"Quando la tempesta sarà finita, probabilmente non saprai neanche tu come hai fatto ad attraversarla e a uscirne vivo. Anzi, non sarai neanche sicuro se sia finita per davvero. Ma su un punto non c'è dubbio. Ed è che tu, uscito da quel vento, non sarai lo stesso che vi è entrato."
(Murakami, Kafka sulla spiaggia)

Ai miei genitori,

ciò che per me è spigolo, linea interrotta, groviglio,

diventa sempre, come per miracolo, un cerchio perfetto.

Ai miei nonni,

che hanno ispirato ogni mia scelta e ogni mio passo.

University of Basilicata

International PhD Program in Applied Biology

Identification and Characterization of Insect Peptides with Antibacterial Activity

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XXXVII Cycle

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Abstract in English

Antimicrobial peptides (AMPs) constitute a chemically and structurally heterogeneous family of molecules produced by a wide range of living organisms, including plants, fish, amphibians, mammals, and insects. Their expression is particularly high in organisms that are frequently exposed to microbial invasions, where they play a key role in innate immune responses. Insects, in particular, represent one of the richest natural sources of AMPs. Over their long evolutionary history, they have developed a highly efficient immune system in which AMPs play a central role in defense against pathogens, allowing them to colonize a wide range of habitats. In recent years, interest in AMPs has significantly increased due to the rise in bacterial strains resistant to conventional antibiotics, positioning these peptides as potential therapeutic alternatives for infections caused by resistant pathogens. In this study, we investigated the antimicrobial activity of peptides extracted from the hemolymph of *Hermetia illucens* larvae (Diptera, Stratiomyidae), an insect known for its high expression of AMPs. Hemolymph samples were collected from larvae infected with *Escherichia coli* (Gram-negative) and *Micrococcus flavus* (Gram-positive), as well as from uninfected larvae, and subsequently treated by organic solvent precipitation. Antimicrobial activity was assessed through microbiological assays, including agar diffusion tests and microdilution assays, which the peptides demonstrated significant activity against pathogenic bacterial strains, including antibiotic-resistant strains. The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were determined for each experimental condition. Mass spectrometry analysis identified 33 antimicrobial peptides, 13 of which were differentially expressed in response to bacterial infection. The two selected peptides, MO_Ab1Lin and MO_Ab4, were chemically synthesized via solid-phase synthesis, which enabled their structural and functional characterization. The synthesized peptides demonstrated significant inhibitory potential in antimicrobial activity assays. Additionally, sequence analysis of the synthesized peptides showed a high degree of homology with peptides from the defensin family. In parallel, a cloning approach was developed to express two other peptides in a heterologous system (*Pichia pastoris*), with the goal of enhancing production and enabling a more detailed characterization of their biological activity. The results of this study highlight the potential of AMPs from *H. illucens*, both natural and synthetic, as promising candidates for the development of new antimicrobial therapies, particularly in the fight against antibiotic-resistant pathogens.

Abstract in Italian

I peptidi antimicrobici (AMPs) costituiscono una famiglia chimicamente e strutturalmente eterogenea di molecole prodotte da un'ampia gamma di organismi viventi, inclusi piante, pesci, anfibi, mammiferi e insetti. La loro espressione è particolarmente elevata negli organismi frequentemente esposti a invasioni microbiche, dove svolgono un ruolo chiave nelle risposte immunitarie innate. Gli insetti, in particolare, rappresentano una delle fonti naturali più ricche di AMPs. Nel corso della loro lunga storia evolutiva, hanno sviluppato un sistema immunitario altamente efficiente in cui gli AMPs giocano un ruolo centrale nella difesa contro i patogeni, permettendo loro di colonizzare una vasta gamma di habitat. Negli ultimi anni, l'interesse verso gli AMPs è aumentato significativamente a causa dell'incremento di ceppi batterici resistenti agli antibiotici convenzionali, posizionando questi peptidi come potenziali alternative terapeutiche per infezioni causate da patogeni resistenti. In questo studio, abbiamo indagato l'attività antimicrobica di peptidi estratti dall'emolinfa delle larve di *Hermetia illucens* (Diptera, Stratiomyidae), un insetto noto per l'elevata espressione di AMPs. I campioni di emolinfa sono stati raccolti da larve infettate con *Escherichia coli* (Gram-negativo) e *Micrococcus flavus* (Gram-positivo), così come da larve non infettate, e successivamente trattati mediante precipitazione con solventi organici. L'attività antimicrobica è stata valutata attraverso saggi microbiologici, inclusi test di diffusione su agar e saggi di microdiluizione, nei quali i peptidi hanno dimostrato un'attività significativa contro ceppi batterici patogeni, inclusi ceppi resistenti agli antibiotici. Sono state determinate la concentrazione minima inibitoria (MIC) e la concentrazione minima battericida (MBC) per ciascuna condizione sperimentale. L'analisi di spettrometria di massa ha identificato 33 peptidi antimicrobici, 13 dei quali erano espressi in modo differenziale in risposta all'infezione batterica. I due peptidi selezionati, MO_Ab1Lin e MO_Ab4, sono stati sintetizzati chimicamente mediante sintesi in fase solida, permettendo la loro caratterizzazione strutturale e funzionale. I peptidi sintetizzati hanno dimostrato un potenziale inibitorio significativo nei saggi di attività antimicrobica. Inoltre, l'analisi delle sequenze dei peptidi sintetizzati ha evidenziato un elevato grado di omologia con peptidi della famiglia delle defensine. Parallelamente, è stato sviluppato un approccio di clonaggio per esprimere altri due peptidi in un sistema eterologo (*Pichia pastoris*), con l'obiettivo di migliorarne la produzione e consentire una caratterizzazione più dettagliata della loro attività biologica. I risultati di questo studio evidenziano il potenziale degli AMPs di *H. illucens*, sia naturali che sintetici, come promettenti candidati per lo sviluppo di nuove terapie antimicrobiche, in particolare nella lotta contro i patogeni resistenti agli antibiotici.

1 Introduction

1.1 The Evolution and Impact of Antibiotics in Medicine

In 1947, S. A. Waksman introduced the term "antibiotic" to refer to a chemical compound produced by microorganisms that has the ability to hinder the growth of bacteria and other microbes. Over time, the meaning of antibiotics has broadened to include any substance that can kill or inhibit the growth of microorganisms (Mohr, 2016). The roots of antibiotics, however, date back to much earlier times; ancient cultures utilised treatments derived from moulds, mushrooms, and plant chemicals to treat diseases, thus marking the origins of antibiotics. Historical records show that ancient Egyptians, Greeks, and Chinese used mouldy bread and other substances with antimicrobial properties to treat infections. These early forms of antibiotics were effective due to the natural production of antibacterial compounds by the moulds and plants used (Money, 2024; Venturella et al., 2021). Nevertheless, the comprehension of infectious diseases and the contribution of microorganisms in generating illness only surfaced through progress in the field of microbiology. Antonie van Leeuwenhoek's groundbreaking research in the 17th century, utilising microscopes to study bacteria, established the foundation for future scientific breakthroughs. During the 19th century, scientists such as Louis Pasteur and Robert Koch provided additional clarification on the germ theory of disease, establishing that microbes were the causative agents of infectious diseases. Koch's research on anthrax and tuberculosis produced a set of conditions, known as the Henle-Koch postulates, for establishing a direct connection between certain microorganisms and diseases. The initial phase of antibiotic development was marked by a multitude of significant findings and improvements, which demonstrated the intersection of scientific investigation, medical necessity, and technological progress (Parija, 2023). Antibiotics have had a profound impact on medicine, leading to the saving of numerous lives and profoundly changing the way infectious diseases are treated. The identification of the initial antibiotic, mycophenolic acid, by Bartolomeo Gosio in 1893 represented a noteworthy achievement. Gosio isolated this compound from Penicillium moulds and proved its efficacy against *Bacillus anthracis*. The primary focus of this discovery was on the treatment of pellagra (Bentley, 2000; Parija, 2023). However, subsequent researchers such as Alexander Fleming, Ernst Duchesne, and others further advanced the knowledge of antibiotics. This eventually led to the widespread use of penicillin and other antibiotics to effectively tackle bacterial diseases (Ligon, 2004).

The identification and large-scale manufacturing of antibiotics during the early 20th century was a crucial milestone in the field of medicine, effectively mitigating the consequences of infectious diseases on human populations. Prior to the introduction of medicines, infectious diseases were a primary factor contributing to mortality rates, with catastrophic pandemics such as the plague, smallpox, and malaria inflicting widespread pain and death. The advent of antibiotics, along with enhanced understanding of infections and cleanliness protocols, revolutionised the field of public health. An illustrative instance is the plague induced by *Yersinia pestis*, which devastated populations throughout history. In the absence of antibiotic treatment, plague outbreaks led to fatality rates ranging from 50% to 90%. Although there have been significant advancements in the field of medicine, the plague continues to pose a risk to public health, and there are worries regarding its possible utilisation as a biological weapon (Hawgood, 2008).

This progression demonstrates the profound influence that antibiotics have had on the field of medicine, namely in the fight against infectious diseases. Natural antibiotics are an extensive and varied collection of bioactive chemicals that have great promise for scientific investigation and the creation of novel medications. The intricate and diverse array of chemicals present in microbes, plants, and fungi offers a fertile basis for the extraction and examination of novel antibiotic substances (Bentley & Bennett, 2003). Streptomyces is a crucial reservoir of natural antibiotics within the domain of bacteria. Their capacity to generate a diverse array of bioactive substances renders research on the identification and isolation of novel molecules exceedingly promising (Quinn et al., 2020). Plants possess intricate defence chemistry and produce a wide variety of antibiotic substances. Oregano essential oil has attracted significant attention due to its antibacterial properties, while olive leaf extract has been extensively studied for its potential therapeutic effects (Martino et al., 2009). Synthetic antibiotics are manufactured through chemical synthesis in a controlled laboratory setting. Linezolid, aminoglycosides, and quinolones are prime illustrations of artificial antibiotics. On the other hand, semi-synthetic antibiotics are obtained from natural sources but are then altered in the laboratory to enhance their effectiveness. Amoxicillin, ampicillin, and methicillin are all examples of semi-synthetic antibiotics (Leisner, 2020).

1.2 Antimicrobial Resistance

Antimicrobial resistance (AMR) is an increasingly widespread problem that pertains to the ability of bacteria to survive exposure to doses of antibiotics that would typically kill or inhibit the growth of susceptible bacteria (Christaki et al., 2019). In recent years, there has been a worrisome surge in antibiotic resistance, primarily caused by improper and indiscriminate use of these drugs. Antibiotic resistance can manifest in various ways: bacteria can develop mechanisms to neutralise antibiotics, modify their targets, or develop alternate metabolic pathways to survive despite antibiotic treatment (Darby et al., 2024). Furthermore, the extensive use of antibiotics in human medicine, veterinary medicine, and agriculture has contributed to the emergence of this issue. The problem closely affects Italy as well, where antibiotic resistance remains among the highest in Europe and among high-income countries. According to the latest data published by the European Centre for Disease Prevention and Control, approximately 11,000 out of the 33,000 deaths caused by antibiotic resistance in Europe each year are recorded in our country (*Antimicrobial Resistance Surveillance in Europe 2023 - 2021 Data*, n.d.).

When normalising the data to the population (as Italy is the third most populous country in Europe after Germany and France), it is revealed that Italy ranks second after Greece in the overall burden of antibiotic-resistant bacterial infections, including the number of deaths per 100,000 inhabitants. For several years, the World Health Organisation has identified antibiotic resistance as a major global health issue. It is predicted that by 2050, antibiotic resistance could be responsible for an additional 10 million direct deaths per year (Figure 1.1), equivalent to the total global deaths from cancer recorded in 2020 (de Kraker et al., 2016). It is estimated that it might become the leading cause of death worldwide and could entail costs exceeding 100 trillion euros. It is, therefore, crucial to implement a strategy for the rational use of antibiotics; only in this way can we ensure the long-term effectiveness of these valuable drugs, safeguarding public health and preventing complications associated with antibiotic resistance. These valuable drugs safeguarding public health and prevent complications associated with antibiotic resistance.

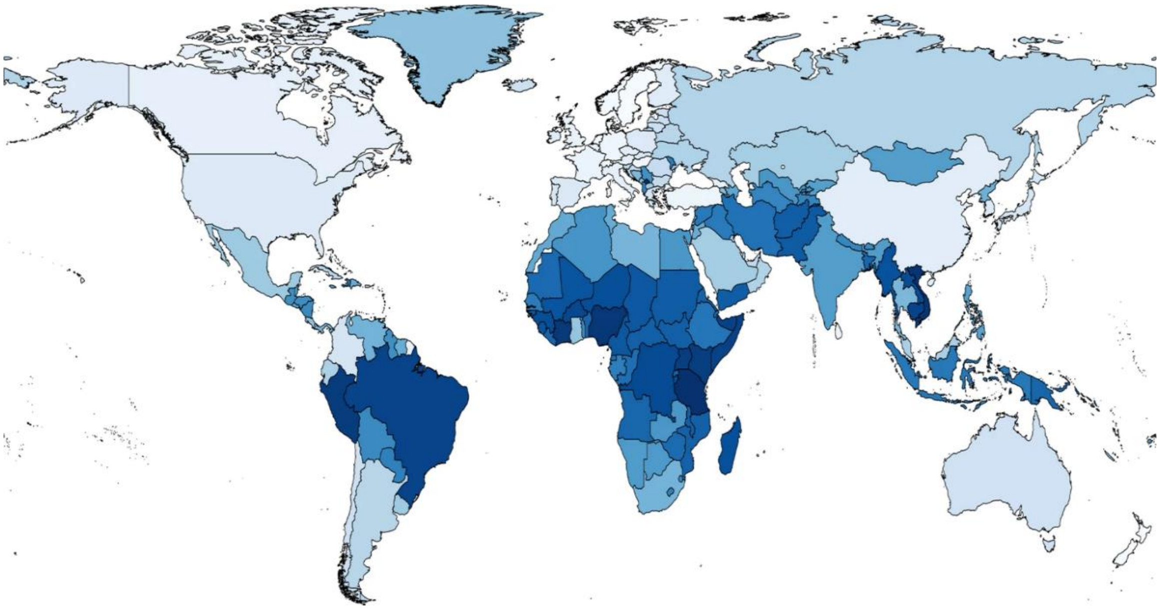

Figure 1.1. Global predictions of antimicrobial resistance (AMR) abundance in all countries and territories in the world. Map colored according to predicted abundance of AMR from light blue (low AMR abundance) to dark blue (high AMR abundance) (Hendriksen et al., 2019).

1.3 History of Antimicrobial Resistance

The history of antibiotic resistance illustrates the ongoing battle between medicine and bacterial infections. Antibiotics have revolutionized medical practice, making many infections treatable. However, resistant bacterial strains emerged almost immediately after antibiotics were introduced. Penicillin, discovered in 1928, was the first antibiotic to efficiently treat bacterial infections in soldiers during World War II. Yet, *Staphylococcus* strains resistant to penicillin were recorded before its widespread use in 1940. Methicillin, marketed in 1959, faced resistance from *Staphylococcus* just a year later (Sengupta et al., 2013). Vancomycin, introduced in 1958 for methicillin-resistant *staphylococci*, encountered resistance in coagulase-negative *staphylococci* by 1979. A decade later, resistance in enterococci was documented (Courvalin, 2006), and by 1997, less-susceptible strains of *S. aureus* appeared in Japan. Tetracycline, released in 1950, showed resistance in Shigella strains by 1959. Levofloxacin, used in 1996, saw pneumococcus bacteria exhibit resistance the same year (Sengupta et al., 2013). While the pharmaceutical industry produced enough new antimicrobial drugs for twenty years, the discovery rate of novel antibiotics has slowed since the 1980s (Parmar et al., 2018). The emergence of antimicrobial resistance, coupled with the scarcity of new antibiotics, has led to a significant rise in infections caused by multidrug-resistant or extensively drug-resistant organisms, posing a substantial challenge in global clinical practice.

1.4 Mechanism of Antimicrobial Resistance

Resistance to antibiotics usually occurs due to the destruction or modification of antibiotics, changes in the target, and reduced antibiotic accumulation caused by decreased permeability or increased efflux (Figure 1.2). Alternatively, antibiotic resistance might arise from the bacterial cell undergoing a worldwide adaptation (Christaki et al., 2019).

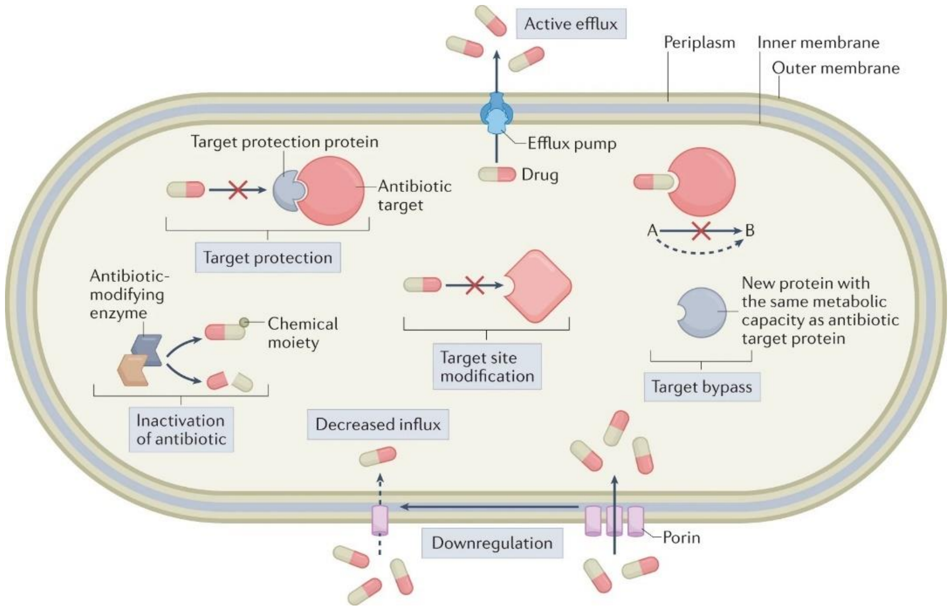

Figure 1.2. Mechanisms of Antimicrobial Resistance. This image illustrates various mechanisms by which bacteria exhibit resistance to antibiotics (Darby et al., 2023).

In this context, bacteria can exhibit antibiotic resistance through intrinsic, acquired, or adaptive mechanisms (J.-H. Lee, 2019) . Intrinsic resistance refers to the resistance that is displayed due to the inherent characteristics of the bacterium. Intrinsic resistance refers to the natural resistance exhibited by organisms without the need for external factors. An example of this is the glycopeptide resistance displayed by Gram-negative bacteria. This resistance is caused by the impermeability of the outer membrane found in the cell envelope of Gram-negative bacteria.

Acquired resistance refers to the ability of a bacterium that was previously sensitive to develop resistance due to either a mutation or the acquisition of new genetic material from an external source through horizontal gene transfer. Horizontal gene transfer can take place through three primary methods. Transformation is a process of genetic recombination where DNA pieces from a deceased bacterium enter a recipient bacterium and become part of its chromosome. Only a limited number of bacteria possess the intrinsic ability to undergo transformation. Transduction is a process where genetic material is transferred from one bacterium to another using a bacteriophage. Conjugation is the primary mechanism of horizontal gene transfer. Bacterial conjugation is the process by which genetic material is transferred from one bacterial cell to another by direct physical contact between the cells. A conjugation bridge is formed between the two bacterial cells, facilitating the transfer of a plasmid from the donor cell to the recipient cell. Multiple resistance genes are frequently found on a single plasmid, allowing for the transmission of multidrug resistance in a single conjugation event. Mobile genetic elements, such as transposons, integrons, and insertion sequences, facilitate the assembly of numerous resistance genes on a single plasmid. Common region refers to the shared portion of a sequence or set of components. ISCR elements are specific genetic elements that are involved in the movement of genes (A. H. Holmes et al., 2016; J.-H. Lee, 2019; Motta et al., 2015; Munita & Arias, 2016; Rizi et al., 2018).

Adaptive resistance refers to the ability of microorganisms to develop resistance to antibiotics due to specific environmental signals, such as stress, growth status, pH, ion concentrations, nutritional circumstances, or exposure to sub-inhibitory amounts of antibiotics. Unlike intrinsic and acquired resistance, adaptive resistance is temporary. However, this resistance typically returns to its initial condition once the triggering signal is eliminated (Fernández et al., 2011; J.-H. Lee, 2019; Motta et al., 2015; Rizi et al., 2018).

Adaptive resistance arises from alterations in gene expression in response to environmental changes and may be due to epigenetic modifications rather than genetic mutations. It is believed that DNA methylation, performed by the DAM methylase enzyme, can create distinct gene expression patterns within a bacterial population, contributing to the variability and epigenetic inheritance of gene expression. This process is crucial for the development of adaptive resistance and involves the regulation of efflux pumps and porins (Motta et al., 2015).

1.4.1 Antibiotic Inactivation

One common method of resistance in numerous disease-causing bacteria is altering or rendering ineffective the antimicrobial medication. Typically, this is achieved through enzymatic activity and does not typically require modifications to the primary components of the bacterial cell (Schaenzer & Wright, 2020). The modification of antibiotics can be broadly categorised into two mechanisms: the inactivation of the antibiotic by degradation or the modification of the antibiotic through the transfer of a chemical group. Both of these processes are prevalent in bacteria because of the mobility of the coding genes (Darby et al., 2023).

Antibiotic inactivation is a significant mechanism of drug resistance, whereby the antibiotic's chemical structure is altered or degraded, resulting in reduced effectiveness. This might hinder the outcome of clinical treatment. Antibiotic inactivation can occur through processes such as the hydrolysis of β-lactam antibiotics by β-lactamases and the binding of tetracycline hydroxylase to render tetracyclines inactive. Beta-lactamases are enzymes that provide resistance to β-lactam drugs by catalysing the hydrolysis of the amide bond inside the β-lactam ring, resulting in the degradation of the drug (Tooke et al., 2019). Beta-lactamases emerged in nature due to result of microbes producing β-lactam antibiotics, and they have been the subject of research since the 1940s. The number of identified β-lactamases is constantly growing. Currently, the Beta Lactamase DataBase documents more than 7,000 unique β-lactamases (Naas et al., 2017).

The concern over resistance to carbapenems is significant due to their high potency as antibiotics. Moreover, the concurrent development of resistance to other β-lactam antibiotics might greatly restrict the available therapeutic choices (L. M. Lima et al., 2020). Extended spectrum β-lactamases (ESBLs) confer resistance to extended-spectrum cephalosporins and monobactams (Nepal et al., 2017). Carbapenem resistance can occur due to the presence of carbapenemase or due to the combination of ESBLs and loss of porin. In 2017, the World Health Organisation (WHO) identified three crucial infections that have developed resistance to carbapenems (Organization, 2022). Carbapenemases, including Klebsiella pneumoniae carbapenemase (KPC, class A), New Delhi metallo-beta-lactamase (NDM, class B), and oxacillinase (OXA, class D), break down penicillins, cephalosporins, and carbapenems, greatly limiting treatment choices.

Continuously, novel iterations of carbapenemase enzymes are being identified, including KPC-55, NDM-19, and OXA-679, which exhibit significant resistance to carbapenems. NDM enzymes, initially identified in India in 2009, have since become prevalent and confer resistance to nearly all β-lactam antibiotics, with the exception of aztreonam. Research has demonstrated that multiple bacterial species commonly found in hospital intensive care units frequently possess antibiotic resistance genes, particularly the β-lactam resistance gene. Multi-drug-resistant plasmids frequently contain β-lactamase, which aids in its transmission between various bacteria. Furthermore, β-lactamases frequently have connections with insertion sequences that have the ability to move their genes and impact their expression. Enzymes, such those in the Tet family, can inactivate medicines such as tetracycline. These enzymes, capable of horizontal gene transfer across bacteria, confer significant resistance to tetracycline and are prevalent in multi-drug-resistant bacteria. Tet (X3/X4/X5) enzymes provide resistance to tigecycline and have been detected in *Enterobacter* and *Acinetobacter* isolates in China. The enzymatic capacity to break down tetracyclines varies, with certain enzymes demonstrating higher efficacy in degrading the substrate.

1.4.2 Antibiotic Modification

Antibiotics can potentially lose their effectiveness when a chemical group is transferred. Enzymes that alter the properties of different antibiotics have been discovered, such as aminoglycosides, macrolides, rifamycin, streptogramins, lincosamides, and phenicols.

Enzymes such as acetyltransferase, phosphotransferase, or nucleotidyltransferase can render aminoglycosides ineffective by modifying the hydroxyl or amine groups of the medication. This modification reduces the drug's affinity for the target. An illustrative instance is ApmA, an enzyme that deactivates apramycin by acetylating it (Ramirez & Tolmasky, 2010). In contrast, lincosamide antibiotics can undergo modification by the action of nucleotidyltransferases, which are produced by the lnu genes and add phosphate groups to the antibiotic. These genes are frequently located on mobile genetic elements, which enable the rapid dissemination of resistance among bacteria (Feßler et al., 2018). Macrolides are subject to changes through phosphotransferase and esterase processes, which hinder their ability to bind effectively to the 50S ribosome. The structure of phosphotransferases has been elucidated, revealing their membership in the same superfamily as the aminoglycosides (Golkar et al., 2018). Acetyltransferase typically modifies phenicol and streptogram antibiotics. An instance of this is chloramphenicol acetyltransferase (CAT), which facilitates the transfer of an acetyl group, hence impeding the binding of chloramphenicol to the ribosome (Gu Liu et al., 2020). Virginiamicin acetyltransferase (Vats) modifies streptograms, hence decreasing the antibiotic's effectiveness (Q. Li et al., 2020). Rifamycin is susceptible to inactivation by ADP-ribosyltransferase, glycosyltransferase, phosphotransferase, and monooxygenase. These enzymes hinder the binding of rifamycin to RNA polymerase and can decrease the vulnerability of the organism, particularly if they are found on mobile genetic elements like plasmids (Stogios et al., 2016). Recent findings have revealed that rifamycin can be linearised by the action of rifamycin monooxygenase (Rox) enzymes, resulting in the disruption of their interaction with RpoB (Koteva et al., 2018).

1.4.3. Target Replacement or Target Bypass

The phenomenon of target replacement involves the alteration of the drug's target site within the microbial cell, rendering the antimicrobial agent ineffective. For instance, bacteria can acquire mutations in genes encoding penicillin-binding proteins (PBPs), which are critical for cell wall synthesis. This alteration can lead to reduced binding affinity for β-lactam antibiotics, such as penicillin, effectively allowing the bacteria to survive despite the presence of these drugs (Aly & Balkhy, 2012). Furthermore, the emergence of extended-spectrum β-lactamases (ESBLs) and carbapenemases exemplifies how bacteria can enzymatically inactivate β-lactam antibiotics, further complicating treatment options (Eiamphungporn et al., 2018). The prevalence of these resistance mechanisms is not limited to a single species; studies have shown that various strains of *Escherichia coli* and other pathogens exhibit similar patterns of resistance, often encoded on mobile genetic elements that facilitate rapid dissemination among bacterial populations (Botrel et al., 2010).

In contrast, target bypass refers to the ability of bacteria to circumvent the action of an antimicrobial agent through alternative pathways or mechanisms. This can occur when bacteria develop resistance through the upregulation of efflux pumps, which actively expel antimicrobial agents from the cell, thereby reducing intracellular concentrations of the drug (Varela et al., 2021). For example, *Pseudomonas aeruginosa* employs multiple efflux systems that contribute to its intrinsic and acquired resistance, complicating treatment regimens (Poole, 2014). Additionally, some bacteria can modify metabolic pathways to bypass the inhibited step targeted by the antibiotic, thus maintaining their viability despite the presence of the drug (Kapoor et al., 2017).

1.4.4 Decreased Permeability of the Bacterial Outer Membrane

To carry out their function, numerous antimicrobial agents must traverse the bacterial cell membrane to access their intended site. The double membrane structure found in Gram-negative bacteria plays a crucial role in their resistance to antibiotics that target Gram-positive pathogens. This structure makes the cellular envelope highly impermeable, posing a significant obstacle to the development of new antimicrobial drugs that can effectively penetrate the bacterial cells (N. N. Mishra et al., 2012). Furthermore, alterations in the envelope's structure, such as the reduction of porins or modifications in the phospholipid and fatty acid composition of the cytoplasmic membrane, might impact a drug's capacity to enter the cell and can contribute to the development of antimicrobial resistance. Gram-positive bacteria do not have an outer membrane, which inherently makes them more susceptible to many antibiotics. However, research has demonstrated that alterations in the composition of the inner membrane, which impacts its fluidity, play a crucial role in decreasing antibiotic permeability. Mycobacteria have an elongated outer lipid layer and a polysaccharide capsule coating, which effectively obstructs the entry of hydrophilic molecules into the cell (Draper, 1998).

Porins serve as the primary pathway for hydrophilic antibiotics, including β-lactams, fluoroquinolones, tetracyclines, and chloramphenicol, to enter the bacterial outer membrane. The quantity and nature of porins present on the outer membrane will influence the penetration of hydrophilic antibiotics and, consequently, the vulnerability of the bacterial cell to these antibiotics (Fernández et al., 2011). Alterations impacting the expression or functionality of porins can result in the development of acquired antibiotic resistance.

These mutations can result in various outcomes, including the loss of porins, alterations in their size or conductance, or decreased production of porins. Alterations in porin expression typically result in a modest amount of antibiotic resistance.

Nevertheless, it is frequently observed that bacterial strains exhibit an amplified impact of alterations in porin expression due to the simultaneous presence of other resistance mechanisms. Essentially, the decreased absorption of the antibiotic caused by alterations in porin expression amplifies the impact of concurrent resistance mechanisms such as efflux pumps or antibiotic-degrading enzymes, resulting in a high level of resistance (Fernández et al., 2011).

1.4.5 Efflux Pumps

Efflux pumps are intricate bacterial systems located on the cytoplasmic membrane that require energy to function. They have the ability to expel harmful chemicals from the cell. The initial discovery of the efflux pump, which removes tetracycline from bacterial cells, was documented in *E. coli* in 1980. This pump was shown to be encoded by a plasmid (Ball et al., 1980; McMurry et al., 1980). Since then, a multitude of instances of efflux systems implicated in antibiotic resistance have been discovered in both Gram-positive and Gram-negative bacteria. Efflux systems have the ability to transport various substances that are not connected to each other. This can lead to the development of multidrug resistance, there are also drug-specific efflux pumps. Most of the time, multidrug efflux mechanisms are encoded in the chromosome of bacteria and can sometimes account for the natural resistance of bacteria to certain antibiotics. Although multidrug efflux mechanisms are widely present in bacteria, only a small number of them provide clinically significant antibiotic resistance. Clinical resistance typically occurs due to mutational events that result in higher pump expression or enhanced pump effectiveness (A. H. Holmes et al., 2016). Substrate-specific efflux pumps are typically found on mobile genetic components, such as genes (Fernández et al., 2011). Substrate-specific efflux pumps, such as those for tetracyclines, macrolides, and chloramphenicol, are exemplified by Poole (Poole, 2005).

1.5 Antimicrobial Peptides: A Promising Alternative to Overcome Antibiotic Resistance

There is currently a growing focus on antimicrobial peptides as a potential source of safe and effective novel medications. These biologically active molecules are derived from non-traditional and undiscovered sources. Antimicrobial peptides (AMPs) are small bioactive proteins naturally produced by all living organisms. They serve as crucial and essential components of the innate immune system, acting as the first line of defence against microbial attacks in eukaryotes. In prokaryotes, AMPs are produced as a competitive strategy to restrict the growth of other microorganisms (Huan et al., 2020). In recent years, there has been significant focus on the use of AMPs as new antimicrobials for treating microbial infections (Moretta et al., 2021). AMPs offer several advantages over conventional antibiotics, including low resistance levels, broad-spectrum activity with minimal harm to the host, synergistic effects on antimicrobial activity when combined with conventional antibiotics, and rapid elimination of microorganisms (Mahlapuu et al., 2016) AMPs features have positioned it as the most promising choice for addressing antibiotic resistance.

1.5.1 History of Antimicrobial Peptides

The initial discovery of AMPs dates back to 1921 when Alexander Fleming observed a lysis process in a bacterial culture obtained from the nasal mucosa of a patient with common acute rhinitis (Fleming, 1932). Dr. Fleming named the agent responsible for bacterial lysis "lysozyme," which is now recognized as a generic term for over 50 different intracellular digestive enzymes contained within specific proteolytic organelles (Bainton, 1981). Immediately following this discovery, Alexander Fleming stated that this bacteriolytic ingredient is distributed throughout the human body.

The first clearly identified AMP was discovered in 1939 by Dubos and his team in a culture of aerobic sporulating bacilli. They isolated an alcohol-soluble substance named tyrothricin, which exhibited bactericidal activity against numerous Gram-negative and Gram-positive bacteria (Dubos, 1939). Later, two distinct components were purified from tyrothricin: tyrocidine, effective against both Gram-positive and Gram-negative bacteria, and gramicidin, which was particularly effective against Gram-positive bacteria when applied topically (Dubos & Hotchkiss, 1941; Mootz & Marahiel, 1997). Initially, interest in AMPs was limited as attention was more focused on the development of new antibiotics. However, with the emergence of antibiotic resistance mechanisms, AMPs have been re-evaluated as potential therapies against infections (Aminov, 2017). Hans G. Boman and his group undertook a series of unique research in the 1970s and 1980s. These studies led to the discovery of antimicrobial peptides derived from insects. These peptides have a significant stimulatory effect on the immune response in Drosophila, as reported by Faye and Lindberg (2016). Boman's keen fascination with the functionality of the human immune system led him to redirect his attention towards the initial defensive mechanisms in the vertebrate immune system that take place prior to the development of antibodies, which are necessary during the early phases of infections. Thus, he utilised insect species, which do not possess lymphocytes and immunoglobulins, as a primary paradigm for his experimental investigations of the immune system using specific stimuli (HULTMARK et al., 1982a). In 1981, Boman and his group discovered and analysed a novel category of antimicrobial peptides (AMPs) known as cecropins. These peptides demonstrate a notable ability to combat Gram-negative bacteria, as demonstrated by their considerable antibacterial action (Steiner et al., 1981). In 1987, Zasloff et al. (1987) identified a new family of AMPs called magainins: these AMPs are composed of two small peptides, each consisting of 23 amino acids. The researchers extracted the amphipathic peptides, which possess a wide range of antibacterial properties against various bacteria, fungus, and protozoa, from the skin of *Xenopus laevis*. In addition, scientists successfully identified the initial mammalian skin AMP in 1994. This AMP is part of a broader category of tiny positively charged antimicrobial substances known as cathelicidins. This AMP has been discovered in humans, as well as in other animals such as birds and marine species including cattle, sheep, pigs, chickens, and certain fish. It demonstrates a wide range of antimicrobial effects against bacteria, viruses, and fungi (Kościuczuk et al., 2012). Without a doubt, this captivating research, along with other studies conducted in the past 60 years that have identified and described various antimicrobial peptides (AMPs), has sparked a widespread effort to fully comprehend the role of AMPs in our natural immune system (Faye & Lindberg, 2016; HULTMARK et al., 1982b; Liddle et al., 1995; Lung et al., 2001; Q. Wu et al., 2018).

1.5.2 Structural Characteristics of AMPs

AMPs are molecules produced by genes that have been conserved throughout evolution. These molecules have a wide range of structures and functions, allowing them to effectively combat different infections in various animals. However, AMPs have certain shared properties: AMPs are short molecules consisting of 15 to 50 amino acids or frequently less than 100 amino acids. They have a positive charge ranging from +2 to +11. They possess an amphipathic structure, characterised by both hydrophobic and hydrophilic areas, enabling them to dissolve readily in aqueous environments (Moretta et al., 2021). Anionic peptides, which possess a net negative charge ranging from -1 to -7, belong to a less prevalent category of antimicrobial peptides. These peptides have been discovered in vertebrates, invertebrates, and plants. These peptides contain several negatively charged residues of aspartic acid and glutamic acid. In mammals, they are present in different organs such as the brain, epidermis, respiratory system, and gastrointestinal tract (Moravej et al., 2018; Moretta et al., 2021). The structural flexibility of most AMPs can be attributed to their amphiphilic character. AMPs are typically categorised into four groups based on their secondary structure (Figure 1.3).

These groups include α-helical linear peptides, β-sheet peptides with two or more disulfide bonds, β-hairpin or β-loop peptides with a single disulfide bond and/or cyclization of the peptide chain, and extended structures (Boparai & Sharma, 2020; Jung & Kang, 2014; Moretta et al., 2021).

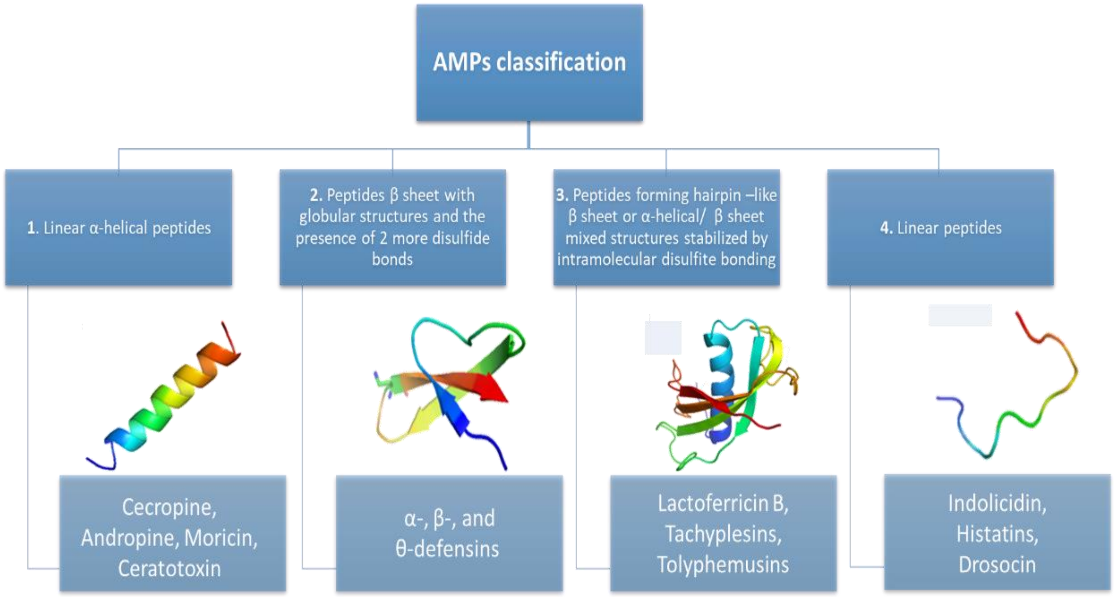

Figure 1.3. Classification of AMPs based on structural characteristics

The α-helical secondary structure limits the number of hydrogen bonds and exposes the hydrophobic groups of the peptide to the lipophilic surfaces of the membrane phospholipids, which helps the peptide pass through the membrane (P. F. Almeida et al., 2012; Nassar et al., 2022). Nevertheless, the α-helical structure of the peptide contributes only partially to its biological activity. Other factors, such as the peptide's flexibility and self-assembly, the balance of hydrophilic and hydrophobic properties, the positive charge of the peptide, and the ionisation of the bacterial membrane, also play a significant role (Juretić & Simunić, 2019; Nassar et al., 2022). The α-helical section is formed by incorporating particular amino acids, such as arginine, alanine, phenylalanine, isoleucine, leucine, and lysine, into the peptide sequence (Phoenix et al., 2013). AMPs, or antimicrobial peptides, are often longer peptides that have the ability to traverse the bacterial membrane. Magainin, derived from the African clawed frog *Xenopus laevis*, is a representative α-helical AMP that hinders the growth of bacteria and fungi and causes the destruction of protozoa (Nassar et al., 2022; Zasloff, 1987).

Upon encountering the aqueous media, the β-sheet family of AMPs assumes an antiparallel β-sheet structure. This is achieved through the creation of disulfide bonds between cysteine residues located on adjacent β-strands (Kier et al., 2015; Moravej et al., 2018). The formation of contiguous β-sheets, which are connected by at least two covalent disulfide bonds, results in the creation of a sturdy structure that accommodates the crucial functional groups of the peptide (Nassar et al., 2022). These functional groups include cationic and hydrophobic groups (Zasloff, 2002). In general, β-sheet AMPs such as human defensins, bactenecin, cathelicidin, protegrin, and tachyplesin are less plentiful than to α-helical peptides. They are found everywhere in different types of organisms (Moravej et al., 2018; Phoenix et al., 2013).

