the meticulous steps taken in taxonomic classification but also aligns with established best practices. The results contribute to the robustness of the

study's microbial composition analysis, enhancing the credibility and reliability of subsequent analyses. Figures 12 and 13 visually depict the results of Kraken 2 classification transcripts, employing KronaTools (version 2.8.1) (URL - 8) for Krona charts and Pavian R package (version 1.0) (URL - 9) for Sneaky visualization networks. These figures provide a detailed insight into the taxonomic composition of the classified microbiota, showing the distribution of microorganisms from domains to species levels in adults (Figure 12 A) and larvae (Figure 12 B), as well as the hierarchical structure of domains, phyla, families, genera, and species in adults (Figure 13 A) and larvae (Figure 13 B), This approach proves particularly valuable in the identification and classification of entomopathogens within the studied microbiomes.

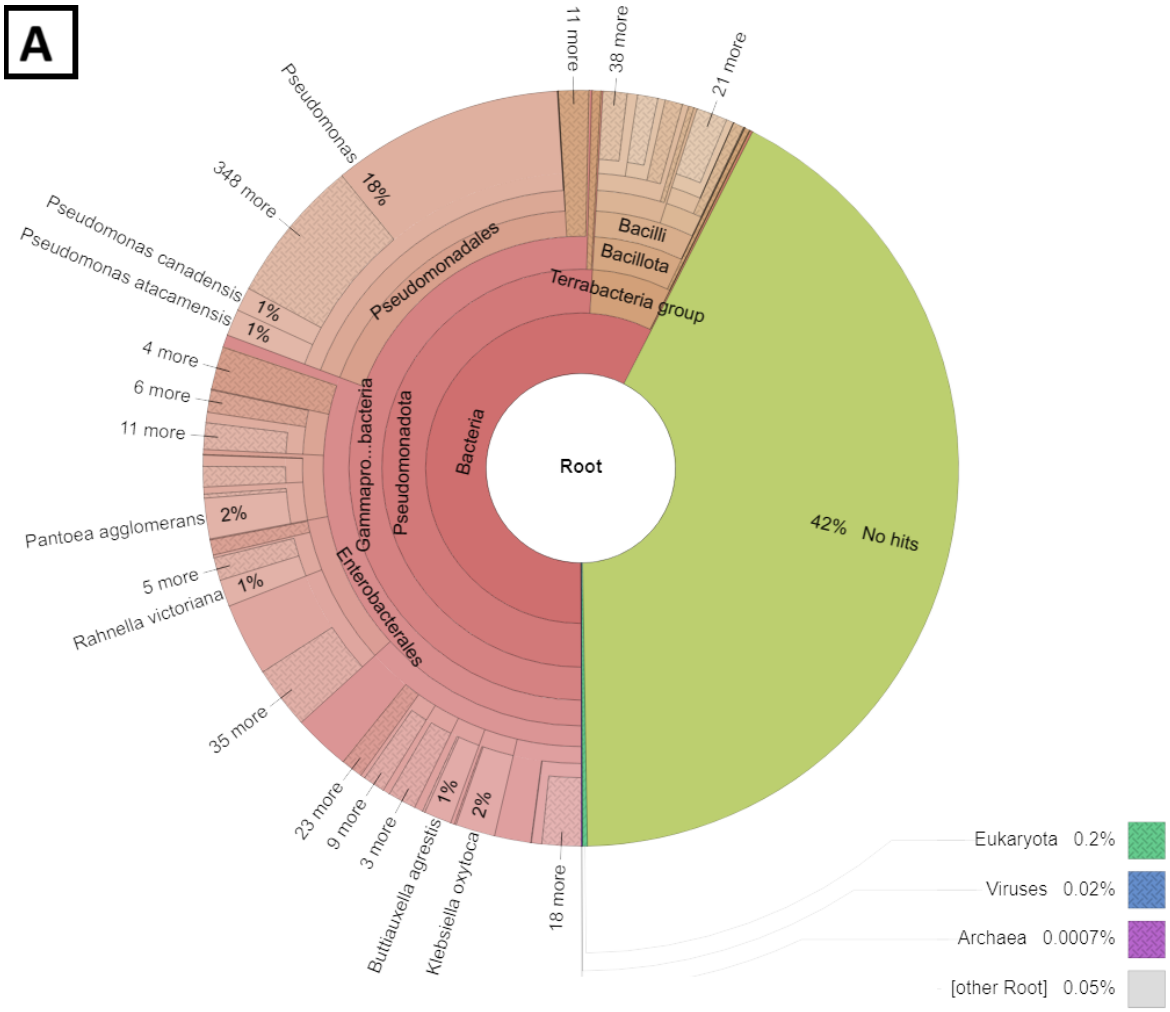
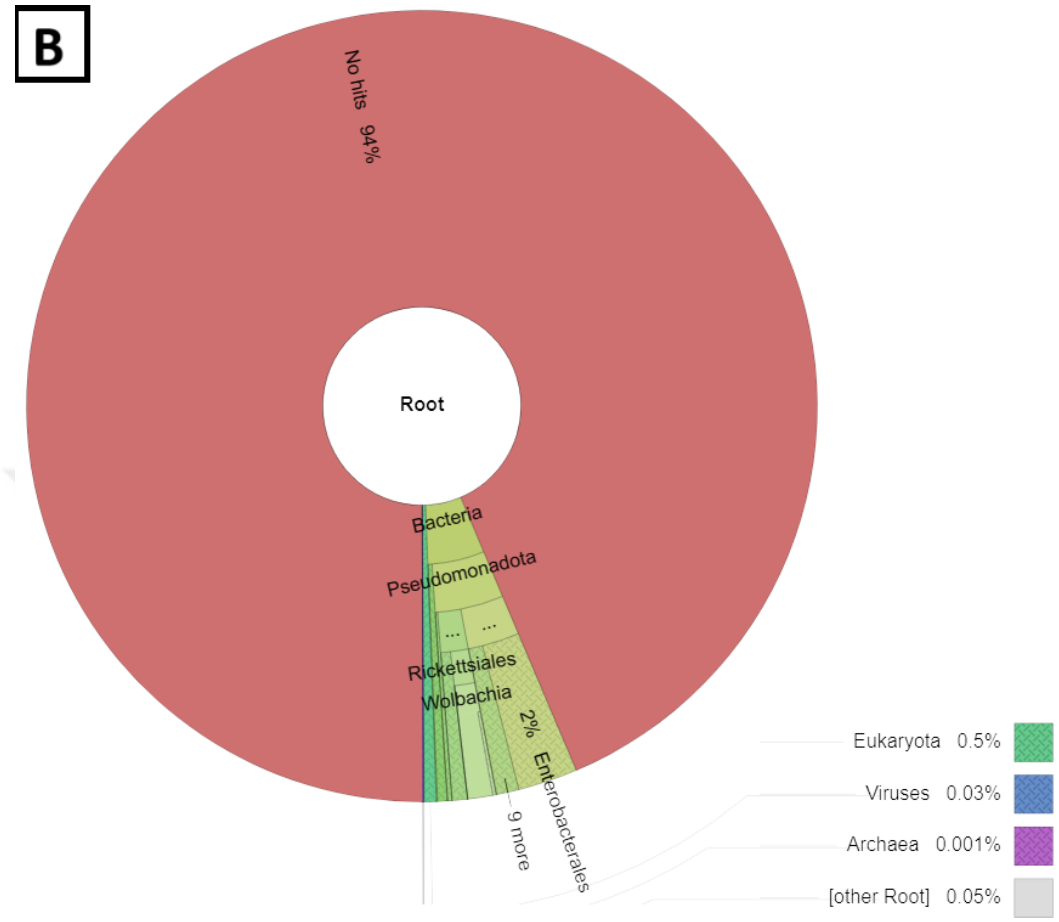


Figure 12. Krona charts of adults and larvae. The figure displays the general composition of the classified microbiota based on the results from the Kraken 2 classification. Specifically, Figure A corresponds to adults, while Figure B pertains to larvae. These charts provide a visual representation of the taxonomic distribution within the microbiomes of adult and larval specimens as determined by the Kraken 2 analysis.