Cyclic or loop peptides are linear AMPs that form a ring-shaped segment as a result of a single disulfide bridge or other types of bonds, such as isopeptide, ester, or amide bonds. This gives rise to what is known as a heterodetic cyclic peptide (Davies, 2003; Powers & Hancock, 2003). Cyclization restricts and reduces the flexibility of amino acid residues, leading to increased structural rigidity. Therefore, a stronger attraction between the ligand and the target site results in a notable enhancement in the biological effectiveness of the peptide (Davies, 2003). In a comparative study, Mika et al. investigated the biological activities of a linear AMP termed BPC194 and its analogue, a cyclic decapeptide that was developed from scratch. The study focused on their effectiveness against the plant diseases *Erwinia amylovora* and *Xanthomonas vesicatoria* (Mika et al., 2011). The cyclic peptide exhibited the adoption of a β-sheet conformation, which resulted in a higher affinity and more pronounced rupture of the bacterial membrane. In contrast, the linear counterpart remained on the surface of the membrane (Mika et al., 2011). In addition, Hirakura et al. investigated the correlation between the variety of structures in antimicrobial peptides (AMPs) and their unique antibacterial effects (Hirakura et al., 2002). The activity of cyclic tachyplesin was compared to that of α-helical magainin in the experiment. The β-sheet cyclic tachyplesin exhibited a significantly higher binding affinity (280 times greater) for the lipopolysaccharide component of the cell membrane compared to its affinity for acidic phospholipids. In contrast, the linear α-helical magainin displayed equal affinity towards both membrane components (Hirakura et al., 2002).

The expanded variant of AMPs lacks a stable or well-defined structural configuration. Nevertheless, they include a high concentration of specific amino acids, including arginine, glycine, histidine, proline, and tryptophan (A. K. Mishra et al., 2018; Powers & Hancock, 2003). Furthermore, the active arrangement of these molecules is determined by their electrostatic contact, namely Van der Waals forces, with the microbial membrane rather than by inherent chemical or physical bonding between amino acid residues (Powers & Hancock, 2003). Thus, the elongated structural arrangement of certain AMPs has a negligible effect on the stability of the microbial membrane (Mika et al., 2011).

1.5.3 The mechanism of action of AMPs

A further classification of antimicrobial peptides can be made based on their mechanism of action. AMPs bind to the phospholipids in the host cell membrane. The interaction between antimicrobial peptides and bacterial cell membranes is an intricate and precise process that encompasses a range of molecular and mechanical factors. The combined forces facilitate the early attachment of antimicrobial peptides to bacterial membranes (Ji et al., 2024). Antimicrobial peptides with a positive charge interact through electrostatic forces with the negatively charged phospholipid groups present on bacterial membranes. This interaction causes the peptides to accumulate on the surface of the membrane. Once the concentration surpasses a specific threshold, the hydrophobic groups can penetrate the lipid bilayer. Peptide concentration, molecular structure, and lipid makeup are crucial components in this process. When antimicrobial peptide molecules are present in high concentrations, they come together on the surface of the membrane, causing changes in its fluidity and structure. This might result in the membrane becoming thinner or the creation of pores. This disruption not only impairs the barrier function of the bacterial cell membrane but also disrupts the equilibrium of material exchange between the interior and exterior of the cell, consequently impacting bacterial development. Antimicrobial peptides can induce cell death by diminishing the proton gradient, resulting in membrane potential loss, impairing ATP synthesis, and interfering with cellular metabolism (Ganesan et al., 2023; Miao et al., 2016). The AMP-host interaction can be broken down into five separate steps: The process of interacting with target cells involves: (1) initial contact with the target cells facilitated by biochemical or biophysical affinity, such as electrostatic or hydrophobic interactions; (2) structural adaptation of the target membrane; (3) accumulation to a functional level; (4) disruption of the target membrane through permeabilization or depolarization, or inducing other direct or indirect anomalies in its function, which can be temporary or permanent; and finally, (5) accessing the ultimate targets either through the membrane or within the cell (Moravej et al., 2018).

AMPs have undergone natural evolution to specifically engage with the membranes of target cells. This first association plays a crucial role in shaping the behaviour of both the peptides and the target cells. The interaction between AMP molecules and biomembranes mostly relies on the biochemical and biophysical characteristics of the peptide and the lipid bilayer (Matsuzaki et al., 1997). Hence, the connection is mostly facilitated by electrostatic forces and/or receptors situated on the membrane. As a result, AMPs with positive charges and bacterial lipid membranes with negative charges form a strong and mutually beneficial interaction. Gram-negative bacteria engage in AMP interaction through a mechanism known as self-promoted uptake. At first, they displace divalent cations that are connected to LPS, causing the microbial membrane to become unstable (Hancock et al., 1991). Some investigations have indicated that the binding affinity of a typical AMP for lipopolysaccharide (LPS) is three times higher than its affinity for divalent cations. Conversely, Gram-positive bacteria are made vulnerable to the antimicrobial properties of positively charged AMPs due to the existence of negatively charged teichoic acid (TA) and teichuronic acid in their cell wall (Figure 1.4). Multiple investigations utilising model membranes have shown that a modest electrochemical gradient is essential for achieving adequate AMP activity (Matsuzaki et al., 1997; Moravej et al., 2018). Receptor-mediated interaction is a significant alternative to electrostatic interaction, which is widely acknowledged as the main mechanism. Nisin exemplifies potent antimicrobial activity at low concentrations, facilitated by a unique receptor-like interaction with lipid II, a membrane-bound constituent implicated in peptidoglycan (PGN) formation. Hence, nisin has significantly higher efficacy against Gram-positive organisms that have a high content of PGN, as opposed to Gram-negative species (Breukink & De Kruijff, 1999). Mersacidin, like nisin, is an AMP that is produced by *Bacillus* spp. and is classified as a lantibiotic. Prior research has demonstrated that mersacidin disrupts transglycosylation and PGN production in Gram-positive bacteria by specifically targeting lipid II. PR-39 belongs to a distinct category of AMPs that exhibit a particular attraction to the SbmA membrane receptor. PR-39 is a peptide composed mainly of proline and arginine. It is classified as a cathelicidin AMP and is found exclusively in mammals. The peptide lacks the ability to create openings in bacterial membranes and is thought to have various targets within cells. These targets include interfering with DNA and protein synthesis by swiftly triggering proteolytic activity, which leads to the breakdown of proteins involved in DNA replication. Conversely, SbmA is an intramembrane protein found in numerous Gram-negative bacteria and is anticipated to function as the transmembrane component of an ATP-binding cassette (ABC) transport mechanism. *Escherichia coli*bacteria that do not have sbmA are more resistant to proline-rich AMPs such as Bac7 because their ability to take up these peptides is diminished, similar to PR-39 (Moravej et al., 2018).

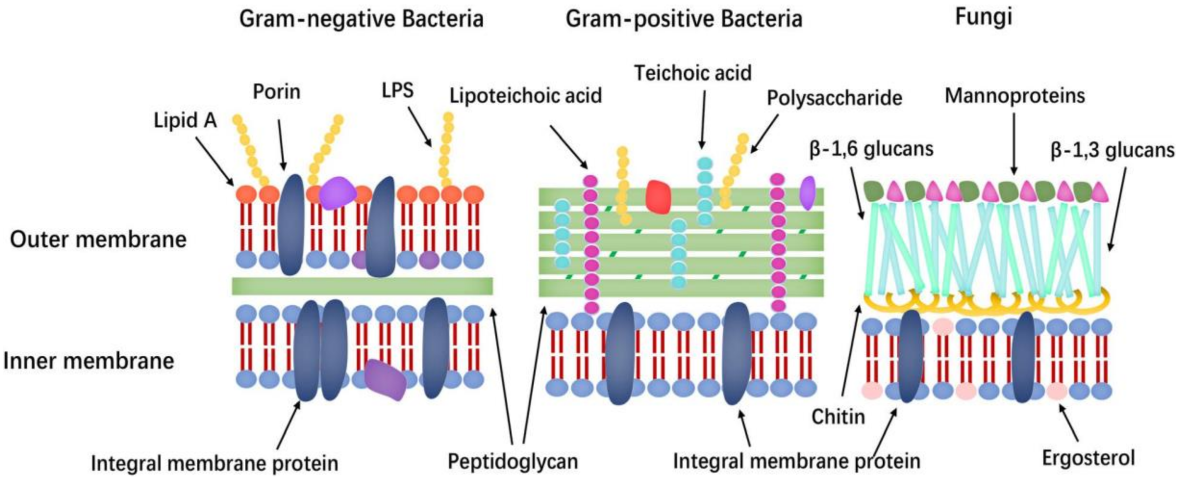

Figure 1.4. Comparison of Gram-negative bacteria, Gram-positive bacteria and fungi cell walls (Huan et al., 2020).

As previously mentioned, AMPs can act against their targets either when located on the membrane or after crossing the membrane. The exact mechanism through which the peptides affect microbial targets and exert their selective toxicity is controversial. Some studies have shown that AMPs self-associate or multimerize after the initial interaction with target membranes. These peptide-peptide and peptide-lipid interactions within the membranes create complex structures associated with specific mechanisms of AMP action. Such peptide complexes can create transmembrane pores or channels that may function selectively or non-selectively. AMPs also act through various mechanisms in different membrane environments; however, after the interaction between membrane lipids and AMPs, the peptides generally enter the host cell and cause cell death (Sitaram & Nagaraj, 1999). It has been reported that subsequent events following peptide-target binding significantly depend on the environmental conditions of the membrane, such as pH, osmotic tolerance, ionic strength, temperature, and viscosity, either individually or in combination. Two different mechanisms of interaction between AMPs and the microbial membrane have been proposed. The first mechanism, known as membranolytic, involves AMPs destabilizing the membrane, leading to the death of the microorganisms due to osmotic shock. The second mechanism, known as non-membranolytic, involves AMPs penetrating the membrane and disrupting normal cellular functions, thereby destabilizing the cell (Luo & Song, 2021).

In the barrel-stave model, a membranolytic mechanism of action, the peptides are of sufficient length to penetrate the membrane in a perpendicular manner and create a pore by passing through the lipid bilayer. The establishment of these pores leads to the creation of a channel that is responsible for the outflow of the cell's cytoplasmic contents and the subsequent collapse of the cell (Lohner & Prossnigg, 2009). The centre lumen of the channel consists of the hydrophobic portions of the peptides, which are encircled by the central section of the phospholipid bilayer. The central section of the channel consists of the hydrophilic segments of the peptides. Alamethicin demonstrates its ability to create pores by employing this model. The hairpin AMP Protegrin-1 has been observed to form stable octameric β-barrels and tetrameric arches (half-barrels) in membranes (Lipkin & Lazaridis, 2015).

In the toroidal pore model, also a membranolytic mechanism, known as the wormhole model, the peptides align vertically through the membrane, forming a pore with a diameter of 1-2 nm. In this model, the pore is composed of both peptides and phospholipids. The AMPs induce the phospholipids from both membrane layers to curve around a central channel, ultimately creating the transmembrane pore (Huan et al., 2020).

In the Carpet-like model, a non-membranolytic mechanism, AMPs are aligned in a parallel orientation with the cell membrane. The hydrophilic ends of the molecules are oriented towards the solution, while the hydrophobic ends are oriented towards the phospholipid bilayer. AMPs bond to the membrane surface extensively and cause disruption to the cell membrane in a manner like a "detergent" (Oren & Shai, 1998). Nevertheless, this process of pore creation necessitates a specific threshold concentration. The activity of the human cathelicidin LL-37 is mediated via this mechanism, and AMPs with a β-sheet structure also contribute to this model. The impact of the AMP cecropin P1 on the bacterial cell membrane was investigated using Fourier-transform infrared spectroscopy (ATR-FTIR) with polarised light; the study by Lyu et al. (2019) discovered that the AMP directly targeted the pathogen's cell memrane, causing destabilisation and eventual destruction of the membrane.

Antimicrobial peptides that specifically target the cell wall do not compromise the structural integrity of the cytoplasmic membrane. Instead, they gain access to the cell by using transmembrane transport. These peptides hinder the creation of bacterial cell walls by reducing the production of chemicals that serve as building blocks for the cell wall, such as lipid II. Lipid II is an important precursor in the formation of cell walls. It is composed of a peptidoglycan component consisting of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM), as well as a peptide chain and a lipid molecule that is connected to it. This structure serves as both a connector in the creation of the cell wall and a crucial component for the transport of peptidoglycans across the cell membrane and the formation of cross-links in the cell wall. Antimicrobial peptides can hinder the production of lipid II, which in turn prevents the transportation of peptidoglycans and weakens the stability of the cell wall, resulting in an inhibitory effect. As an illustration, gallidermin specifically interacts with lipid II by attaching its initial and secondary thioether rings to the pyrophosphate of lipid II (Panina et al., 2021). Cochrane et al. found that the lipopeptide Tridecaptin A1 kills bacteria by attaching to the lipid II, a precursor of the cell wall, on the bacterial cell membrane. This attachment disrupts the proton motive force (Cochrane et al., 2016). Zhao et al. developed a novel antimicrobial peptide named TL19 that exhibits enhanced antibacterial properties compared to peptides with a single lipid II binding site, as it binds to two distinct lipid II binding sites. In addition to targeting lipid II, several antimicrobial peptides have the ability to directly interact with peptidoglycan chains, thereby impacting the structure and function of the cell wall by the degradation or destruction of these chains (Konstantinova et al., 2022; X. Zhao et al., 2020).

Antimicrobial peptides that specifically target the cell wall also have an impact on its stability and integrity by activating particular enzymes and signalling pathways within the cell. They enhance the permeability of the cell wall and affect it internally by triggering the release of lysosomes and activating autolysins, hence impeding cell wall expansion. Research has demonstrated that Triton X-100 stimulates autolysins and enhances the permeability of the cell wall (Hamzah et al., 2018). Yasir and his colleagues identified the cationic antimicrobial peptide Mel4, which triggers the release of autolysins, resulting in the demise of *Staphylococcus aureus* cells (Yasir et al., 2019).

Antimicrobial peptides have the ability to interact with certain cell wall components, such as the outer membrane, in Gram-negative bacteria, resulting in an increase in its permeability. This compromises the bacteria's barrier function, rendering them more vulnerable to external assaults. Magnesium peptides derived from the skin of African clawed frogs increase the permeability of the outer membrane of Gram-negative bacteria. This leads to the release of intracellular potassium ions and the creation of vesicular structures on the cell surface, resulting in an inhibitory effect. Antimicrobial peptides have the ability to disrupt the production of wall teichoic acid (WTA) in Gram-positive bacteria, which ultimately hinders their growth (K. Zhang et al., 2021).

Antimicrobial peptides can hinder bacterial cell wall formation and restrict bacterial cellular respiration. Cell wall synthesis is a metabolically demanding process. When antimicrobial peptides interfere with bacterial respiration and decrease ATP production, they impact cell wall synthesis (Lorenzon et al., 2019). Xia et al. discovered that the synthetic cationic peptide, D11, improves the binding to lipopolysaccharides and membrane phospholipids. This enhances the membrane permeability, making it easier for antibiotics to enter the cell. As a result, the proton motive force (PMF) is disrupted, affecting the respiratory chain. This disruption leads to the generation of reactive oxygen species (ROS) and ultimately causes the cell death (Xia et al., 2021).

The mechanisms by which certain antimicrobial peptides traverse the cell membrane are not completely comprehended. In addition to the receptor-mediated transmembrane translocation mechanism, it has been shown that certain antimicrobial peptides can directly enter the cell by exploiting flaws in the membrane barrier, resulting in the formation of a circular breach. These peptides have intracellular inhibitory effects such as blocking the production of DNA and RNA, interfering with important metabolic enzymes and cellular respiration, impairing the repair of nucleic acids, and preventing cell division. Antimicrobial peptides hinder the process of DNA and RNA synthesis, which leads to the disruption of genetic information transmission in bacterial cells. This, in turn, affects the replication of bacterial genetic material and the generation of proteins. Proteins play a crucial role in the structure and function of cells, encompassing processes such as metabolism, cell repair, and defence systems. Hence, the activity of antimicrobial peptides not only hinders bacterial proliferation but also induces metabolic disruptions and physiological dysfunctions within the cell. Buforin II enters the bacterial cell membrane and prevents the reproduction of genetic material, specifically DNA and RNA. This inhibition is mainly due to the interaction between Buforin II and the N-terminal region of histone H2A, which is crucial for the replication process (Parker et al., 2016). TO17 exerts its action against *Staphylococcus aureus* by penetrating the cell via the cell membrane and initiating the destruction of DNA and RNA, resulting in swift cell death (He et al., 2018). *Bacillus anthracis* (Bac) interacts with the intracellular protein transporter SbmA and is internalised, leading to the inhibition of 70S ribosome synthesis and thus, the suppression of bacterial reproduction. Bac5 and Bac7 exert inhibitory effects on protein and RNA synthesis, as well as respiration, in *Escherichia coli*and *Klebsiella pneumoniae*, resulting in a reduction in ATP levels (Mardirossian et al., 2018). Upon entering the cells, antimicrobial peptides have the ability to influence the function of enzymes that are important for energy generation and cellular metabolism. Certain antimicrobial peptides have the ability to directly attach to crucial enzymes in the respiratory chain, effectively obstructing the transfer of electrons and the creation of ATP. Decreasing ATP levels can greatly diminish bacterial activity. Antimicrobial peptides can disrupt enzyme activity in several metabolic pathways, such as those involved in the production and breakdown of carbohydrates, lipids, and proteins, so impacting cell development. In addition, antimicrobial peptides can induce metabolic imbalances and physiological dysfunctions within the cell, resulting in the buildup of toxic intermediates and further harm to the cell's physiological condition. Gramicidin, derived from Bacillus subtilis, exerts its effect by inhibiting phospholipid synthase and cytochrome in bacterial cells, leading to a disruption in ATP generation and therefore impacting bacterial respiration (S. Kim et al., 2020; Pavithrra & Rajasekaran, 2020).

Antimicrobial peptides can hinder bacterial nucleic acid damage repair mechanisms, hence exerting their inhibitory effects. These peptides have the ability to directly attach to DNA repair enzymes, hinder their activity, or disrupt signal transduction pathways involved in DNA repair. This interference can impede the bacterial response to DNA damage, resulting in genetic instability and cellular demise. Recent studies have shown that the antimicrobial peptide BTP-001 hinders DNA repair by decreasing the activity of many enzymes involved in the TCA cycle (Singleton et al., 2023).

Moreover, studies have demonstrated that antimicrobial peptides have the ability to impede cell division and obstruct the cell cycle. Temporin L has inhibited cell division in *Escherichia coli*by interacting with the tubulin FtsZ, which possesses GTPase activity(Di Somma, Canè, et al., 2021). Lcn972 inhibits cell division by selectively binding to lipid II in many cell wall precursors, hence impacting the development of the septum (Tymoszewska & Aleksandrzak-Piekarczyk, 2021).

1.6 Applications of Antimicrobial Peptides

AMPs are increasingly recognized for their potential applications across various fields, including medicine, agriculture, and food preservation. In the medical field, AMPs are being explored as novel therapeutic agents to overcome antibiotic-resistant infections. The emergence of multidrug-resistant (MDR) pathogens has created an urgent need for new antimicrobial strategies. AMPs, due to their diverse structures and mechanisms, offer a viable solution. Research has shown that AMPs can effectively target and disrupt bacterial membranes, leading to cell lysis and death, which is a mechanism less likely to induce resistance compared to traditional antibiotics Fare clic o toccare qui per immettere il testo.(Koo & Seo, 2019; X. Li et al., 2022). Furthermore, AMPs such as nisin and polymyxin B have already found clinical applications, demonstrating their efficacy in treating infections caused by resistant strains (Bruni et al., 2016; Herbel, 2016). In addition to their direct antimicrobial properties, AMPs also exhibit immunomodulatory effects, enhancing the host's immune response. This dual action is particularly beneficial in treating infections where the immune system is compromised. Studies have indicated that certain AMPs can stimulate the production of cytokines, thereby promoting an effective immune response against pathogens(X. Li et al., 2022; Rodríguez et al., 2021). The potential of AMPs in treating viral infections, including emerging threats like COVID-19, has also been highlighted, showcasing their versatility as therapeutic agents (Koo & Seo, 2019; X. Li et al., 2022).The agricultural sector is another promising area for the application of AMPs. Plant-derived AMPs have been identified as effective agents against phytopathogens, contributing to plant defense mechanisms. For instance, AMPs from plants such as *Sambucus nigra* have demonstrated antimicrobial activity against aquaculture pathogens, indicating their potential use in sustainable agriculture and aquaculture practices (Álvarez et al., 2018; Chaturvedi et al., 2018) Moreover, the genetic engineering of crops to enhance

AMP production could lead to increased resistance against diseases, reducing the reliance on chemical pesticides and promoting environmentally friendly farming practices (Porto et al., 2018; Sampedro & Valdivia, 2013). In the food industry, AMPs are being investigated for their potential to enhance food safety and preservation. Antimicrobial peptides derived from milk proteins, for example, have shown promise in inhibiting the growth of foodborne pathogens, thereby extending the shelf life of dairy products (Benkerroum, 2010; Bruni et al., 2016). The incorporation of AMPs into food packaging materials is also being explored, as these peptides can provide an additional layer of protection against microbial contamination. This application not only improves food safety but also aligns with consumer demand for natural preservatives (Benkerroum, 2010; Bruni et al., 2016). The veterinary field is witnessing a growing interest in the use of AMPs to combat infections in livestock and pets. The rise of antibiotic resistance in veterinary medicine has prompted researchers to seek alternatives that can effectively manage infections without contributing to resistance issues. AMPs such as lactoferrin-derived peptides have been studied for their ability to control bacterial infections in animals, offering a potential solution for maintaining animal health while minimizing antibiotic use. Furthermore, the application of AMPs in aquaculture has been particularly noteworthy, as they can help prevent infections in fish, thereby improving overall yield and sustainability in fish farming (Chaturvedi et al., 2018; Masso-Silva & Diamond, 2014).

1.7 Sources of Antimicrobial Peptides

AMPs can be sourced from mammals, amphibians, microorganisms, and insects (Figure 1.5):

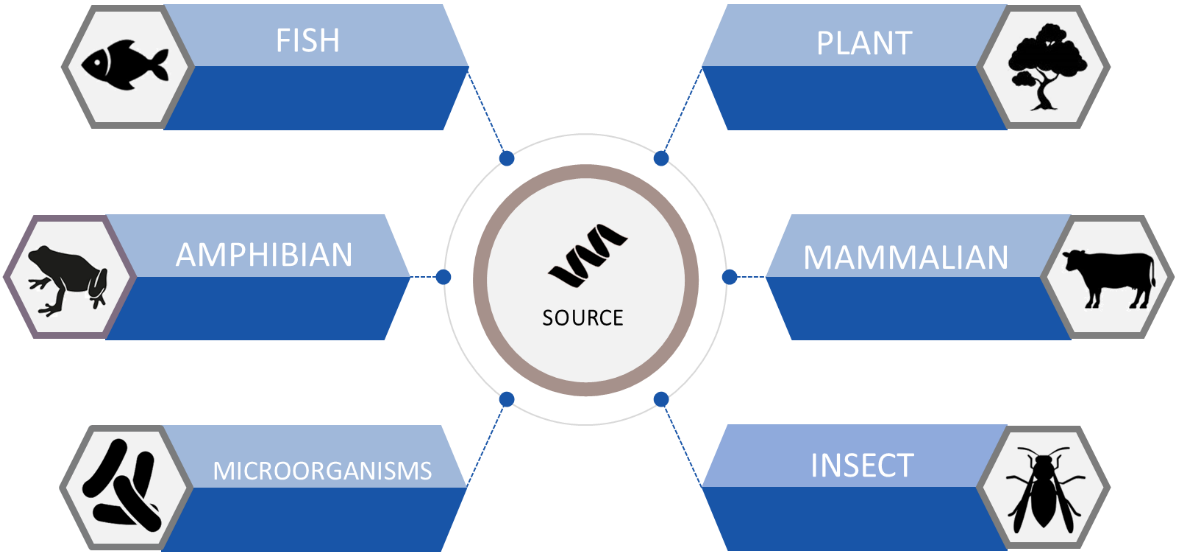

Figure 1.5. Illustrative diagram representing the diverse biological sources for the extraction and study of bioactive compounds, including fish, amphibians, microorganisms, plants, mammals, and insect. Adapted from (Huan et al., 2020).

1.7.1 Mammalian Antimicrobial Peptides

Mammalian antimicrobial peptides are prevalent in humans and other mammals, including sheep, cattle, and other vertebrates. The primary categories of mammalian AMPs consist of cathelicidins and defensins. Defensins can be categorised into α-, β-, and θ-defensins based on the arrangement of the disulfide bonds. The α-defensins contain three disulfide bridges connecting the Cys1-Cys6, Cys2-Cys4, and Cys3-Cys5 residues. These connections stabilise the helical structure of α-defensin. β-Defensins have three disulfide bonds connecting residues Cys1-Cys5, Cys2-Cys4, and Cys3-Cys6. This arrangement contributes to the formation of a β-sheet structure. θ-Defensins, which are cyclic, contain three disulfide bonds connecting residues Cys1-Cys6, Cys2-Cys5, and Cys3-Cys4. This configuration results in a compact cyclic structure (Reddy et al., 2004). Human host defence peptides (HDPs) have differential expressions during distinct phases of human development, providing protection against microbial illnesses. For instance, the cathelicidin LL-37, which is a widely recognised AMP originating from the human body, is typically found in the skin of newborns. On the other hand, human beta-defensin 2 (hBD-2) is more commonly observed in older individuals rather than in younger ones (Gschwandtner et al., 2014). HDPs are present in various anatomical regions, including the dermis, eyes, ears, oral cavity, respiratory tract, lungs, gastrointestinal system, and urinary canal. Additionally, antimicrobial AMPs in human breast milk play a crucial role in enhancing the health and survival of breastfed infants by reducing disease incidence and mortality rates (Field, 2005). Of particular interest, casein 201, a peptide derived from β-casein 201–220 aa, has been shown to exist in different concentrations in preterm and full-term human colostrum, as observed by Zhang et al. in 2017 (F. Zhang et al., 2017). Dairy products serve as a significant reservoir of AMPs, which are produced by breaking down milk through enzymatic hydrolysis. Various AMPs have been discovered in different portions of α-lactalbumin, β-lactoglobulin, lactoferrin, and casein. One particularly well-known peptide that has been isolated is lactoferricin B (LfcinB) (Huan et al., 2020; Sibel Akalin, 2014).

1.7.2 Amphibians Antimicrobial Peptides

Amphibian antimicrobial peptides have a crucial function in safeguarding amphibians from infections, which have caused a worldwide decrease in amphibian populations (Huan et al., 2020; Rollins-Smith, 2009). Frogs are the primary producers of amphibian AMPs, with magainin being the most well-known AMP derived from frogs. The skin of frogs from the genera *Xenopus*, *Silurana*, *Hymenochirus*, and *Pseudhymenochirus* is known to produce significant quantities of AMPs (Michael Conlon & Mechkarska, 2014). Additionally, the peptide cancrin, identified by its amino acid sequence GSAQPYKQLHKVVNWDPYG, has been recognized as the first AMP to be discovered in the marine amphibian *Rana cancrivora.* (Lu et al., 2008).

1.7.3 Microorganisms Antimicrobial Peptides

Microorganisms, including bacteria and fungi, have the capability to generate AMPs. Prominent examples of these AMPs are nisin and gramicidin, which are produced by species such as *Lactococcus lactis*, *Bacillus subtilis*, and *Bacillus brevis* (Cao et al., 2019). The biological production of AMPs has gained traction due to the high costs associated with their chemical synthesis. Expression systems, including yeast species like *Pichia pastoris* and *Saccharomyces cerevisiae*, as well as bacteria like *Escherichia coli* and *B. subtilis*, along with plant-based systems, have been employed to produce these peptides (Huan et al., 2020; Parachin et al., 2012). However, producing AMPs in *E. coli* presents challenges due to issues like toxicity, proteolytic breakdown, and difficulties in purification (Huan et al., 2020; H. Yu et al., 2015).

1.6.3 Plants Antimicrobial Peptides

In addition, a significant number of antimicrobial peptides (AMPs) have been obtained and separated from the stems, seeds, and leaves of plants. These AMPs are categorised into various families, such as thionins, defensins, and cyclotides (Tang et al., 2018). There is a rising recognition of the significance of marine resources, leading to an increasing interest in marine-derived antimicrobial peptides (AMPs). While the majority of marine antimicrobial peptides (AMPs) have been examined in laboratory settings, a significant number of these AMPs have demonstrated encouraging outcomes in live organisms. As an example, As-CATH4 demonstrates an immunostimulatory impact in living organisms and has the ability to increase the anti-infective properties of medications when used together (Semreen et al., 2018). Myticusin-beta is an AMP that is involved in the immune response. It is derived from *Mytilus coruscus* and has potential as a substitute for antibiotics (R et al., 2020). Moreover, GE33, often referred to as pardaxin, is an AMP found in marine organisms. The GE33-based vaccination has been shown to effectively boost the immune response against tumours in mice, as reported in a study by Huang et al (Huang et al., 2013).

1.8 Insects as a Source of Antimicrobial Peptides

Insects are the primary source of AMPs due to their extensive biodiversity, although all living organisms produce these peptides. Insects, lacking adaptive immunity, depend on the synthesis of broad-spectrum chemicals called AMPs to effectively protect themselves against infections. Insects have developed a wide spectrum of innate immune responses throughout their evolutionary history as a result of their feeding on various substrates and occupying distinct environments. Thus, insects, with their vast array of over a million documented species, serve as an abundant and limitless reservoir of AMPs (Q. Wu et al., 2018).

Insect AMPs are classified into three main groups based on their amino acid sequence and structure. The first group includes linear peptides with an α-helix structure, lacking cysteine residues, such as cecropins. The second group consists of peptides with secondary structures stabilized by the presence of cysteine residues forming disulfide bridges; defensins belong to this group, characterized by a structure stabilized by 3 or 4 disulfide bridges and comprising three domains, including a flexible N-terminal loop. The third group comprises peptides rich in proline and/or glycine residues (Makarova et al., 2018; Q. Wu et al., 2018). Among insects most extensively studied AMPs are cecropins, drosocin, attacins, diptericins, defensins, ponericins, drosomycin, and metchnikowin. Peptides rich in glycine and proline exhibit a high level of activity against Gram-negative bacteria (Rozgonyi et al., 2009). Defensins, on the other hand, show specificity in targeting Gram-positive bacteria, while cecropins are active against both Gram-positive and Gram-negative bacteria (Józefiak & Engberg, 2017; Q. Wu et al., 2018). The characteristics and activity of these peptides will be further discussed in the following sections; however, it is important to note that the discovery of novel peptides remains a possibility.

1.8.1 Defensins

Defensins are a group of compact, positively charged peptides that contain a high amount of arginine (Ganz & Lehrer, 1995). Defensins are not exclusive to insects, and the current number of known defensins exceeds 300. These peptides are composed of 18-45 amino acids and contain 6-8 conserved cysteine residues, and exhibit an N-terminal loop, followed by an α-helix, and then an antiparallel β-sheet. (Manniello et al., 2021; Thomma et al., 2002). Defensins bind to the cell membrane or create pore-like flaws in the membrane, causing the release of necessary ions and nutrients (Tay et al., 2011; Zhu & Gao, 2013).

Insect defensins are particularly effective against Gram-positive bacteria such as *S. aureus*, *Bacillus subtilis*, *M. luteus*, and *B. megaterium*. However, some of them have also shown antimicrobial activity against Gram-negative bacteria like *E. coli*. Insect defensins have been extracted from many insect orders, including Diptera, Hymenoptera, Coleoptera, Trichoptera, Hemiptera, and Odonata (Manniello et al., 2021; Tay et al., 2011).

Royalisin is an AMP extracted from the royal jelly of *Apis mellifera*. The molecule is composed of 51 amino acids, with three disulfide bonds formed by six cysteine residues. This arrangement results in a compact and spherical structure for the molecule (Fujiwara et al., 1990). Royalisin is a protein that has both hydrophobic and hydrophilic regions, and its C-terminal portion contains a high concentration of amino acids that carry an electric charge.

This peptide has the ability to suppress the growth of Gram-positive bacteria and fungi (Bíliková et al., 2001; Q. Wu et al., 2018).

1.8.2 Cecropins

Cecropins are a prominent family of AMPs originally discovered in insects, particularly in the hemolymph of the giant silk moth, *Hyalophora cecropia*. These peptides play a crucial role in the innate immune response, providing a first line of defense against a variety of pathogens, including bacteria, fungi, and viruses. The structure of cecropins typically features an α-helical conformation, which is essential for their antimicrobial activity. This structural characteristic allows them to interact effectively with microbial membranes, leading to cell lysis and death (H. Lee et al., 2023; C.-L. Wu et al., 2022). The antimicrobial efficacy of cecropins has been extensively studied, revealing their broad-spectrum activity against both Gram-positive and Gram-negative bacteria. For instance, cecropin A has demonstrated potent antibacterial effects against multidrug-resistant strains of *E. coli*, highlighting its potential as a therapeutic agent in combating antibiotic resistance (H. Lee et al., 2023; Silvestro & Axelsen, 2000). In addition to their antibacterial properties, cecropins have shown antifungal activity, making them versatile candidates for various applications in medicine and agriculture (Hu et al., 2013). Cecropins exhibit not only antimicrobial properties but also anti-inflammatory effects. Research indicates that cecropin A can modulate inflammatory responses by regulating the expression of cyclooxygenase-2 (COX-2) and influencing signaling pathways such as the mitogen-activated protein kinase (MAPK) pathway. This dual functionality suggests that cecropins could be beneficial in treating infections where inflammation is a significant concern, potentially reducing the need for additional anti-inflammatory medications.

Cecropin B is a peptide composed of 35 amino acids and, compared to other members of the same family, it exhibits the most pronounced antimicrobial activity (Srisailam et al., 2000). The administration of cecropin B has been shown to reduce mortality in rats with *E. coli* infections (Giacometti et al., 2001). Additionally, it demonstrates significant antifungal activity against *C. albicans* (Andrä et al., 2001).

Cecropin D, structurally similar to cecropins A and B (HULTMARK et al., 1982b), is expressed in detectable concentrations in the hemolymph following a bacterial infection, but its expression occurs later compared to cecropins A and B (Gudmundsson et al., 1991). A recombinant form of cecropin D, produced through expression in *Pichia pastoris*, has proven to be effective against both Gram-positive and Gram-negative bacteria (Guo et al., 2012). Phosphorylation of the lysine residue at the C-terminal end has been shown to enhance its antibacterial activity (S.-I. Park et al., 2013). Additionally, cecropin D exhibits significant antiviral activity in the treatment of porcine reproductive and respiratory syndrome virus (PRRSV) infections (Liu et al., 2015).

Lucilin, a cecropin consisting of 36 amino acid residues, has been detected as a partial gene sequence in the larvae of *Lucilia sericata* (Téllez & Castaño-Osorio, 2014). The fusion protein, GWLK-Lucilin-CPD-His8, exhibits promising efficacy against multidrug-resistant (MDR) E. coli (Téllez & Castaño-Osorio, 2014). The cecropin derived from Musca domestica has demonstrated potential as a bactericidal agent against clinical isolates of *E. coli* (X. Lu et al., 2012).

1.8.3 Attacins

Attacins are AMPs characterized by a high content of glycine residues. They were first isolated from the hemolymph of pupae of *H. cecropia* (Hultmark et al., 1983). These proteins are initially expressed as pre-pro-proteins, containing a signal peptide, a propeptide region, an N-terminal attacin domain, and two glycine-rich domains, referred to as G1 and G2 domains. Attacins can be divided into two groups: the first group, which includes attacins A-D, is rich in basic amino acid residues, while attacins E and F contain acidic amino acid residues. These proteins exhibit strong activity against Gram-negative bacteria such as *E. coli* (Carlsson et al., 1991) but show less intense action against Gram-positive bacteria. More specifically, attacins function by inhibiting the synthesis of membrane proteins essential for the division of Gram-negative bacteria. Attacins have been identified in numerous insects, including *Bombyx mori*, *Glossina morsitans* (tsetse fly), *Heliothis virescens*, *Trichoplusia ni*, *Samia cynthia ricini* (wild silk moth), and *Musca domestica* (housefly).

1.8.4 Lebocins

Lebocins are a group of antimicrobial peptides (AMPs) that were first isolated from the immunized hemolymph of *B. mori* (Hara & Yamakawa, 1995). These peptides, composed of approximately 30 amino acids, are rich in proline residues and require O-glycosylation for their full activity, primarily against *Acinetobacter sp.* and *E. coli* (G. Liu et al., 2000). Lebocins exhibit antimicrobial activity against both Gram-positive and Gram-negative bacteria, as well as against certain fungi (Manniello et al., 2021).

1.8.5 Drosocins

Drosocins are AMPs synthesized by the insect *Drosophila melanogaster* (Bulet et al., 1993). These peptides are composed of 19 amino acids, are rich in proline residues, and undergo O-glycosylation, a process crucial for their antimicrobial activity, similar to what is observed in lebocins (McManus et al., 1999). Drosocins exhibit significant antimicrobial activity against both Gram-positive bacteria, such as *M. luteus* (Lele et al., 2015), and certain Gram-negative strains, such as *E. coli*.

Apidaecin IB and drosocin have substantial similarity in their sequences and modes of interaction, although they do not possess any ability to generate pores (Gobbo et al., 2002). Apidaecins serve as the primary constituents of the honeybee's humoral defence mechanism to combat microbial intrusion (Gobbo et al., 2002). The N-terminal alteration of apidaecins not only increases the binding affinity with unknown intracellular targets but also improves the efficacy of cell penetration (Matsumoto et al., 2010). The antibacterial action against *Pseudomonas aeruginosa* is enhanced by the repetition of the N-terminal motifs Ile-Orn and Trp-Orn (Bluhm et al., 2016) .

1.8.6 Diptericins

Diptericins are a class of AMPs particularly well-studied in *Drosophila melanogaster*. They play a crucial role in the innate immune response against Gram-negative bacteria. The expression of diptericin is primarily regulated by the immune deficiency (IMD) signalling pathway, which is activated in response to peptidoglycan from bacterial cell walls. This activation leads to the transcription of diptericin and other AMPs, forming a vital part of the insect's defense mechanism against pathogens (Hanson et al., 2016; Y. S. Kim et al., 2000).

Research has shown that diptericin is specifically induced by Gram-negative bacterial infections. For instance, demonstrated that diptericin expression could be measured using a luciferase reporter assay in Drosophila S2 cells, confirming its induction upon stimulation with purified Gram-negative bacterial peptidoglycan (Okugawa et al., 2009). Furthermore, noted that excreted-secreted products from certain bacteria could provoke a significant increase in diptericin expression in adult Drosophila, highlighting the peptide's role as a readout for the IMD pathway (Kenney et al., 2019). This pathway is essential for the production of various AMPs, including diptericin, which is particularly effective against Gram-negative bacteria (Hanson et al., 2016; Y. S. Kim et al., 2000).

The regulation of diptericin expression is complex and can be influenced by various factors. For example, reported that certain hormones could suppress the immune response in adult Drosophila, leading to decreased transcription of diptericin and other AMPs (Jones et al., 2010). Additionally, the expression of diptericin is subject to post-transcriptional regulation, as indicated by the presence of regulatory elements in its mRNA (H. Chen et al., 2004). This suggests that the immune response is finely tuned and can be modulated by both transcriptional and post-transcriptional mechanisms.

Moreover, diptericin's role extends beyond mere antimicrobial activity; it is also involved in the broader context of the insect immune system. The production of diptericin and other AMPs is part of a multi-faceted immune response that includes phagocytosis and the activation of proteolytic cascades (Hao et al., 2001; Imler & Bulet, 2005). The interplay between different AMPs, such as defensins and cecropins, further illustrates the complexity of the immune response in insects (Buonocore et al., 2021; Imler & Bulet, 2005) .

1.8.7 Metchnikowin

The discovery of this peptide dates back to 1995 and was made by a group of Russian scientists. The peptide was named Metchnikowin in honor of E. Metchnikow. The structure of Metchnikowin is characterized by a proline-rich sequence, which is crucial for its antimicrobial activity. This peptide is composed of 26 amino acids and exhibits strong in vitro activity against fungal pathogens, such as *Fusarium graminearum*, making it a potential candidate for biotechnological applications in crop protection (Rahnamaeian et al., 2009). The mechanism of action for Metchnikowin involves disrupting the integrity of microbial membranes, which is a common strategy employed by many AMPs (Pal & Wu, 2009).

Research has shown that Metchnikowin is robustly induced upon activation of the Toll signaling pathway, which is a critical component of the immune response in *Drosophila* (Lindsay et al., 2018). This pathway is responsible for the transcriptional regulation of various AMPs, including Metchnikowin, in response to microbial challenges. The expression of Metchnikowin and other AMPs can be influenced by various factors, including the presence of pathogens and the activation of pattern recognition receptors that detect microbial components (Aggarwal et al., 2008).

1.8.8 Ponericins

Isolated from the venom of the ant *Pachycondyla goeldii*, ponericins exhibit a range of biological activities, including antibacterial and insecticidal effects, making them a valuable subject of study in the field of entomology and antimicrobial research. The structural characteristics of ponericins also warrant attention. They are typically composed of cationic and amphipathic sequences, which facilitate their interaction with microbial membranes, leading to cell lysis (Bulet & Stocklin, 2005). This structural similarity to other insect AMPs, such as cecropins, suggests that ponericins may share common evolutionary origins and functional roles within the insect immune system (Buonocore et al., 2021). Ponericins have been shown to possess membrane-perturbing properties, which contribute to their antimicrobial activity against both Gram-positive and Gram-negative bacteria (Nixon et al., 2021).

1.8.9 Jelleines

Jelleins are a group of peptides that have been extracted from the royal jelly of Apis mellifera. These molecules are composed of 8-9 amino acids and possess a positive charge of +2 at the C-terminal end (Romanelli et al., 2011). Four antimicrobial peptides (AMPs) have been isolated from bee royal jelly: Jelleines-I–III demonstrated antibacterial properties against several microorganisms, including yeasts, fungi, Gram-positive bacteria, and Gram-negative bacteria (Fontana et al., 2004; Jia et al., 2018). These peptides do not have any similarities with other bee AMPs. The gelin molecules are currently undergoing characterisation (BĂRNUłIU et al., 2011).

1.8.10 Apisimin

Apisimin is an AMP derived from honeybee royal jelly, specifically from the species *A. mellifera*. It is characterized by a serine-valine-rich structure, which distinguishes it from other AMPs that typically contain cysteine residues, a common feature in many antimicrobial peptides. The absence of cysteine in apisimin does not preclude its role in immune defense, as other AMPs in honeybee hemolymph also lack this amino acid and still exhibit antimicrobial properties (Bíliková et al., 2002). The significance of apisimin in the immune response of honeybees is underscored by its transcriptional regulation during pathogen exposure. Studies have shown that apisimin, along with other AMPs such as abaecin and defensin, is upregulated in response to infections, particularly those caused by *Ascosphaera apis*, a pathogen affecting honeybee larvae (B. Xu et al., 2019). This upregulation is part of a broader immune response that involves various signaling pathways, including the Toll and IMD pathways, which are crucial for the activation of immune genes in insects (B. Xu et al., 2019). Moreover, apisimin has been identified as one of the key AMPs present in the honeybee's head and hypopharyngeal glands, particularly in nurse bees that are responsible for feeding larvae destined to become queens. This suggests that apisimin may play a role not only in direct antimicrobial activity but also in the overall health and development of the colony (Vojvodic et al., 2014). The expression of apisimin is particularly pronounced in forager bees, where it is co-expressed with enzymes involved in nectar processing, indicating a multifaceted role in both nutrition and immunity (Vannette et al., 2015).

1.8.11 Pyrrhocorins

Cociancich et al. (1994) discovered pyrrhocorin, a proline-rich found in the hemolymph of the lygaeid bug *Pyrrhocoris apterus*. The peptide consisting of 20 amino acids interacts with the fungal heat shock protein DnaK, which is associated with antimicrobial action (Kragol et al., 2001). The primary mechanism of action for pyrrhocorins, like many other insect AMPs, involves disrupting the integrity of bacterial cell membranes, leading to cell lysis and death (Stączek et al., 2023).

Pyrrhocorins has the ability to bind to and enhance the ATPase activity of the molecular chaperone DnaK (Chesnokova et al., 2004; Kragol et al., 2002). Boxell et al. (Boxell et al., 2008) showed that pyrrhocorin can function as a carrier in the process of delivering peptides across the cell membrane of the parasite *Cryptosporidium parvum*. Efficient transduction enhances target validation and aids in the development of peptide-based medicines for this significant human pathogen. The process of forming a ring structure in the structural components of pyrrhocorin is crucial for the antibacterial effectiveness of the original peptide (Rosengren et al., 2004).

1.8.12 Persulcatusin

Persulcatusin is an AMP derived from the hard tick *Ixodes persulcatus*, this peptide plays a significant role in the innate immune response of ticks, particularly against various pathogenic bacteria, including drug-resistant strains such as *S. aureus* (Miyoshi, Isogai, et al., 2016; Miyoshi, Saito, et al., 2016). The characterization of persulcatusin has revealed that it exhibits a cationic nature, which is a common feature among many AMPs, allowing it to interact effectively with negatively charged bacterial membranes, leading to cell lysis and death(Miyoshi et al., 2017). The structure of persulcatusin is indicative of its functional capabilities. It has been shown to adopt an α-helical conformation, a characteristic shared with other insect AMPs such as cecropins and defensins (Lamberty et al., 2001; Stączek et al., 2023) . This structural feature is crucial for its antimicrobial activity, as it facilitates the peptide's insertion into bacterial membranes, disrupting their integrity (Miyoshi, Isogai, et al., 2016; Stączek et al., 2023). Moreover, persulcatusin has demonstrated efficacy against both Gram-positive and Gram-negative bacteria, highlighting its broad-spectrum antimicrobial potential (Miyoshi et al., 2017; Miyoshi, Saito, et al., 2016).

1.8.13 Melittin

Melittin, a prominent antimicrobial peptide derived from honeybee venom *A. mellifera*, has garnered significant attention due to its diverse biological activities, particularly its antimicrobial properties. Comprising approximately 50% of bee venom, melittin exhibits a range of effects, including antibacterial, antifungal, antiviral, and anti-inflammatory activities (Nainu et al., 2021). Its amphipathic α-helical structure enables it to interact effectively with microbial membranes, leading to membrane disruption and cell lysis, which is a critical mechanism underlying its antimicrobial action (Elkhateeb et al., 2021; Laverty et al., 2011). Research has demonstrated that melittin exhibits potent antibacterial activity against both Gram-positive and Gram-negative bacteria, with notable efficacy against

MRSA (Fischer & Guichard, 2010; Zolfagharian et al., 2016). The mechanism of action involves the formation of pores in bacterial membranes, which disrupts membrane integrity and leads to cell death (Shi et al., 2016; Uppu et al., 2016). Studies have shown that melittin's activity is influenced by its amino acid composition, particularly the presence of hydrophobic residues such as phenylalanine and tryptophan, which are crucial for its antibacterial and hemolytic properties (Elkhateeb et al., 2021; J. Lee & Lee, 2014). Furthermore, melittin selective toxicity towards prokaryotic cells over eukaryotic cells has been highlighted, making it a promising candidate for therapeutic applications (Zolfagharian et al., 2016; (Nainu et al., 2021; Zolfagharian et al., 2016) et al., 2021). In addition to its antibacterial properties, melittin has been investigated for its potential in treating various diseases. For instance, it has shown efficacy against *Trypanosoma cruzi*, the causative agent of Chagas disease, promoting selective autophagic death in certain forms of the parasite (Souza et al., 2016). Moreover, its anti-inflammatory properties have been explored in the context of various inflammatory diseases, suggesting a multifaceted role in health and diseas(Elkomy, 2023). However, the clinical application of melittin is limited by its cytotoxicity and rapid degradation, necessitating further research to enhance its therapeutic potential while minimizing adverse effects (Rahnama et al., 2022).

1.9 Insects immune System

Insects, similar to other invertebrates, lack an adaptive immune system. Nevertheless, numerous insects possess a robust innate immune system that enables them to survive in environments with significant microbial pressure, protecting them from pathogens such as bacteria, fungi, and viruses(Strand, 2008). The immune system of insects differs greatly from that of vertebrates, yet it exhibits a remarkable level of complexity. Insects have a first line of defense that consists of physical barriers like the exoskeleton and cuticular secretions. These barriers effectively block the entry of diseases. Once the pathogens successfully overcome these barriers, the insect's immune system is triggered and reacts with a sequence of innate and acquired reactions. The arthropods possess an innate immune system that consists of cellular and humoral components. The cellular defense is facilitated by the hemocytes. Nevertheless, the categorization and nomenclature of hemocytes forms varied among various insect species. For instance, *Drosophila* has three categories of cells: plasmatocytes, crystalline cells, and lamellocytes. In contrast, some species like *Aedes* mosquitoes have a broader variety of cell types, including proemocytes, adipoemocytes, oenocitoids, thrombocytoids, plasmatocytes, and granulocytes. However, there is no consensus among researchers about the precise immune functions of these different cell types. As a result, some researchers have chosen to differentiate between hemocytes and immunocytes (U. Müller et al., 2008).

1.9.1 Cellular Components of the Insect Innate Immune System: Plasmatocytes, Crystal Cells, Lamellocytes, and Granulocytes

In insects, a broad variety of different cell types exists that support the innate immune response against invading pathogens:

-*Plasmatocytes*

Plasmatocytes constitute the major hemocyte population (comprising 90–95% in Drosophila) among arthropods (R. M. Rizki & Rizki, 1984) . These cells, typically spherical and ranging from 8–10 μm in diameter (Lanot et al., 2001), are distinguished by a large cytoplasm rich in lysosomes and an extensive endoplasmic reticulum (Evans et al., 2003). They possess adhesive properties and have the ability to migrate along chemokine gradients. Plasmatocytes fulfill multiple roles in development and immune defense: they are phagocytic, engulfing apoptotic debris during development and pathogens during immune responses. Furthermore, they secrete various extracellular matrix proteins that are crucial for tissue remodeling during morphogenesis and produce antimicrobial peptides (AMPs) (Evans et al., 2003). Within the communication network of the innate immune system, they function as messengers, alerting the fat body to the presence of infectious agents and thereby initiating a robust AMP response. This signaling involves the cytokine unpaired-3, which stimulates AMP production via the JAK/STAT pathway. Additionally, plasmatocytes are believed to detect parasitization and promote significant differentiation of lamellocytes through the posterior signaling center (PSC) (Meister & Lagueux, 2003).

-*Crystal Cells*

Crystal cells represent approximately 5% of *Drosophila* hemocytes during the embryonic and larval stages (Lanot et al., 2001). They are named for the crystalline inclusions within their cytoplasm and are characterized by the expression of two out of the three prophenoloxidase (proPO) genes in *Drosophila* (Pinheiro & Ellar, 2006). These inclusions contain components of the proPO system, which are essential for processes such as wound healing and melanization. The presence of phenoloxidase (PO) activity in the hemolymph of adult *Drosophila* is a notable feature, which is absent in mutants that are unable to develop crystal cells during maturation (T. M. Rizki et al., 1985).

-*Lamellocytes*

Lamellocytes are found exclusively in larvae and are rarely present in uninfected larvae, but they undergo marked differentiation in response to parasitization. Unlike plasmatocytes and crystal cells, lamellocytes are inducible cells. These cells are flattened and can reach sizes of up to 20 µm, exhibiting adhesive properties (Lanot et al., 2001), and they are involved in the cellular encapsulation of large foreign bodies that cannot be phagocytosed. Lamellocytes appear to initiate the melanization process in the absence of crystal cells (Irving et al., 2005a). Similar to crystal cells, lamellocytes disappear during metamorphosis (Meister & Lagueux, 2003).

-*Granulocytes*

In addition to plasmatocytes, other immune-relevant cell types such as granulocytes exist in various insect species. Granulocytes contain small granules and circulate as hemocytes involved in phagocytosis, albeit to a lesser extent than plasmatocytes. Their primary function is encapsulating foreign particles and clearing apoptotic cells.

1.9.2 Control of Pathogens: Opsonization, Encapsulation, or Phagocytosis, Production of Antimicrobial Peptides

-*Opsonization*

Opsonization of pathogens primarily facilitates their recognition and subsequent phagocytosis by phagocytes. This process of aggregation allows for the ingestion of a larger number of pathogens. Additionally, opsonization prevents the entry of intracellular pathogens such as bacteria and viruses by interfering with the interaction with specific receptors necessary for their internalization. Furthermore, it can activate the prophenoloxidase (proPO) system in insects, highlighting the crucial role of opsonizing molecules in coordinating both humoral and cellular effector systems (Marieshwari et al., 2023). Insects employ this mechanism to compensate for the absence of antibody-mediated processes typical in vertebrates. Various proteins with opsonizing properties have been identified in insects, including C-type lectins, C-reactive proteins, and complement-like proteins (Ao et al., 2007; X.-Q. Yu et al., 1999; X.-Q. Yu & Kanost, 2000).

C-type lectins are carbohydrate-binding proteins capable of agglutinating cells or precipitating glycoconjugates. In insects, these lectins are often referred to as agglutinins or hemagglutinins. Many insect species possess constitutive agglutinins, whose synthesis can be induced by antigenic stimulation and is primarily carried out by hemocytes. For example, *Drosophila* encodes more than 30 C-type lectins (Tanji et al., 2006). Detailed studies on three C-type lectins from *Drosophila* have shown that the expression of DL1 increases in larvae following injury. The recombinant lectins DL2 and DL3 bind to certain Gram-negative bacteria and agglutinate *Escherichia coli*. DL2 and DL3 are not only secreted but are also found on the surface of hemocytes. Due to their distinctive binding properties, lectins can bind to a wide range of microorganisms through various carbohydrate-binding domains, similar to an antibody-like mechanism (Ao et al., 2007; Tanji et al., 2006).

-*Cellular Encapsulation*

Cellular encapsulation is a defense mechanism that involves immobilizing parasites, fungi, and protozoa of sizes large enough to evade the phagocytic activity of individual immune cells. In Drosophila, a significant proliferation and differentiation of precursors into lamellocytes is observed, followed by the release of these lamellocytes into the hemolymph (Lanot et al., 2001). The lamellocytes attach to the surface of the parasite, forming a multilayered capsule around the invader. Initially, this capsule is stabilized by the formation of septate junctions between the lamellocytes, likely mediated by integrins (Irving et al., 2005a). The capsule often darkens due to melanization, catalyzed by phenoloxidase (PO), which oxidizes phenols into quinones that polymerize into melanin. This phenomenon is mainly observed when the immune response is directed towards living organisms, thereby subjecting the parasite to the toxic by-products of melanization. Since lamellocytes are absent in adults, cellular encapsulation is limited to larvae, although a similar cell-free mechanism, called humoral encapsulation, remains functional in adults (Irving et al., 2005b).

-*Phagocytosis*

Phagocytosis leads to the killing of organisms as the engulfment is followed by the formation of phagolysosomes (Uribe-Querol & Rosales, 2020). The process of phagocytosis is initiated either by the strong binding of a foreign structure to cell surface receptors or by the recognition of opsonized microorganisms. Specifically, the recognition of microorganisms by the innate immune system occurs through mechanisms mediated by Toll receptors. Proteins that recognize peptidoglycan, known as peptidoglycan recognition proteins

(PGRPs), can detect bacteria and their cell wall components. The activation of these receptors triggers an intracellular cascade that leads to the rearrangement of the cytoskeleton at the binding site. This actin-dependent process enables the phagocyte to surround the target (Royet et al., 2011; Uribe-Querol & Rosales, 2020). In *Drosophila*, the CD36 homologue encoded by the croquemort gene is crucial for the uptake of apoptotic remnants during embryogenesis. It also mediates the phagocytosis of *S. aureus*, indicating the presence of multifunctional receptors (Franc et al., 1999). As mentioned above, PGRPs also participate in the phagocytosis of microorganisms. While PGRP-LC is involved in the engulfment of Gram-negative bacteria in Schneider cells, PGRP-SA appears to function as an opsonizing protein that enhances the phagocytosis of Gram-positive bacteria. In *A. gambiae*, transcriptional silencing of the TEP1 gene, which encodes a complement-like thiolester-containing protein, reduces the phagocytosis of the Gram-negative bacterium *E. coli* by blood cell lines. The binding of TEP1 includes a thiolester bond and thus seems to occur in a manner similar to that of C3. Drosophila has four expressed tep genes, and an additional two DNA sequences related to complement-like proteins in its genome (though they appear to be pseudogenes); tep1, 2, and 4 are upregulated in response to immune challenges in larvae, and tep2 and 4 in adults, and they are presumed to be secreted (Dziarski & Gupta, 2006; Yanagawa et al., 2017). After internalization, the phagosome is directed to fuse with primary lysosomes. The formation of the phagolysosome depends on several fission and fusion events involving lysosomes and endosomes. Within this compartment, the pathogen is killed due to the acidic environment, digestive enzymes, antimicrobial peptides (AMPs), and reactive oxygen intermediates (ROIs), similar to the well-known role of ROIs in killing bacteria in mammalian macrophages and neutrophils (Uribe-Querol & Rosales, 2020).

1.9.3 Humoral Components of the Insect Innate Immune System

Insects exhibit humoral responses that encompass melanization, clotting, and the release of antimicrobial peptides.

-Clotting

Coagulation is a crucial part of the innate immune system and facilitates haemostasis by triggering the creation of an insoluble clot in the hemolymph of insects. Given that insects possess an open circulatory system, clotting process plays an essential role in their immune system. Insects possess a remarkably effective coagulation mechanism that effectively prevents the loss of hemolymph, limits the spread of infection, and compartmentalises the hemocoel. In addition, coagulation serves to restrict potential tissue damage resulting from other immune reactions by confining the activity to the specific locations where the wound or pathogen entry (Dushay, 2009; Sheehan et al., 2018). For example, in Drosophila, the coagulation cascade involves a series of coagulation factors in three stages to form a hardened clot. First, a primary clot forms through the degranulation of hemocytes, where an aggregate consisting of hemocytes, cellular debris, and extracellular matrix is established. For example, in *Drosophila*, the coagulation cascade involves a series of factors in three stages to form a hardened clot. Initially, a primary clot forms through the degranulation of hemocytes, resulting in an aggregate composed of hemocytes, cellular debris, and extracellular matrix (Cerenius & Söderhäll, 2010; Rowley & Ratcliffe, 1976). In addition, the prophenoloxidase system and transglutaminases become activated, leading to the formation of cross-links and the hardening of the clot (Eleftherianos & Revenis, 2010). Ultimately, plasmatocytes are enlisted to effectively seal the clot (D. Li et al., 2002). The insect clot is mostly composed of hemolectin, which is the most abundant protein. However, there are several other components that also play a role in the creation of a stable clot. When transglutaminase is activated due to wounds or infections, it interacts with its substrates, Fondue and Eig71Ee, resulting in the production of a solid clot through covalent cross-linking. Lipophorin, similar to the lipid transporter found in mammals, also plays a role in the formation of the insect clot. Additionally, the activation of phenoloxidase by the proPO system works with transglutaminase in the ultimate formation of cross-links in the clot. Furthermore, it has a primary role in directly eliminating pathogens by means of melanization (Eleftherianos & Revenis, 2010; D. Li et al., 2002).

-*Melanization*

Melanin synthesis in insects is vital for various functions, including the innate immune system, pigmentation of the exoskeleton, sclerotization, and wound healing. In insects, the production of melanin is contingent upon the activation of the prophenoloxidase (proPO) activation system. This mechanism is promptly stimulated by the entrance of pathogens or injury to the cuticle. When the proPO system is activated, it can immediately destroy a pathogen by producing toxic compounds. Alternatively, the system has the ability to trigger phagocytosis and encapsulation of the invading pathogen or coagulation of the hemolymph (Cerenius et al., 2008). The process of melanin formation, initiated by the prophenoloxidase (proPO) system, is facilitated by the redox enzyme phenoloxidase. In insects, melanization is a complex process that requires precise regulation since it produces toxic and reactive substances that might injure the host. ProPO activation can be initiated by pathogen-associated molecular patterns (PAMPs) such as bacterial lipopolysaccharide and peptidoglycan or by the interaction of fungal β-1,3-glucan with their corresponding pathogen recognition receptors. In addition, proPO activation can occur autonomously, without the involvement of pathogen-associated molecular patterns (PAMPs), such as in the case of wounds and the presence of cells undergoing abnormal programmed cell death (apoptosis) (Cerenius et al., 2008; Y.-Y. Chen et al., 2014; Sheehan et al., 2018). The activation of the prophenoloxidase (proPO) system triggers a cascade of serine proteases, with phenoloxidase (PO) being activated by apolipophorin III and inhibited by lysozyme and anionic peptide-2 in *Galleria mellonella* (S. Y. Park et al., 2005). Consequently, the phenoloxidase activation system is initiated when the prophenoloxidase activating enzyme (ppA) is converted from its inactive form (pro-ppA) to its active form (ppA). The active form of ppA catalyzes the proteolytic cleavage of prophenoloxidase (proP) into phenoloxidase (PO).

Active PO is involved in the hydroxylation of monophenols, followed by the oxidation of phenols to form quinones. Finally, the polymerization of quinones, catalyzed by phenoloxidase-monophenol-L-dopa, leads to the formation of melanin (A. Lu et al., 2014; P. Zhao et al., 2007). Excluding the final step where melanin is produced, the proPO system shows similarities to the vertebrate complement system. In both systems, cytotoxic and opsonic components are produced. Although melanization is a vital component of the insect immune system, it must be tightly regulated by specific protease inhibitors to ensure accurate melanin deposition and reduce the toxicity associated with melanin overproduction (Cerenius et al., 2008; Sheehan et al., 2018).

1.10 *Hermetia illucens* life cycle

Insects, lack an adaptive immune system and rely on the production of broad-spectrum molecules, such as AMPs, to defend against infections effectively.

The insect *Hermetia illucens*, commonly referred to as the Black Soldier Fly, is of particular significance in this context. It is a dipteran species that belongs to the family *Stratiomyidae.* Originally confined to tropical and subtropical regions of the America, it has recently become prevalent worldwide.

H. illucens undergoes a complex life cycle that spans approximately 38 to 55 days, consisting of five distinct stages: egg, larval stage, prepupal stage, pupa, and adult (Figure 1.6) (Padmanabha et al., 2020).

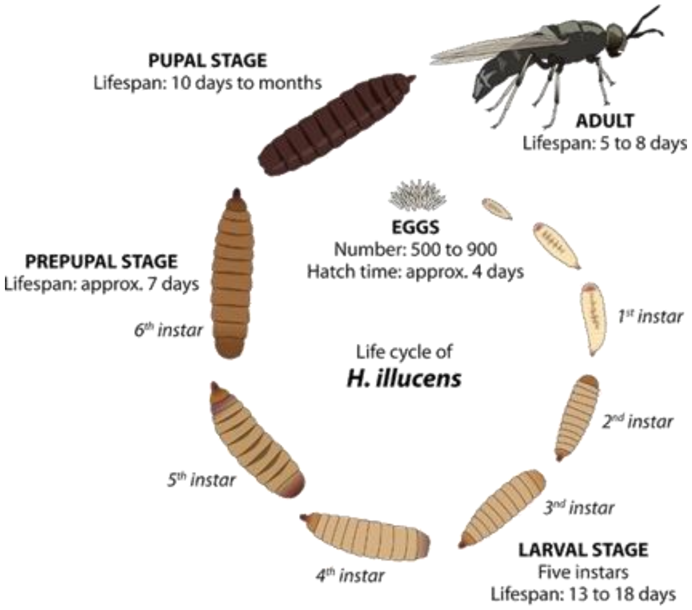

Figure 1.6. The life cycle of *H. illucens*. The different stages of the development of *H. illucens* are shown, as well as the average duration for each of these different stages (De Smet et al., 2018).

1.10.1 Egg Stage

The life cycle begins when the female *H.illucens* lays between 550 and 900 eggs. These eggs are typically deposited in crevices near decaying organic matter, such as compost piles or animal manure, which will serve as a food source for the emerging larvae. The incubation period for the egg averages about four days, although this can vary significantly depending on environmental factors such as temperature, humidity, and geographic location (L. A. Holmes et al., 2013; Padmanabha et al., 2020).

1.10.2 Larval Stage

Upon hatching, the larvae, which initially measure about 1.8 mm, immediately start feeding voraciously on a wide variety of organic materials. This includes animal manure, decaying fruits and vegetables, and other food wastes. The larval stage is the longest and most active period of the life cycle, during which the larvae grow significantly, reaching sizes of up to 20 mm (Figure 1.7). At this stage, they are considered mature larvae. The larvae go through several instars, or molts, as they grow and develop. During these instars, they are highly efficient at converting organic waste into biomass, which is one of the reasons why *H. illucens* is being researched for waste management and sustainable protein production (Tomberlin et al., 2002, 2009).

Figure 1.7. Dorsal (left) and ventral (right) views of sixth instar larvae of the black soldier fly, *H. illucens*. Credit: Bianca Diclaro, University of Florida

1.10.3 Prepupal Stage

When the larvae reach the sixth instar, they undergo a process called melanization. This involves the darkening of the cuticle, indicating the transition to the prepupal stage. During this phase, the larvae stop feeding and empty their digestive tracts in preparation for pupation. The prepupae then migrate away from their food source, seeking out dry and sheltered locations to pupate. This migration is crucial as it reduces the risk of predation and environmental hazards (L. A. Holmes et al., 2013).

1.10.4 Pupal Stage

The pupal stage lasts between 7 and 10 days, during which the larvae are immobile and do not feed (Figure 1.8). During this phase, significant internal structural reorganization occurs as the insect transitions from the larval to the adult form. The pupae remain in their dry and protected environment until they are ready to emerge as adults. The duration of the pupal stage can vary depending on environmental conditions but typically lasts at least eight days (L. A. Holmes et al., 2013).

Figure 1.8. Pupa of the black soldier fly, *H. illucens*. Credit: James Castner, University of Florida

1.10.5 Adult Stage

The adult Black Soldier Fly emerges from the pupal case after completing metamorphosis. The adults are primarily focused on reproduction and do not feed on solid food. Instead, they rely on the fat reserves accumulated during the larval stage and consume only water. The adult stage is relatively short compared to the larval and pupal stages, and adults are often observed mating in flight. Shortly after mating, females begin to lay eggs, thus completing the life cycle (Tomberlin et al., 2002).

Figure 1.9. Adult black soldier fly, *H. illucens* (Linnaeus). Credit: Lyle J. Buss, University of Florida

1.11 *H.illucens* as a Source of Antimicrobial Peptides

The significant increase in the incidence of drug-resistant bacteria and the reduction in the production of new antibiotics effective against multidrug-resistant strains represent a serious global concern. In this context, numerous studies have investigated the immune system of *Hermetia illucens*, highlighting it as a promising approach to address this issue.

H. illucens is recognized not only for its ability to bioconvert organic waste and as a supplement in aquaculture feed, but also as a source of numerous AMPs (Vogel et al., 2018). This capability arises from its environmental adaptation, as *H. illucens* larvae feed on decomposing organic substrates often characterized by high bacterial loads (A. Müller et al., 2017). In recent years, research has demonstrated that *H. illucens* can express more than 50 genes encoding AMPs(Van Moll et al., 2022). Additionally, recent studies have shown that AMP production can be optimized through techniques aimed at stimulating the insect's immune system. This stimulation can occur either via the direct injection of microorganisms into the hemocoel or through modifications to the larval feeding substrate(Zdybicka-Barabas et al., 2017).

An example of this immune stimulation was described by Vogel et al. (2018), who altered the larvae’s diet by adding bacteria or organic additives, such as lignin sulfonate and sunflower oil, and subsequently analyzed larval extracts and the immunity-related transcriptome. The high-protein diet enriched with brewery spent grains caused the most significant variation in gene expression, followed by a vegetable oil-based diet. Both diets induced a more robust immune response compared to bacterial supplementation alone. However, bacterial supplementation significantly increased the expression of over half of the identified AMP-encoding genes, including attacins, cecropins, defensins, diptericins, and knottin-like peptides.

Park et al. (2015) identified a new defensin-like peptide in *H. illucens* larvae immunized via piercing with a thin needle immersed in *S. aureus*. The purified DLP4 peptide exhibited antimicrobial activity against Gram-positive bacteria. In another study, Park and Yoe (2017) induced and purified a cecropin-like peptide (CLP1) from the hemolymph of immunized larvae, which displayed activity against Gram-negative bacteria. Subsequently, it was revealed that the DLP3 peptide exhibits activity against both Gram-positive and Gram-negative bacteria. The expression of CLP1 and DLP4 was absent in the larvae’s bodies before immunization but was primarily detected in the fat body after treatment with *S. aureus*.

Shin and Park (2019) described another AMP, a new attacin derived from *H. illucens*. The larvae immunized with *E. coli* induced the production of attacin, which exhibited antibacterial activity against *E. coli* and methicillin-resistant *S. aureus* (MRSA). Elhag et al. (2017) identified seven new gene fragments encoding antimicrobial peptides, including cecropinZ1, sarcotoxins, and stomoxins, expressed as fusion proteins and tested against Gram-positive, Gram-negative bacteria, and fungi.

Recent studies by Xu et al. (2020) led to the identification of new AMPs derived from *H. illucens*, such as Hidefensin-1, Hidiptericin-1, and HiCG13551, all capable of inhibiting the growth of pathogenic bacteria such as *S. aureus* and *E. coli in vitro*.

Finally, Moretta et al. (2020) conducted a transcriptomic analysis of *H. illucens* larvae, identifying fifty-seven potentially active peptides. Four peptides with high antimicrobial scores in all prediction programs were subsequently selected and chemically synthesized: Hill_BB_C6571, Hill_BB_C16634, Hill_BB_C46948, and Hill_BB_C7985. All synthesized peptides demonstrated activity against *E. coli*.

2. Aims of the study

The increasing resistance to antimicrobial drugs, resulting from inappropriate use in human medicine, veterinary practices, and agriculture, has led to the development of resistant microorganisms responsible for hard-to-treat diseases for which no adequate therapies are available. Concurrently, there is a slowdown in the development of new innovative drugs, creating a concerning scenario where healthcare services may no longer be able to respond effectively to demands. Therefore, it is crucial to focus on identifying new potentially effective molecules.

Recent scientific advancements have highlighted the potential of antimicrobial peptides (AMPs), small bioactive molecules found across a wide range of organisms. These peptides have demonstrated significant antimicrobial activity against gram-positive bacteria, gram-negative bacteria, and yeasts, including strains resistant to conventional antibiotics. Insects, with their vast biodiversity and ability to synthesize antimicrobial peptides in response to infections, represent one of the most promising sources of these molecules.

This research project aims to explore pharmacologically active principles derived from insects to develop antimicrobial peptides as a new category of safe and effective molecules. The goal is to offer innovative therapeutic solutions for human health protection by leveraging sustainable production processes and natural sources. This research could lay the groundwork for novel strategies in combating resistant infections, significantly contributing to public health preservation.

3. Materials and methods

3.1 *Hermetia illucens* Rearing

Hermetia illucens larvae were provided by Xflies s.r.l (Potenza, Italy). After egg hatching, larvae were fed on a standard Gainesville diet (30% alfalfa, 50% wheat bran, 20% corn meal) (Hogsette, 1992) at 70% moisture under controlled conditions of temperature (27 ± 1.0 °C), relative humidity (70% ± 5%) and photoperiod (12L:12D (h)) (Scieuzo et al., 2023).

3.2 *H. illucens* Larval Infection and Hemolymph Collection

Escherichia coli (Gram negative, LMG:2092 strain) and *Micrococcus flavus* (Gram positive, DSM 19079) were incubated in 10 mL of Luria Bertani (LB) broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl), at 37 °C for 24 h, under shaking. A total of 1 mL of each bacterial culture was inoculated into a fresh LB broth, incubated at 37 °C and used for the experiment once the optical density (OD) at 600 nm reached 1. Last instar larvae of *H. illucens* were firstly washed with sterile water and then infected via a capillary dipped into the cell suspension of *E. coli* or *M. flavus* (DANG et al., 2006; Elhag et al., 2017) in order to stimulate the production of different antimicrobial peptides (AMPs) and to induce a higher expression level of constitutively expressed AMPs. Following the bacterial challenge, larvae were left in a controlled chamber at 27 °C for 24 h. A group of uninfected larvae was used as control. For each treatment, 100 larvae were used, yielding a total volume of approximately 1 mL of hemolymph. To facilitate the spill of hemolymph, larval abdomens were punctured by a sterile capillary and the hemolymph from infected and uninfected larvae was collected, using a pipette (Gilson, Middleton, WI, USA), in ice-cold tubes, containing a fixed-minimum quantity of l-ascorbic acid (0.015 g) (Merck Millipore, Burlington, MA, USA), to prevent hemolymph melanization. To recover only the plasma and remove the cellular components, the extracted hemolymph was subjected to centrifugation at 10,000 rcf for 5 min at 4 °C. The recovered supernatant (cell-free hemolymph) was stored at −80 °C until use.

3.3 Isolation of Peptide Fraction by Organic Solvent Precipitation

In order to separate the putative AMPs in the hemolymph from the higher molecular weight proteins, the plasma recovered from both uninfected and infected larvae was subjected to a precipitation protocol with methanol (Merck Millipore, Burlington, MA, USA), acetic acid (Merck Millipore, Burlington, MA, USA) and water in a 90:1:9 v/v ratio. The plasma and solvent were mixed in a 1:9 v/v ratio and then centrifuged for 45 min at 16,000 rcf at 4 °C. The obtained supernatant, containing compounds with a molecular weight lower than 30 kDa, was vacuum dried to remove the organic solvents and resuspended in a volume of sterile water equal to the original plasma volume. All samples were subsequently stored at 4 °C until the next use.

3.4 Protein Quantification via Bradford Assay

The protein concentrations of all samples were quantified with Bio-Rad Protein Assay, Dye Reagent Concentrate (Bio-Rad, Hercules, CA, USA), according to the Bradford method (Bradford, 1976). To calculate the concentration of the proteins of interest, a standard calibration using known concentrations of the Bovine Serum Albumin (BSA) protein (Merck Millipore, Burlington, MA, USA) was set up. The absorbance of the samples was measured at a wavelength of 595 nm using a spectrophotometer (Thermo Scientific, Waltham, MA, USA).

3.5 Bacterial strains and Culture Conditions

E. coli (LMG:2092), *M. flavus* (DSM 19079) were cultured on Luria-Bertani Broth and Luria-Bertani agar (Oxoid ltd); *Pseudomonas aeruginosa* (ATCC® 9027™) and *Proteus mirabilis* were cultured on Brain Heart Infusion (BHI) broth and Brain Heart Infusion (BHI) agar (Oxoid ltd); *Staphylococcus aureus* (ATCC® 6538™), *Salmonella* subsp. *enterica* serovar. *Thypimurium* (ATCC® 14028GFP™), *Enteroinvasive Escherichia coli* (EIEC) (ATCC® 43893™), *Enterococcus faecalis* (ATCC® 9027™), *Staphylococcus epidermidis* (ATCC® 35984™), *Streptococcus pyogenes, carbapenem-resistant Klebsiella pneumoniae, methicillin- resistant Staphylococcus aureus* were cultured on Tryptic Soy Broth (TSB) and Tryptic Soy Agar (TSA) (Oxoid ltd); *Candida albicans* (ATCC® 10231™) was cultured on Sabouraud Dextrose Broth (SDB) and Sabouraud Dextrose Agar (SDA) (Oxoid ltd). The bacterial strains of *P. mirabilis*, *S. pyogenes*, carbapenem-resistant *K. pneumoniae*, and methicillin-resistant *S. aureus* were clinically isolated at the Microbiology and Virology Service of the University Hospital of the University of Campania "Luigi Vanvitelli" and from Institute for Pulmonary Diseases of Vojvodina in Serbia.

3.6 Evaluation of the Peptide Fraction Antibacterial Activity via Bioautography (SDS Gel Overlay Method) Experiment

The antibacterial activity of the peptide fraction (PF) recovered from the plasma of infected and uninfected larvae was also evaluated via a bioautography experiment (Zdybicka-Barabas et al., 2017). Briefly, two polyacrylamide gels were prepared (4% stacking, 12% running); one of the two gels was stained with a solution of Blue Coomassie (Merck Millipore, Burlington, MA, USA) in order to visualize the bands corresponding to the peptide samples, while the second gel was washed with Triton X-100 (Bio Rad, Hercules, CA, USA) at 2.5% for 1 h to remove the SDS and with Tris-HCl 50 mM pH 7.5 for 2 h to allow the renaturation of the peptides; finally, the gel was incubated in LB culture medium for 1 h. At the end of the incubation in LB, solid nutrient LB-agar culture medium (0.7%) containing *E. coli* or *M. flavus* cells was transferred onto the gel and incubated for 24 h at 37 °C. For each experimental condition, 20 μL of sample was loaded.

3.7 Evaluation of the Antibacterial Activity of Peptide Fraction via Agar Diffusion Assay

The *in vitro* evaluation of the antimicrobial activity of peptide fraction was conducted using the agar diffusion assay. A colony of each bacterial strain was transferred to the appropriate culture medium and incubated overnight at 37°C under constant shaking. Subsequently, 100 μL of the bacterial suspension, with an optical density of 0.3 at 600 nm (OD600), was uniformly distributed on plates containing solid medium using a sterile cotton swab. After absorption of the suspension, each sample, corresponding to the peptide fractions of hemolymph extracted from both infected and uninfected larvae, was dispensed onto the plates. Sterile water was used as a negative control. All tests were performed in triplicate, and the plates were incubated overnight at 37°C.

3.8 Evaluation of the Peptide Fraction Antibacterial Activity via Microdilution Assay

Microdilution assays were conducted on peptide fraction to determine the minimum inhibitory concentration (MIC). The MIC is the lowest concentration of an antimicrobial agent capable of inhibiting the growth of a microorganism *in vitro* after 12-24 hours of incubation. This value is obtained by testing a standard concentration of microorganisms against a series of scalar dilutions of the antimicrobial substance (Andrews, 2001). Specifically, the microdilution method was used with 96-well microplates, where scalar doses of the antimicrobial agent were added, followed by inoculation with the bacterial strain at a constant concentration. After incubation, bacterial growth was assessed based on turbidity. For the assay, eight serial dilutions were prepared (0.750 µg/µL, 0.375 µg/µL, 0.187 µg/µL, 0.0937 µg/µL, 0.0468 µg/µL, 0.0234 µg/µL, 0.0117 µg/µL, 0.0058 µg/µL). The bacterial strains tested against the peptide fraction samples were diluted to an optical density at 600 nm of 0.3. In each well of the microplate, 100 µL of medium and 100 µL of the antimicrobial agent were added to the first row, with an initial concentration of 0.750 µg/µL. Serial dilutions were then performed directly in the plate, and 1 µL of bacterial culture at OD 0.3 was added to each well. The controls used included a negative control with only the medium, a positive control with the medium and 1 µL of bacterial culture at OD 0.3, and a substance control with 100 µL of medium and 100 µL of the antimicrobial agent being tested.

3.9 Evaluation of the Peptide Fraction Antibacterial Activity via Minimum Bactericidal Assay

The minimum bactericidal concentration (MBC) is the lowest concentration of an antimicrobial agent required to kill 99.9% of the initial microbial population after a specified period of incubation, typically 24 hours. In other words, while the minimum inhibitory concentration (MIC) indicates the lowest concentration of an antimicrobial that inhibits the visible growth of a microorganism, the MBC measures the antimicrobial's ability to actually kill the microorganism.

Determining the MBC is a subsequent step following the determination of the MIC. After identifying the MIC, samples are taken from the wells containing antimicrobial concentrations equal to or greater than the MIC and are inoculated onto agar plates without antimicrobials. These plates are then incubated, and bacterial growth is observed. The MBC is the lowest concentration of the antimicrobial agent that shows no visible bacterial growth on the agar plates, indicating that 99.9% of the initial bacterial population has been killed (Balouiri et al., 2016).

Based on the obtained results, MBC assays were developed for the concentrations at which a reduction in bacterial growth was observed. From each of the wells, 100 µL of the bacterial suspension was taken. In seven tubes, each containing 900 µL of PBS, 100 µL of the bacterial suspension was added, and seven serial dilutions were performed. From the most diluted sample, 10 µL of each dilution was plated onto solid media; each dilution was plated in triplicate. The following day, colony counting was performed, and the results were expressed in CFU/mL.

3.10 SDS-PAGE and *In Situ* Hydrolysis

The peptide fraction extracted from *H. illucens* larvae infected with *E. coli*, *M. flavus* and from uninfected larvae (control) was fractionated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In detail, at 15 µL for each protein extract, the loading buffer 1X, composed of 2% SDS (Bio-Rad, Hercules, CA, USA), 50 mM TRIS-HCl pH 6.8 (Merck Millipore, Burlington, MA, USA), 10% Glycerol (Merck Millipore, Burlington, MA, USA) and bromophenol blue (Bio-Rad, Hercules, CA, USA), was added. They were separated on a 20% SDS-PAGE gel. After the run, the gel was stained with GelCode™ Blue Safe Protein Stain (Thermo Fisher Scientific, Waltham, MA, USA) and destained with Milli-Q water. A total of 3 bands for each condition (*E. coli*, *M. flavus*, control) were cut and *in situ* hydrolyzed with trypsin as previously described (Butturini et al., 2016). Peptide mixtures were extracted in 0.2% formic acid (HCOOH) (Merck Millipore, Burlington, MA, USA) and acetonitrile (ACN) (Merck Millipore, Burlington, MA, USA) and vacuum dried via a SpeedVac System (Thermo Fisher Scientific, Waltham, MA, USA).