Figure 12: Continued



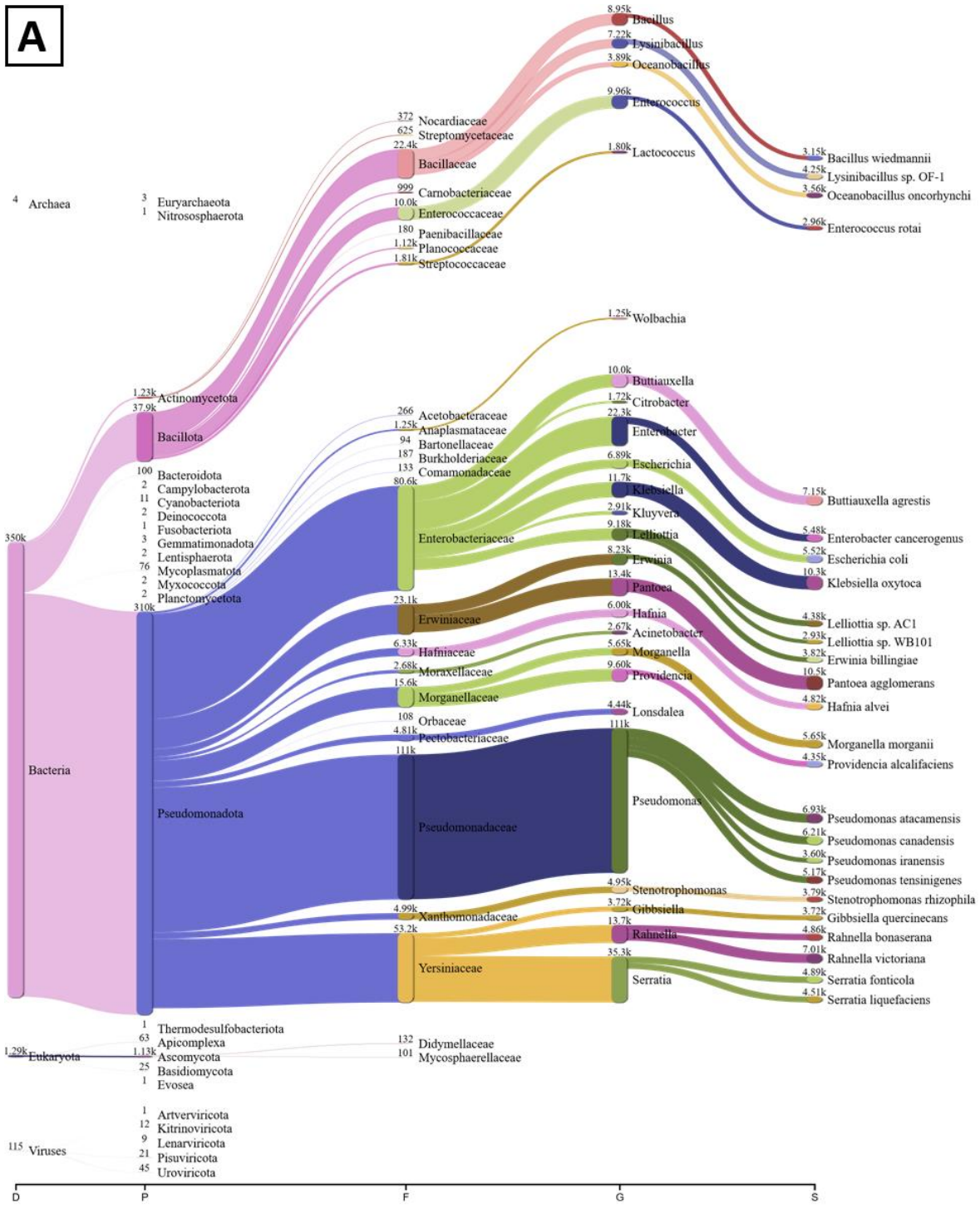
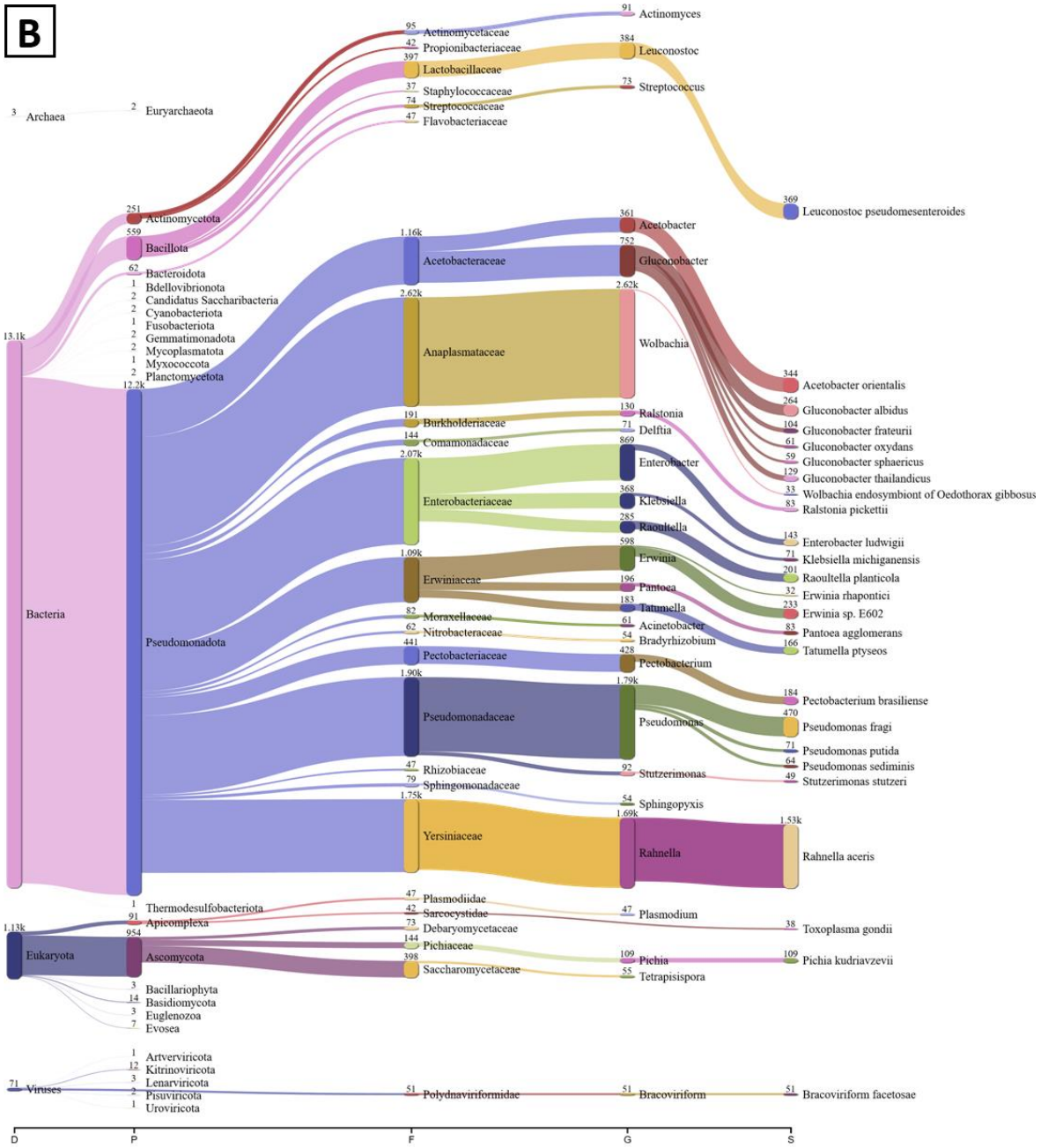


Figure 13. Pavian Sneaky visualization networks reveal the taxonomic composition of the classified microbiota. illustrating the hierarchical structure from the domain to the species level. The visualization encompasses domain (D), phylum (P), family (F), genus (G), and species (S). Figure A corresponds to adults, while Figure B pertains to larvae, providing a comprehensive view of the taxonomic relationships within the microbiomes of adult and larval specimens identified through Kraken 2 analysis.

Figure 13: Continued



Figures 12 and 13 visually depict the results of Kraken 2 classification transcripts, providing detailed insights into the taxonomic composition of the microbiota in adults and larvae. These figures show the distribution of microorganisms from domains to species levels, highlighting the hierarchical structure of domains, phyla, families, genera, and species in both adults (Figure 13 A) and larvae (Figure 13 B). The taxonomic distribution of

microbial transcripts in adult and larval specimens of *R. cerasi* reveals distinct differences in their microbiota composition. In adults, bacteria were the dominant microorganisms, comprising 57.5% (349,698 transcripts) of the classified transcripts. Among the bacterial phyla, *Pseudomonadota* was the most abundant, with 309,708 classified transcripts. Within *Pseudomonadota*, the families *Enterobacteriaceae* (80,582 transcripts), *Pseudomonadaceae* (111,325 transcripts), and *Yersiniaceae* (53,178 transcripts) were the most abundant. Other notable families included *Xanthomonadaceae* (4,988 transcripts), *Pectobacteriaceae* (4,812 transcripts), *Moraxellaceae* (2,676 transcripts), *Morganellaceae* (15,590 transcripts), *Hafniaceae* (6,332 transcripts), *Erwiniaceae* (23,068 transcripts), and *Anaplasmataceae* (1,249 transcripts).

In comparison, larvae exhibited a different microbiota composition, with bacteria accounting for 5.8% (13,149 transcripts) of the classified transcripts. Within the bacterial phyla, *Pseudomonadota* was the most abundant, with 12,158 classified transcripts. The families *Yersiniaceae* (1,753 transcripts), *Pseudomonadaceae* (1,897 transcripts), *Erwiniaceae* (1,085 transcripts), *Enterobacteriaceae* (2,073 transcripts), and *Anaplasmataceae* (2,616 transcripts) were prominent in larvae.

Fungi were present in both adults and larvae but were more abundant in adults, comprising 0.2% (1,194 transcripts) of the classified transcripts in adults and 0.44% (987 transcripts) in larvae. *Ascomycota* was the dominant fungal phylum in both adults and larvae, with 1,128 and 954 classified transcripts, respectively. Among the fungal families, *Didymellaceae* (132 transcripts) and *Mycosphaerellaceae* (101 transcripts) were prominent in adults, while *Saccharomycetaceae* (398 transcripts) was abundant in larvae.

In adults *Apicomplexa* is the dominant protozoan phylum with 63 transcripts, mainly represented families by *Sarcocystidae* (31 transcripts) and *Plasmodiidae* (30 transcripts). In larvae also *Apicomplexa* is the dominant protozoan phylum with 91 transcripts, mainly in families with *Sarcocystidae* (42 transcripts) and *Plasmodiidae* (47 transcripts).

Viruses were present in low abundance in both adults (115 transcripts) and larvae (71 transcripts), with *Uroviricota* being the dominant viral phylum with 45 transcripts in adults and *Kitrinoviricota* with 12 transcripts in larvae. The family *Autographiviridae* (28 transcripts) was prominent in adults, while *Polydnaviriformidae* (51 transcripts) was dominant in larvae.

The analysis also identified several abundant species in both adult and larval samples of *R. cerasi*. For the adult samples, the most abundant species included *Serratia liquefaciens*

(4,505 transcripts), *Stenotrophomonas rhizophila* (3,791 transcripts), *Serratia fonticola* (4,885 transcripts), *Rahnella victoriana* (7,006 transcripts), *Rahnella bonaserana* (4,863 transcripts), *Pseudomonas tensinigenes* (5,172 transcripts), *Pseudomonas iranensis* (3,601 transcripts), *Pseudomonas canadensis* (6,209 transcripts), *Pseudomonas atacamensis* (6,926 transcripts), *Providencia alcalifaciens* (4,346 transcripts), *Pantoea agglomerans* (10,537 transcripts), *Morganella morganii* (5,647 transcripts), *Lelliottia sp. WB101* (2,927 transcripts), *Lelliottia sp. AC1* (4,382 transcripts), *Klebsiella oxytoca* (10,272 transcripts), *Hafnia alvei* (4,821 transcripts), *Gibbsiella quercinecans* (3,723 transcripts), *Escherichia coli* (5,519 transcripts), *Erwinia billingiae* (3,824 transcripts), *Enterobacter cancerogenus* (5,480 transcripts), and *Buttiauxella agrestis* (7,151 transcripts) in the *Pseudomonadota* phylum. In the *Bacillota* phylum, *Oceanobacillus oncorhynchi* (3,559 transcripts), *Lysinibacillus sp. OF-1* (4,248 transcripts), *Enterococcus rotai* (2,961 transcripts), and *Bacillus wiedmannii* (3,145 transcripts) were among the most abundant species.

In adults, the fungal species *Ascochyta rabiei* was among the most abundant, with 132 transcripts. For protozoa, *Toxoplasma gondii* was the most abundant, with 31 transcripts. Among viruses, *Bracoviriform facetosae* was the most abundant, with 25 transcripts.

For the larval samples, the most abundant bacterial species included *Rahnella aceris* (1,534 transcripts), *Pseudomonas fragi* (470 transcripts), *Acetobacter orientalis* (344 transcripts), *Erwinia sp. E602* (233 transcripts), *Gluconobacter albidus* (264 transcripts), *Raoultella planticola* (201 transcripts), *Pectobacterium brasiliense* (184 transcripts), *Tatumella ptyseos* (166 transcripts), *Enterobacter ludwigii* (143 transcripts), *Gluconobacter thailandicus* (129 transcripts), and *Gluconobacter frateurii* (104 transcripts) in the *Pseudomonadota* phylum. In the *Bacillota* phylum, *Leuconostoc pseudomesenteroides* (369 transcripts) was among the most abundant species.

Among the fungal species, *Pichia kudriavzevii* (109 transcripts) was the most abundant in the larval samples. For viruses in the larval sample, *Bracoviriform facetosae* was the most abundant with 51 transcripts.

These species-level classifications provide further insights into the specific microbial composition of adult and larval *R. cerasi* samples, highlighting the diverse range of microorganisms present in each life stage.