3.11. LC-MS/MS Analysis and Protein Identification

Each peptide mixture was dissolved in 10 µL of 0.2% HCOOH (Merck Millipore, Burlington, MA, USA) and analyzed via nano LC-MS/MS on an LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) coupled to a nanoLC system nano Easy II. Each peptide mixture was concentrated and desalted onto a trapping column (C18 Easy Column L = 2 cm, ID = 100 mm, Nano Separations, Nieuwkoop, the Netherlands), and then fractionated on a C18 reverse-phase capillary column (C18 Easy Column L = 20 cm, ID = 7.5 µm, 3 µm, (Nano Separations, Nieuwkoop, The Netherlands) with a flow rate of 250 nL/min. The gradient used for peptide elution ranged from 10% to 60% of eluent B in 69 min (Fusco et al., 2020). Eluents A and B have the following composition: 2% ACN LC-MS grade and 0.2% HCOOH, and 95% ACN LC-MS grade and 0.2% HCOOH, respectively. The MS/MS method was set up in a data-dependent acquisition mode (DDA), with a full scan ranging from 300 to 1800 m/z range, followed by fragmentation in CID modality of the top 5 ions (MS/MS scan) selected by intensity and charge state (+2, +3, +4 charges), and applying a dynamic exclusion time of 40 s (Salvia et al., 2022). The peak list generated was uploaded in Mascot software (version 2.4.0) and research was performed by using the in-house database named the "*Hermetia illucens* database". The parameters for protein identification were as follows: "trypsin" as an enzyme with at least one missed cleavage, "carbamidomethyl" as a fixed modification, "oxidation of Met" and "pyro-Glu at N-term if Gln" as variable modifications, 0.6 Da as MS/MS tolerance and 10 ppm as peptide tolerance. Scores threshold of matches for MS/MS data was fixed at 10 for all peptides.

3.12 Solid Phase Peptide Synthesis

The chemical synthesis of two antimicrobial peptides identified as MO_Ab1Lin and MO_Ab4 was performed. Both peptides were synthesized on a 0.05 mmol scale using solid-phase peptide synthesis (SPPS) with an automated orthogonal Fmoc/tBu protection strategy and magnetic induction heating. The instrument used was the PurePep® Chorus® (Gyros Protein Technologies, Tucson, Arizona). The resin used for the syntheses of MO_Ab1Lin and MO_Ab4 was Fmoc-Asn(Trt)-Wang TG resin with a substitution degree of 0.23 mmol/g. Solid-phase synthesis proceeds, contrary to natural synthesis, from the C-terminus to the N-terminus and involves the repetition of a cycle consisting of:

- Deprotection of the α-amino group from the Fmoc group
- Washing with Dimethylformamide (DMF)
- Coupling with the subsequent Fmoc-amino acid, forming an amide bond

The Fmoc deprotection was performed at 90°C for 1 minute using a 20% (v/v) piperidine solution in DMF. The coupling system, which enabled the formation of new amide bonds, included 5 equivalents each of Diisopropylcarbodiimide (DIC), Ethyl Cyanohydroxyiminoacetate (Oxyma Pure), and the appropriately side-chain-protected Fmoc-amino acid. The reaction was conducted cyclically at 90°C for 2 minutes in DMF. The resin bearing the finished product was then treated with a solution of trifluoroacetic acid, triisopropylsilane, and water in a 95:2.5:2.5 (v/v/v) ratio for 3 hours at room temperature to detach the product from the resin and simultaneously remove all protective groups from the side chains. The peptide solution in TFA was filtered from the spent resin, and the product was precipitated by adding a counter-solvent (diethyl ether at 0°C) and separated from the supernatant by centrifugation. The solid was vacuum dried, then dissolved in water, and finally lyophilized.

3.13 Oxidative Folding

The peptide MO_Ab4 was subjected to the oxidative folding protocol, which is based on the work of Kent and collaborators (Dhayalan et al., 2017). The linear peptide was dissolved at a concentration of 0.05 mg/mL in a buffer consisting of 1.5 M Guanidine Hydrochloride, 20 mM Tris, 8 mM L-Cysteine, and 1 mM Cystine Hydrochloride at pH 7.6.

The reaction was conducted at room temperature and monitored via LC-MS: the chromatographic peak corresponding to the peptide with formed disulfide bonds displayed a different retention time compared to the linear peptide, and the relative mass of the peak decreased by approximately 6 units.

Upon completion, the reaction was quenched by adding acetic acid to adjust the pH to 3. At this point, the solution was loaded using an external pump onto a C18 cartridge (SNAP Ultra C18, Biotage, Uppsala, Sweden, 60g), and the product was eluted using the CombiFlash NextGen300 plus instrument (Teledyne Isco, Lincoln NE, USA) with an isocratic solvent composition of 40% B in A (A: 0.1% TFA in water, B: 0.1% TFA in acetonitrile).

3.14 HPLC Purification and Characterization

The peptides MO_Ab1Lin and MO_Ab4 were subjected to purification by preparative HPLC using a Waters Alliance system (model 2487 Dual λ Absorbance detector, model 600 pump) equipped with a Phenomenex Aqua 5 µm C18 200 Å column. The eluent system used was A: 0.1% TFA in water and B: 0.1% TFA in acetonitrile, with a gradient of 20% to 70% B in A over 30 minutes. The analytical system consisted of a Waters Alliance 2565 coupled with an ESI Waters ZQ mass spectrometer. The analytical column used was the SUPELCO BIOShell A160 Peptide C18 100x3 mm, 2.7 µm. The eluent system comprised A: 0.1% (v/v) TFA in Milli-Q water and B: 0.1% (v/v) TFA in acetonitrile.

3.15 Molecular cloning of C-7081 and C-158 genes: Polymerase Chain Reaction products

The C-158 and C-7081 peptides, previously identified in the H. illucens transcriptome (Vogel et al., 2018), were analyzed in Moretta et al. through bioinformatics strategies and functionally annotated as Defensin by the Blast2Go software (Moretta et al., 2020). Because of their high prediction scores for antimicrobial activity and their sequence length, they were selected for in vitro studies. The genes encoding the peptides were amplified from transcripts of H. illucens larvae using primers specific to the DNA fragment of interest. The forward primer was designed with an EcoRI restriction site at the 5' end. In comparison, the reverse primer included a NotI restriction site at the 5' end of the stop codon and a 6-histidine tag at the 3' end of the stop codon for subsequent purification.

Table 2.1. Primer sequences for the molecular cloning of peptides C-158 and C-7081

HILL_BB C158 FOR	5’-GAATTCGCCACCTGTGACCTGTTG-3’
HILL_BB C158 REV	5’-GCGGCCGCTTAATGATGATGATGATGATGTCGGCAGACACAGACAAGT-3’
HILL_BB C7081 FOR	5’- GAATTCGCCACCTGTGACCTAATAA-3’
HILL_BB C7081 REV	5-GCGGCCGCTTAATGATGATGATGATGATGGCGGCAAATGCAGATAAGG -3’

The amplification of the sequence was carried out using Platinum™ Taq DNA Polymerase, High Fidelity, which is ideal for amplifying DNA fragments requiring high yields and robust amplification. High fidelity is ensured by a blend of Platinum™ Taq DNA Polymerase and a proofreading enzyme (3′→5′ exonuclease activity) from *Pyrococcus* species GB-D polymerase. The specificity of the PCR was enhanced by incorporating the automatic "hot-start" Platinum™ technology. The amplification reaction was performed in a 25 µL reaction volume containing 1 µL of template, 2.5 µL of 10X High Fidelity PCR Buffer, 0.5 µL of 10 mM dNTP, 3 µL of $MgCl_2$, 0.5 µL of 10 µM forward primer, 0.5 µL of 10 µM reverse primer, 0.1 µL of Platinum™ Taq DNA Polymerase, High Fidelity, and 18.9 µL of sterile H_2O. The reaction mixture underwent 30 cycles of amplification in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems) with the following parameters: initial denaturation at 94°C for 30 seconds, denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds, and extension at 68°C for 1 minute, followed by a hold at 4°C. After amplification, 0.2 µL of DreamTaq DNA Polymerase (5 U/µL, Thermo Scientific, USA) was added, and a final extension cycle was conducted at 72°C for 10 minutes to add single adenines to the 3' ends of the synthesized fragments, necessary for cloning into the pCRII-TOPO plasmid vector. An aliquot of the amplification product was verified by agarose gel electrophoresis at a concentration of 0.8%. The gel was prepared by dissolving 0.8 g of agarose in 100 mL of 1X TAE buffer, heating the solution to approximately 65°C, and cooling it to around 45°C before adding 5.0 µL of ethidium bromide to a final concentration of 0.05 µL/mL. The sample was prepared for electrophoresis by mixing 1 µL of Bromophenol Blue (BBF, Sigma Aldrich®) with 10 µL of the PCR product. Electrophoresis was conducted at 90 V for 60 minutes.

3.16 Cloning of C-7081 and C-158 into the pCR2.1-TOPO Vector

The DNA fragment obtained via Polymerase Chain Reaction (PCR) was cloned into the pCR2.1-TOPO plasmid vector (Figure 2.1), which was subsequently used to transform competent bacterial cells. The cloning was performed using the “TOPO® TA-Cloning” kit (Invitrogen, Carlsbad, California, USA), which utilizes the TA cloning mechanism. This mechanism relies on the 3’ transferase activity of certain polymerases, including the DreamTaq DNA Polymerase used in the PCR reaction, which adds single adenines to the 3’ ends of the synthesized fragments during the final extension phase. During the cloning reaction, the adenines at the 3’ ends of the insert pair with the terminal thymines at the vector's cloning site. The ligation between the insert and the vector is facilitated by Topoisomerase I, which is covalently bound to the vector, making the ligation reaction rapid and efficient compared to other kits that require the addition of T4 DNA Ligase to the ligation mixture. For the ligation, the PCR product, Salt Solution, and TOPO® Vector were incubated at room temperature for 20 minutes. Four microliters of the vector-insert reaction mixture were used to transform an aliquot of 50 µL of TOP10 chemically competent *Escherichia coli* cells (Invitrogen, Carlsbad, California, USA) via heat shock. The heat shock facilitates the entry of the plasmid into the cells. The cells, with 3 µL of vector-insert added, were incubated on ice for 15 minutes, followed by a heat shock at 42°C for 45 seconds and an immediate transfer back to the ice for an additional 15 minutes. After the heat shock, 850 µL of SOC Medium was added for a final volume of 1 mL. The transformed cells were then incubated at 37°C for 1 hour with constant agitation before being plated onto solid LB agar medium containing 50 µg/mL ampicillin for the selection of transformed clones. The plate was pre-warmed at 37°C for 10 minutes, and 40 µL of X-Gal (40 mg/mL) (Sigma–Aldrich, St. Louis, MO, USA) was added to facilitate blue/white screening. This screening allows discrimination between colonies containing the insert within the vector and those where the plasmid has self-ligated. The cloned fragment disrupts the Open Reading Frame of the ß-Galactosidase (lacZ) gene, which is otherwise transcribed and translated if the plasmid re-circularizes. The active enzyme hydrolyses the chromogenic substrate X-Gal, turning these colonies blue, whereas colonies with the correctly inserted fragment remain white. The plates with the transformed cells were incubated at 37°C for 16 hours. At the end of the 16-hour incubation period, the colonies were ready for further analysis.

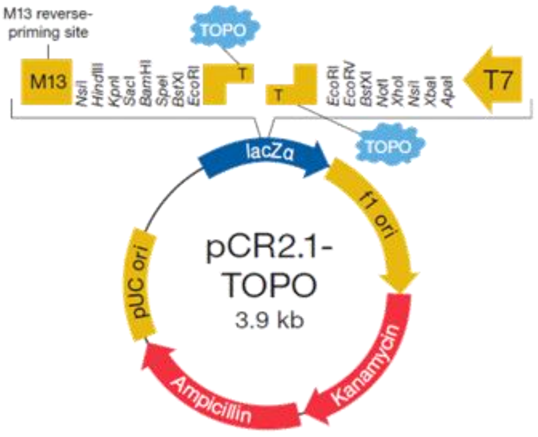

Figure 2.1. pCR™2.1 TOPO® map. The map shows the features of the pCR™2.1 TOPO® vector.

3.17 Colony PCR

To assess the positivity of clones, both blue and white colonies obtained from the blue/white screening were subjected to colony PCR. This technique allows for the rapid identification of whether the insert is present in the vector. The colony PCR was set up following the protocol described below. Five microliters were taken from each inoculum and mixed with 50 µL of MilliQ water (osmotic shock). The samples were then incubated at room temperature for 10 minutes in a sterile hood. Following this incubation, the samples underwent a denaturation cycle at 95°C for 5 minutes and were then placed on ice (thermal shock). After the osmotic and thermal shock, a standard PCR reaction was set up in a 25 µL reaction volume. The reaction mixture included 5 µL of lysis solution, 2.5 µL of buffer, 0.5 µL of dNTPs, 0.75 µL of forward primer (10 µg/µL), 0.75 µL of reverse primer (10 µg/µL), 0.25 µL of DreamTaq DNA Polymerase (5U/µL, Thermo Scientific, USA), and 15.25 µL of distilled water. A negative control was also prepared, consisting of all the reaction components except the lysis solution. The reaction mixture was subjected to 35 amplification cycles with the following parameters: initial denaturation at 94°C for 5 minutes, followed by denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 1 minute and 30 seconds. This was followed by a final extension at 72°C for 10 minutes, and the reaction was then held at 4°C until further analysis. At the end of the amplification, a 5 µL aliquot of the PCR product was taken and verified by agarose gel electrophoresis at a concentration of 0.8%. An aliquot of 500 µL from the positive colonies inoculum was transferred to 5 mL of liquid LB medium containing 50 µg/mL ampicillin and incubated with constant agitation at 37°C for 16 hours to extract the plasmid DNA.

3.18 Extraction of C-7081-pCR2.1-topo and C-158-pCR2.1-topo via Mini-Prep

Plasmid DNA was extracted from each inoculum of transformed cells using the "FastPlasmid Mini Kit-250 Preps" (5Prime, Hamburg, Germany) according to the following protocol: the cell suspension was centrifuged for 1 minute at 13,200 rpm to pellet the cells and remove the culture medium. To lyse the cells, 400 µL of Lysis Buffer was added, and the solution was vortexed for 30 seconds to enhance the lysis process. The mixture was incubated at room temperature for 3 minutes. The solution was then transferred to a spin column and centrifuged for 1 minute at 13,200 rpm. Subsequently, 400 µL of Wash Buffer was added to wash the membrane-bound plasmid DNA. This step was repeated twice, each time for 1 minute at 13,200 rpm, to ensure complete removal of unbound substances. The spin column was then transferred to a new tube, and 50 µL of Elution Buffer was added directly to the filter to elute the membrane-bound plasmid DNA. The column was centrifuged for 1 minute at 13,200 rpm to collect the eluted DNA. The plasmid DNA extracted from the transformed cells was quantified using a NanoDrop™ OneC (Thermo Scientific, Waltham, Carlsbad CA, USA) and subsequently analyzed by agarose gel electrophoresis at a concentration of 0.8%.

3.19 Analysis by Digestion of C-7081-pCR2.1-TOPO and C-158-pCR2.1-TOPO constructs

To excise the insert from the cloning vector, the construct was digested with endonucleases that cut outside the exogenous sequence. EcoRI and NotI (Biolabs, Ipswich, Massachusetts, USA) were used for the digestion. At least 70 ng of the sample is required to visualize a DNA fragment on an agarose gel. The amount of plasmid DNA required to obtain a visible insert on the gel (3 µg) was calculated based on the vector/insert ratio. The digestion was performed in a mixture containing 6.67 µL of recombinant plasmid DNA for C7081 and 13.2 µL for C158 (equivalent to 3 µg), 1.5 µL of NEB Buffer 3 (10X), 1 µL of NotI (20,000 U/mL), 1 µL of EcoRI (20,000 U/mL), and H_2O to a final volume of 15 µL. The reaction took place at 37°C for 2 hours, and the digestion was verified by electrophoresis on a 0.8% agarose gel.

3.20 Extraction of C7081-pCR2.1-TOPO and C158-pCR2.1-TOPO by Midi-Prep

The recombinant cloning plasmid (pCR$^{®}$2.1-TOPO) was extracted from the positive colony to digest the fragment of interest and clone it into the expression vector. To obtain a substantial amount of the insert, a larger quantity of recombinant vector than that obtained by miniprep was required. Therefore, a midiprep of the recombinant vector was performed. An aliquot of 1 mL from the recombinant colony inoculum was added to 4 mL of liquid LB containing 50 µg/mL ampicillin. The pre-inoculum was incubated with agitation at 37°C for 8 hours. This pre-inoculum was then transferred to 50 mL of liquid LB containing 50 µg/mL ampicillin and incubated at 37°C for 16 hours with agitation. For extraction, the “HiPure

Plasmid Midiprep" kit (Invitrogen, Carlsbad, California, USA) was used, following the protocol described below. Twenty-five milliliters of inoculum were collected and centrifuged at 4°C for 10 minutes at 4,000 rpm. The supernatant was discarded. The step was repeated to achieve a final inoculum volume of 50 mL. After discarding the supernatant, 4 mL of resuspension buffer was added, and the pellet was vortexed to resuspend. Then, 4 mL of lysis buffer were added, mixed gently, and left at room temperature for 5 minutes. Subsequently, 4 mL of precipitation buffer were added and mixed gently. The mixture was centrifuged at 30°C for 30 minutes at 15,000 rpm. During the centrifugation, a column from the kit was placed in a sterile Falcon tube, and 10 mL of equilibration buffer were added, allowing it to pass through by gravity. After centrifugation, the supernatant was carefully transferred to the column, avoiding the pellet. The solution was allowed to pass through the column by gravity. Then, 10 mL of wash buffer were added and allowed to pass through by gravity, a step that was repeated twice. The column was transferred to a sterile Falcon tube, and 5 mL of elution buffer were added, allowing it to pass through by gravity. In the Falcon tube, 3.5 mL of isopropanol was added. The sample was aliquoted into six 1.5 mL tubes and centrifuged at 4°C for 30 minutes at 13,200 rpm. The supernatant was discarded, and the pellet was washed with 500 µL of 70% ethanol, followed by centrifugation at 4°C for 5 minutes at 13,200 rpm. The supernatant was discarded again, and the pellet was allowed to dry. The pellet was resuspended in 20 µL of H_2O and left on ice for about two hours, then the samples were combined into a single tube. The extracted DNA was quantified using a NanoDrop™ OneC (Thermo Scientific, Waltham, Carlsbad CA, USA) and evaluated via agarose gel electrophoresis (0.8% gel).

3.21 Sequencing of C-7081-pCR2.1-TOPO and C-158-pCR2.1-TOPO

Following digestion, the plasmid DNA was sent to the Macrogen Europe Sequencing Service (Amsterdam, The Netherlands). The sample was prepared with 5 µL of plasmid DNA (100 ng/µL) and 5 µL of the specific oligonucleotide (5-10 pmole/µL). In this case, the M13 Forward primer, designed upstream of the plasmid's polylinker region, was used instead of the sequence-specific primers used for fragment amplification. Using oligonucleotides located upstream of the fragment of interest ensures optimal sequencing. The sequences of the forward and reverse M13 primers are shown in the Table 3.2.

Table 3.2. Sequences of the forward (M13 FOR) and reverse (M13 REV) primers used for sequencing.

Primer	Sequence
M13 FOR primer	5'-GTAAAACGACGGCCAG-3'
M13 REV primer	5'-CAGGAAACAGCTATGAC-3'

3.22 Digestion of C-7081-pCR2.1-TOPO and C-158-pCR2.1-TOPO and Purification from Agarose Gel

The recombinant plasmid, purified via midi-prep, was digested as previously described. The fragment was recovered from a 0.8% agarose gel using the "Freeze N Squeeze™ DNA gel extraction spin columns" kit (Bio-Rad Laboratories, USA). The band of interest was excised using a scalpel and transferred to a spin column. After cooling at -20°C for 5 minutes, the column was centrifuged at 13,200 rpm for 3 minutes, and the eluate was collected in a new tube. This step was repeated twice, and the eluates were combined into a single tube for quantification. The DNA was precipitated with 3 M sodium acetate (1/10 of the volume) and three volumes of 96% ethanol, then stored at -80°C for at least 2 hours. The sample was centrifuged for 20 minutes at 16,000 g, followed by two washes with 70% ethanol, each with centrifugation at 12,000 g for 5 minutes at 4°C. The pellet was dried, resuspended in 25 µL of distilled H_2O, and quantified using a NanoDrop™ OneC (Thermo Scientific, Waltham, Carlsbad CA, USA).

3.23 Cloning of C-7081 and C-158 into the Expression Vector pPIC9K

The pPIC9K expression plasmid vector (Invitrogen) (Figure 2.2) was initially digested with EcoRI and NotI and dephosphorylated to prevent self-ligation. The cohesive ends generated by the same enzymes on both the vector and insert ensured the directional insertion of the C-7081 and C-158 genes. The reaction, catalyzed by T4 DNA ligase, facilitated the formation of phosphodiester bonds between the ends of the vector and the insert. The ligation of the fragment into the linearized vector was carried out at 14°C for 16 hours, using a vector/insert ratio of 1:3. The ligation mixture contained 2.7 ng of insert, 30 ng of vector, 1 µL of 10X buffer, 2 µL of T4 DNA ligase (diluted 1:10 to 4 U/µL), and sterile H_2O to a final volume of 10 µL. This mixture was then used to transform 150 µL of *E. coli* TOP 10 cells via heat shock, as described in paragraph 2.16. The transformed cells were plated on LB agar containing 50 µg/mL ampicillin and incubated at 37°C for 16 hours. The following day, ten colonies were selected and grown in 5 mL of LB liquid medium containing 50 µg/mL ampicillin, with constant agitation at 37°C for 16 hours. Plasmid DNA was extracted to verify the success of the cloning.

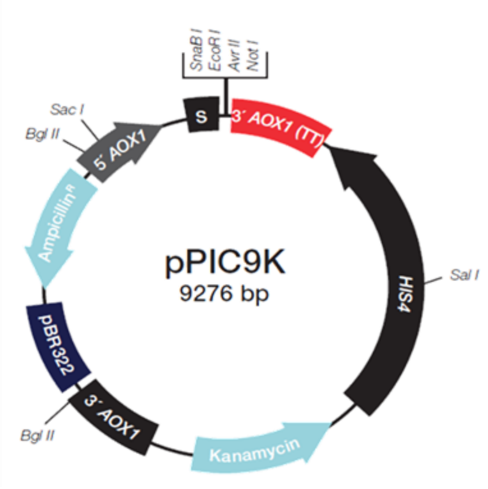

Figure 3.2. pPPIC9K map. The map shows the features of the pPPIC9K vector.

3.24 Screening of Transformed Colonies

The pPIC9K vector (Invitrogen) contains the ampicillin resistance gene as a selectable marker (Figure 2.2), allowing only colonies that have acquired the plasmid to grow on LB agar plates with 50 µg/mL ampicillin. To distinguish colonies containing recombinant vectors from those with self-ligated vectors, the colonies were screened using PCR and digestion. The PCR was performed using oligonucleotides flanking the insertion site of C-158 and C-7081 on the pPIC9K vector, as described in paragraph 3.17.

The sequences of the primers used in the PCR are listed in Table 3.3 below:

Table 3.3. Sequences of the 5’ AOX and α-Factor primers used for screening colonies containing recombinant vectors.

Primer Name	Sequence (5' -> 3')
Primer 5' AOX	5´-GACTGGTTCCAATTGACAAGC-3´
Primer α-Factor	5´-TACTATTGCCAGCATTGCTGC-3´

3.25 Extraction of C7081-pPIC9K and C158-pPIC9K via Midi-Prep

For the constructs C7081-pPIC9K and C158-pPIC9K, a large-scale inoculation was performed, followed by plasmid extraction using the Midi-prep procedure, as described in section 3.20 in order to transform the extracted DNA into suitable expression cells.

3.26 Linearization of C7081-pPIC9K and C158-pPIC9K constructs and transformation

The obtained plasmid DNA was subsequently linearized using the enzyme SacI to prepare it for transformation into *Pichia pastoris* GS115 (Invitrogen) yeast cells via electroporation. For both constructs, the linearization process was carried out as follows: a digestion mixture was prepared containing 50 µg of plasmid DNA, 2 µL of SacI enzyme, and 10 µL of Buffer 1 (10X). The digestion reaction was incubated at 37°C for 8 hours. The success of the linearization was assessed after incubation by electrophoresis on a 0.8% agarose gel. Subsequently, transformation into *P. pastoris* (strain GS115) was performed by electroporating 10 µg of linearized plasmid under the following conditions:

- 200 Ω
- 1.5 kV
- 25 µF

The transformed colonies were screened both by an additional colony PCR, using primers specific for the insert and the vector (α-factor and AOX1), and through a screening with geneticin (G418) at various concentrations (0.5, 1, 1.5, 2, 4 mg/mL). The sequences of the primers used are listed in Table 3.4 below:

Table 3.4. Sequences of the primers used for screening colonies after transformation in *Pichia pastoris* GS115.

Primer Name	Sequence (5' -> 3')
5' AOX1	5'-GACTGGTTCCAATTGACAAGC-3'
3' AOX1	5'-GCAAATGGCATTCTGACATCC-3'
α-factor	5'-TACTATTGCCAGCATTGCTGC-3'

3.27 Statistical Analysis

All experiments were performed in triplicates (three independent biological replicates) and results were expressed as means ± standard error. Data were analysed via GraphPad Prism 6.0 software (GraphPad Software, Inc., La Jolla, CA, USA) using one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test.

4. Results

4.1 Evaluation of Samples Concentration

The concentration of the samples obtained following precipitation with organic solvents was evaluated via the Bradford assay. The values obtained are shown in the following table:

Table 4.1. Concentrations of the samples obtained via precipitation with organic solvents from hemolymph extracted from uninfected larvae or larvae infected with *E. coli* or *M. flavus*. Data are expressed as mean ± standard errors of three independent biological replicates.

	Uninfected Larvae	Larvae Infected with *E. coli*	Larvae Infected with *M. flavus*
Precipitation with organic solvents	1.05 ± 3 µg/ µL	1.33 ± 2 µg/ µL	2.19 ± 2 µg/µL

4.2 Evaluation of the Antibacterial Activity of Peptide Fraction of the Hemolymph via Bioautography (SDS Gel Overlay Method) Assay

An electrophoretic analysis of the infected and uninfected samples, treated with methanol, acetic acid, and water in a 90:1:9 ratio v/v was performed. Three identical gels (12% acrylamide) were prepared. At the end of the electrophoretic run, one of the gels was stained with Coomassie Blue, while on the other gels, a bioautography test against *E. coli* and *M. flavus* was performed. Results in Figure 3.1a and Figure 4.2a show the presence of low molecular weight bands, around 10 kDa. Figure 4.1b and Figure 4.2b show an inhibition zone in correspondence with low molecular weight bands relative to the peptide fraction obtained following precipitation of the hemolymph extracted from all samples and tested against *E. coli* and *M. flavus*, respectively. Figure 4.1c and Figure 4.2c show the overlay between the gel and the inhibition zone observed on bioautography, to confirm that the obtained inhibition comes from peptides around 10 kDa.

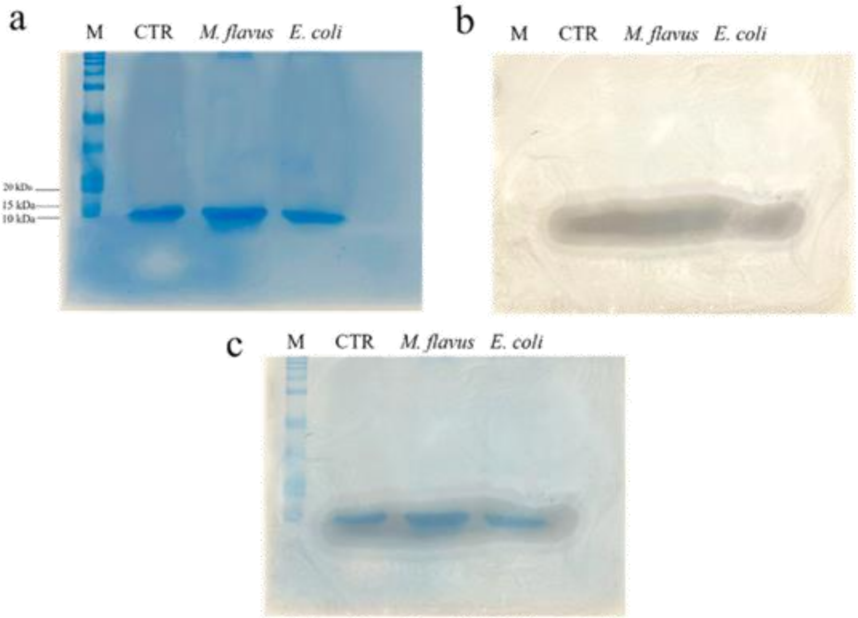

Figure 4.1. SDS-PAGE (**a**) and bioautography (**b**) performed against *E. coli* of the samples obtained following precipitation with organic solvents. In (**c**), an overlay of the previous images is presented. M = marker, “All Blue Standards Biorad” (Biorad, Hercules, CA, USA). CTR = peptide fraction from uninfected larvae; *M. flavus* = peptide fraction from larvae infected with *M. flavus*; *E. coli* = hemolymph from larvae infected with *E. coli*. The experiments were carried out in triplicate (three independent biological replicates).

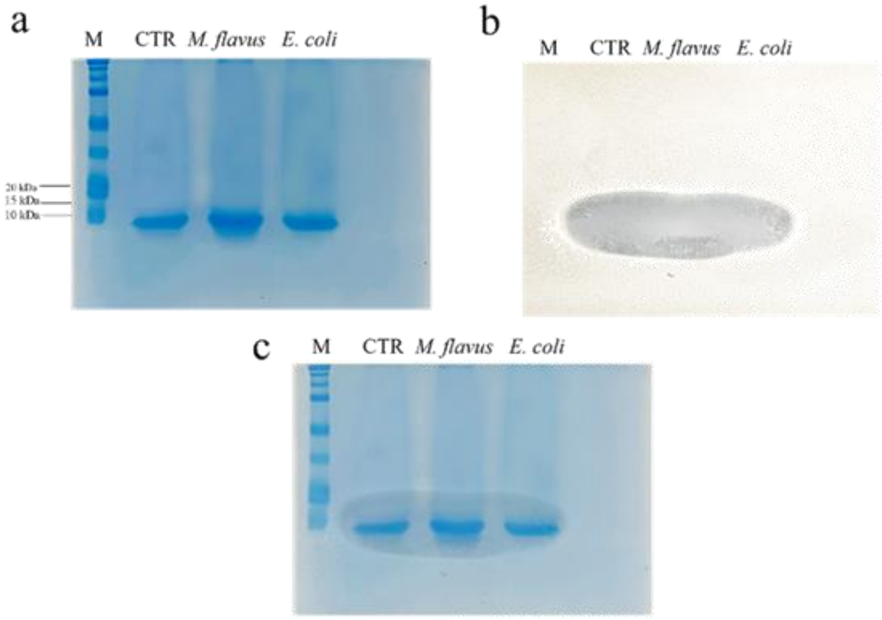

Figure 4.2. SDS-PAGE (**a**) and bioautography (b) performed against *M. flavus* of the samples obtained following precipitation with organic solvents. In (**c**), an overlay of the previous images is presented. M = marker, “All Blue Standards

Biorad" (Biorad, Hercules, CA, USA). CTR = hemolymph from uninfected larvae; *M. flavus* = hemolymph from larvae infected with *M. flavus*; *E. coli* = hemolymph from larvae infected with *E. coli*. The experiments were carried out in triplicate (three independent biological replicates).

4.3 Evaluation of the Antibacterial Activity of Peptide Fraction against *E. coli*

The antimicrobial activity of the peptide fraction from the hemolymph of *H. illucens* larvae was evaluated against 15 bacterial strains, including 6 that are antibiotic-resistant. Additionally, its antifungal properties were tested against a fungal strain following precipitation with organic solvents. For each bacterial strain, antimicrobial efficacy was measured using three methods: the agar diffusion assay, minimum inhibitory concentration (MIC) assay, and minimum bactericidal concentration (MBC) assay.

In particular, the effectiveness of the peptide fraction against *E. coli* was investigated. This Gram-negative bacterium, part of the Enterobacteriaceae family, is commonly found in the intestinal tract of warm-blooded animals, including humans. While generally beneficial, certain pathogenic strains of *E. coli* can cause infections such as urinary tract infections, gastroenteritis, sepsis, and neonatal meningitis (Gould, 2010).

4.3.1 Evaluation of the Antibacterial Activity of Peptide Fraction via Agar Diffusion Assay

Peptide fractions, obtained from the hemolymph of uninfected larvae and larvae infected with *E. coli* and *M. flavus*, were precipitated using a methanol/acetic acid/water solution (90:1:9 v/v ratio). These fractions were then subjected to the agar diffusion test to assess their antibacterial activity against *E. coli*.

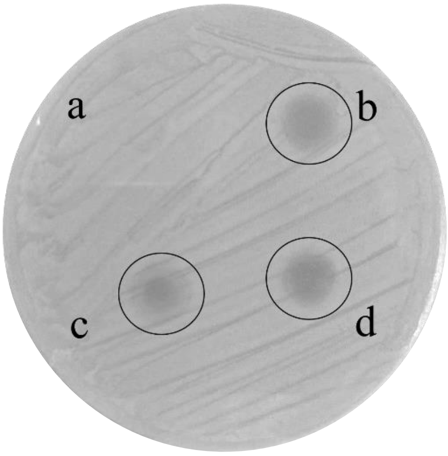

Figure 4.3. Agar diffusion assay of peptide fractions obtained via precipitation with organic solvents, performed against *E. coli*. (**a**) H_2O, negative control; (**b**) peptide fraction from larvae infected with *E. coli*; (**c**) peptide fraction from uninfected larvae; (**d**) peptide fraction from larvae infected with *M. flavus*. The experiments were carried out in triplicate (three independent biological replicates).

The results showed an inhibition zone surrounding all the samples tested against *E. coli*, confirming that the peptide fractions demonstrated antimicrobial activity (Figure 4.3).

4.3.2 Evaluation of the Peptide Fraction Antibacterial Activity via Microdilution Assay

Based on the qualitative results from the agar diffusion assay, a MIC assay was developed to determine the minimum inhibitory concentration of the peptide fraction extracted from the hemolymph of *H. illucens* larvae against *E. coli*.

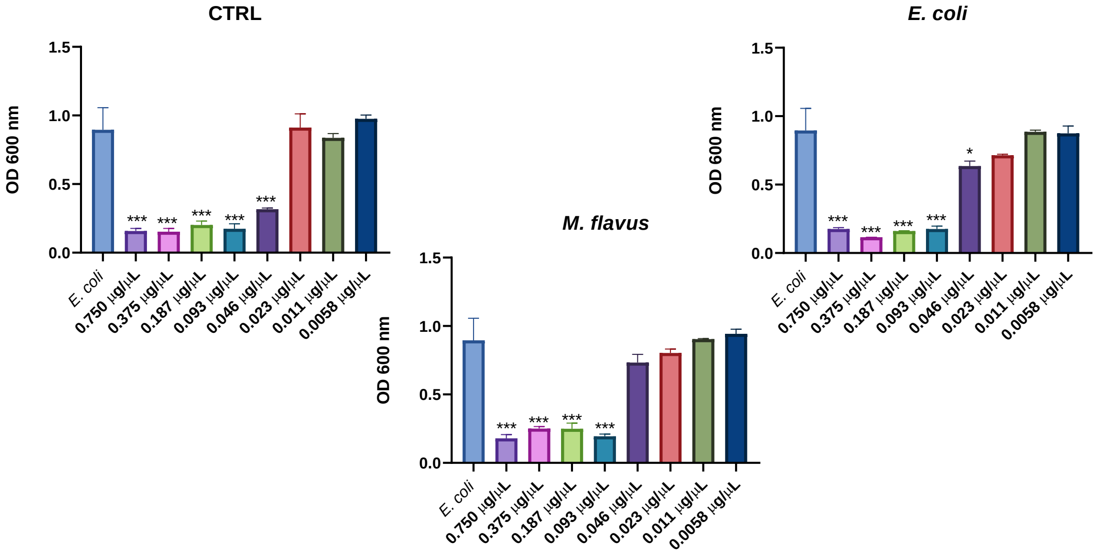

Figure 4.4. Microdilution assay against *E. coli* performed with the peptide fractions obtained via precipitation with organic solvents. CTRL = peptide fractions from uninfected larvae; *E. coli* = peptide fractions from larvae infected with *E. coli*; *M. flavus* = peptide fractions from larvae infected with *M. flavus*. The first bars represent the untreated *E. coli* cell culture. Data are expressed as means ± standard error of three independent biological replicates, and comparisons between treatments and control were performed by one-way ANOVA followed by Bonferroni post hoc test (* $p < 0.1$, ** $p<0.001$*** $p < 0.0001$).

The results demonstrated a reduction in bacterial growth across all samples. Specifically, the MIC value for the peptide fraction from both control larvae and those infected with *E. coli* was 0.046 µg/µL, while the fraction from larvae infected with *M. flavus* showed a higher MIC value of 0.093 µg/µL (Figure 4.4).

4.3.3 Evaluation of the Antibacterial Activity of Peptide Fraction via Minimum Bactericidal Concentration Assay

Following the results of the MIC assays, an MBC assay was performed to evaluate the peptide fraction from *H. illucens* larvae. The aim was to determine which concentrations inhibited bacterial growth and exhibited bactericidal activity.

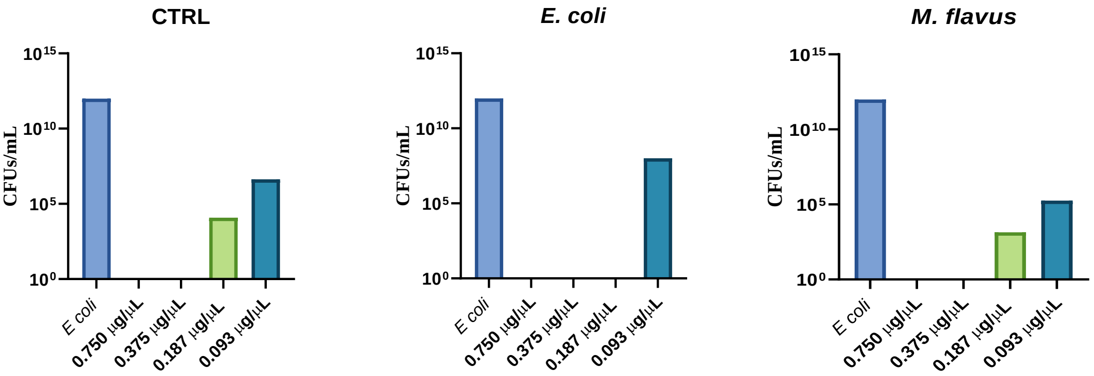

Figure 4.5. Minimum Bactericidal Concentration assay against *E. coli* performed with the peptide fractions obtained via precipitation with organic solvents. CTRL = peptide fractions from uninfected larvae; *E. coli* = peptide fractions from larvae infected with *E. coli*; *M. flavus* = peptide fractions from larvae infected with *M. flavus*. The first bars represent the untreated *E. coli* cell culture.

The results showed that all samples tested displayed bactericidal activity. Specifically, the MBC for the peptide fraction from the hemolymph of control larvae and those infected with *M. flavus* was 0.375 µg/µL, while the fraction from larvae infected with *E. coli* had an MBC of 0.187 µg/µL (Figure 4.5).

4.4 Evaluation of the Antibacterial Activity of Peptide Fraction against *M. flavus*

The antimicrobial properties of a peptide fraction, obtained by precipitating the hemolymph of *H. illucens* larvae using organic solvents, were assessed to evaluate its effectiveness against *M. flavus*. This Gram-positive bacterium, belonging to the Micrococcaceae family, is commonly found in the environment, including soil, water, air, and human skin. While generally harmless, *M. flavus* can act as an opportunistic pathogen, particularly in immunocompromised individuals. Infections associated with *M. flavus* are relatively uncommon but can include skin and wound infections and in some cases, bacteremia. It has also been implicated in nosocomial infections, often linked to contaminated surfaces or medical equipment in hospital settings (X.-Y. Liu et al., 2007).