3.4.7. Conformations of Entomopathogens Transcripts

To confirm the presence of classified entomopathogens, we meticulously searched the Kraken 2 report. Kraken2 (URL - 7) analysis revealed the presence of various entomopathogens in both adults and larvae samples:

In adults samples, Bacteria including *Bacillus thuringiensis* (100 contigs), *Lysinibacillus sphaericus* (53 contigs), *Paenibacillus* (162 contigs), *Photorhabdus* (3 contigs), *Serratia entomophila* (82 contigs), *Serratia proteamaculans* (49 contigs), *Pseudomonas fluorescens* (750 contigs), *Pseudomonas aeruginosa* (27 contigs), *Enterococcus gallinarum* (61 contigs), and *Raoultella terrigena* (14 contigs) were identified. Viruses with *Iflaviridae* (5 contigs), as well as fungi with *Fusarium* (71 contigs), were also found as shown in Table 11.

Table 11. BLASTx results of entomopathogens in adult samples.

Class	Entomopathogen	Contigs	Alignment Length	Identity %	Bit Score	E-Value
Bacteria	*Bacillus thuringiensis*	100	11 - 514	26.263 - 100	21.9 - 926	0 -7.87E-06
	Lysinibacillus sphaericus	53	26 - 590	88.764 - 100	55.8 - 1190	0 - 9.59E-10
	Paenibacillus	162	22 - 373	69.231 - 100	64.6 - 723	0 - 7.85E-06
	Serratia entomophila	82	25 - 271	67.47 - 100	57 – 521	7.99E-178 - 3.18E-09
	Serratia proteamaculans	49	22 - 477	71.642 - 100	48.5 - 932	0 - 1.90E-06
	Pseudomonas fluorescens	750	18 - 524	38.931 - 100	40.4 - 1000	0 - 3.53E-06
	Pseudomonas aeruginosa	27	67 - 385	77.885 - 100	54.3 - 795	0 - 5.64E-07
	Enterococcus gallinarum	61	34 - 452	94.118 - 100	65.5 - 917	0 - 2.47E-14
	Raoultella terrigena	14	42 - 689	59.574 - 100	59.7 - 1311	0 - 9.72E-11

Table 11: Continued

Viruses	*Iflaviridae*	5	82 - 208	91.346 - 100	178 - 405	2.05E-55 - 1.65E-132
Fungi	*Fusarium*	71	22 - 647	56.41 - 100	44.7 - 1123	0 - 3.74E-06

In larvae samples, bacteria including *Raoultella terrigena* (6 contigs), *Pseudomonas fluorescens* (7 contigs), and *Pseudomonas aeruginosa* (6 contigs), as shown in Table 12.

Table 12. BLASTx results of entomopathogens in larva samples.

Class	Entomopathogen	Contigs	Alignment Length	Identity %	Bit Score	E-Value
Bacteria	*Pseudomonas fluorescens*	7	66 - 88	96.97 - 100	112 - 176	1.08E-55 - 3.93E-29
	Pseudomonas aeruginosa	6	42 - 125	97.619 - 100	88.2 - 260	8.46E-87 - 4.32E-19
	Raoultella terrigena	6	36 - 104	100 - 100	76.6 - 217	2.79E-68 - 5.97E-19

We then extracted the transcripts associated with these microorganisms. Utilizing Kraken Tools (version 1.2), we conducted a targeted search based on microorganisms recognized as entomopathogens in the existing literature. Subsequently, to validate these findings, we subjected the extracted entomopathogen transcripts to a local BLASTx analysis using version 2.14.0. For each identified entomopathogen, we obtained the corresponding protein sequences from the NCBI database and conducted separate BLASTx analyses against the NCBI data, utilizing an e-value threshold of 1e-5. We then meticulously filtered the results and retained only the best BLASTx hits for each transcript query identifier by eliminating those with low identity and high e-values, ensuring the accuracy and reliability of our findings.

The figures (14, 15, 16, 17) visualize the BLASTx confirmation results for various entomopathogens in adult and larvae samples. These plots depict –log (E-value) on the y-axis and bit score on the x-axis, with points colored according to identity. Each point in these plots represents a transcript (contig) identified in the BLASTx analysis.

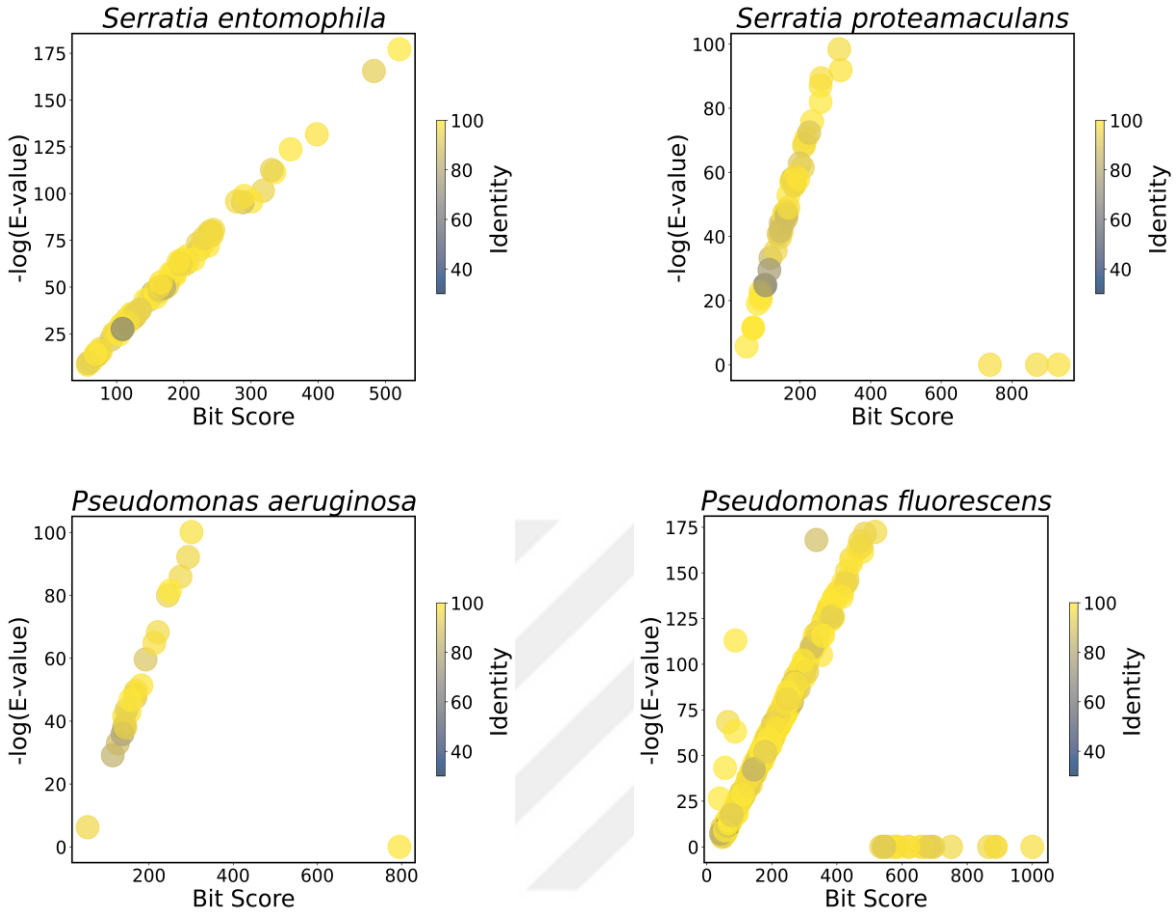
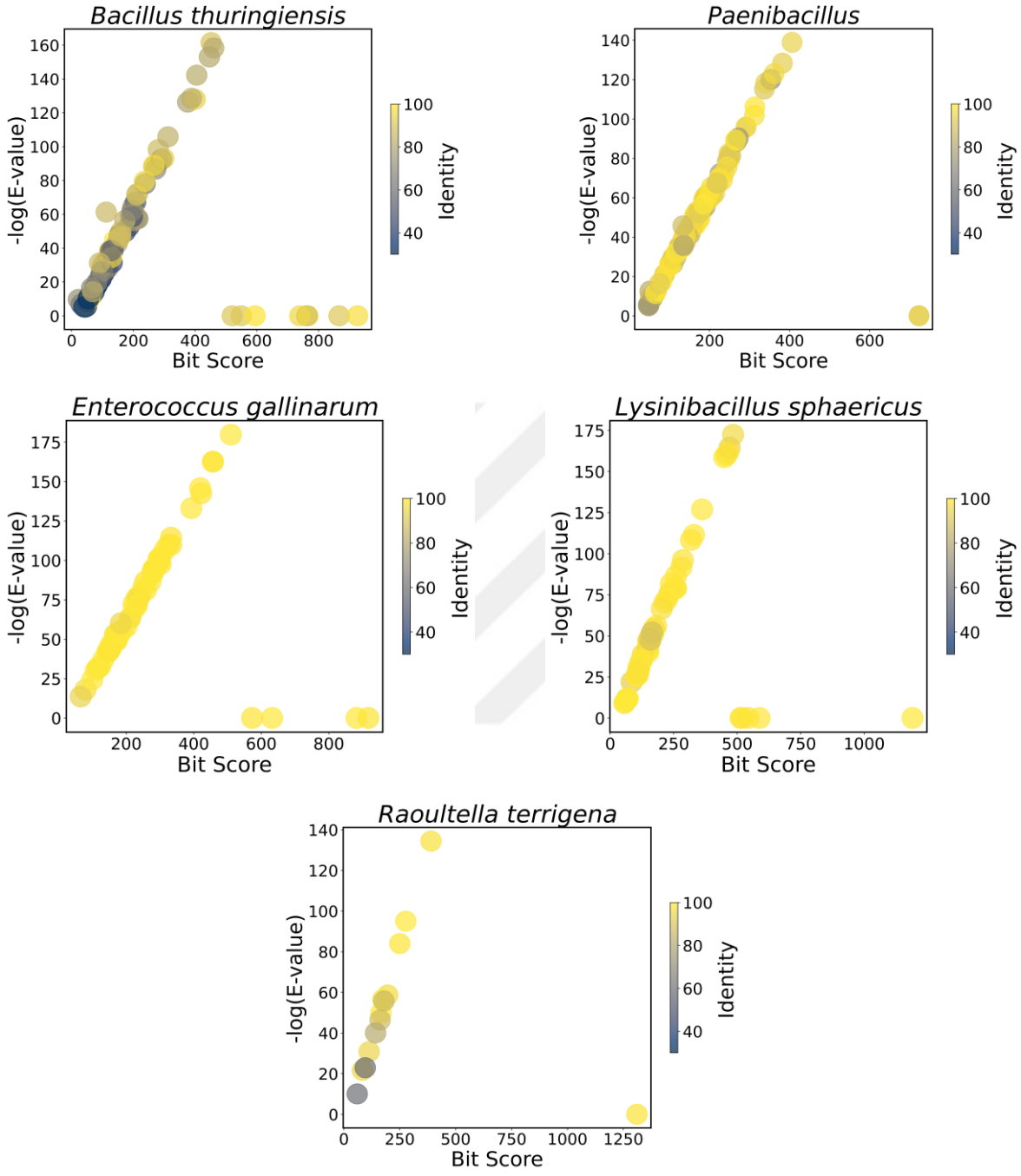


Figure 14. BLASTx analysis of bacterial entomopathogens in adults chery fruit fly. This figure presents the BLASTx results for bacterial entomopathogens found in adults samples. The entomopathogens identified include *Bacillus thuringiensis* 100 contigs (transcripts), *Lysinibacillus sphaericus* 53 contigs (transcripts), *Serratia entomophila* 82 contigs (transcripts), *Serratia proteamaculans* 49 contigs (transcripts), *Pseudomonas fluorescens* 750 contigs (transcripts), *Pseudomonas aeruginosa* 27 contigs (transcripts), *Enterococcus gallinarum* 61 contigs (transcripts), and *Raoultella terrigena* 14 contigs (transcripts).

Figure 14: Continued



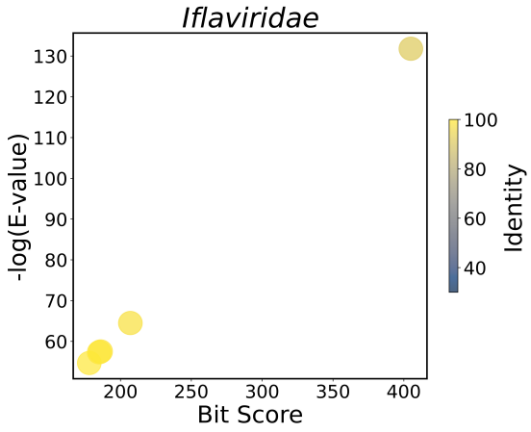


Figure 15. BLASTx analysis of viral entomopathogens in adults chery fruit fly This figure displays the BLASTx results for viral entomopathogen in adult samples. The identified entomopathogen includes *Iflaviridae* 5 contigs (transcripts).

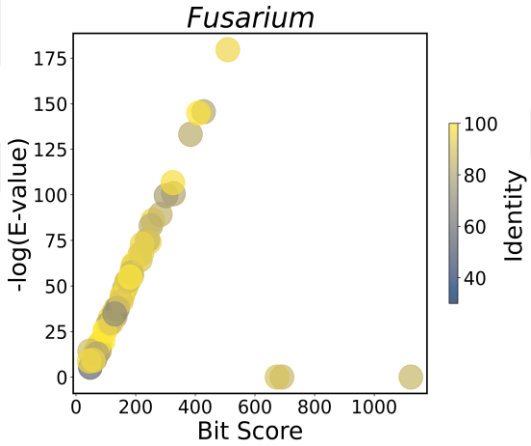


Figure 16.BLASTx analysis of fungal entomopathogens in adults chery fruit fly. The BLASTx results for fungal entomopathogens in adult samples are depicted in this figure. The identified fungal entomopathogen is *Fusarium* 71 contigs (transcripts).