4.4.1 Evaluation of the Antibacterial Activity of Peptide Fraction via Agar Diffusion Assay

Peptide fractions were analyzed for their antibacterial activity against *M. flavus* using an agar diffusion assay. These fractions were obtained from the hemolymph of both uninfected larvae and larvae infected with *E. coli* or *M. flavus*, precipitated using a methanol/acetic acid/water solution (90:1:9 v/v).

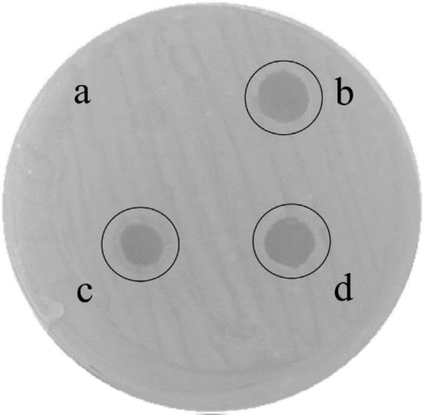

Figure 4.6. Agar diffusion assay of peptide fractions obtained via precipitation with organic solvents, performed against *M. flavus*. (**a**) H_2O, negative control; (**b**) peptide fraction from larvae infected with *E. coli*; (**c**) peptide fraction from uninfected larvae; (**d**) peptide fraction from larvae infected with *M. flavus*. The experiments were carried out in triplicate (three independent biological replicates).

The assay results of the assay indicated the presence of clear inhibition zones around all the samples tested, providing evidence that the peptide fractions exhibited significant antimicrobial activity against *M. flavus* (Figure 4.6).

4.4.2 Evaluation of the Peptide Fraction Antibacterial Activity via Microdilution Assay

The results of the agar diffusion assay demonstrated inhibition zones for all the samples analysed. Therefore, MIC assays were carried out to determine the minimum inhibitory concentration of the peptide fractions obtained from the hemolymph of *H. illucens* larvae, precipitated with organic solvents, against *M. flavus*.

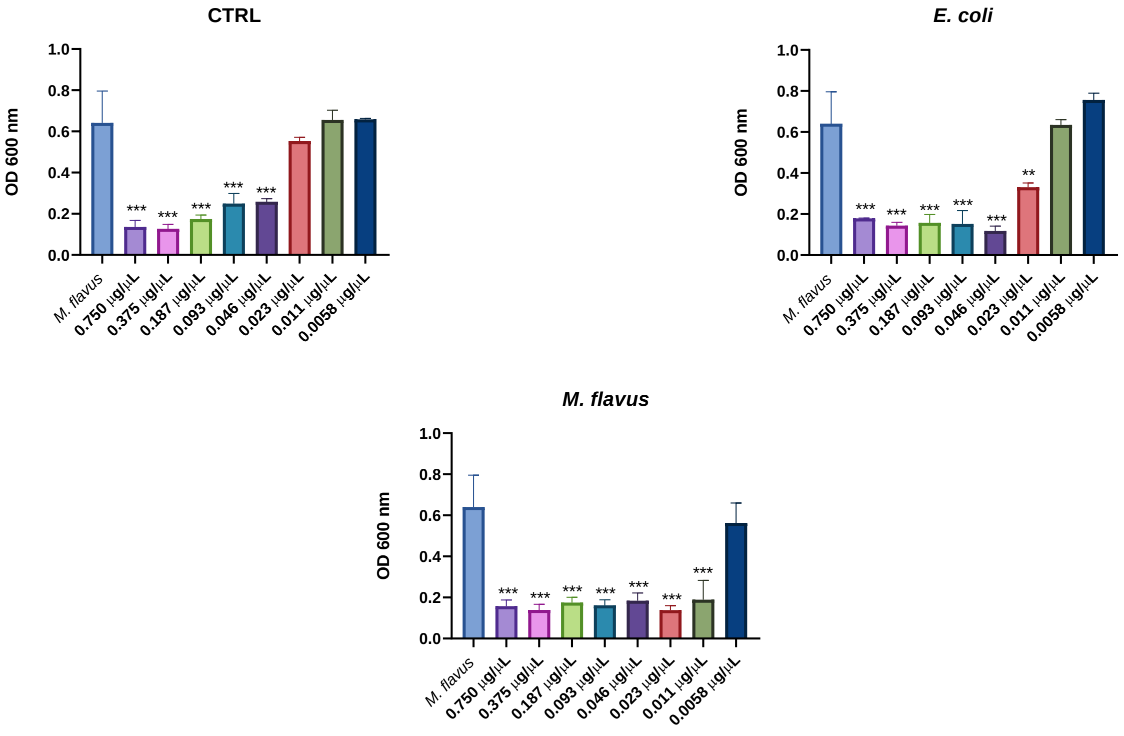

Figure 4.7. Microdilution assay against *M. flavus* performed with the peptide fractions obtained via precipitation with organic solvents. CTRL = peptide fractions from uninfected larvae; *E. coli* = peptide fractions from larvae infected with *E. coli*; *M. flavus* = peptide fractions from larvae infected with *M. flavus*. The first bars represent the untreated *M. flavus* cell culture. Data are expressed as means ± standard error of three independent biological replicates, and comparisons between treatments and control were performed by one-way ANOVA followed by Bonferroni post hoc test (* $p < 0.1$, ** $p<0.001$***$p < 0.0001$).

The results clearly demonstrate that all peptide fractions tested exhibit significant antimicrobial activity (Figure 4.7). Specifically, the MIC for the peptide fraction from the hemolymph of control larvae was determined to be 0.046 µg/µL. In contrast, the MIC value for the peptide fraction extracted from the hemolymph of larvae infected with *E. coli* was 0.023 µg/µL, while the MIC for the fraction from larvae infected with *M. flavus* was the lowest, at 0.011 µg/µL.

4.4.3 Evaluation of the Antibacterial Activity of Peptide Fraction via Minimum bactericidal Concentration Assay

An MBC assay was performed against *M. flavus* using the peptide fraction precipitated with organic solvents from the hemolymph extracted from *H. illucens* larvae to determine the minimum bactericidal concentration.

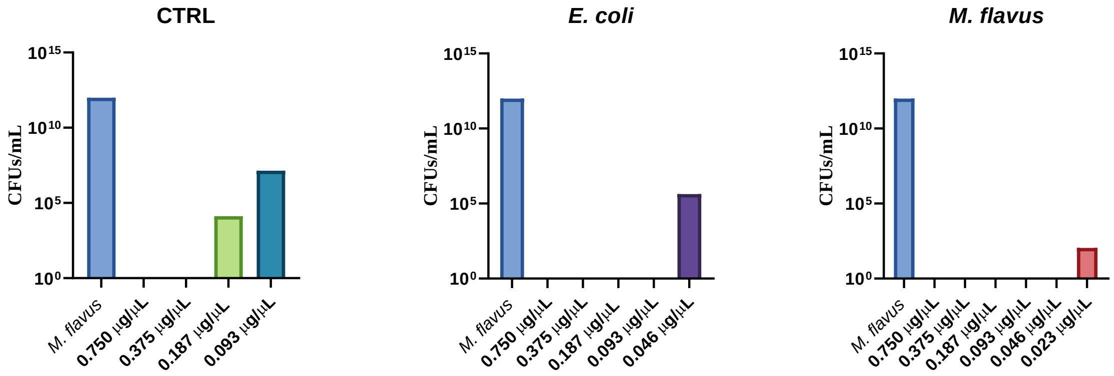

Figure 4.8. Minimum Bactericidal Concentration against *M. flavus* performed with the peptide fractions obtained via precipitation with organic solvents. CTRL = peptide fractions from uninfected larvae; *E. coli* = peptide fractions from larvae infected with *E. coli*; *M. flavus* = peptide fractions from larvae infected with *M. flavus*. The first bars represent the untreated *E. coli* cell culture.

The results indicate that all samples exhibited bactericidal activity (Figure 4.8). In particular, the MBC for the peptide fraction derived from the hemolymph of control larvae was 0.375 µg/µL. The fraction from larvae infected with *E. coli* demonstrated a notably lower MBC value of 0.093 µg/µL. The most pronounced bactericidal effect, with an MBC of 0.046 µg/µL, was observed for the peptide fraction obtained from larvae infected with *M. flavus*, indicating its higher antimicrobial potency.

4.5. Evaluation of the Antibacterial Activity of Peptide Fraction against EIEC

Based on the promising results obtained against *E. coli* and *M. flavus*, the antimicrobial activity of the peptide fraction extracted from *H. illucens* larvae was tested against various pathogenic bacterial strains. This antimicrobial analysis was conducted in collaboration with the University of Campania "Luigi Vanvitelli" and the University of Novi Sad in Serbia. Initially, the antimicrobial activity was evaluated against *Enteroinvasive Escherichia coli* (EIEC), a Gram-negative bacterium from the Enterobacteriaceae family. EIEC is characterized by its ability to invade and replicate within the epithelial cells of the colon, causing infections that resemble bacillary dysentery. Common infections associated with EIEC include watery diarrhea, abdominal cramps, fever, and in more severe cases, dysentery with bloody stools and pronounced intestinal inflammation (van Den Beld et al., 2019).

4.5.1 Evaluation of the Antibacterial Activity of Peptide Fraction via Agar Diffusion Assay

The peptide fractions obtained from the hemolymph of both uninfected larvae and larvae infected with *E. coli* or *M. flavus*, precipitated using a methanol/acetic acid/water solution (90:1:9 v/v), were first evaluated for their antibacterial activity against *Enteroinvasive Escherichia coli* (EIEC) through an agar diffusion assay.

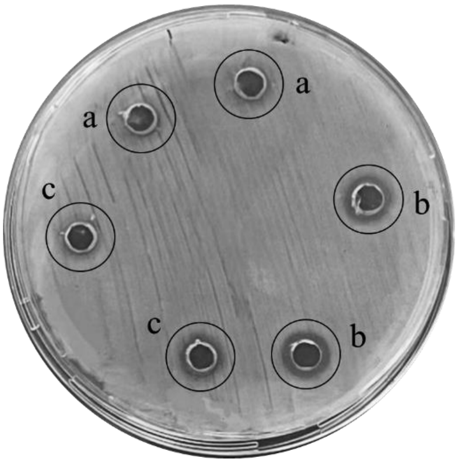

Figure 4.9. Agar diffusion assay of peptide fractions obtained via precipitation with organic solvents, performed against EIEC. (**a**) peptide fraction from uninfected larvae; (**b**) peptide fraction from larvae infected with *E. coli*; (**d**) peptide fraction from larvae infected with *M. flavus*. The experiments were carried out in triplicate (three independent biological replicates).

The results revealed clear inhibition zones around all the tested samples, including those obtained from control larvae and larvae infected with *E. coli* and *M. flavus*. Notably, the inhibition zones surrounding the peptide fractions from larvae infected with *E. coli* were significantly larger, suggesting enhanced antimicrobial efficacy in these fractions compared to those from uninfected larvae or larvae infected with *M. flavus* (Figure 4.9).

4.5.2 Evaluation of the Peptide Fraction Antibacterial Activity via Microdilution Assay

The positive outcomes of the agar diffusion assay led to the execution of a MIC assay to evaluate the peptide fraction obtained from *H. illucens* larvae, precipitated using organic solvents. This assay aimed to determine the minimum inhibitory concentration of the peptide fraction when tested against EIEC.

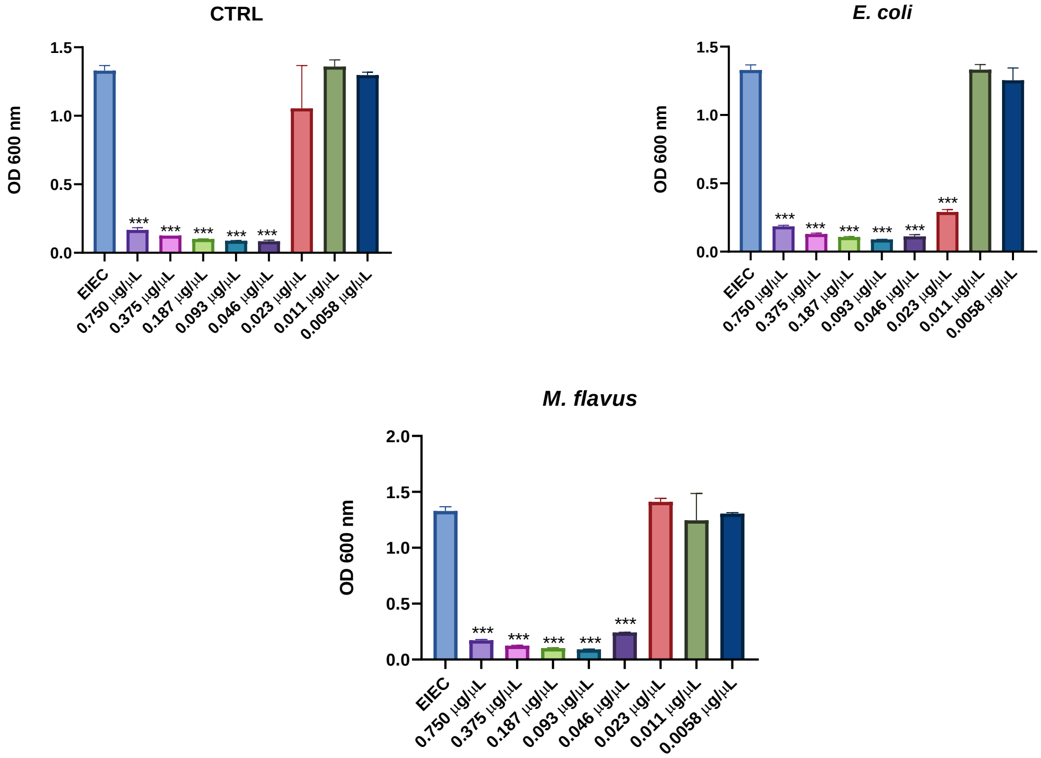

Figure 4.10. Microdilution assay against EIEC performed with the peptide fractions obtained via precipitation with organic solvents. CTRL = peptide fractions from uninfected larvae; *E. coli* = peptide fractions from larvae infected with *E. coli*; *M. flavus* = peptide fractions from larvae infected with *M. flavus*. The first bars represent the untreated EIEC cell culture. Data are expressed as means ± standard error of three independent biological replicates, and comparisons between treatments and control were performed by one-way ANOVA followed by Bonferroni post hoc test (* $p < 0.1$, ** $p<0.001$*** $p < 0.0001$).

The results of the microdilution assay (Figure 4.10) confirmed antimicrobial activity in all three conditions analyzed. Specifically, the MIC value for the peptide fraction obtained from the hemolymph of control larvae and those infected with *M. flavus* was 0.046 µg/µL, whereas the MIC for the peptide fraction derived from larvae infected with *E. coli* was lower, at 0.023 µg/µL, indicating greater potency in this sample.

4.5.3 Evaluation of the Antibacterial Activity of Peptide Fraction via Minimum Bactericidal Concentration Assay

An MBC assay was implemented to identify the concentrations of the peptide fraction derived from the hemolymph of *H. illucens* larvae that, in addition to reducing bacterial growth, also exhibited bactericidal activity. This assay determined the minimum concentration of the peptide fractions necessary to exert a bactericidal effect against EIEC, thus providing a more comprehensive evaluation of their antimicrobial efficacy.

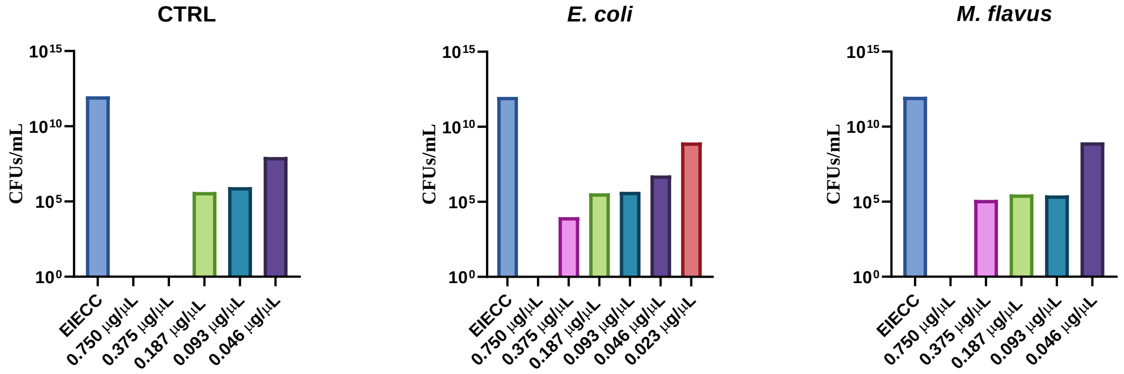

Figure 4.11. Minimum Bactericidal Concentration assay against EIEC performed with the peptide fractions obtained via precipitation with organic solvents. CTRL = peptide fractions from uninfected larvae; *E. coli* = peptide fractions from larvae infected with *E. coli*; *M. flavus* = peptide fractions from larvae infected with *M. flavus*. The first bars represent the untreated EIEC cell culture.

The results of the assay (Figure 4.11) show that bactericidal activity was observed in all the samples, though at different concentrations. Specifically, the MBC value for the peptide fraction obtained from control larvae was 0.375 µg/µL. In comparison, the MBC value for the peptide fractions derived from the hemolymph of larvae infected with *E. coli* and *M. flavus* was higher, measured at 0.750 µg/µL.

4.6 Evaluation of the Antibacterial Activity of Peptide Fraction against *P. aeruginosa*

The antimicrobial activity of the peptide fraction extracted from the hemolymph of *H. illucens* larvae was also tested against *P. aeruginosa*. This bacterium, belonging to the family Pseudomonadaceae and part of the ESKAPE pathogens group, is known to cause severe infections, particularly in immunocompromised individuals. Major infections associated with *P. aeruginosa* include pulmonary infections, such as ventilator-associated pneumonia, urinary tract infections, skin and soft tissue infections, as well as bacteremia and sepsis (Moore & Flaws, 2011).

4.6.1 Evaluation of the Antibacterial Activity of Peptide Fraction via Agar Diffusion Assay

The peptide fractions extracted from plasma, precipitated using a methanol/acetic acid/water mixture (90:1:9 v/v), from both uninfected larvae and those infected with *E. coli* or *M. flavus*, were first evaluated using an agar diffusion assay to determine their antibacterial activity against *P. aeruginosa.*

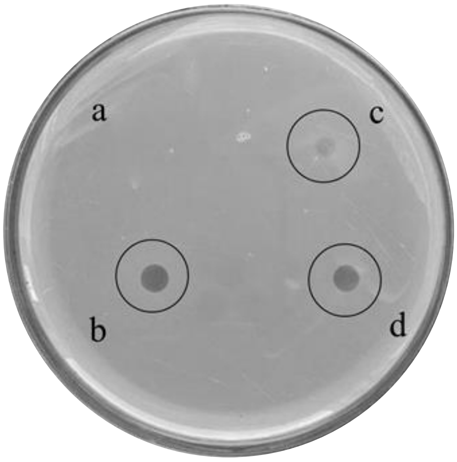

Figure 4.12. Agar diffusion assay of peptide fractions obtained via precipitation with organic solvents, performed against *P. aeruginosa*. (**a**) H_2O, negative control; (**b**) peptide fraction from larvae infected with *E. coli*; (**c**) peptide fraction from uninfected larvae; (**d**) peptide fraction from larvae infected with *M. flavus*. The experiments were carried out in triplicate (three independent biological replicates).

The results of the agar diffusion assay (Figure 4.12) indicate that all tested peptide fractions exhibited antibacterial activity against *P. aeruginosa*. However, there were significant differences in the intensity of the inhibition zones among the various samples. The peptide fraction extracted from uninfected larvae produced a visible inhibition zone, but it was less pronounced compared to the fractions obtained from infected larvae. In contrast, the peptide fractions derived from larvae infected with *E. coli* and *M. flavus* displayed larger and more intense inhibition zones, suggesting a higher antibacterial efficacy.

4.6.2 Evaluation of the Peptide Fraction Antibacterial Activity via Microdilution Assay

Based on the results obtained from the agar diffusion assay, the MIC assay was subsequently performed on the peptide fraction extracted from the hemolymph of *H. illucens* larvae. The purpose of this assay was to determine the minimum inhibitory concentration required to inhibit the growth of *P. aeruginosa*.

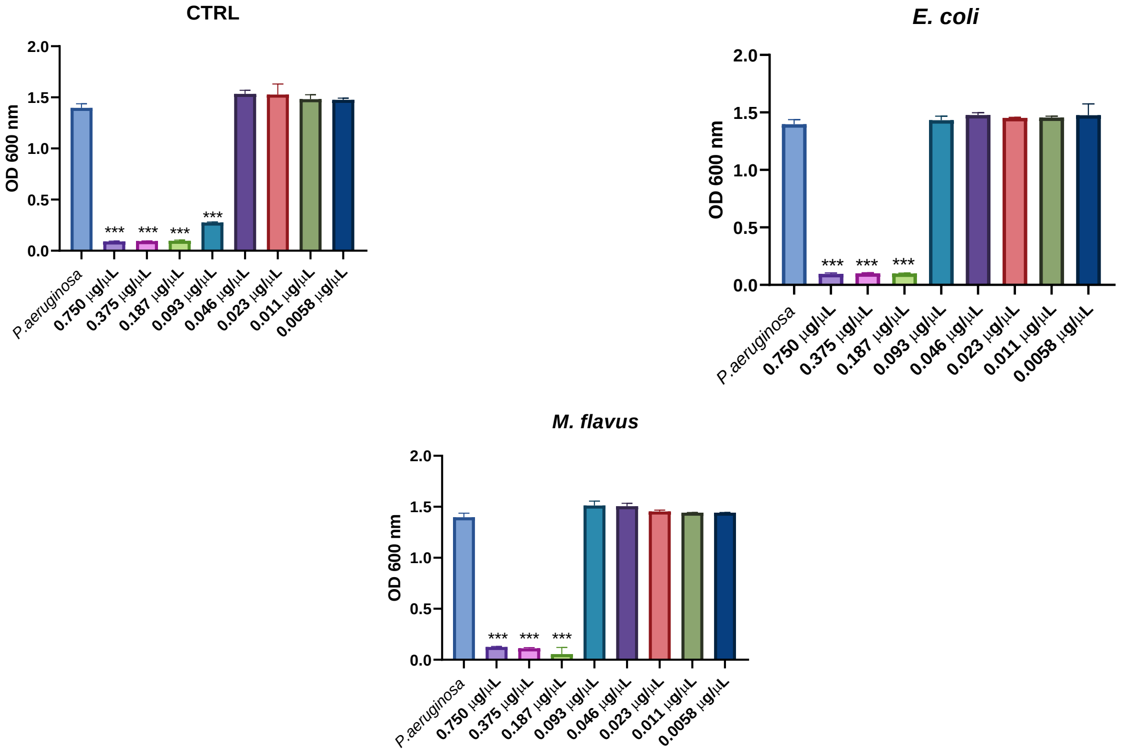

Figure 4.13. Microdilution assay against *P. aeruginosa* performed with the peptide fractions obtained via precipitation with organic solvents. CTRL = peptide fractions from uninfected larvae; *E. coli* = peptide fractions from larvae infected with *E. coli*; *M. flavus* = peptide fractions from larvae infected with *M. flavus*. The first bars represent the untreated *P. aeruginosa* cell culture. Data are expressed as means ± standard error of three independent biological replicates, and comparisons between treatments and control were performed by one-way ANOVA followed by Bonferroni post hoc test (* $p < 0.1$, ** $p<0.001$*** $p < 0.0001$).

All samples analyzed were able to inhibit the growth of *P. aeruginosa*, though with varying MIC values. The control sample, derived from the hemolymph of uninfected larvae, exhibited a MIC of 0.093 µg/µL. In contrast, the peptide fractions obtained from larvae infected with *E. coli* and *M. flavus* displayed a higher MIC value of 0.187 µg/µL (Figure 4.13).

4.6.3 Evaluation of the Antibacterial Activity of Peptide Fraction via Minimum Bactericidal Concentration Assay

Based on the results obtained from the MIC assay, the MBC assay was performed to determine the minimum bactericidal concentration of the peptide fractions extracted from the hemolymph of *H. illucens* larvae that had induced a reduction in bacterial growth. This assay was specifically conducted to assess the effect on *P. aeruginosa*, in order to identify the concentration required to achieve effective bactericidal activity.

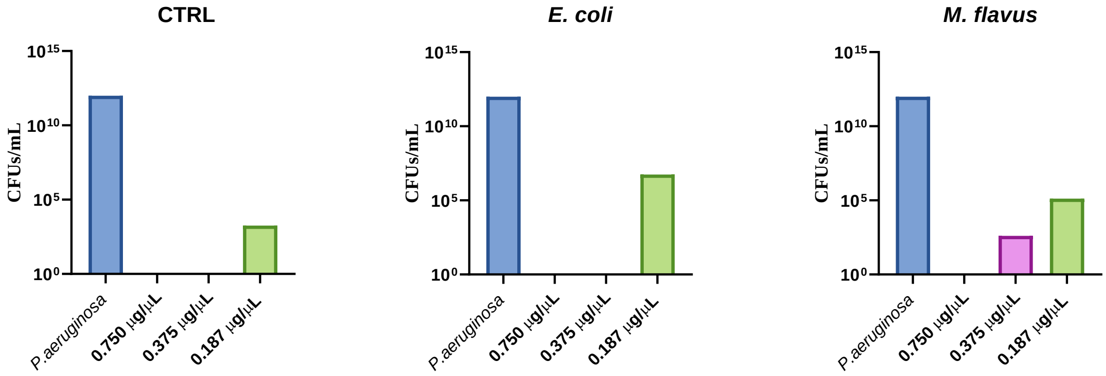

Figure 4.14. Minimum Bactericidal Concentration against *P. aeruginosa* performed with the peptide fractions obtained via precipitation with organic solvents. CTRL = peptide fractions from uninfected larvae; *E. coli* = peptide fractions from larvae infected with *E. coli*; *M. flavus* = peptide fractions from larvae infected with *M. flavus*. The first bars represent the untreated *P. aeruginosa* cell culture.

The MBC value for the peptide fraction obtained from the hemolymph of control larvae and infected with *E. coli* was 0.375 µg/µL. Meanwhile, the peptide fraction extracted from larvae infected with *M. flavus* exhibited a higher MBC value of 0.750 µg/µL (Figure 4.14). These results indicate differences in the bactericidal efficacy of the peptide fractions depending on the infection status of the larvae, with the peptide fraction from *M. flavus*-infected larvae requiring a higher concentration to achieve effective bactericidal activity against *P. aeruginosa*.

4.7 Evaluation of the Antibacterial Activity of Peptide Fraction against *S. aureus*

The antimicrobial activity of peptide fraction was tested against *S. aureus*, a Gram-positive bacterium belonging to the Staphylococcaceae family and the ESKAPE group of pathogens, similar to *P. aeruginosa*. *S. aureus* can cause various infections, ranging from common skin infections such as boils and impetigo to more severe conditions like pneumonia, bacteremia, osteomyelitis, and endocarditis. In some cases, it produces toxins that can lead to toxic shock syndrome or scalded skin syndrome (Lima et al., 2015).

4.7.1 Evaluation of the Antibacterial Activity of Peptide Fraction via Agar Diffusion Assay

The peptide fractions extracted from the hemolymph of *H. illucens* larvae, both uninfected and infected with *E. coli* or *M. flavus*, precipitated using a methanol/acetic acid/water solution (90:1:9 v/v), were evaluated for their antibacterial activity against *Staphylococcus aureus* using an agar diffusion assay.

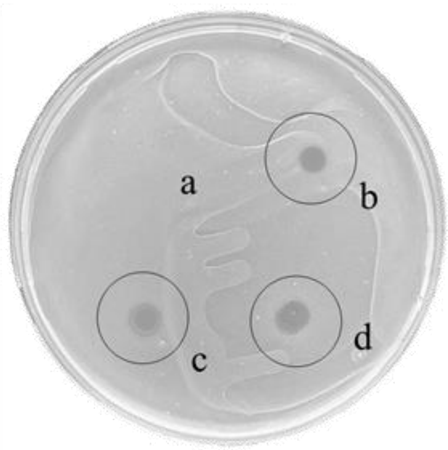

Figure 4.15. Agar diffusion assay of peptide fractions obtained via precipitation with organic solvents, performed against *S. aureus*. (**a**) H_2O, negative control; (**b**) peptide fraction from larvae infected with *E. coli*; (**c**) peptide fraction from uninfected larvae; (**d**) peptide fraction from larvae infected with *M. flavus*. The experiments were carried out in triplicate (three independent biological replicates).

The results showed clear inhibition zones for all tested peptide fractions, indicating antibacterial activity. Specifically, inhibition zones were observed for the fractions derived from uninfected larvae and those obtained from larvae infected with *E. coli* and *M. flavus* (Figure 4.15), suggesting that all tested samples effectively inhibited the growth of *S. aureus*.

4.7.2 Evaluation of the Peptide Fraction Antibacterial Activity via Microdilution Assay

Microdilution assay was performed to determine the minimum inhibitory concentration of the peptide fractions obtained through organic solvent precipitation from *H. illucens* larvae when tested against *S. aureus*.

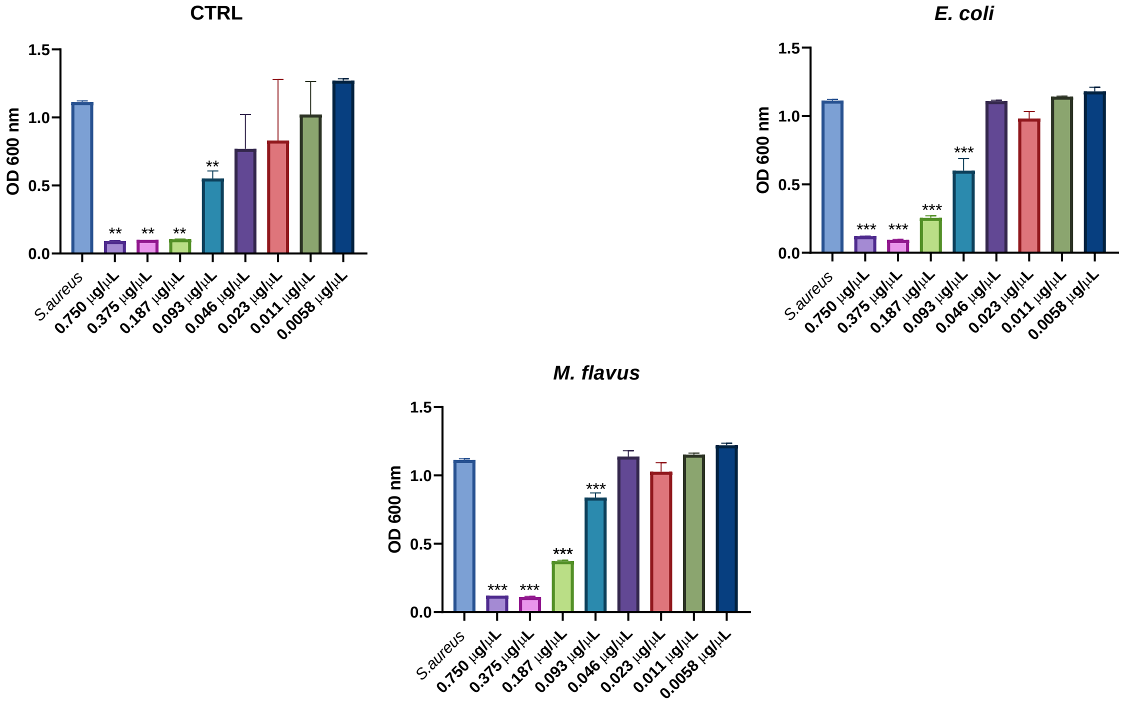

Figure 4.16. Microdilution assay against *S. aureus* performed with the peptide fractions obtained via precipitation with organic solvents. CTRL = peptide fractions from uninfected larvae; *E. coli* = peptide fractions from larvae infected with *E. coli*; *M. flavus* = peptide fractions from larvae infected with *M. flavus*. The first bars represent the untreated *S. aureus* cell culture. Data are expressed as means ± standard error of three independent biological replicates, and comparisons between treatments and control were performed by one-way ANOVA followed by Bonferroni post hoc test (* $p < 0.1$, ** $p<0.001$*** $p < 0.0001$).

All three samples analysed inhibited the growth of *S. aureus*. The MIC value for the peptide fraction derived from the hemolymph of both control larvae and larvae infected with *E. coli* or *M. flavus* was consistently 0.093 µg/µL (Figure 4.16). These findings indicate that all tested fractions exhibited comparable antibacterial efficacy against *S. aureus*, regardless of the larvae's infection status.

4.7.3 Evaluation of the Antibacterial Activity of Peptide Fraction via Minimum Bactericidal Concentration Assay

MBC assays were performed to identify the minimum bactericidal concentration for the peptide fractions obtained from the hemolymph of *H. illucens* larvae that exhibited a reduction in bacterial growth. These assays specifically aimed to evaluate the bactericidal effect on *S. aureus*.

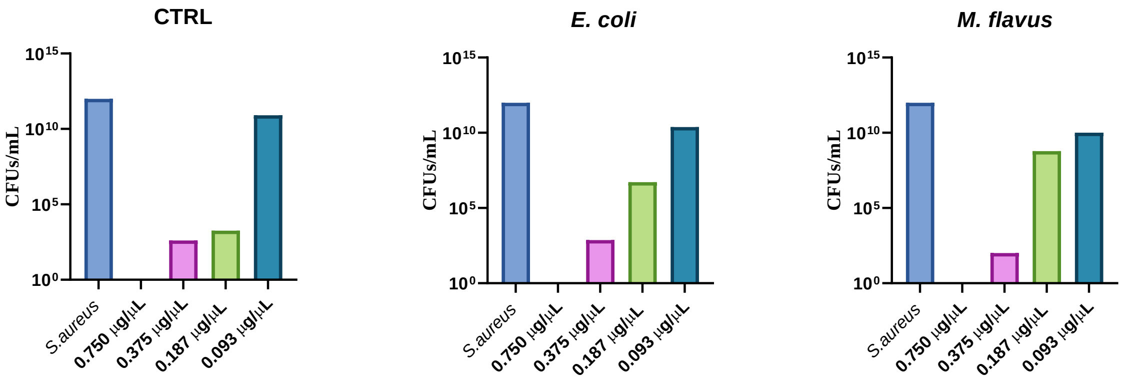

Figure 4.17. Minimum Bactericidal Concentration assay against *S. aureus* performed with the peptide fractions obtained via precipitation with organic solvents. CTRL = peptide fractions from uninfected larvae; *E. coli* = peptide fractions from larvae infected with *E. coli*; *M. flavus* = peptide fractions from larvae infected with *M. flavus*. The first bars represent the untreated *S. aureus* cell culture.

The results showed that all three samples effectively inhibited the growth of *S. aureus*. The MBC value remained consistent across all samples, with an MBC of 0.750 µg/µL observed for the peptide fraction obtained from the hemolymph of control larvae, as well as for those obtained from larvae infected with *E. coli* and *M. flavus* (Figure 4.17). These findings suggest that the peptide fractions, regardless of the infection status of the larvae, exhibit a uniform bactericidal activity against *S. aureus*.

4.8 Evaluation of the Antibacterial Activity of Peptide Fraction against *S. Typhimurium*

Antimicrobial assays were performed on the peptide fractions extracted from *H. illucens* larvae against *Salmonella enterica* subsp. *enterica* serovar *Typhimurium*, a Gram-negative bacterium belonging to the Enterobacteriaceae family. *S. Typhimurium* is responsible for approximately 50% of gastrointestinal infections and is a major cause of foodborne illness in industrialized countries (Galán, 2021).

4.8.1 Evaluation of the Antibacterial Activity of Peptide Fraction via Agar Diffusion Assay

The peptide fractions obtained from the hemolymph of *H. illucens* larvae, precipitated using a methanol/acetic acid/water solution (90:1:9 v/v), from both uninfected larvae and larvae infected with *E. coli* or *M. flavus*, were initially evaluated for their antibacterial activity against *S. Typhimurium* using an agar diffusion assay.

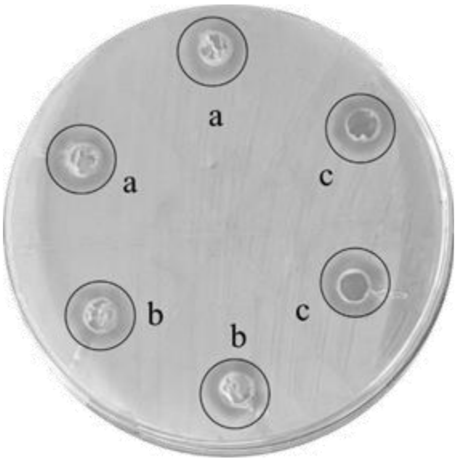

Figure 4.18. Agar diffusion test of peptide fractions obtained via precipitation with organic solvents, performed against *S. Typhimurium* (**a**) peptide fraction from uninfected larvae; (**b**) peptide fraction from larvae infected with *E. coli*; (**c**) peptide fraction from larvae in fected with *M. flavus*. The experiments were carried out in triplicate (three independent biological replicates).

The results of the agar diffusion assay demonstrated the presence of inhibition zones for all tested peptide fractions, indicating antibacterial activity against *S. Typhimurium*. Specifically, inhibition zones were observed for the peptide fraction obtained from the hemolymph of uninfected larvae and for the fractions derived from larvae infected with *E. coli* and *M. flavus* (Figure 4.18). These findings suggest that all peptide fractions, regardless of the larvae's infection status, exhibited an inhibitory effect on the growth of *S. Typhimurium.*

4.8.2 Evaluation of the Peptide Fraction Antibacterial Activity via Microdilution Assay

Based on the results obtained from the agar diffusion assay, a microdilution assay was performed to determine the minimum inhibitory concentration of the peptide fraction extracted from the hemolymph of *H. illucens* larvae, precipitated using organic solvents, against *S. Typhimurium*. This assay aimed to quantitatively assess the antimicrobial potency of the peptide fraction and identify the lowest concentration required to inhibit the growth of *S. Typhimurium*.

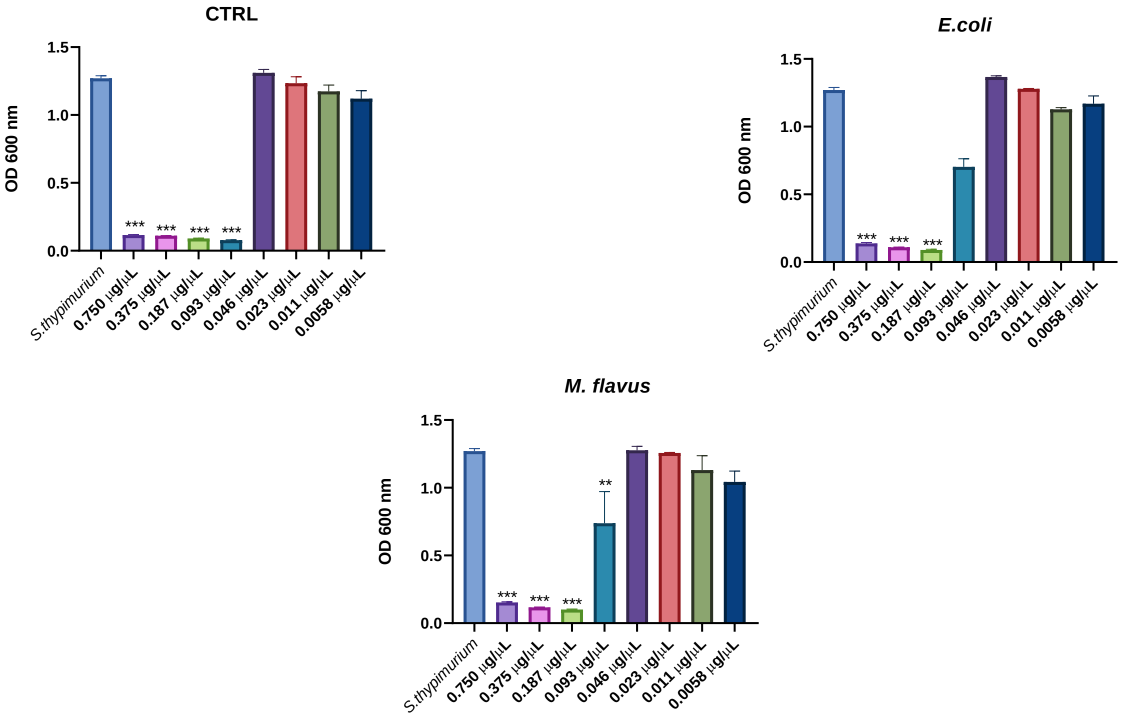

Figure 4.19. Microdilution assay against *S. Typhimurium* performed with the peptide fractions obtained via precipitation with organic solvents. CTRL = peptide fractions from uninfected larvae; *E, coli* = peptide fractions from larvae infected with *E. coli*; *M. flavus* = peptide fractions from larvae infected with *M. flavus*. The first bars represent the untreated *S. typhimurium* cell culture. Data are expressed as means ± standard error of three independent biological replicates, and comparisons between treatments and control were performed by one-way ANOVA followed by Bonferroni post hoc test (* $p < 0.1$, ** $p<0.001$*** $p < 0.0001$).

The results of the MIC assay revealed that all peptide fractions exhibited identical antimicrobial activity against *S. Typhimurium*. The MIC value was consistent across all conditions, with a concentration of 0.093 µg/µL observed for the peptide fractions derived from the hemolymph of control larvae and from larvae infected with *E. coli* and *M. flavus* (Figure 4.19)

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4.8.3 Evaluation of the Antibacterial Activity of Peptide Fraction via Minimum Bactericidal Concentration Assay

Subsequently, the MBC assay was performed to determine the minimum bactericidal concentration of the peptide fraction obtained from the hemolymph of *H. illucens* larvae. The assay was specifically carried out for the concentrations that induced a reduction in bacterial growth against *S. Typhimurium.* The aim was to identify the lowest concentration required to achieve effective bactericidal activity, thereby completing the evaluation of the antimicrobial potential of the peptide fraction.

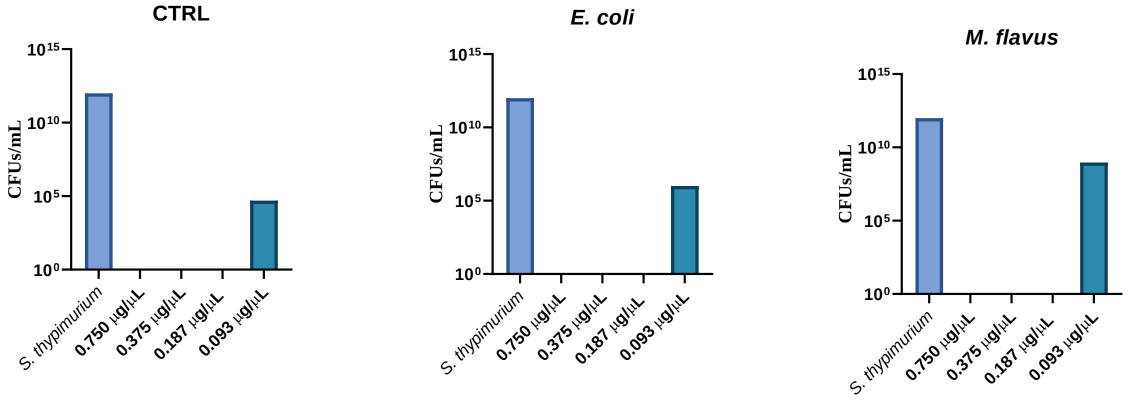


Figure 4.20. Minimum bactericidal concentration against *S. Thypimurium* performed with the peptide fractions obtained via precipitation with organic solvents. CTRL = peptide fractions from uninfected larvae; *E. coli* = peptide fractions from larvae infected with *E. coli*; *M. flavus* = peptide fractions from larvae infected with *M. flavus*. The first bars represent the untreated *S. Thypimurium* cell culture.

The results of the MBC assay demonstrated that the minimum bactericidal concentration was 0.187 µg/µL across all conditions tested (Figure 4.20). This concentration was consistent for the peptide fraction obtained from the hemolymph of control larvae and the fractions derived from larvae infected with *E. coli* and *M. flavus*. These findings suggest that the bactericidal efficacy of the peptide fractions is uniform, irrespective of the infection status of the larvae, indicating a consistent bactericidal activity against *S. Typhimurium.*

4.9 Evaluation of the Antibacterial Activity of Peptide Fraction against *S. pyogenes*

Antimicrobial assays were performed on the peptide fraction derived from *H. illucens* larvae against *Streptococcus pyogenes*, a Gram-positive bacterium belonging to the Streptococcaceae family. *S. pyogenes* is associated with a range of infections, including streptococcal pharyngitis (strep throat), scarlet fever, impetigo, cellulitis, necrotizing fasciitis, toxic shock syndrome, rheumatic fever, and post-streptococcal glomerulonephritis (Efstratiou & Lamagni, 2022).

4.9.1 Evaluation of the Antibacterial Activity of Peptide Fraction via Agar Diffusion Assay

The peptide fractions extracted from the hemolymph of *H. illucens* larvae, both uninfected and infected with *E. coli* or *M. flavus*, precipitated using a methanol/acetic acid/water solution (90:1:9 v/v), were evaluated for their antibacterial activity against *Streptococcus pyogenes* using an agar diffusion assay.

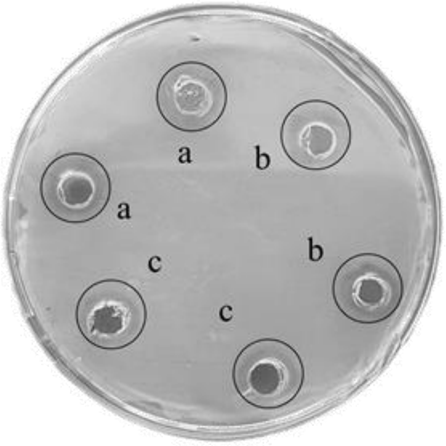


Figure 4.21. Agar diffusion assay of peptide fractions obtained via precipitation with organic solvents, performed against *S. pyognes* (**a**) peptide fraction from uninfected larvae; (**b**) peptide fraction from larvae infected with *E. coli*; (**c**) peptide fraction from larvae infected with *M. flavus*. The experiments were carried out in triplicate (three independent biological replicates).

The agar diffusion assay results showed clear inhibition zones for all three samples tested against *S. pyogenes* (Figure 4.21).

4.9.2 Evaluation of the Peptide Fraction Antibacterial Activity via Microdilution Assay

Subsequently, a microdilution assay was performed to determine the minimum inhibitory concentration of the peptide fraction obtained from the hemolymph of *H. illucens* larvae, precipitated using organic solvents, against *S. pyogenes*. The aim of the assay was to identify the lowest concentration of the peptide fraction required to effectively inhibit the growth of *S. pyogenes*.

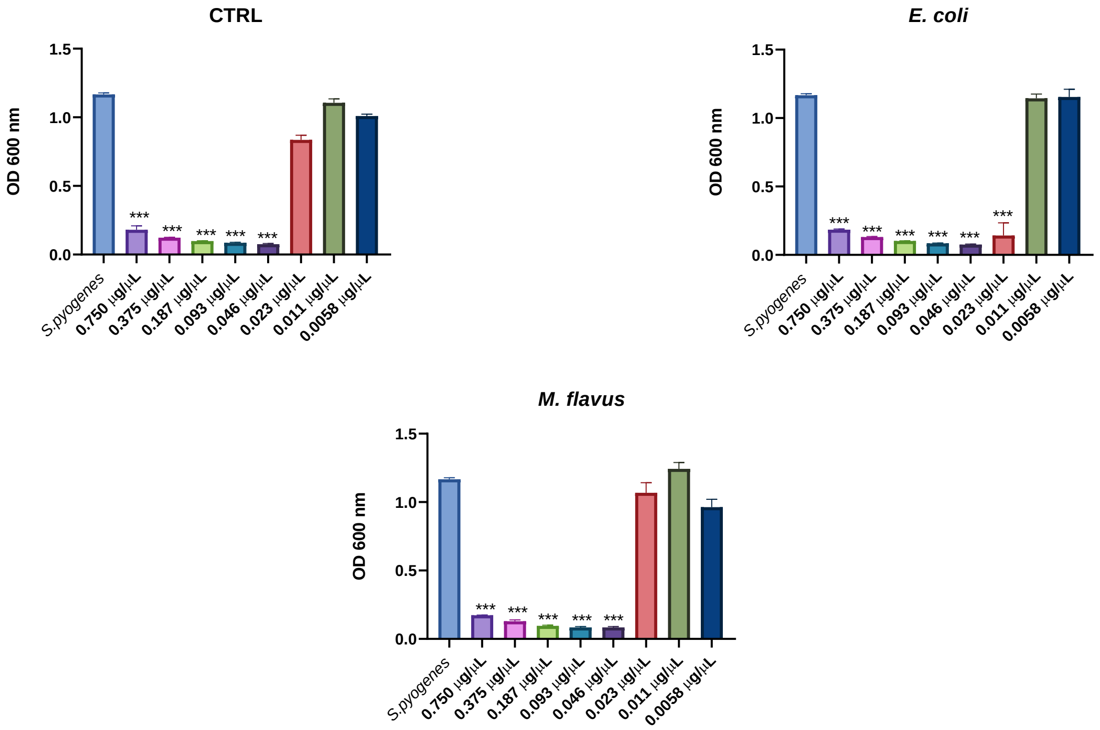


Figure 4.22. Microdilution assay against *S. pyogenes* performed with the peptide fractions obtained via precipitation with organic solvents. CTRL = peptide fractions from uninfected larvae; *E. coli* = peptide fractions from larvae infected with *E. coli*; *M. flavus* = peptide fractions from larvae infected with *M. flavus*. The first bars represent the untreated *S. pyogenes* cell culture. Data are expressed as means ± standard error of three independent biological replicates, and comparisons between treatments and control were performed by one-way ANOVA followed by Bonferroni post hoc test (* $p < 0.1$, ** $p<0.001$ *** $p < 0.0001$).

The MIC assay results revealed that all peptide fractions analyzed exhibited antimicrobial activity against *S. pyogenes*. The MIC value for the precipitated fraction obtained from the hemolymph of both control larvae and larvae infected with *M. flavus* was 0.046 µg/µL. In contrast, the MIC value for the peptide fraction obtained from larvae infected with *E. coli* was lower, at 0.023 µg/µL (Figure 4.22). These findings suggest that the peptide fraction derived from *E. coli*-infected larvae exhibited a higher antimicrobial potency against *S. pyogenes* compared to the other samples.

4.9.3 Evaluation of the Antibacterial Activity of Peptide Fraction via Minimum Bactericidal Concentration Assay

Based on the results obtained from the MIC assay, an MBC assay was subsequently performed to determine the minimum bactericidal concentration of the peptide fraction extracted from the hemolymph of *H. illucens* larvae against *S. pyogenes*. The assay focused specifically on the concentrations that had demonstrated bacterial growth inhibition, aiming to identify the lowest concentration required to achieve bactericidal activity.

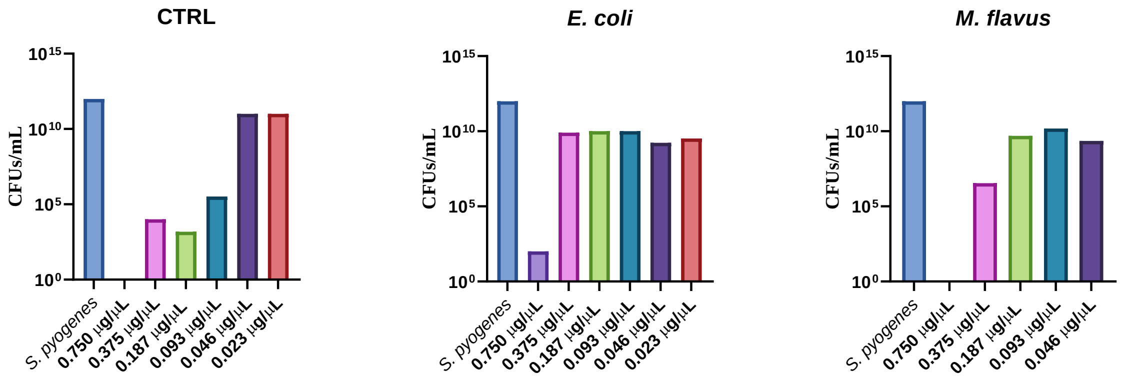


Figure 4.23. Minimum bactericidal concentration against *S. pyogenes* performed with the peptide fractions obtained via precipitation with organic solvents. CTRL = peptide fractions from uninfected larvae; *E. coli* = peptide fractions from larvae infected with *E. coli*; *M. flavus* = peptide fractions from larvae infected with *M. flavus*. The first bars represent the untreated *S. pyogenes* cell culture.

The MBC assay results indicate that not all conditions analyzed exhibited bactericidal activity. Specifically, only the peptide fraction obtained from control larvae and the fraction derived from the hemolymph of larvae infected with *M. flavus* demonstrated bactericidal activity at a concentration of 0.750 µg/µL. In contrast, the peptide fraction obtained from the hemolymph of larvae infected with *E. coli* did not show bactericidal activity at any of the concentrations tested (Figure 4.23).

4.10 Evaluation of the Antibacterial Activity of Peptide Fraction against *E. faecalis*.

Antimicrobial assays were performed on the peptide fraction extracted from *H. illucens* larvae against *Enterococcus faecalis*, a Gram-positive bacterium from the Enterococcaceae family. This bacterium is commonly found in the human gastrointestinal tract but is recognized as an opportunistic pathogen, particularly in hospitals. *E. faecalis* can cause a range of nosocomial infections, including urinary tract infections, endocarditis, and sepsis, with an increased risk in immunocompromised patients (Beganovic et al., 2018).

4.10.1 Evaluation of the Peptide Fraction Antibacterial Activity via Agar Diffusion Assay

The peptide fractions recovered following precipitation with methanol/acetic acid/water (90:1:9 v/v) from the hemolymph of both uninfected larvae and larvae infected with *E. coli* or *M. flavus* were first analyzed using an agar diffusion test to evaluate their antibacterial activity against *E. faecalis*.

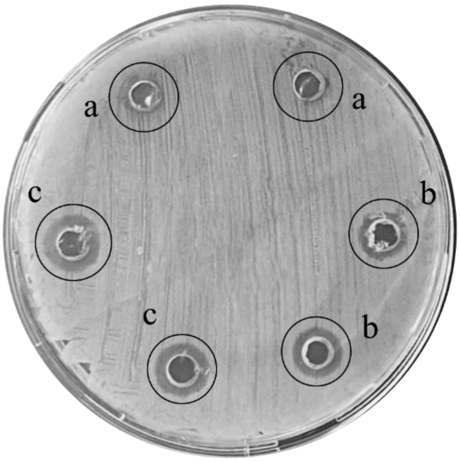


Figure 4.24. Agar diffusion test of peptide fractions obtained via precipitation with organic solvents, performed against *E. faecalis*. (**a**) peptide fraction from uninfected larvae; (**b**) peptide fraction from larvae infected with *E. coli*; (**c**) peptide fraction from larvae infected with *M. flavus*. The experiments were carried out in triplicate (three independent biological replicates).

The results of the agar diffusion test revealed inhibition zones for all three peptide fractions analyzed, indicating antibacterial activity against *E. faecalis*. Notably, the peptide fraction derived from larvae infected with *M. flavus* exhibited the largest and most pronounced inhibition zone, suggesting a higher antimicrobial activity compared to the fractions obtained from uninfected larvae and those infected with *E. coli* (Figure 4.24).

4.10.2 Evaluation of the Peptide Fraction Antibacterial Activity via Microdilution Assay

The minimum inhibitory concentration assay was conducted on hemolymph samples precipitated with organic solvents to determine the MIC value against *E. faecalis*. The aim of this assay was to evaluate the antimicrobial potency of the peptide fractions and identify the lowest concentration required to inhibit the growth of *E. faecalis*, providing a quantitative assessment of the samples antibacterial activity.

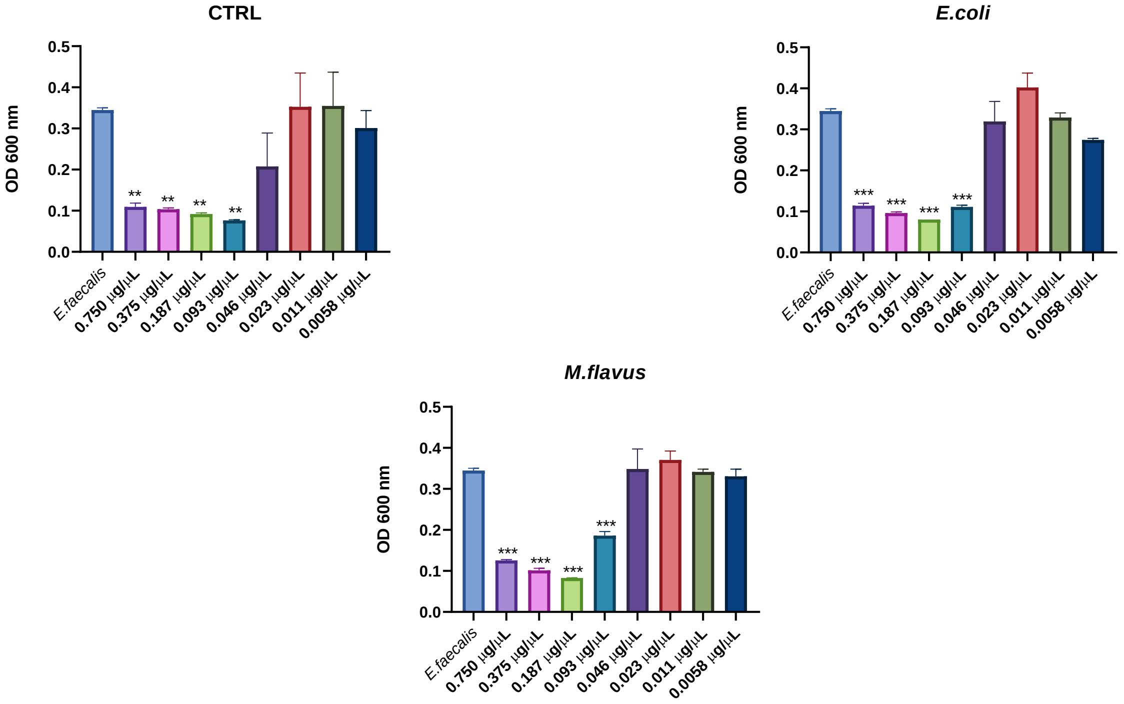


Figure 4.25. Microdilution assay against *E. faecalis* performed with the peptide fractions obtained via precipitation with organic solvents. CTRL = peptide fractions from uninfected larvae; *E. coli* = peptide fractions from larvae infected with *E. coli*; *M. flavus* = peptide fractions from larvae infected with *M. flavus*. The first bars represent the untreated *E, faecalis* cell culture. Data are expressed as means ± standard error of three independent biological replicates, and comparisons between treatments and control were performed by one-way ANOVA followed by Bonferroni post hoc test (* $p < 0.1$, ** $p<0.001$*** $p < 0.0001$).

The results of the MIC assay demonstrated that all the conditions analyzed exhibited antimicrobial activity against *E. faecalis*. Specifically, the MIC value for the peptide fractions derived from the hemolymph of both uninfected larvae and larvae infected with *E. coli* and *M. flavus* was determined to be 0.093 µg/µL (Figure 4.25). These findings suggest that the antimicrobial potency of the peptide fractions remains consistent across the different conditions, regardless of the larvae infection status.

4.10.3 Evaluation of the Antibacterial Activity of Peptide Fraction via Minimum Bactericidal Concentration Assay

A minimum bactericidal concentration assay was performed to determine the MBC value of the peptide fraction extracted from the hemolymph of *H. illucens* larvae against *E. faecalis*. This assay aimed to identify the lowest concentration of the peptide fraction required to achieve bactericidal activity, thereby complementing the findings of the MIC assay and providing a comprehensive evaluation of the antimicrobial potential of the peptide fraction.

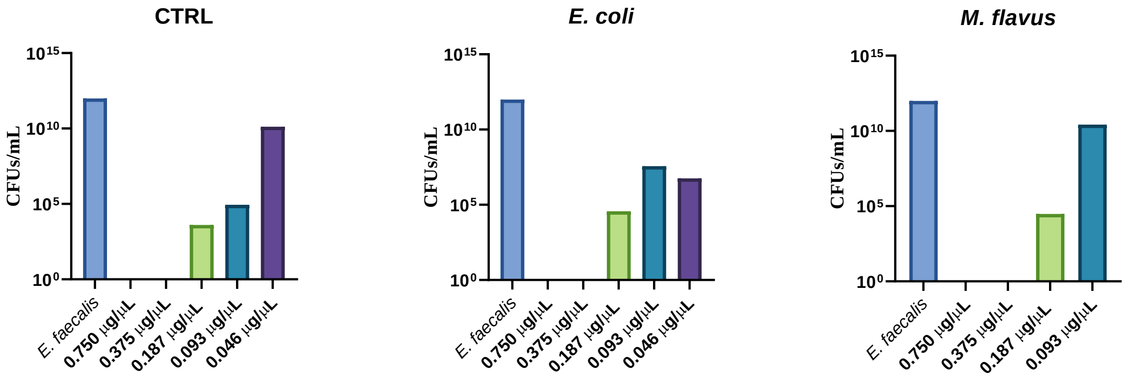


Figure 4.26. Minimum Bactericidal Concentration assay against *E. faecalis* performed with the peptide fractions obtained via precipitation with organic solvents. CTRL = peptide fractions from uninfected larvae; *E. coli* = peptide fractions from larvae infected with *E. coli*; *M. flavus* = peptide fractions from larvae infected with *M. flavus*. The first bars represent the untreated *E. faecalis* cell culture.

The results of the MBC assay demonstrated that all conditions analyzed exhibited bactericidal activity against *E. faecalis*. Specifically, the peptide fraction obtained from the hemolymph of control larvae, as well as the fractions derived from larvae infected with *E. coli* and *M. flavus*, showed a minimum bactericidal concentration of 0.375 µg/µL (Figure 4.26). These findings indicate that the bactericidal efficacy of the peptide fractions remains consistent across all tested conditions, regardless of the larvae's infection status.

4.11 Evaluation of the Antibacterial Activity of Peptide Fraction against *S. epidermidis*

Antimicrobial assays were performed on the peptide fraction derived from *H. illucens* larvae against *Staphylococcus epidermidis*, a Gram-positive bacterium primarily colonizing human skin and nasal passages. Although typically harmless, *S. epidermidis* can act as an opportunistic pathogen, leading to nosocomial infections, particularly in immunocompromised individuals or those with implanted medical devices such as catheters and prostheses. This bacterium is frequently associated with infections of medical implants and conditions like ocular keratitis and endophthalmitis (Severn & Horswill, 2023)

4.11.1 Evaluation of the Antibacterial Activity of Peptide Fraction via Agar Diffusion Assay

The peptide fractions obtained from the hemolymph of both uninfected larvae and larvae infected with *E. coli* or *M. flavus*, precipitated using a methanol/acetic acid/water solution (90:1:9 v/v), were initially evaluated using an agar diffusion test to assess their antibacterial activity against *S. epidermidis*.

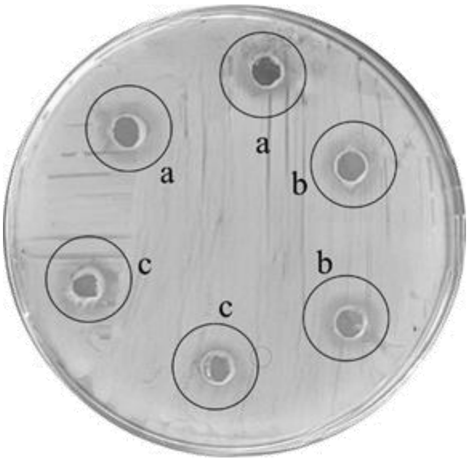


Figure 4.27. Agar diffusion test of peptide fractions obtained via precipitation with organic solvents, performed against *S. epidermidis*. (**a**) peptide fraction from uninfected larvae; (**b**) peptide fraction from larvae infected with *E. coli*; (**c**) peptide fraction from larvae infected with *M. flavus*. The experiments were carried out in triplicate (three independent biological replicates).

The agar diffusion assay results showed inhibition zones for all three peptide fractions. The fractions, derived from the hemolymph of uninfected larvae and larvae infected with *E. coli* and *M. flavus*, exhibited antibacterial activity against *S. epidermidis*, as evidenced by the clear zones of inhibited bacterial growth surrounding the samples (Figure 4.27).

4.11.2 Evaluation of the Peptide Fraction Antibacterial Activity via Microdilution Assay

To determine the minimum inhibitory concentration, peptide fractions derived from *H. illucens* larvae were tested using a microdilution assay. These fractions, precipitated using organic solvents, were evaluated for their antimicrobial activity against *S. epidermidis*.

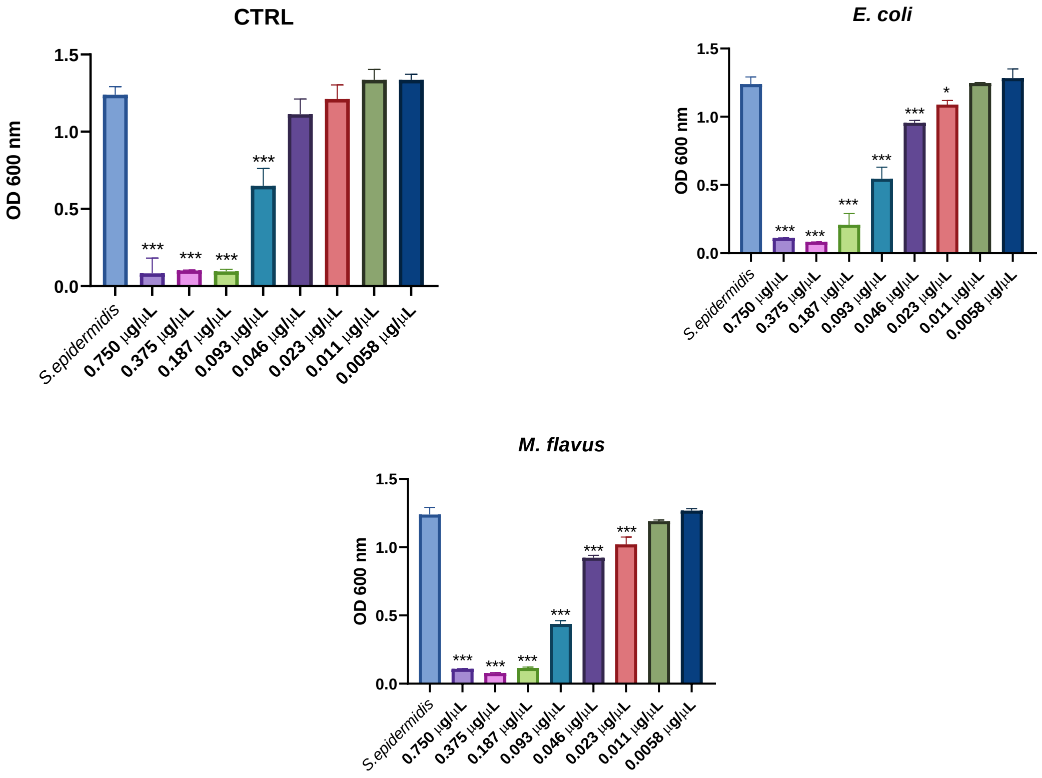


Figure 4.28. Microdilution assay against *S. epidermidis* performed with the peptide fractions obtained via precipitation with organic solvents. CTRL = peptide fractions from uninfected larvae; *E. coli* = peptide fractions from larvae infected with *E. coli*; *M. flavus* = peptide fractions from larvae infected with *M. flavus*. The first bars represent the untreated *S. epidermidis* cell culture. Data are expressed as means ± standard error of three independent biological replicates, and comparisons between treatments and control were performed by one-way ANOVA followed by Bonferroni post hoc test (* $p < 0.1$, ** $p<0.001$*** $p < 0.0001$).

The MIC assay results demonstrated uniform antimicrobial activity across all three conditions analysed. The peptide fraction obtained from control larvae and those derived from larvae infected with *E. coli* and *M. flavus* exhibited the same minimum inhibitory concentration (MIC) of 0.093 µg/µL against *S. epidermidis*. These findings suggest that the antimicrobial potency of the peptide fractions is consistent regardless of the larvae's infection status (Figure 4.28).

4.11.3 Evaluation of the Antibacterial Activity of Peptide Fraction via Minimum Bactericidal Concentration Assay

Based on the results obtained from the microdilution assay, an MBC assay was performed to determine the minimum bactericidal concentration of the peptide fraction obtained from *H. illucens* larvae, specifically for the conditions where a reduction in bacterial growth was observed, against *S. epidermidis*.

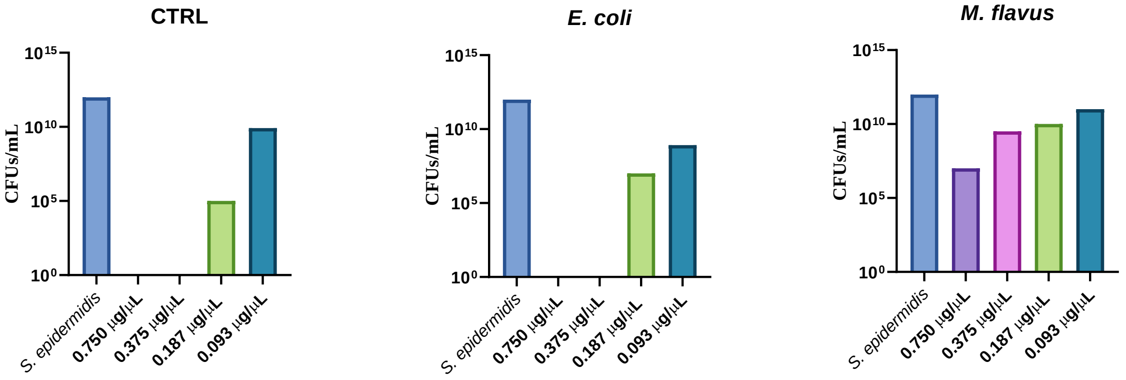


Figure 4.29. Minimum Bactericidal Concentration assay against *S. epidermidis* performed with the peptide fractions obtained via precipitation with organic solvents. CTRL = peptide fractions from uninfected larvae; *E. coli* = peptide fractions from larvae infected with *E. coli*; *M. flavus* = peptide fractions from larvae infected with *M. flavus*. The first bars represent the untreated *S. epidermidis* cell culture.

The results of the MBC assay revealed differences in bactericidal activity among the three samples analyzed. Specifically, the minimum bactericidal concentration for the peptide fractions obtained from the hemolymph of control larvae and larvae infected with *E. coli* was 0.375 µg/µL. In contrast, none of the tested concentrations exhibited bactericidal activity for the peptide fraction derived from larvae infected with *M. flavus* (Figure 4.29).

4.12 Evaluation of the Antibacterial Activity of Peptide Fraction against *P. mirabilis*

The antimicrobial activity of the peptide fraction obtained from *H. illucens* larvae was tested against *Proteus mirabilis*, a Gram-negative bacterium from the Enterobacteriaceae family. *P. mirabilis* is primarily responsible for urinary tract infections (UTIs), particularly in individuals with long-term urinary catheters or other urological abnormalities. This microorganism is also associated with wound infections, pneumonia, bacteraemia, and, in rare cases, biliary tract infections (Armbruster et al., 2018).

4.12.1 Evaluation of the Antibacterial Activity of Peptide Fraction via Agar Diffusion Assay

The peptide fractions isolated from plasma, precipitated with a methanol/acetic acid/water solution (90:1:9 v/v ratio), from both uninfected larvae and those infected with *E. coli* or *M. flavus*, were initially tested using an agar diffusion assay to assess their antibacterial activity against *P. mirabilis*.

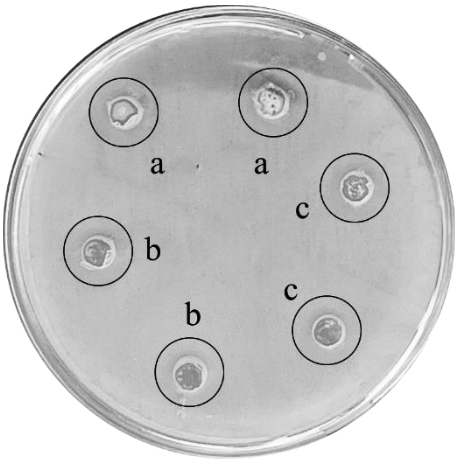


Figure 4.30. Agar diffusion test of peptide fractions obtained via precipitation with organic solvents, performed against *P. mirabilis*. (**a**) peptide fraction from uninfected larvae; (**b**) peptide fraction from larvae infected with *E. coli*; (**c**) peptide fraction from larvae infected with *M. flavus*. The experiments were carried out in triplicate (three independent biological replicates).

The results of the agar diffusion assay did not reveal any appreciable inhibition zones for any of the tested samples (Figure 4.30). This suggests that, under the experimental conditions used, none of the peptide fractions exhibited significant antimicrobial activity detectable by this method. A possible explanation could be that the peptide fractions exert a bacteriostatic effect, inhibiting bacterial growth without causing direct bacterial cell death.

4.12.2 Evaluation of the Hemolymph Antibacterial Activity via Microdilution Assay

Although the results of the agar diffusion assay suggested a potential lack of antimicrobial activity in the peptide fraction obtained from the hemolymph of *H. illucens* larvae, the MIC assay was nonetheless performed to determine the minimum inhibitory concentration required to inhibit the growth of *P. mirabilis*.

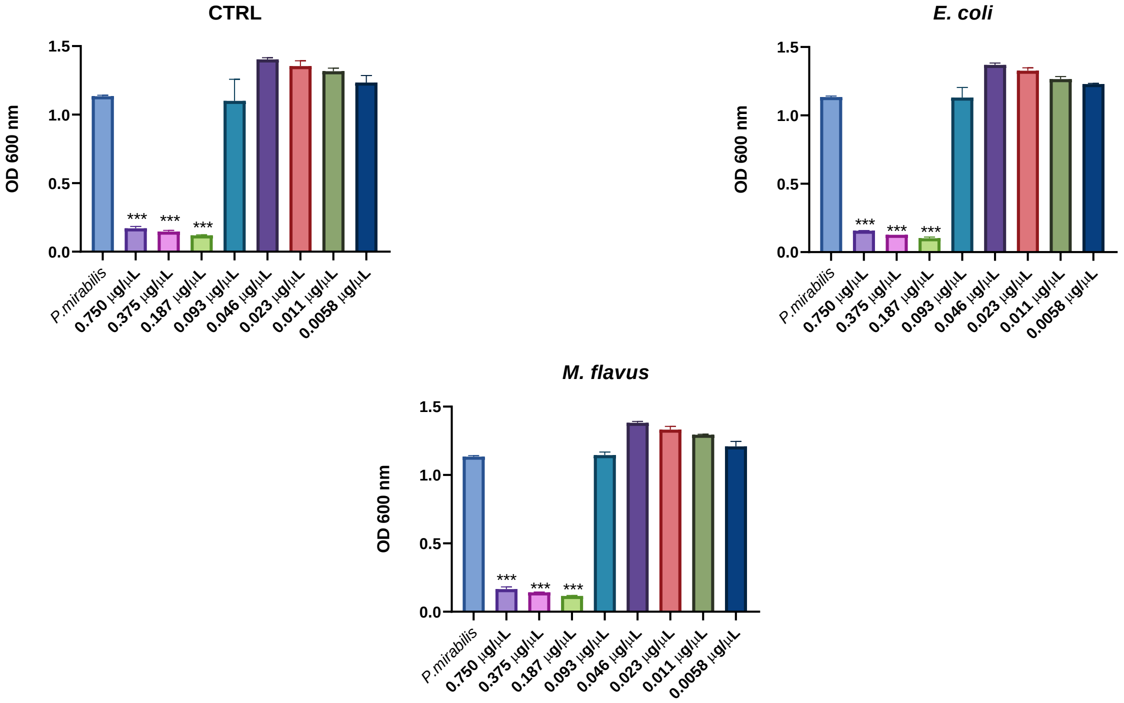


Figure 4.31. Microdilution assay against *P. mirabilis* performed with the peptide fractions obtained via precipitation with organic solvents. CTRL = peptide fractions from uninfected larvae; *E. coli* = peptide fractions from larvae infected with *E. coli*; *M. flavus* = peptide fractions from larvae infected with *M. flavus*. The first bars represent the untreated *P.mirabilis* cell culture. Data are expressed as means ± standard error of three independent biological replicates, and comparisons between treatments and control were performed by one-way ANOVA followed by Bonferroni post hoc test (* $p < 0.1$, ** $p<0.001$*** $p < 0.0001$).

The minimum inhibitory concentration assay results showed a significant reduction in the growth of *P. mirabilis* under all experimental conditions tested. The peptide fraction obtained from the hemolymph of control larvae and the fraction obtained from the hemolymph of larvae infected with *E. coli* and *M. flavus* demonstrated effective antimicrobial activity. In both fractions, the MIC value was determined to be 0.187 µg/µL (Figure 4.31).

4.12.3 Evaluation of the Antibacterial Activity of Peptide Fraction via Minimum Bactericidal Concentration Assay

The minimum bactericidal concentration assay was performed on hemolymph samples precipitated with organic solvents obtained from *H. illucens* larvae against *P. mirabilis* to determine the minimum concentration required to exert a bactericidal effect on *P. mirabilis.*

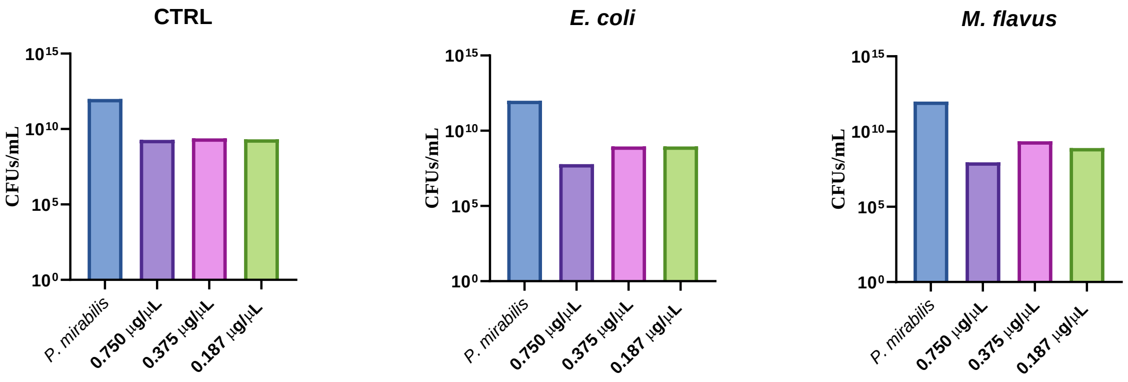


Figure 4.32. Minimum Bactericidal Concentration against *P. mirabilis* performed with the peptide fractions obtained via precipitation with organic solvents. CTRL = peptide fractions from uninfected larvae; *E. coli* = peptide fractions from larvae infected with *E. coli*; *M. flavus* = peptide fractions from larvae infected with *M. flavus*. The first bars represent the untreated *P. mirabilis* cell culture.

The minimum bactericidal concentration assay results indicate that none of the concentrations tested in the analyzed samples exhibited bactericidal activity (Figure 4.32). This suggests that, although the peptide fractions may exert an inhibitory effect on bacterial growth at certain concentrations, they are not potent enough to completely eradicate the tested bacteria. These findings are consistent with the results of the agar diffusion assay, where no significant inhibition zones were observed.

4.13 Evaluation of the Antibacterial Activity of Peptide Fraction against Carbapenem-resistant *K. pneumoniae*

Klebsiella pneumoniae carbapenem-resistant (CRKP) is a Gram-negative bacterium belonging to the Enterobacteriaceae family. This bacterium is characterized by its resistance to carbapenems, a class of broad-spectrum antibiotics often used as a last resort for treating severe infections. Infections caused by CRKP pose a significant threat to public health, particularly in healthcare settings. CRKP can cause various nosocomial infections, including pneumonia, bloodstream infections, and urinary tract infections (González-Romero et al., 2024). These infections are associated with high mortality rates, ranging from 38% to 54.5% (Almeida, 2013). The antimicrobial activity was tested on five different strains of *K. pneumoniae*, each with distinct resistance profiles to carbapenems. Each strain is identified by a specific code corresponding to the patient from whom it was isolated, reflecting varying resistance profiles among the patients.

4.14.1 Evaluation of the Antibacterial Activity of Peptide Fraction via Agar Diffusion Assay against CRKP 70131

The peptide fractions extracted from plasma, precipitated using a methanol/acetic acid/water mixture (90:1:9 v/v ratio), from both uninfected larvae and larvae infected with *E. coli* or *M. flavus*, were first evaluated with an agar diffusion test to determine their antibacterial effect against CRKP 70131.