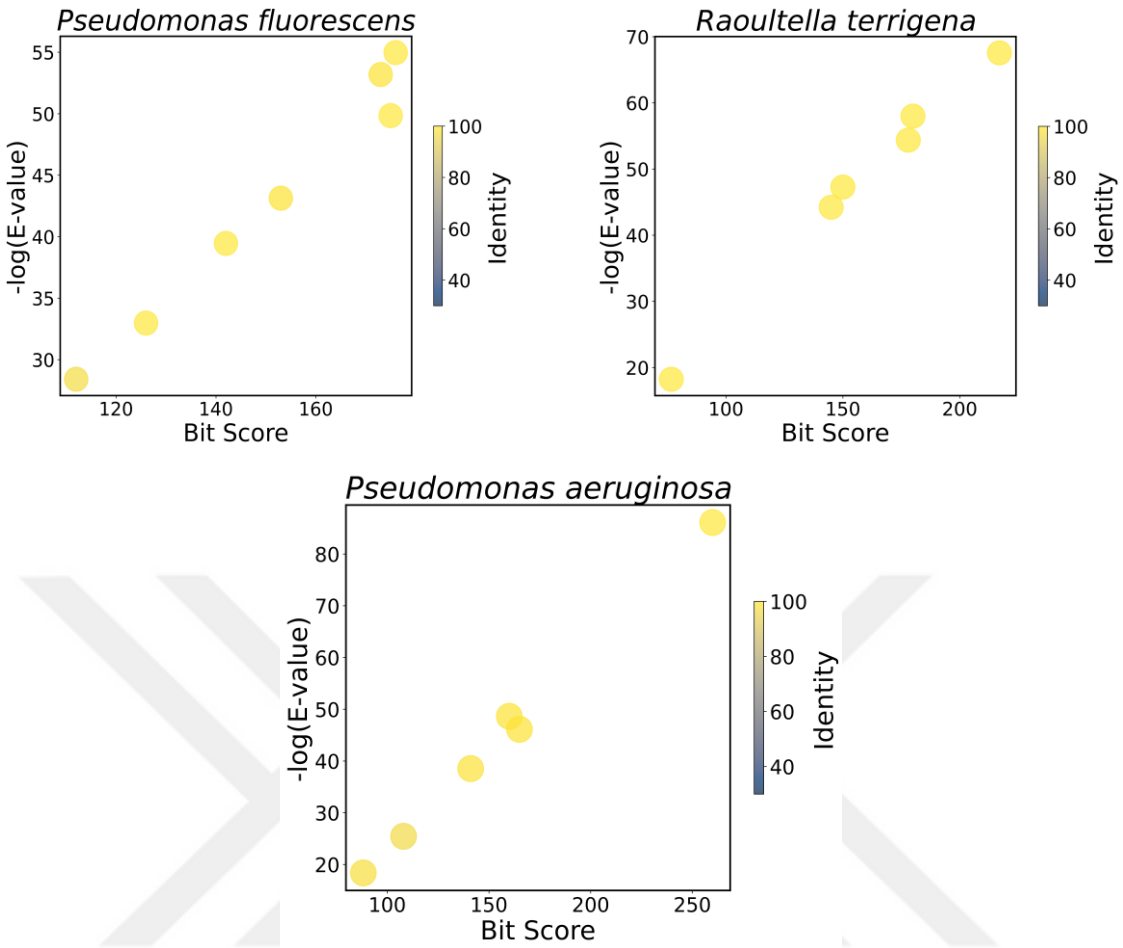


Figure 17. BLASTx analysis of bacterial entomopathogens in larval chery fruit fly.The BLASTx results for bacterial entomopathogens in larvae samples are presented in this figure. The identified bacterial entomopathogens include *Pseudomonas fluorescens* 7 contigs (transcripts), *Pseudomonas aeruginosa* 6 contigs (transcripts), and *Raoultella terrigena* 6 contigs (transcripts).

In adult samples, bacterial entomopathogens with a small number of transcripts were identified. *Bacillus subtilis* was found with 3 contigs (transcripts) exhibiting bit scores of 190, 160, and 213, E-values of 5.46E-59, 3.13E-45, and 1.69E-65, and identities of 88.35, 90.588, and 95.327, respectively. *Photorhabdus* was also identified with 3 contigs, showing bit scores of 134, 65.9, and 94.4, E-values of 4.25E-39, 8.11E-15, and 3.38E-24, and identities of 80.597 and 100 (for two contigs), respectively. *Yersinia entomophaga* was represented by a single transcript with an identity of 100, a bit score of 124, and an expected value of 3.99E-36.

However, for viral entomopathogens with a small number of transcripts were identified as *Baculoviridae*, with 2 contigs. These contigs showed identities of 96.667 and 98.571, bit scores of 64.7 and 152, and expected values of 6.90E-46 and 2.27E-14,

respectively. In adult samples also, a fungal entomopathogen with a small number of transcripts was found: *Aspergillus fumigatus*, represented by a single transcript with an identity of 95.789, a bit score of 184, and an expected value of 6.03E-60.

In larvae samples, *Serratia proteamaculans* were identified with just one transcript, showing an identity of 88.406, a bit score of 131, and an expected value of 8.13E-37. Additionally, in larvae samples, a fungal entomopathogen with a small number of transcripts was found. *Aspergillus fumigatus*, represented by a single transcript with an identity of 81.69, a bit score of 127, and an expected value of 2.54E-34.

4. DISCUSSION

Previous studies have highlighted the importance of microorganisms in the biology and management of cherry fruit flies (*R. cerasi*). Būda and colleagues (2022) investigated the responses of *R. cerasi* to volatile organic compounds (VOCs) from sour cherry fruit, identifying potential attractants for these flies. Daniel and Wyss (2009) studied the susceptibility of different life stages of *R. cerasi* to entomopathogenic fungi, emphasizing the potential of fungi like *Beauveria bassiana* and *Isaria fumosorosea* in controlling *R. cerasi* populations. Building on this research, Daniel and Wyss (2010) conducted field applications of *Beauveria bassiana*, demonstrating its potential for reducing fruit infestation by *R. cerasi*.

In this study, we utilized RNA-Seq datasets to perform *de novo* transcriptome assembly of the insect host, enabling us to gain insights into the composition of its associated microbiome. Omics techniques have revolutionized our ability to study entire microbial communities in their natural habitats, overcoming previous limitations associated with individual species studies in the laboratory. Acquiring benefit from the fact that the metatranscriptomic approaches also provide information about which genes the microbial community is expressing. The sequence matches most likely represent some of the most active microbes associated with the sample. By analyzing transcriptome data derived from RNA, we were able to identify the most active microbes associated with *R. cerasi.*

In the current study, the metatranscriptome analysis focused on identifying the composition and abundance of microbiota, as well as detecting entomopathogens, in both larvae and adults of *R. cerasi.*

The taxonomic distribution of microbial transcripts in adult and larval *R. cerasi* specimens highlights distinct differences in microbiota composition. Adults exhibited 57.8% microbial classified sequences, whereas larvae showed only 6.39% microbial classified species. Due to the host filtering step, not all host transcripts were completely filtered out, likely due to the low quality of the available genome for *R. cerasi* and the classification performed with the microbiome database. This was particularly notable for larvae, and because of their enclosed fruit habitat, diet, life stage, and the local environment. These factors are highly significant in explaining the structuring of the microbial community composition (Gurung et al., 2019; Engel & Moran, 2013; Mikaelyan et al., 2015) .

Bacteria dominated in both adult and larval stages of *R. cerasi*, consistent with findings in other insect species (Li et al., 2022; Kowallik & Mikheyev, 2021). The presence of fungi, viruses, and protozoa in lower abundance aligns with observations in various insect microbiomes (Chen et al., 2018; Xie et al., 2019; Kaltenpoth & Flórez, 2020). Archaea were minimal in both life stages and this could be due to the feeding resources of *R. cerasi* (Engel & Moran, 2013).

In adult *R. cerasi*, *Pseudomonadota* was the most abundant bacterial phylum, which is in line with studies on other insects where *Pseudomonadota* has been reported as a dominant phylum (Lixiang et al., 2023). The families *Enterobacteriaceae*, *Pseudomonadaceae*, and *Yersiniaceae* were particularly abundant, similar to their prevalence in other insect microbiomes (Martoni et al., 2023; Magagnoli et al., 2022). The presence of other notable families included *Xanthomonadaceae*, *Pectobacteriaceae*, *Moraxellaceae*, *Morganellaceae*, *Hafniaceae*, *Erwiniaceae*, and *Anaplasmataceae* in adult *R. cerasi* aligns with their occurrence in other insect microbiomes (Vecherskii et al., 2022; Moro et al., 2021; Peral-Aranega et al., 2023; Kaur et al., 2022; Martoni et al., 2023; Shell & Rehan, 2022; Kang et al., 2014).

Larval *R. cerasi* showed a microbiota composition distinct from that of adults, with a lower proportion of bacterial transcripts. However, bacteria still dominated the microbiota, which is consistent with observations in other insect (Vecherskii et al., 2022; Peral-Aranega et al., 2023; Kaur et al., 2022; Martoni et al., 2023; Magagnoli et al., 2022; Lixiang et al., 2023). The prevalence of *Pseudomonadota* and families *Yersiniaceae*, *Pseudomonadaceae*, *Erwiniaceae*, *Enterobacteriaceae*, and *Anaplasmataceae* in larvae mirrors their abundance in other insects.

The *Anaplasmataceae* family thant has been detected in adults and larvae, which includes small obligate intracellular α-proteobacteria, most closely related to mitochondria, These bacteria infect invertebrate hosts that are abundant and ubiquitous in the environment (i.e., ticks, insects) (Dahmani et al., 2017; Pruneau et al., 2014).

Pseudomonadaceae family which was detected in adults and larvae as *Pseudomonas spp.* are aerobic, Gram-negative bacteria that are prevalent in soils. They are especially well-suited for plant root colonization, and several strains show plant growth-promoting and/or biocontrol action against numerous plant diseases (Roquigny et al., 2017).

Erwiniaceae, detected in both adult and larval samples, are bacteria often associated with insects. These bacteria are assumed to primarily aid in nutrition by breaking down plant

material and fixing nitrogen. They can also hinder the growth of entomopathogenic fungi and are known to produce antibiotics (Cambronero-Heinrichs et al., 2023).

Enterobacteriaceae family was found in both adults and larvae. Investigations imply that interactions with arthropods played a role in the evolution of the *Enterobacteriaceae*. Aphids, psyllids, scale insects, whiteflies, weevils, and other insects all carry maternally transmitted *Enterobacteriaceae.* They play a vital function in the environment by participating in vegetative processes and spreading widely through insect vectors. Many species have been identified, some of which are related to specific disease processes. Some established species are increasingly being found in novel infectious disease situations and syndromes (Janda & Abbott, 2021; Moran et al., 2005).

Additionally, the *Yersiniaceae* family was detected in both adult and larval samples, comprising pathogenic and non-pathogenic species. *Yersiniaceae* is known for its pathogenicity and has been implicated in causing disease in the larvae of the New Zealand grass grub (Guern et al., 2020).

Fungi were more abundant in adult *R. cerasi* compared to larvae, with *Ascomycota* being the dominant phylum in both stages. This is in line with studies on other insects where *Ascomycota* has been reported as an insect fungal phylum (Mohammed et al., 2018; G. Gao et al., 2018). The presence of families like *Didymellaceae* and *Mycosphaerellaceae* in adult *R. cerasi* is consistent with their occurrence in other insect fungal communities (Pyszko et al., 2023). The abundance of *Saccharomycetaceae* in larval *R. cerasi* is also observed in other insect larvae (Klüber et al., 2022).

Ascomycota is a fungal phylum with several species that infect and kill insects are well-studied models for investigating the physiological interactions between fungi and insects, and they serve as biological controls for insect pests (Shang et al., 2015; T. Ahmad et al., 2019). On the other hand, the members of *Mycosphaerellaceae* are typically considered plant pathogens (Beule et al., 2017; Sánchez Márquez et al., 2011).

Protozoa, mainly represented by the phylum *Apicomplexa*, were present in both adults and larvae, with families *Sarcocystidae* and *Plasmodiidae* being dominant. This aligns with studies on other insects where *Apicomplexa* has been reported in other insects (Lantová et al., 2010; Lantova & Volf, 2014). *Plasmodiidae* contains the genus *Plasmodium*, responsible for malaria, transmitted through infected female *Anopheles* mosquitoes (Paton et al., 2019). It was detected with both stages but with relatively low identity and high E-values.

Sarcocystidae is also reported as including genera, causing diseases like toxoplasmosis in humans (Cabral et al., 2021). A group of *Apicomplexan* parasites, are known to infect invertebrates and have received attention for parasite-host coevolution and biological control of insects (Dias et al., 2017; Valigurová & Florent, 2021; Rueckert & Devetak, 2017; Logan et al., 2012; Hughes & Libersat, 2018).

Viruses were present in low abundance in adults and larvae (115 transcripts 71 transcripts, respectively). *Uroviricota* (double-stranded DNA bacteriophages) and *Kitrinoviricota* (positive-strand RNA viruses that commonly infect plants) being dominant viruses phylums in adults and larvae, respectively (Bernadus et al., 2023; Fuji et al., 2022). The presence of families *Autographiviridae* (double-stranded DNA bacteriophages) was dominated viruses in adults, and the most dominated viruses in larvae was with *Polydnaviriformidae* (double-stranded DNA viruses) family. This is consistent with their occurrence in other insect viromes (López-Cuevas et al., 2022; Antoine et al., 2021; Starchevskaya et al., 2023).

Autographiviridae, a virus family that infects bacteria, has received little attention in terms of its potential for biological pest control. Some study suggests that bacteriophages could be used as biological control agents for insects, In a study using bacteria and bacteriophage cocktails to control houseflies found that certain phages from the *Autographiviridae* family may be useful against insects (K. Zhang et al., 2024). Further research is required to fully understand the potential of *Autographiviridae* phages in insect biological control.

Polydnaviriformidae family is a polyphyletic assemblage of nonorganismal mobile genetic elements (MGEs) derived from several different groups of large DNA viruses of insects and recognized as insect viriforms specifically parasitoid wasps (Koonin et al., 2021; van Oers et al., 2023; Prado et al., 2023).

RNA viruses, mainly those classified under *Riboviria*, are also common in viromes and contribute majorly to the viral makeup in adults (Hernández-Pelegrín et al., 2022; da Silva Ferreira et al., 2020). Due to their lower abundance compared to other clades, they are not represented in the results.

These classifications offer insights into the microbial compositions of adult and larval *R. cerasi* samples, revealing a diverse range of microorganisms in each life stage. This information is vital for identifying and nominating potential entomopathogens within the microbiota composition of *R. cerasi*.

In this study, we used metatranscriptome analysis to detect entomopathogens in both larvae and adults of *R. cerasi*. The results showed that the most abundant bacterial entomopathogens were *Pseudomonas fluorescens, Serratia entomophila, Serratia proteamaculans, Enterococcus gallinarum, Lysinibacillus sphaericus,* and *Pseudomonas aeruginosa* in adults. These entomopathogens showed high abundances and good BLASTx hits, confirming their presence and abundance. *Bacillus thuringiensis* also had a significant number of contigs, although with relatively lower identity. *Raoultella terrigena* had relatively low transcript abundances but showed good BLASTx results.