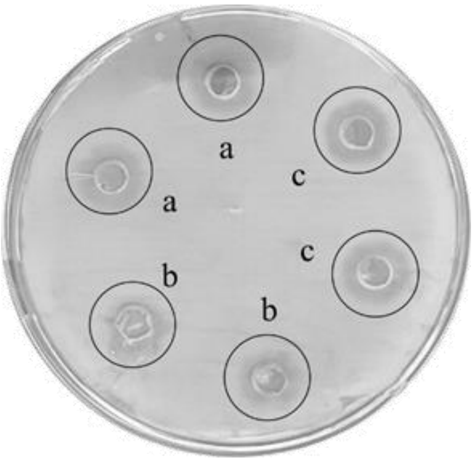


Figure 4.34. Agar diffusion test of peptide fractions obtained via precipitation with organic solvents, performed against CRKP 70131. (**a**) peptide fraction from uninfected larvae; (**b**) peptide fraction from larvae infected with *E. coli*; (**c**) peptide fraction from larvae infected with *M. flavus*. The experiments were carried out in triplicate (three independent biological replicates).

The results of the agar diffusion assay against carbapenem-resistant CRKP 70131 revealed the presence of inhibition zones for all three conditions analyzed. Notably, inhibition zones were observed for the peptide fraction obtained from uninfected larvae and the fraction derived from the hemolymph of *H. illucens* larvae infected with *E. coli* and *M. flavus* (Figure 4.33).

4.14.2 Evaluation of the Peptide Fraction Antibacterial Activity via Microdilution Assay against CRKP 70131

Based on the positive results obtained from the agar diffusion assay, the microdilution assay was performed to determine the minimum inhibitory concentration of the peptide fraction extracted from the hemolymph of *H. illucens* larvae, tested against the CRKP 70131 strain. The aim of the assay was to identify the minimum concentration required to effectively inhibit the growth of the carbapenem-resistant strain.

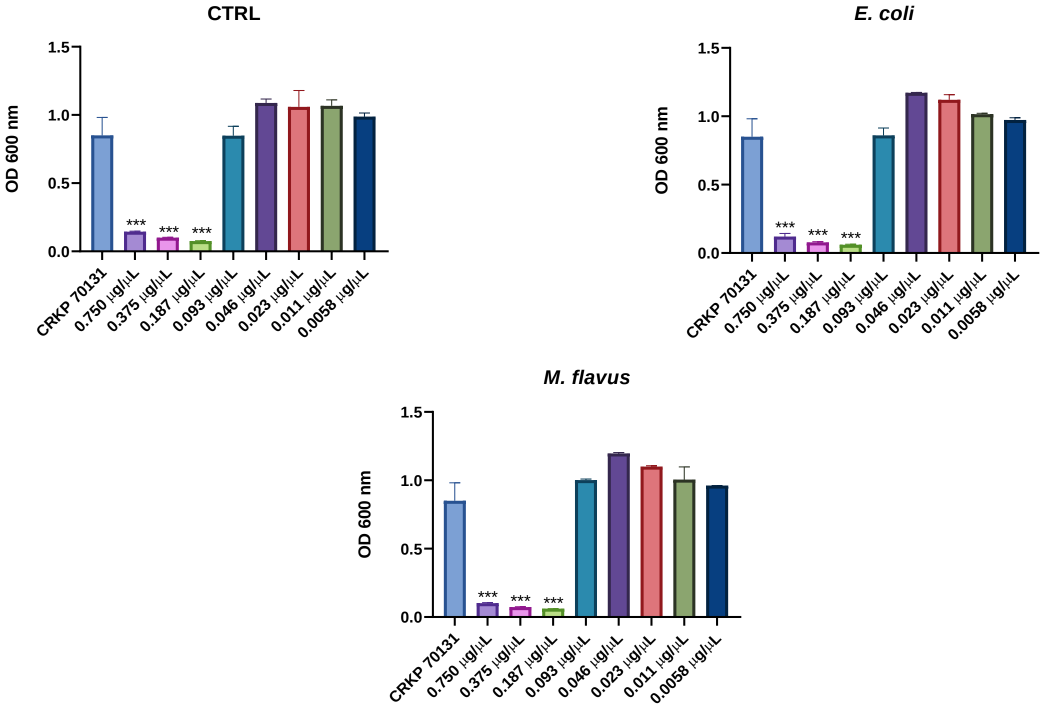


Figure 4.34. Microdilution assay against CRKP 70131 performed with the peptide fractions obtained via precipitation with organic solvents. CTRL = peptide fractions from uninfected larvae; *E. coli* = peptide fractions from larvae infected with *E. coli*; *M. flavus* = peptide fractions from larvae infected with *M. flavus*. The first bars represent the untreated CRKP 70131 cell culture. Data are expressed as means ± standard error of three independent biological replicates, and comparisons between treatments and control were performed by one-way ANOVA followed by Bonferroni post hoc test (* $p < 0.1$, ** $p<0.001$*** $p < 0.0001$).

The MIC assay results demonstrated antimicrobial activity for all three samples analysed. Specifically, the MIC value for all samples tested was 18.7 µg/µL (Figure 4.34). These findings suggest that the peptide fractions consistently inhibit the growth of the CRKP 70131 strain across all samples.

4.14.3 Evaluation of the Antibacterial Activity of Peptide Fraction via Minimum Bactericidal Concentration Assay against CRKP 70131

The minimum bactericidal concentration assay was performed against the multidrug-resistant bacterial strain CRKP 70131 to determine the minimum bactericidal concentration of the peptide fraction extracted from the hemolymph of *H. illucens* larvae.

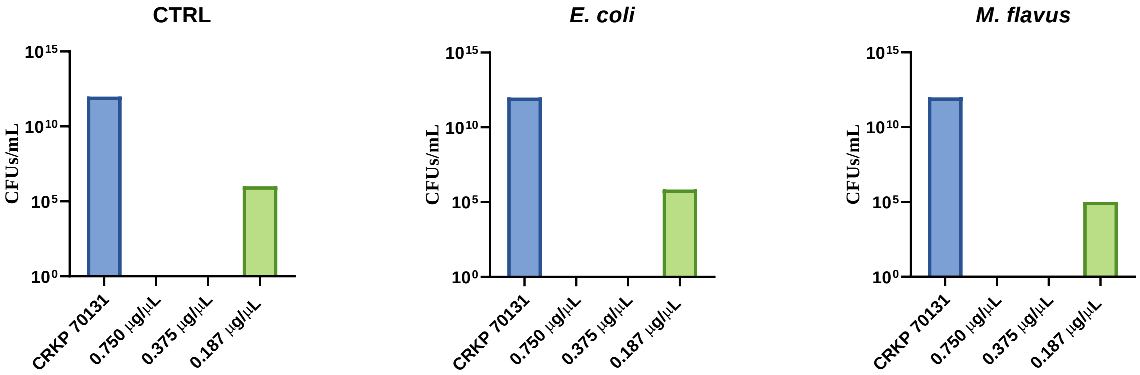


Figure 4.35. Minimum Bactericidal Concentration against CRKP 70131 performed with the peptide fractions obtained via precipitation with organic solvents. CTRL = peptide fractions from uninfected larvae; *E. coli* = peptide fractions from larvae infected with *E. coli*; *M. flavus* = peptide fractions from larvae infected with *M. flavus*. The first bars represent the untreated CRKP 70131 cell culture.

The MBC assay results demonstrated bactericidal activity under all conditions analyzed. Specifically, both the peptide fraction obtained from uninfected larvae and the fractions derived from the hemolymph of larvae infected with *E. coli* and *M. flavus* exhibited a minimum bactericidal concentration of 0.375 µg/µL (Figure 4.35). These findings indicate a consistent bactericidal activity across the tested peptide fractions.

4.14.4 Evaluation of the Antibacterial Activity of Peptide Fraction via Agar Diffusion Assay against CRKP 72895

The peptide fractions obtained from plasma, precipitated using a methanol/acetic acid/water mixture (90:1:9 v/v), from both uninfected larvae and larvae infected with *E. coli* or *M. flavus*, were initially analyzed through an agar diffusion test to assess their antibacterial activity against CRKP 72895.

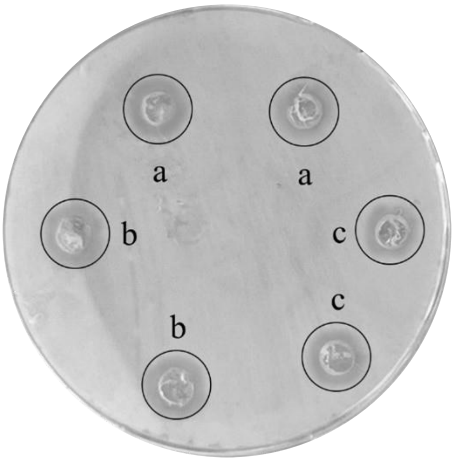


Figure 4.36. Agar diffusion test of peptide fractions obtained via precipitation with organic solvents, performed against CRKP 72895. (**a**) peptide fraction from uninfected larvae; (**b**) peptide fraction from larvae infected with *E. coli*; (**c**) peptide fraction from larvae infected with *M. flavus*. The experiments were carried out in triplicate (three independent biological replicates).

The results of the agar diffusion test showed the presence of appreciable inhibition zones corresponding to the peptide fraction obtained from uninfected larvae and the fraction derived from larvae infected with *E. coli*. In contrast, less intense inhibition zones were observed for the peptide fraction obtained from the hemolymph of larvae infected with *M. flavus* (Figure 4.36).

4.14.5 Evaluation of the Peptide Fraction Antibacterial Activity via Microdilution Assay against CRKP 72895

Following the positive outcomes of the agar diffusion assay, a microdilution assay was carried out to determine the minimum inhibitory concentration (MIC) of the peptide fraction extracted from the hemolymph of *H. illucens* larvae. This assay was conducted against the

CRKP 72895 strain to identify the lowest concentration needed to effectively inhibit the growth of the carbapenem-resistant strain.

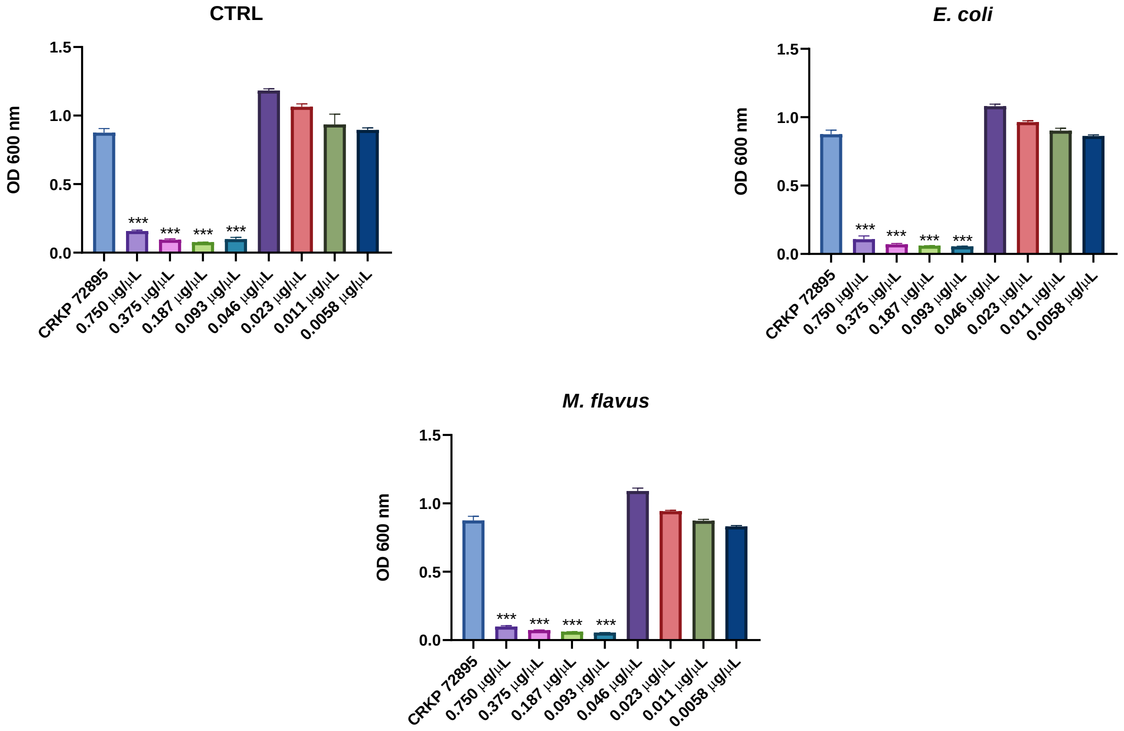


Figure 4.37. Microdilution assay against CRKP 72895 performed with the peptide fractions obtained via precipitation with organic solvents. CTRL = peptide fractions from uninfected larvae; *E. coli* = peptide fractions from larvae infected with *E. coli*; *M. flavus* = peptide fractions from larvae infected with *M. flavus*. The first bars represent the untreated CRKP 72895 cell culture. Data are expressed as means ± standard error of three independent biological replicates, and comparisons between treatments and control were performed by one-way ANOVA followed by Bonferroni post hoc test (* $p < 0.1$, ** $p<0.001$*** $p < 0.0001$).

The MIC assay results demonstrated antimicrobial activity for all three samples analysed. Specifically, the MIC value for all conditions tested was 0.093 µg/µL (Figure 4.37). These findings suggest that, regardless of the experimental condition, the peptide fractions exhibited a consistent ability to inhibit the growth of the CRKP 72895 strain.

4.14.6 Evaluation of the Antibacterial Activity of Peptide Fraction via Minimum Bactericidal Concentration Assay against CRKP 72895

An MBC assay was conducted against the multidrug-resistant bacterial strain CRKP 72895 to determine the minimum bactericidal concentration of the peptide fraction extracted from the hemolymph of *H. illucens* larvae.

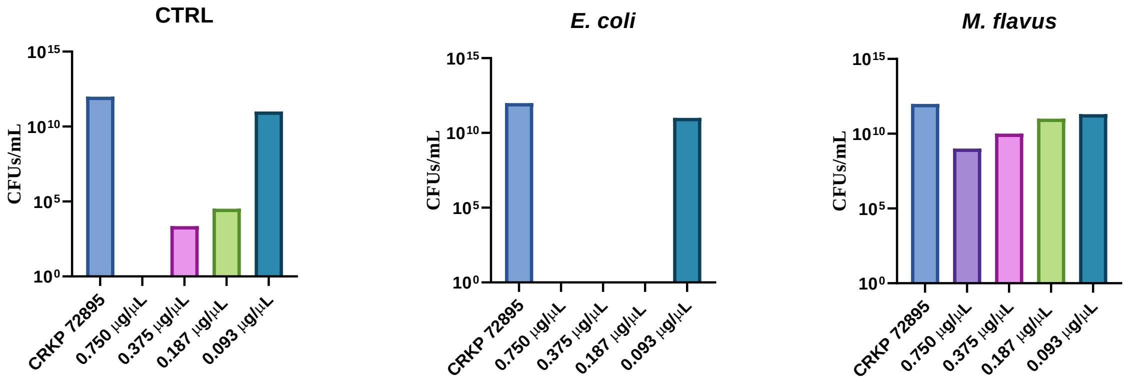


Figure 4.38. Minimum bactericidal concentration against CRKP 72895 performed with the peptide fractions obtained via precipitation with organic solvents. CTRL = peptide fractions from uninfected larvae; *E. coli* = peptide fractions from larvae infected with *E. coli*; *M. flavus* = peptide fractions from larvae infected with *M. flavus*. The first bars represent the untreated CRKP 72895 cell culture.

The MBC results indicate that not all conditions analysed exhibited bactericidal activity. Specifically, the minimum bactericidal concentration for the peptide fraction obtained from the hemolymph of control larvae was 0.750 µg/mL. For the peptide fraction obtained from the hemolymph of larvae infected with *E. coli*, the MBC value was 0.187 µg/mL. Lastly, no bactericidal activity was observed for the peptide fraction obtained from the hemolymph of larvae infected with *M. flavus* (Figure 4.38).

4.14.7 Evaluation of the Antibacterial Activity of Peptide Fraction via Agar Diffusion Assay against CRKP 70461

The peptide fractions isolated from plasma, precipitated with a methanol/acetic acid/water solution (90:1:9 v/v), from both uninfected larvae and those infected with *E. coli* or *M. flavus*, were first evaluated using an agar diffusion assay to determine their antibacterial activity against CRKP 70461.

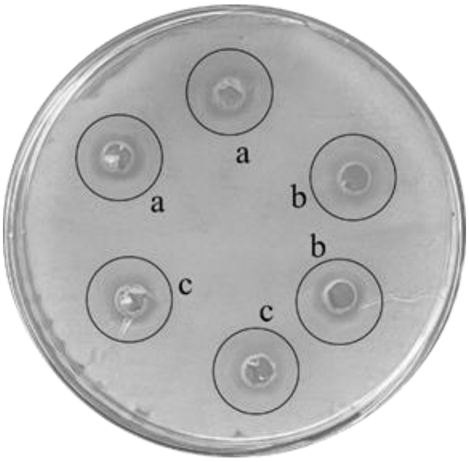


Figure 4.39. Agar diffusion test of peptide fractions obtained via precipitation with organic solvents, performed against CRKP 70461. (**a**) peptide fraction from uninfected larvae; (**b**) peptide fraction from larvae infected with *E. coli*; (**c**) peptide fraction from larvae infected with *M. flavus*. The experiments were carried out in triplicate (three independent biological replicates).

The results obtained from the agar diffusion assay revealed the presence of inhibition zones in all experimental conditions analysed, confirming the antimicrobial activity of the peptide fractions extracted from the hemolymph of *H. illucens* larvae. In particular, inhibition zones were observed both for the peptide fraction extracted from the hemolymph of uninfected larvae and for those obtained from the hemolymph of larvae infected with *E. coli* and *M. flavus* (Figure 4.39).

4.14.8 Evaluation of the Peptide Fraction Antibacterial Activity via Microdilution Assay against CRKP 70461

The microdilution assay was employed to assess the antimicrobial activity of the peptide fraction extracted from the hemolymph of *H illucens* larvae against the CRKP 70461 strain.

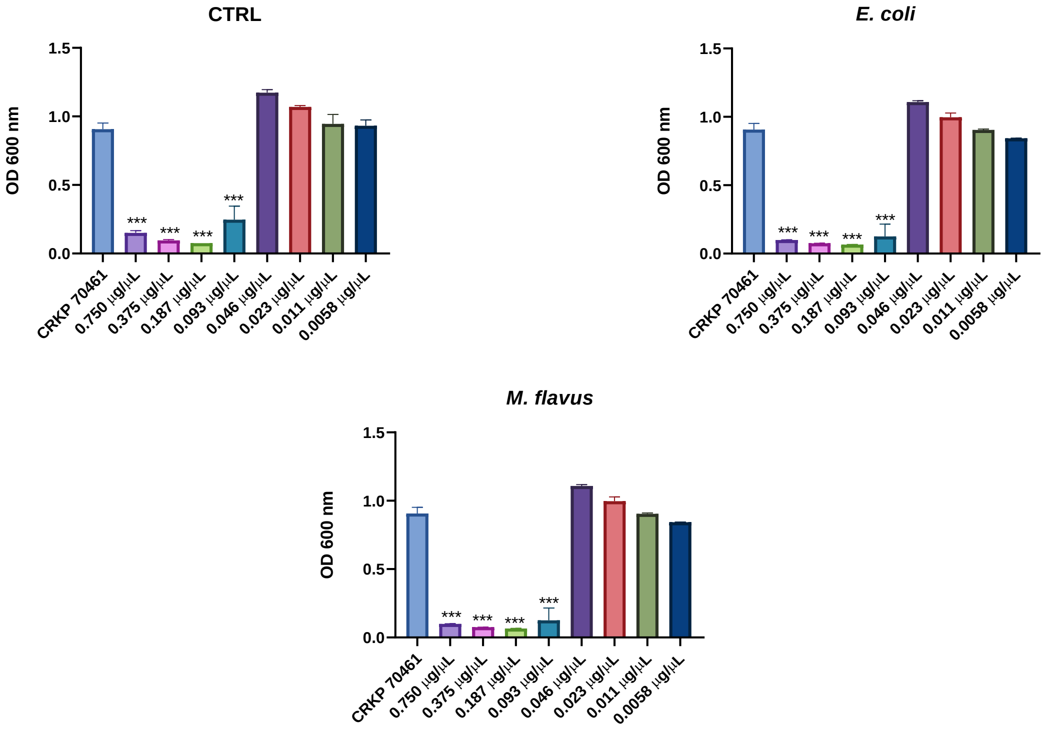


Figure 4.40. Microdilution assay against CRKP 70461 performed with the peptide fractions obtained via precipitation with organic solvents. CTRL = peptide fractions from uninfected larvae; *E. coli* = peptide fractions from larvae infected with *E. coli*; *M. flavus* = peptide fractions from larvae infected with *M. flavus*. The first bars represent the untreated CRKP 70461 cell culture. Data are expressed as means ± standard error of three independent biological replicates, and comparisons between treatments and control were performed by one-way ANOVA followed by Bonferroni post hoc test (* $p < 0.1$, ** $p<0.001$*** $p < 0.0001$).

The MIC assay results revealed antimicrobial activity across all three samples analysed. The MIC value for each condition tested consistently measured 0.093 µg/µL (Figure 4.40). These findings confirm that the peptide fractions exhibited a uniform ability to inhibit the growth of the CRKP 70461 strain under all tested conditions.

4.14.9 Evaluation of the Antibacterial Activity of Peptide Fraction via Minimum Bactericidal Concentration Assay against CRKP 70461

An MBC assay was conducted to determine the minimum bactericidal concentration of the peptide fraction obtained from *H. illucens* larvae, precipitated with organic solvents, against the CRKP 70461 strain. The MBC assay was performed on the concentrations of the peptide fractions where a reduction in bacterial growth was observed to assess the minimum concentration required for bactericidal activity.

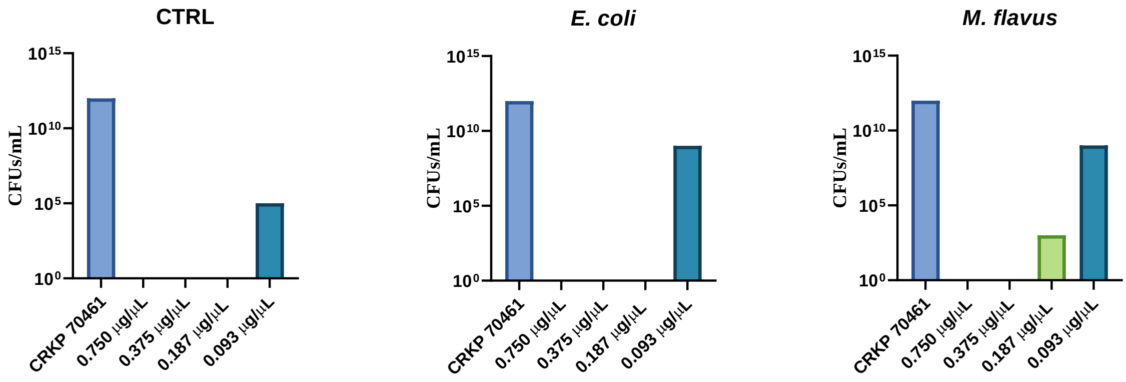


Figure 4.41. Minimum Bactericidal Concentration against CRKP 70461 performed with the peptide fractions obtained via precipitation with organic solvents. CTRL = peptide fractions from uninfected larvae; *E. coli* = peptide fractions from larvae infected with *E. coli*; *M. flavus* = peptide fractions from larvae infected with *M. flavus*. The first bars represent the untreated CRKP 70461 cell culture.

The MBC assay results show that all the conditions analysed exhibit bactericidal activity. Specifically, the MBC value for the peptide fraction precipitated with organic solvents, obtained from control larvae and larvae infected with *E. coli*, is 0.187 µg/µL. In contrast, the MBC value for the fraction obtained from larvae infected with *M. flavus* is 0.375 µg/µL (Figure 4.41).

4.14.10 Evaluation of the Antibacterial Activity of Peptide Fraction via Agar Assay Diffusion Assay against CRKP 5

The peptide fractions isolated from plasma, precipitated using a methanol/acetic acid/water mixture (90:1:9 v/v), from both uninfected larvae and those infected with *E. coli* or *M. flavus*, were first analysed with an agar diffusion assay to evaluate their antibacterial activity against CRKP 5.

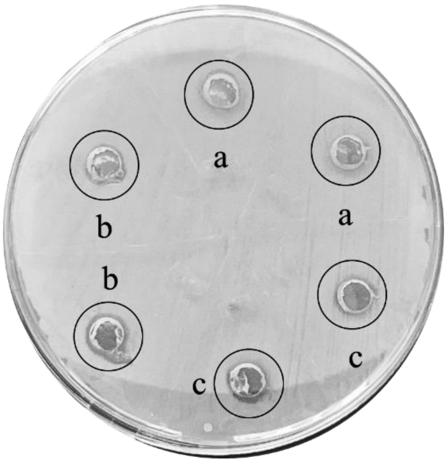


Figure 4.42. Agar diffusion test of peptide fractions obtained via precipitation with organic solvents, performed against CPKR 5. (**a**) peptide fraction from uninfected larvae; (**b**) peptide fraction from larvae infected with *E. coli*; (**c**) peptide fraction from larvae infected with *M. flavus*. The experiments were carried out in triplicate (three independent biological replicates).

The results of the agar diffusion assay revealed the presence of inhibition zones for both the peptide fraction obtained from uninfected larvae and the fraction derived from larvae infected with *E. coli* and *M. flavus* (Figure 4.42).

4.14.11 Evaluation of the Peptide Fraction Antibacterial Activity via Microdilution Assay against CRKP 5

The antimicrobial activity of the peptide fraction extracted from the hemolymph of *H. illucens* larvae was evaluated using the microdilution assay against the CRKP 5 strain.

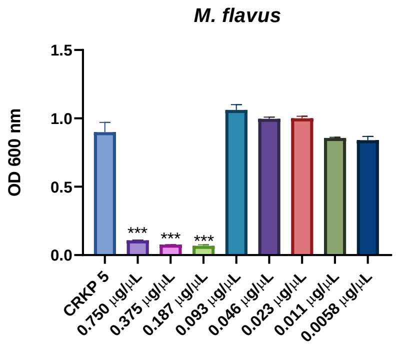


Figure 4.44. Microdilution assay against CRKP 5 performed with the peptide fractions obtained via precipitation with organic solvents. CTRL = peptide fractions from uninfected larvae; COLI = peptide fractions from larvae infected with *E. coli*; FLAVUS = peptide fractions from larvae infected with *M. flavus*. The first bars represent the untreated CRKP 5 cell culture. Data are expressed as means ± standard error of three independent biological replicates, and comparisons between treatments and control were performed by one-way ANOVA followed by Bonferroni post hoc test (* $p < 0.1$, ** $p<0.001$*** $p < 0.0001$).

The MIC assay results demonstrated antimicrobial activity for all three samples analysed. Notably, the MIC value for all conditions tested was once again 18.7 µg/mL (Figure 4.43).

4.14.12 Evaluation of the Antibacterial Activity of Peptide Fraction via Minimum Bactericidal Concentration Assay against CRKP 5

An MBC assay was performed to determine the minimum bactericidal concentration of the peptide fraction obtained from *H. illucens* larvae, precipitated with organic solvents, against the CRKP 5 strain.

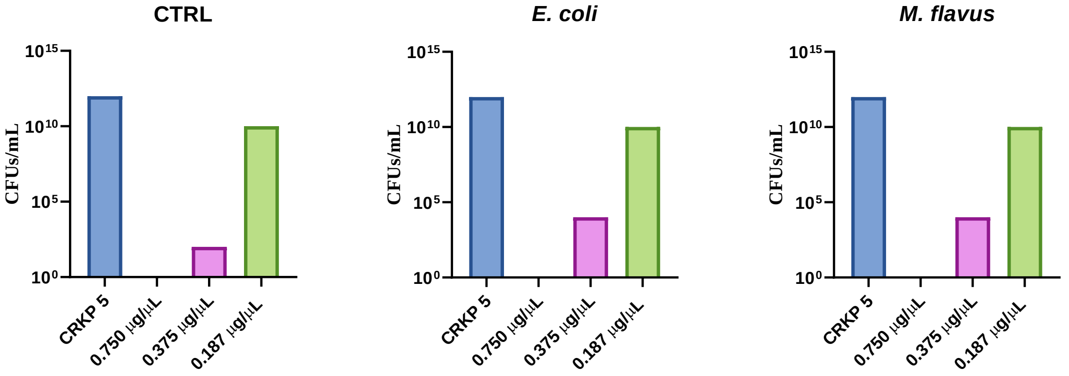


Figure 4.44. Minimum bactericidal concentration against CRKP 5 performed with the peptide fractions obtained via precipitation with organic solvents. CTRL = peptide fractions from uninfected larvae; *E. coli* = peptide fractions from

larvae infected with *E. coli*; *M. flavus* = peptide fractions from larvae infected with *M. flavus*. The first bars represent the untreated CRKP 5 cell culture.

The MBC assay results show that all the conditions analysed exhibit bactericidal activity. Specifically, both the peptide fraction obtained from the hemolymph of uninfected larvae and that obtained from larvae infected with *E. coli* and *M. flavus* have an MBC value of 0.750 µg/mL (Figure 4.44).

4.14.13 Evaluation of the Antibacterial Activity of Peptide Fraction via Agar Diffusion Assay against CRKP 742

The peptide fractions obtained from plasma, precipitated with a methanol/acetic acid/water solution (90:1:9 v/v), from both uninfected larvae and those infected with *E. coli* or *M. flavus*, were initially examined using an agar diffusion assay to assess their antibacterial effect against CRKP 742.

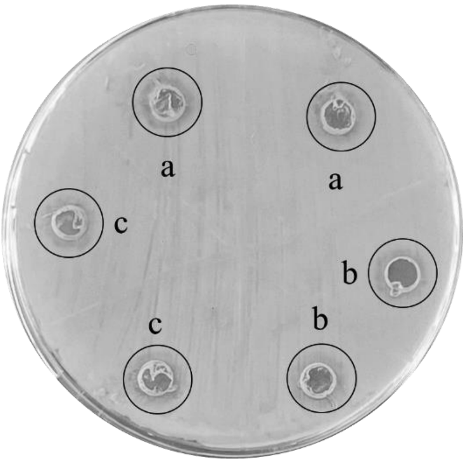


Figure 4.45. Agar diffusion test of peptide fractions obtained via precipitation with organic solvents, performed against CRKP 742. (**a**) peptide fraction from uninfected larvae; (**b**) peptide fraction from larvae infected with *E. coli*; (**c**) peptide fraction from larvae infected with *M. flavus*. The experiments were carried out in triplicate (three independent biological replicates).

The agar diffusion assay results showed inhibition zones for both the peptide fraction extracted from uninfected larvae and the fraction obtained from larvae infected with *E. coli* and *M. flavus* (Figure 4.45). These results suggest that both peptide fractions demonstrated significant antimicrobial activity, as evidenced by the agar diffusion test.

4.14.14 Evaluation of the Peptide Fraction Antibacterial Activity via Microdilution Assay against CRKP 742

The antimicrobial activity of the peptide fraction extracted from the hemolymph of *H. illucens* larvae was evaluated against the CRKP 742 strain using the microdilution assay to determine the minimum inhibitory concentration.

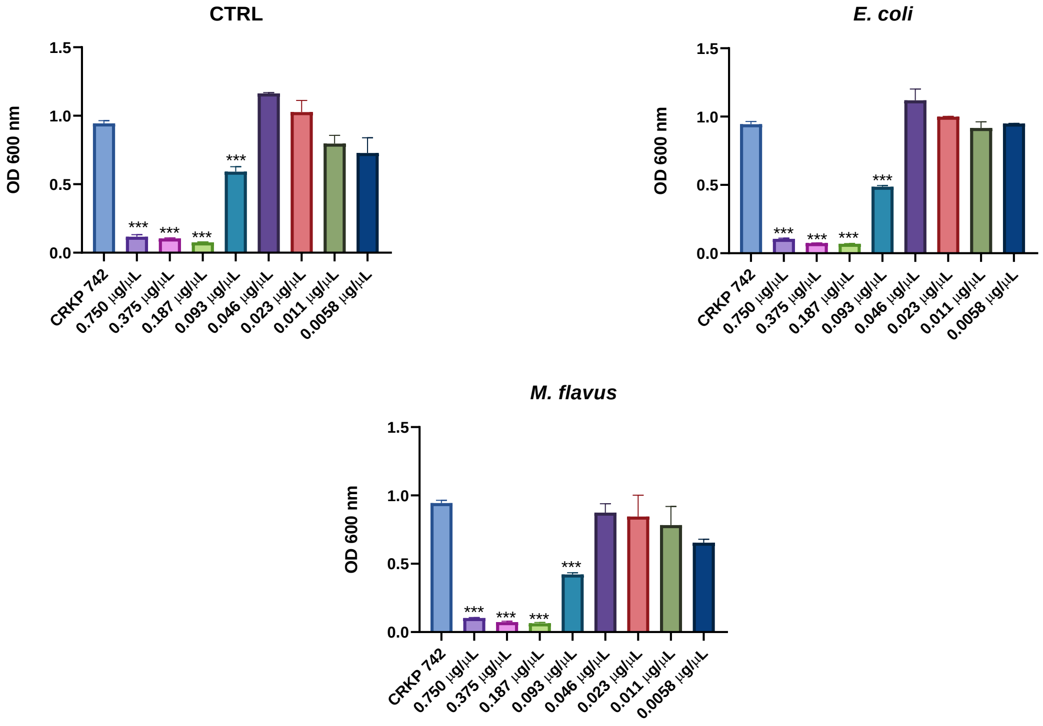


Figure 4.46. Microdilution assay against CRKP 742 performed with the peptide fractions obtained via precipitation with organic solvents. CTRL = peptide fractions from uninfected larvae; *E. coli* = peptide fractions from larvae infected with *E. coli*; *M. flavus* = peptide fractions from larvae infected with *M. flavus*. The first bars represent the untreated CRKP 742 cell culture. Data are expressed as means ± standard error of three independent biological replicates, and comparisons between treatments and control were performed by one-way ANOVA followed by Bonferroni post hoc test (* $p < 0.1$, ** $p<0.001$*** $p < 0.0001$).

The MIC assay results demonstrated antimicrobial activity for all three samples analysed. Specifically, the MIC value for all conditions tested was once again 0.093 µg/mL (Figure 4.46). These findings confirm that the peptide fractions consistently inhibit bacterial growth across all conditions examined.

4.14.15 Evaluation of the Antibacterial Activity of Peptide Fraction via Minimum Bactericidal Concentration Assay against CRKP 742

An MBC assay was performed to determine the minimum bactericidal concentration of the peptide fraction obtained from *H. illucens* larvae, precipitated with organic solvents, against the CRKP 742 strain.

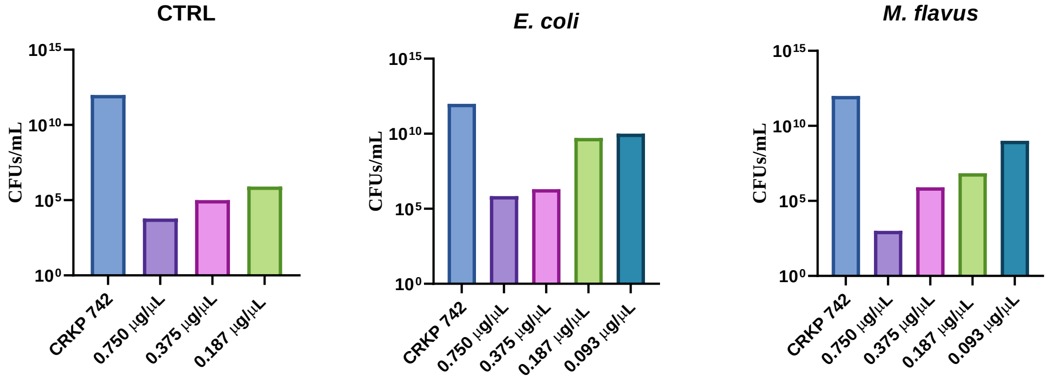


Figure 4.47. Minimum Bactericidal Concentration against CRKP 742 performed with the peptide fractions obtained via precipitation with organic solvents. CTRL = peptide fractions from uninfected larvae; *E. coli* = peptide fractions from larvae infected with *E. coli*; *M. flavus* = peptide fractions from larvae infected with *M. flavus*. The first bars represent the untreated CRKP 742 cell culture.

The MBC assay results showed that none of the analyzed conditions exhibited bactericidal activity (Figure 4.47).

4.14 Evaluation of the Antibacterial Activity of Peptide Fraction against Methicillin-resistant *Staphylococcus aureus*

The antimicrobial activity of the peptide fraction obtained through organic solvent precipitation from the hemolymph of *H. illucens* larvae was analysed against *Staphylococcus aureus*, a Gram-positive pathogen capable of causing a wide range of infections, from mild skin infections to severe systemic diseases. Methicillin-resistant strains, commonly referred to as MRSA (Methicillin-Resistant *Staphylococcus aureus*), belong to the Staphylococcaceae family. MRSA is particularly concerning due to its ability to resist many β-lactam antibiotics, making these infections challenging to treat. It is responsible for several serious infections, including skin and soft tissue infections, pneumonia, endocarditis, osteomyelitis, and sepsis. The growing prevalence of MRSA emphasizes the need for novel antimicrobial agents, and thus, the exploration of peptide fractions from *H. illucens* presents a promising avenue for combating resistant bacterial strains (Pevsner, 2015; Taylor, 2013).

4.14.1 Evaluation of the Antibacterial Activity of Peptide Fraction via Agar Diffusion Assay

The peptide fractions extracted from hemolymph, precipitated using a methanol/acetic acid/water mixture (90:1:9 v/v), from both uninfected larvae and larvae infected with *E. coli* or *M. flavus*, were first analysed through an agar diffusion assay to evaluate their antibacterial activity against MRSA.

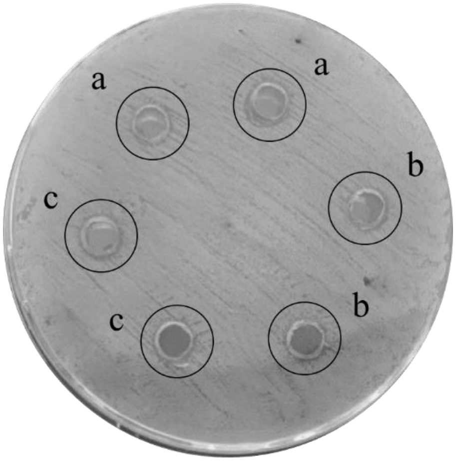


Figure 4.48. Agar diffusion test of peptide fractions obtained via precipitation with organic solvents, performed against MRSA (**a**) peptide fraction from uninfected larvae; (**b**) peptide fraction from larvae infected with *E. coli*; (**c**) peptide fraction from larvae infected with *M. flavus*. The experiments were carried out in triplicate (three independent biological replicates).

The results obtained from the agar diffusion assay showed that none of the three analysed samples produced inhibition zones. This finding was consistent for the peptide fraction extracted from uninfected larvae and for the those obtained from larvae infected with *E. coli* and *M. flavus* (Figure 4.48). The absence of inhibition zones suggests that the tested fractions did not exhibit detectable antimicrobial activity against MRSA under the experimental conditions employed.

4.14.2 Evaluation of the Hemolymph Antibacterial Activity via Microdiluition Assay

A MIC assay was performed on the peptide fraction of hemolymph precipitated with organic solvents, testing it MRSA strains.