The results for larvae samples indicate the presence of just the bacterial entomopathogens although with lower abundance. They include *Pseudomonas fluorescens* (7 contigs), *Pseudomonas aeruginosa* (6 contigs), and *Raoultella terrigena* (6 contigs), exhibited good BLASTx hits but with a low number of contigs (transcripts), suggesting a lower prevalence or abundance of these entomopathogens.

Pseudomonas fluorescens and *Bacillus thuringiensis* are well-known for their biocontrol capabilities against insect pests. *Pseudomonas fluorescens,* versatile biocontrol agent has been documented in diverse insects. *Bacillus thuringiensis* is the most studied entomopathogenic species for biological control of insect pests, and some of the toxin-producing strains have shown high mortality against specific insects compared to conventional insecticides used in the microbial pest management, primarily known for their insecticidal crystal proteins targeting lepidopteran and other insect pests (Pietri & Liang, 2018; Chakrabarty et al., 2020).

Serratia entomophila and *Serratia proteamaculans*, known to cause amber disease in the New Zealand grass grub, *Costelytra zealandica*, are highly specific to their host, affecting only larvae of this particular scarab species. Infected larvae stop feeding within 1–3 days after consuming the pathogenic bacteria. Normally dark, the larvae gut clears, and levels of major gut digestive enzymes (trypsin and chymotrypsin) drop significantly. This state can persist for 1–3 months before bacteria invade the haemocoel, leading to rapid insect death (Hurst et al., 2007).

Lysinibacillus sphaericus, commonly found in natural environments, possesses larvicidal properties against mosquitoes. Its sporulation stage produces BinA/BinB crystals, which are the primary agents of mosquitocidal activity. Commercial formulations of *L. sphaericus*, such as 2362 (VectoLex® and Spherimos®), C3-41 (Jianbao®), 1593 (Spicbiomoss®), and 2297 (VectoLex G®), have significantly contributed to the integrated

control of mosquitoes and mosquito-borne diseases, including malaria, dengue fever, filariasis, and yellow fever (Geng et al., 2021; Berry, 2012).

Pseudomonas aeruginosa is known for its broad-spectrum inhibitory effects on various organisms and its pathogenicity to mammals, insects, and nematodes. One of its secondary metabolites, hydrogen cyanide, is thought to contribute to its entomopathogenic properties. Studies have shown that cyanide of bacterial origin can inhibit the cytochrome c oxidase (CCO) of the respiratory chain in insects like the termite *Odontotermes obesus*. *Pseudomonas spp*., known for producing hydrogen cyanide, can effectively kill macroscopic insect pests through cyanide poisoning. This mechanism offers a novel approach to managing insect pests, particularly social insects like termites, by targeting their respiration rather than relying on infection or predation. This method may explain the high mortality rates observed in termites exposed to *P. aeruginosa* via bacterial-treated wood blocks, suggesting a combination of ingestion and contact intoxication (Chin et al., 2021).

Enterococcus gallinarum and *Raoultella terrigena* are not typically considered entomopathogenic bacteria, and there is limited information on their entomopathogenicity activity. However, studies have shown that *Enterococcus gallinarum* is effective against the turnip moth *Agrotis segetum*, and *Raoultella terrigena* can cause mortality in the adult green shield bug *Palomena prasina* (Gouli et al., 2021).

In terms of viral entomopathogens were in adults including *Iflaviridae* with 5 contigs, which showed acceptable BLASTx results. Additionally, *Baculoviridae* was identified with very low number of transcripts just 2 contigs, and showing high identities and low expected values.

Iflaviridae is a family of entomopathogenic viruses that primarily infect insects. These viruses are characterized by their positive-sense, single-stranded RNA genome. They have a wide host range and are detected in various insect species across different orders (Jakubowska et al., 2015). *Iflavirus* infections can vary in severity, ranging from asymptomatic to causing developmental abnormalities or death, as seen with deformed wing virus in honey bees. Interestingly, *Iflaviruses* have also been found in parasitic mites (Coyle et al., 2018; Daveu et al., 2021).

Baculoviridae is a family of large DNA viruses known for infecting a wide range of insect pests. They are characterized by their small size and predominantly double-stranded DNA structure, which contains genes crucial for virus replication. *Baculoviruses* are ideal

for targeted insect control due to their species-specific nature, posing no harm to plants, mammals, birds, fish, or non-target insects (Clem & Passarelli, 2013).

Additionally, of the identified fungal entomopathogens in adults, just *Fusarium* was the most detected. *Fusarium* species are known to exhibit entomopathogenic properties. Several *Fusarium* species have been reported as entomopathogens. However, it must be noted that not all *Fusarium* species are entomopathogenic, and the majority of them are known as plant pathogens. To effectively utilize *Fusarium* species as biological control agents, it is essential to identify and develop strains that exclusively exhibit entomopathogenic traits without damaging crops or other non-target organisms (Sharma & Marques, 2018).

5. CONCLUSION

The meticulous collection and analysis of *Rhagoletis cerasi* metatranscriptome specimens have provided valuable insights into the microbiota composition of this species, particularly regarding the potential role of entomopathogens in their ecology. The integration of advanced techniques such as Kraken 2 and BLASTx analysis has ensured the accuracy and reliability of our findings, offering a comprehensive overview of the entomopathogens present in both adults and larvae.

Our results indicate a distinct composition and abundance of microbiota and entomopathogens between the two life stages, underscoring the significance of developmental stage and habitat in microbiota studies. For adults, promising candidates for biological control include *Pseudomonas fluorescens, Serratia entomophila, Serratia proteamaculans, Enterococcus gallinarum, Lysinibacillus sphaericus,* and *Pseudomonas aeruginosa* as bacterial entomopathogens, and *Fusarium* as a potential fungal entomopathogen. However, for larvae, although bacterial entomopathogens such as *Pseudomonas fluorescens, Pseudomonas aeruginosa*, and *Raoultella terrigena* exhibited good BLASTx hits, their low abundance in transcripts suggests further investigation is warranted.

This study lays a solid foundation for future research into the ecological dynamics of entomopathogens in *R. cerasi* populations and their potential for use in sustainable pest management strategies. The insights gained not only enhance our understanding of the microbiota composition of *R. cerasi* but also provide valuable information for the development of targeted pest control methods.

6. RECOMMENDATIONS

Based on the identified entomopathogens in *R. cerasi*, we recommend further investigation into their potential as biological control agents. Specifically, the isolated entomopathogens, such as *Pseudomonas fluorescens, Serratia entomophila, Serratia proteamaculans, Enterococcus gallinarum, Lysinibacillus sphaericus, Pseudomonas aeruginosa,* and *Fusarium*, should be isolated in the laboratory and studied for their efficacy in controlling *R. cerasi* populations.

Isolating these entomopathogens would involve culturing them under controlled laboratory conditions, characterizing their growth requirements, and assessing their virulence towards *R. cerasi.* The efficacy of these entomopathogens can be further evaluated through field trials to determine their impact on *R. cerasi* populations and their potential as biological control agents.

Furthermore, the entomopathogens that showed promising BLASTx hits but were found in low abundance in transcripts should also be isolated and studied. These entomopathogens may play a significant role in the ecology of *R. cerasi* and could potentially be valuable biological control agents.

Overall, the isolation and study of these entomopathogens have the potential to provide sustainable pest management strategies for controlling *R. cerasi* populations, reducing the reliance on chemical pesticides, and minimizing environmental impacts.

7. REFERENCES

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