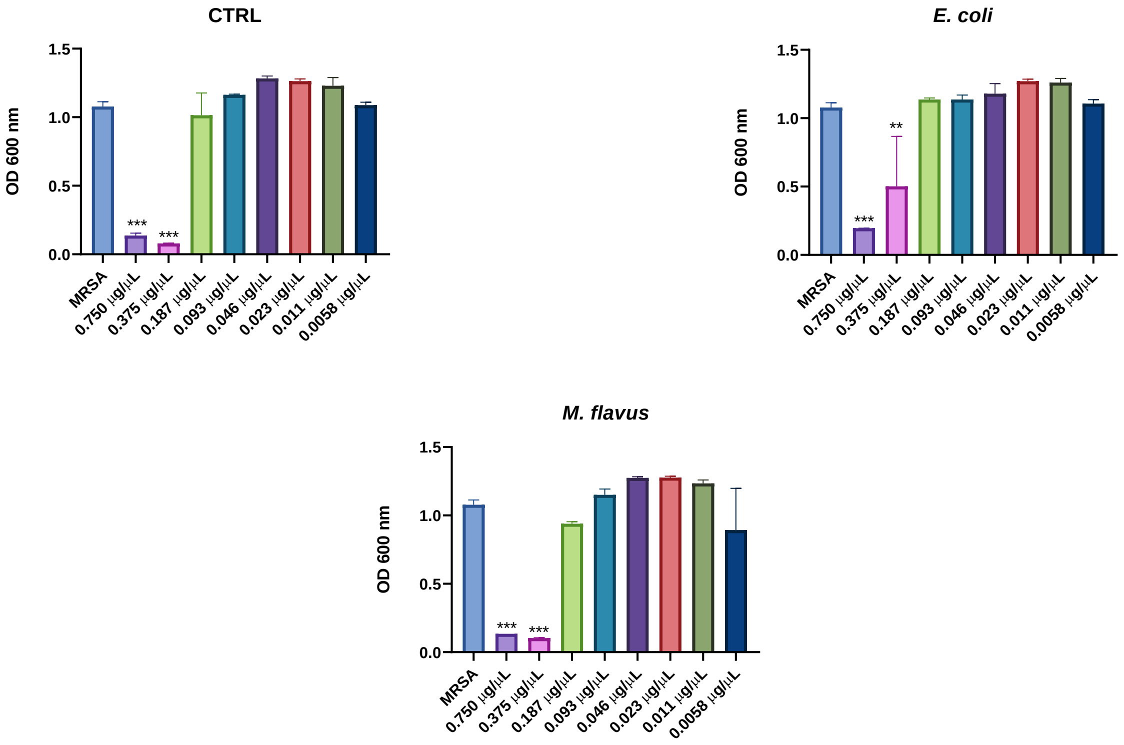


Figure 4.49. Microdilution assay against MRSA performed with the peptide fractions obtained via precipitation with organic solvents. CTRL = peptide fractions from uninfected larvae; *E. coli* = peptide fractions from larvae infected with *E. coli*; *M. flavus* = peptide fractions from larvae infected with *M. flavus*. The first bars represent the untreated MRSA cell culture. Data are expressed as means ± standard error of three independent biological replicates, and comparisons between treatments and control were performed by one-way ANOVA followed by Bonferroni post hoc test (* $p < 0.1$, ** $p<0.001$*** $p < 0.0001$).

The results of the MIC assay demonstrated that all the experimental conditions tested exhibited antimicrobial activity against the bacterial strain. Specifically, the MIC value for all three peptide fractions, including those derived from uninfected larvae and from larvae infected with *E. coli* and *M. flavus*, was found to be 0.375 µg/µL (Figure 4.49). This consistent MIC value across the different samples indicates uniform antimicrobial efficacy, irrespective of the infection status of the larvae from which the peptide fractions were extracted.

4.14.3 Evaluation of the Peptide Fraction Antibacterial Activity via Minimum Bactericidal Concentration Assay

An MBC assay was performed to determine the minimum bactericidal concentration of the peptide fraction obtained from *H. illucens* larvae, precipitated with organic solvents, against the MRSA strain.

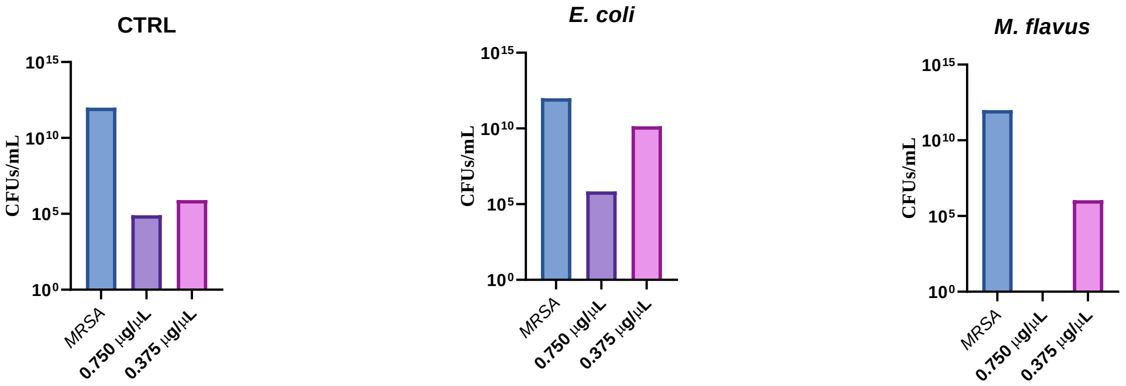


Figure 4.50. Minimum Bactericidal Concentration against MRSA performed with the peptide fractions obtained via precipitation with organic solvents. CTRL = peptide fractions from uninfected larvae; *E. coli* = peptide fractions from larvae infected with *E. coli*; *M. flavus* = peptide fractions from larvae infected with *M. flavus*. The first bars represent the untreated MRSA cell culture.

The results of the MBC assay indicate that bactericidal effects were not observed in all the conditions tested. Only the peptide fraction obtained from the hemolymph of *H. illucens* larvae infected with *M. flavus* showed bactericidal activity, with an MBC value of 0.750 µg/µL, demonstrating its ability to eliminate the bacterial strain at this concentration. In contrast, the peptide fractions derived from uninfected larvae and those infected with *E. coli* did not exhibit bactericidal effects under the same conditions (Figure 4.50).

4.15 Evaluation of the Peptide Fraction Antifungal activity against *C. albicans*

The antifungal activity of the peptide fraction obtained from the hemolymph of *H. illucens* larvae was evaluated against *Candida albicans*, an opportunistic fungus belonging to the Saccharomycetaceae family. Infections caused by *C. albicans* can manifest in various forms, such as oral and vulvovaginal candidiasis, characterized by white plaques and itching, as well as severe systemic infections, particularly in immunocompromised patients. This fungus, normally present in the human body, becomes pathogenic under conditions of immune imbalance or following antibiotic use.

4.15.1 Evaluation of the Peptide Fraction Antifungal Activity via Agar Diffusion Assay

The peptide fractions extracted from hemolymph, precipitated using a methanol/acetic acid/water mixture (90:1:9 v/v), from both uninfected larvae and larvae infected with *E. coli* or *M. flavus*, were first analysed through an agar diffusion assay to evaluate their antibacterial activity against *C. albicans*.

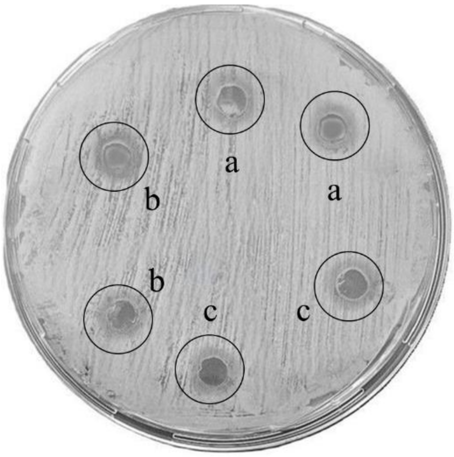


Figure 4.51. Agar diffusion test of peptide fractions obtained via precipitation with organic solvents, performed against *C.albicans* (**a**) peptide fraction from uninfected larvae; (**b**) peptide fraction from larvae infected with *E. coli*; (**c**) peptide fraction from larvae infected with *M. flavus*. The experiments were carried out in triplicate (three independent biological replicates).

The results obtained from the agar diffusion assay show the presence of inhibition zones for all three conditions tested. Specifically, inhibition zones were observed for the peptide fraction extracted from the hemolymph of *H. illucens* larvae, both in the uninfected state and in the fractions obtained from larvae infected with *E. coli* and *M. flavus* (Figure 4.51. The presence of inhibition zones indicates that the tested peptide fractions exhibit antimicrobial activity against the strain of *C. albicans*.

4.15.2 Evaluation of the Peptide Fraction Antifungal Activity via Microdiluition Assay

The minimum inhibitory concentration assay was performed to evaluate the antifungal activity of the peptide fraction obtained by organic solvent precipitation from the hemolymph of *H. illucens* larvae against *C. albicans*. The objective of the MIC determination was to identify the lowest concentration of the peptide fraction capable of inhibiting fungal growth.

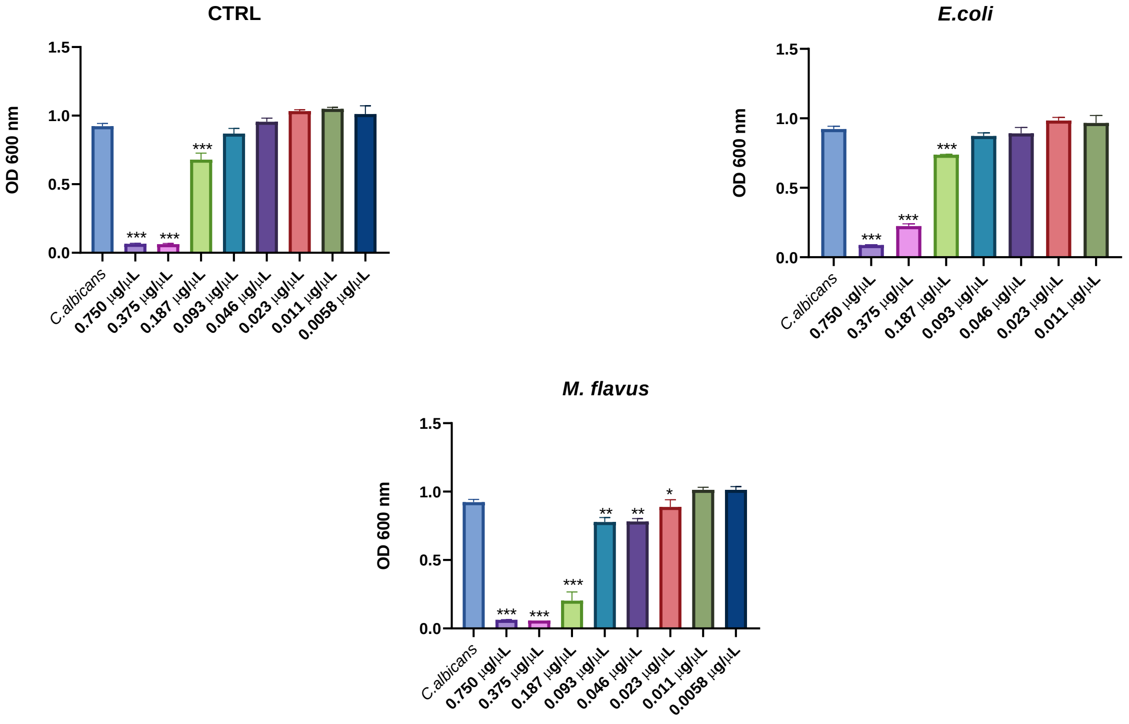


Figure 4.52. Microdilution assay against *C. albicans* performed with the peptide fractions obtained via precipitation with organic solvents. CTRL = peptide fractions from uninfected larvae; *E. coli* = peptide fractions from larvae infected with *E. coli*; *M. flavus* = peptide fractions from larvae infected with *M. flavus*. The first bars represent the untreated *C. albicans* cell culture. Data are expressed as means ± standard error of three independent biological replicates, and comparisons between treatments and control were performed by one-way ANOVA followed by Bonferroni post hoc test (* $p < 0.1$, ** $p<0.001$*** $p < 0.0001$).

The results demonstrated a reduction in *C. albicans* growth under all conditions analyzed. Specifically, the MIC value for the peptide fraction obtained from the hemolymph of control larvae and those infected with *E. coli* was 0.375 µg/µL. In contrast, the MIC value for the peptide fraction derived from the hemolymph of larvae infected with *M. flavus* was 0.187 µg/µL (Figure 4.52).

4.15.3 Evaluation of the Peptide Fraction Antifungal Activity via Minimum Bactericidal Concentration

An MBC assay was performed to determine the minimum fungicidal concentration of the peptide fraction obtained from *H. illucens* larvae, precipitated with organic solvents, against the *C. albiacans* strain.

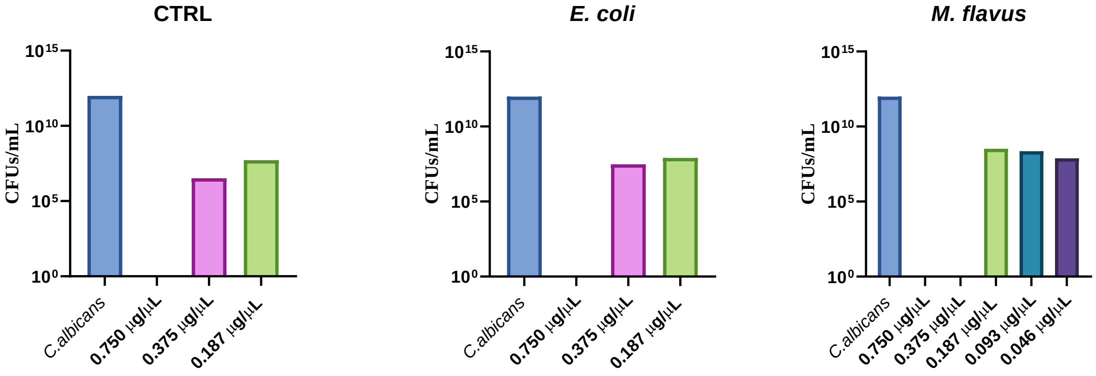


Figure 4.53. Minimum Bactericidal Concentration against *C. albicans* performed with the peptide fractions obtained via precipitation with organic solvents. CTRL = peptide fractions from uninfected larvae; *E. coli* = peptide fractions from larvae infected with *E. coli*; *M. flavus* = peptide fractions from larvae infected with *M. flavus*. The first bars represent the untreated *C. albicans* cell culture.

The MBC assay results show that all the conditions analysed exhibit bactericidal activity against *C. albicans*. Specifically, the MBC value for the fraction precipitated from the hemolymph of control larvae and larvae infected with *E. coli* is 0.750 µg/µL, while the MBC value for the peptide fraction obtained from larvae infected with *M. flavus* is 0.375 µg/µL (Figure 4.53).

4.16 Mass Spectrometry Analysis

After SDS analysis, bands were *in situ* hydrolyzed via trypsin, and the peptide mixtures were analyzed via LC-MS/MS. The raw data from mass spectrometry analysis were converted to mgf files and then inserted into the MASCOT software for protein identification. The protein database used consists of contigs containing putative protein sequences derived from *H. illucens* transcriptomes. Six putative protein sequences, each with a single reading frame, are presented for each contig.

We identified 33 AMPs (Figure 4.54): 20 expressed in all the analysed conditions, 6 absent in control and expressed only after infection with *E. coli* or *M. flavus*, 1 differentially expressed after infection of *E. coli* and 6 differentially expressed after infection with *M. flavus*. The 6 AMPs differentially expressed after the infection of both bacteria were 4 defensins, 1 attacin and 1 uncharacterized protein; the AMPs expressed after *M. flavus* infection were 4 cecropins and 2 defensins, while the differentially AMP expressed after *E. coli* infection was a defensin.

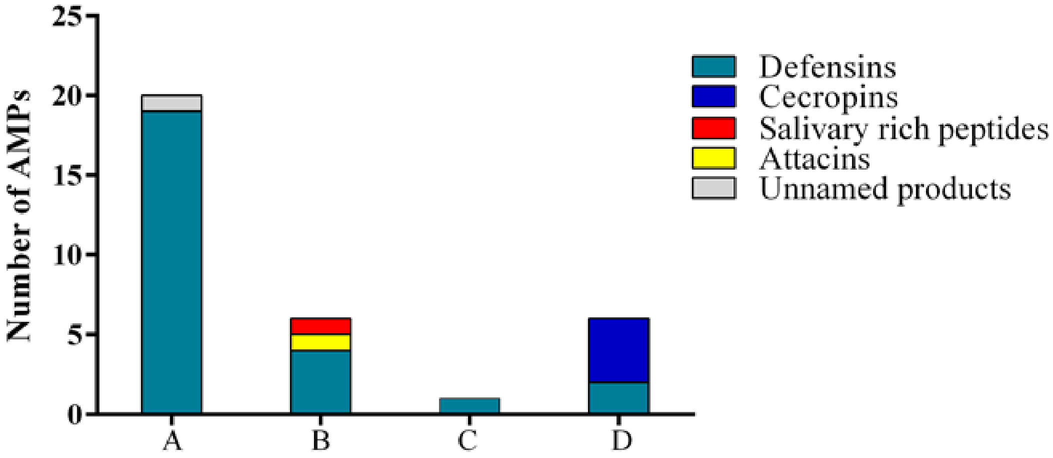


Figure 4.54. Number and classes of AMPs identified via the LC-MS/MS in different experimental conditions: A = peptides identified both in infected and uninfected larvae; B = peptides identified in larvae infected with *E. coli* or *M. flavus*; C = peptide identified exclusively in larvae infected with *E. coli*; D = peptides identified exclusively in larvae infected with *M. flavus***.**

4.17 Solid Phase Peptide Synthesis

Thanks to the collaboration with Professor Papini from the Department of Chemistry "Ugo Schiff" at the University of Florence, two antimicrobial peptides were synthesized successfully. After determining the amino acid sequences of each peptide (Table 2), online software was employed to perform a series of computational analyses, including the calculation of molecular masses, isoelectric points, net charges at pH 7, and estimated water solubility. These parameters were selected due to their relevance in the functional characterization of antimicrobial peptides. The sequence analysis of the two peptides, conducted using the BLAST software (https://blast.ncbi.nlm.nih.gov/Blast.cgi), revealed significant homology with other peptides belonging to the defensin family (Pevsner, 2015). Specifically, the peptide MO_Ab1Lin exhibited over 90% homology with a defensin-like peptide from *H. illucens* and with peptide C13326, also from *H. illucens*. Similarly, peptides MO_Ab4 and MO_Ab4Lin showed over 90% homology with a defensin from *Sitophilus oryzae* and more than 80% homology with a defensin from *Sitophilus zeamais*. These high levels of homology suggest that both peptides may share functional characteristics with these well-known antimicrobial peptides.

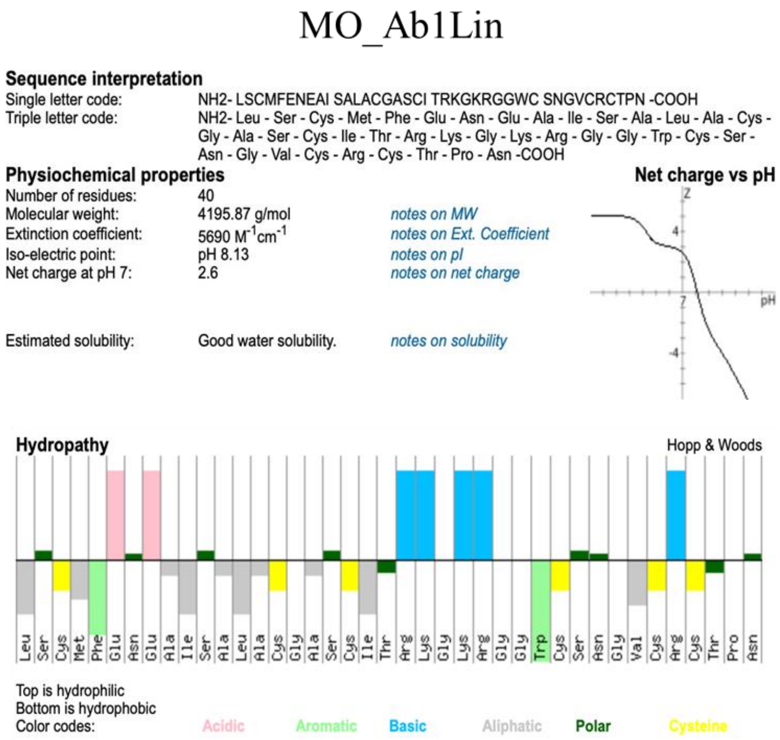
Table 4.2. Peptide sequences with corresponding molecular masses, isoelectric points, and disulfide bond positions. The amino acid sequences of the peptides have been partially masked for confidentiality reasons. The masked portions, indicated by 'X', do not affect the overall understanding of the peptides' properties and characteristics. The complete sequences may be provided upon request or will be disclosed in future publications.

Int. Ref.	**Sequence**	**Disulfide Bridges**	**n° AA**	**MW**	**pI**
MO_Ab1Lin	LSCMFXXXXX SALACXXXXX TRKGKXXXXX SNGVCXXXXX	No	40	4195,87	8,13
MO_Ab4	ATCDLXXXXX KGFKLXXXXX AAHCLXXXXX GGHCNXXXXX VCRN	(3-34) (20-40) (24-42)	44	4624,33	10,53

Table 4.3. Bioinformatics analysis of MO_Ab1Lin and MO_Ab4 peptides

		MO_Ab1LIn	**MO_Ab4**
CAMP$_{R3}$	SVM	0.942	0.952
	RFC	0.825	0.8895
	ANN	AMP	AMP
ANTI FP	Score	-0.15299094	-0.33031345
	Prediction	Non-Antifungal	Non-Antifungal
APD3	Length	40	44
	Total Hydrophobic Ratio	45%	45%
	Total Net Charge	+ 3	+3.5
	Boman Index	1.42 kcal/mol	1.21 kcal/mol

The peptides have been analysed with the CAMP R3 (Collection of Anti-Microbial Peptides) database (https://www.camp.bicni rrh.res.in/) to predict the putative antimicrobial activity and the Antifp (Antifungal peptide Prediction) server (https://webs.iiitd.edu.in/raghava/antifp) to predict their putative antifungal activity. Then their physicochemical properties were evaluated through the Antimicrobial Peptide Database Calculator and Predictor (APD3) (https://aps.unmc.edu/prediction) (Table 4.3).



MO_Ab1Lin

Sequence interpretation

Single letter code:	NH2- LSCMFENEAI SALACGASCI TRKGKRGGWC SNGVCRCTPN -COOH
Triple letter code:	NH2- Leu - Ser - Cys - Met - Phe - Glu - Asn - Glu - Ala - Ile - Ser - Ala - Leu - Ala - Cys - Gly - Ala - Ser - Cys - Ile - Thr - Arg - Lys - Gly - Lys - Arg - Gly - Gly - Trp - Cys - Ser - Asn - Gly - Val - Cys - Arg - Cys - Thr - Pro - Asn -COOH

Physiochemical properties

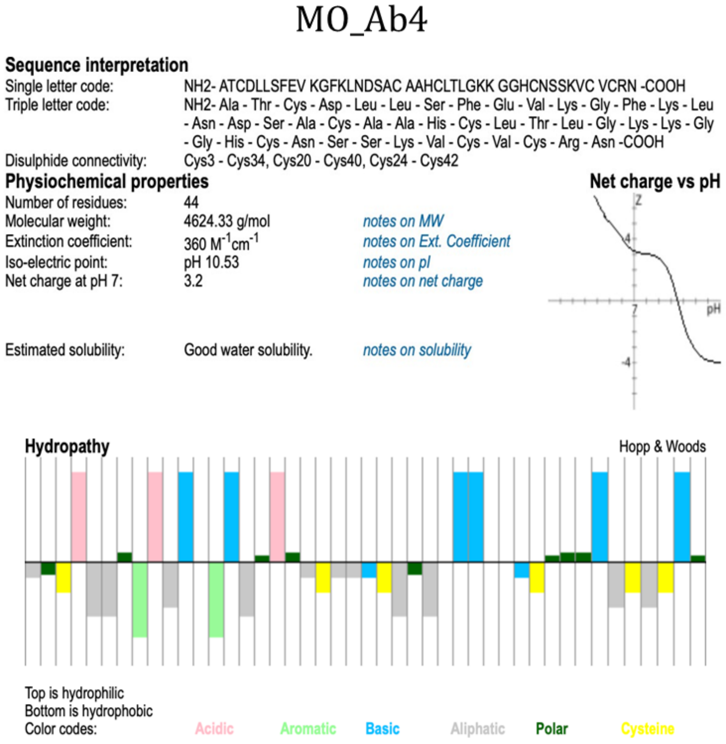
Number of residues:	40	
Molecular weight:	4195.87 g/mol	notes on MW
Extinction coefficient:	5690 $M^{-1}cm^{-1}$	notes on Ext. Coefficient
Iso-electric point:	pH 8.13	notes on pI
Net charge at pH 7:	2.6	notes on net charge
Estimated solubility:	Good water solubility.	notes on solubility

Net charge vs pH

Hydropathy Hopp & Woods

Top is hydrophilic
Bottom is hydrophobic
Color codes: Acidic Aromatic Basic Aliphatic Polar Cysteine

Figure 4.55. Calculation of the molecular mass, isoelectric points, net charges at pH 7, and estimation of water solubility for the peptide MO_Ab1Lin.



MO_Ab4

Sequence interpretation

Single letter code:	NH2- ATCDLLSFEV KGFKLNDSAC AAHCLTLGKK GGHCNSSKVC VCRN -COOH
Triple letter code:	NH2- Ala - Thr - Cys - Asp - Leu - Leu - Ser - Phe - Glu - Val - Lys - Gly - Phe - Lys - Leu - Asn - Asp - Ser - Ala - Cys - Ala - Ala - His - Cys - Leu - Thr - Leu - Gly - Lys - Lys - Gly - Gly - His - Cys - Asn - Ser - Ser - Lys - Val - Cys - Val - Cys - Arg - Asn -COOH
Disulphide connectivity:	Cys3 - Cys34, Cys20 - Cys40, Cys24 - Cys42

Physiochemical properties

Number of residues:	44	
Molecular weight:	4624.33 g/mol	notes on MW
Extinction coefficient:	360 $M^{-1}cm^{-1}$	notes on Ext. Coefficient
Iso-electric point:	pH 10.53	notes on pI
Net charge at pH 7:	3.2	notes on net charge
Estimated solubility:	Good water solubility.	notes on solubility

Net charge vs pH

Hydropathy Hopp & Woods

Top is hydrophilic
Bottom is hydrophobic
Color codes: Acidic Aromatic Basic Aliphatic Polar Cysteine

Figure 4.56. Calculation of the molecular mass, isoelectric points, net charges at pH 7, and estimation of water solubility for the peptide MO_Ab4 and MO_Ab4.

Both peptides demonstrate good water solubility, which was a decisive factor in their selection for chemical synthesis. This property enables efficient synthesis and subsequent use in antimicrobial activity tests, making them strong candidates for developing new peptides with antimicrobial potential (Figure 4.55 and Figure 4.56).

Both peptides were synthesized via solid-phase synthesis using an automated Fmoc/tBu orthogonal protection strategy. This method, widely regarded for its efficiency and precision, facilitates the stepwise assembly of peptides on a solid support. The application of Fmoc (Fluorenylmethyloxycarbonyl) for amino group protection and tBu (tert-Butyl) for side-chain protection ensures that the synthesis proceeds with high fidelity, thereby minimizing side reactions and enabling the production of peptides with accurate sequences and high purity. Following chemical synthesis, high-performance liquid chromatography (HPLC) analysis was conducted on the crude peptides MO_Ab1 Lin (Figure 4.57) and MO_Ab4 (Figure 4.59) using a SUPELCO BIOShell A160 Peptide C18 column (100x3 mm, 2.7 µm). The chromatographic analysis was carried out using a gradient elution ranging from 20% to 70% of solvent B in solvent A over a 10-minute period. The mobile phases employed consisted of 0.1% (v/v) trifluoroacetic acid (TFA) in mQ water (solvent A) and 0.1% (v/v) TFA in acetonitrile (solvent B). The retention time (Rt) for the crude peptide MO_Ab1Lin was 5.93 minutes, whereas the Rt for the MO_Ab4 peptide was 5.47 minutes. Mass spectrometry (MS) analysis was subsequently performed using an ESI Waters ZQ instrument to validate the chromatographic data, which confirmed the molecular weights of the peptides (Figure 4.58, Figure 4.60). The chromatograms and mass spectra are presented below, and the corresponding data are summarized in Table 4.4.

Table 4.4 Data related to crude products.

Int. Ref.	*Rt (min)*	*MW calcd*	*MW osservato*
MO_Ab1 crude	5,93	4195,87	4195,74
MO_Ab4 crude	5,47	4630,37	4630,80

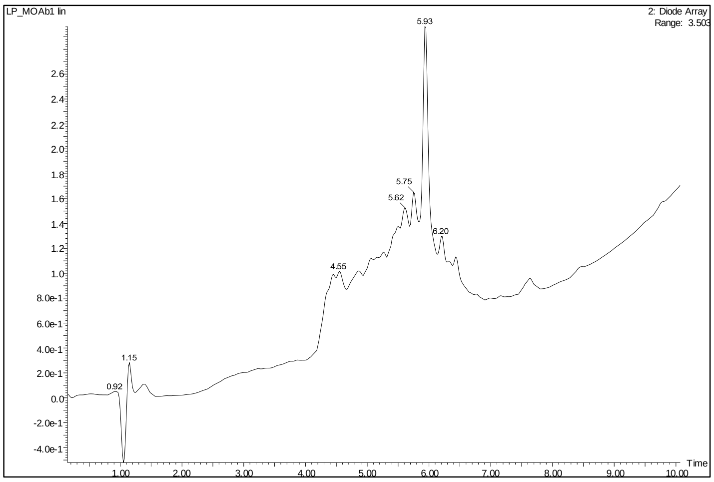


Figure 4.57. Chromatogram of crude MO_Ab1Lin analysed using SUPELCO BIOShell A160 Peptide C18 100x3mm, 2.7μm. Gradient 20%-70% B in A over 10 minutes. A: 0.1% (v/v) TFA in mQ water; B: 0.1% (v/v) TFA in Acetonitrile. Crude MO_Ab1 Rt: 5.94.

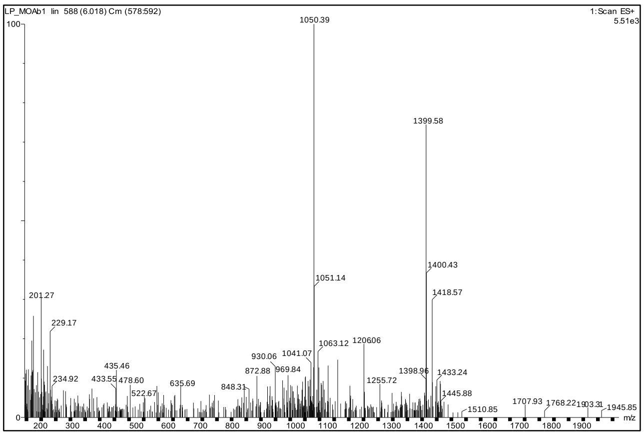


Figure 4.58. Mass spectrum of crude MO_Ab1Lin obtained using an ESI Waters ZQ instrument.

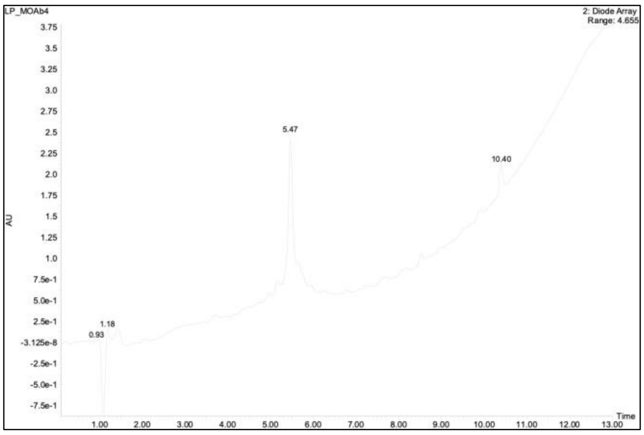


Figure 4.59. Chromatogram of crude MO_Ab4 analysed using SUPELCO BIOShell A160 Peptide C18 100x3mm, 2.7 µm. Gradient 20%-70% B in A over 10 minutes. A: 0.1% (v/v) TFA in mQ water; B: 0.1% (v/v) TFA in Acetonitrile. Crude MO_Ab4 Rt: 5.47.

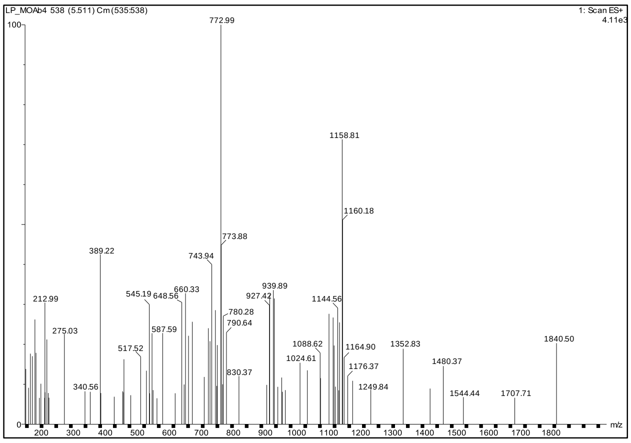


Figure 4.60. Mass spectrum of crude MO_Ab4 obtained using an ESI Waters ZQ instrument.

4.18 Oxidative folding

Following the synthesis, the MO_Ab4 peptide underwent oxidative folding to facilitate the formation of disulfide bonds. The reaction was monitored using LC-MS, which revealed that the chromatographic peak corresponding to the peptide with formed disulfide bonds had a distinct retention time of 5.30 minutes (Figure 4.61). Additionally, the relative molecular mass (Figure 4.62) of the peptide decreased by approximately 6 units, consistent with the loss of three pairs of hydrogen atoms from thiols during the formation of the disulfide bridges, as detailed in the table 4.5. This decrease in mass and shift in retention time indicate successful disulfide bond formation, which is crucial for the peptide's proper folding and stability.

Table.4.5. Data related to the peptide after the oxidative folding process.

Int. Ref.	***Rt (min)***	***MW calcd***	***MW osservato***
MO_Ab4 folded	5,30	4624,33	4624,38

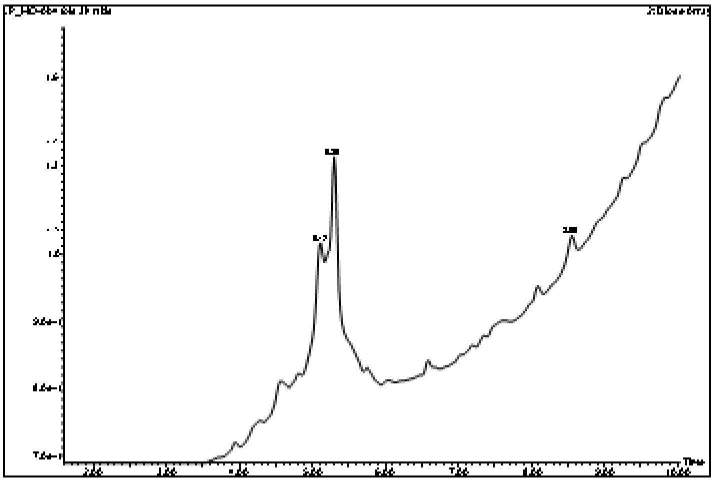


Figure 4.61. Chromatogram of MO_Ab4 after 3 hours of folding, analysed using SUPELCO BIOShell A160 Peptide C18 100x3mm, 2.7µm. Gradient 20%-70% B in A over 10 minutes. A: 0.1% (v/v) TFA in mQ water; B: 0.1% (v/v) TFA in Acetonitrile. MO_Ab4 Rt: 5.30.

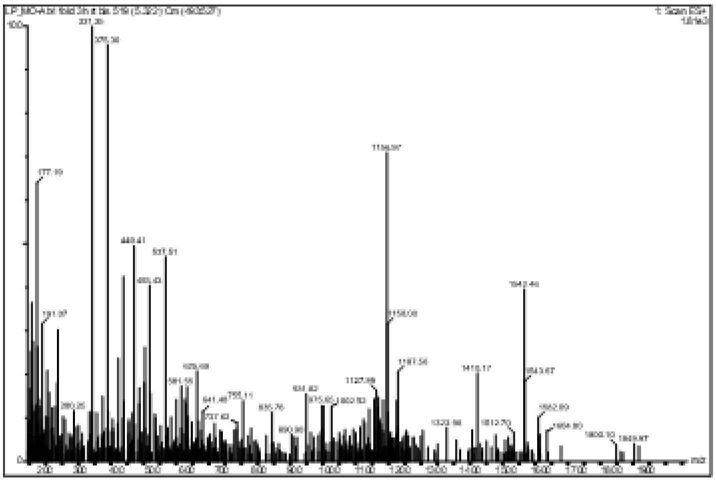


Figure 4.62. Mass spectrum of MO_Ab4 after 3 hours of folding, obtained using an ESI Waters ZQ instrument.

4.19 HPLC purification and characterization

The peptides MO_Ab1 Lin, MO_Ab4 were purified using preparative HPLC with a SUPELCO BIOShell A160 Peptide C18 column (100x3mm, 2.7µm). The purification process employed a gradient ranging from 20% to 70% of solvent B in solvent A over 10 minutes. The mobile phases consisted of 0.1% (v/v) TFA in mQ water (solvent A) and 0.1% (v/v) TFA in acetonitrile (solvent B). The retention time (Rt) for the MO_Ab1Lin peptide was 4.15 minutes (Figure 4.64), while for the MO_Ab4 peptide, it was 4.87 minutes (Figure 66), as confirmed by mass spectrometry analysis using an ESI Waters ZQ instrument (Figure

4.65, Figure 4.67). The final characterization data are presented in Table 4.6, followed by the chromatograms and mass spectra.

Table.4.6. Data related to the final characterization of peptides MO_Ab1Lin and MO_Ab4.

Int. Ref.	*Rt (min)*	*MW calc*	*MW observed*	*Purity*
MO_Ab1Lin	4,15	4195,87	4196,12	98,8
MO_Ab4	3,87	4624,33	4624,32	99,6

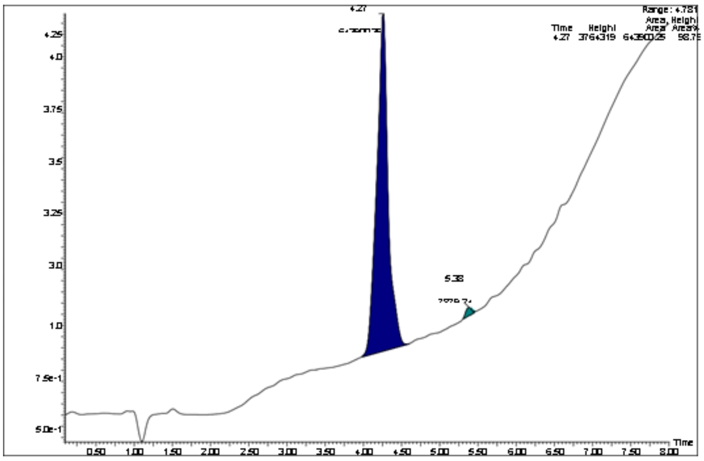


Figure 4.64. Chromatogram of MO_Ab1Lin analysed with SUPELCO BIOShell A160 Peptide C18 100x3mm, 2.7µm. Gradient 20%-70% B in A over 10 minutes. A: 0.1% (v/v) TFA in mQ water; B: 0.1% (v/v) TFA in acetonitrile. MO_Ab1Lin Rt: 4.27.

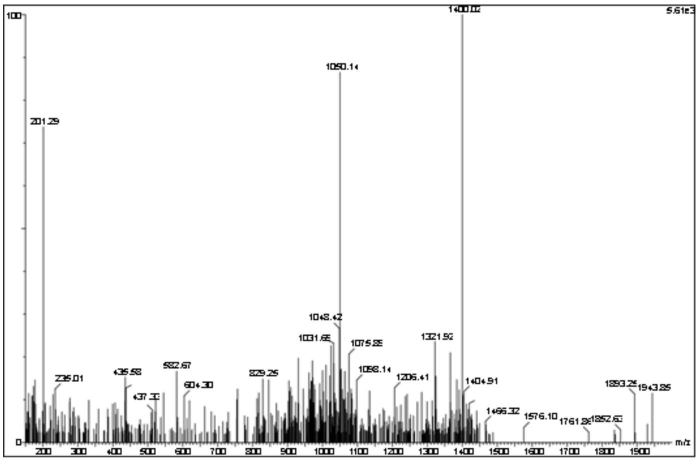


Figure 4.65. Mass spectrum of MO_Ab1Lin obtained using an ESI Waters ZQ instrument.

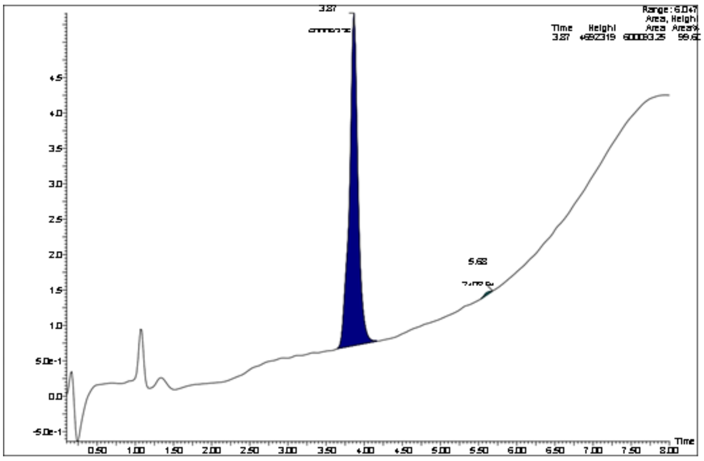


Figure 4.66. Chromatogram of MO_Ab4 analyzed with SUPELCO BIOShell A160 Peptide C18 100x3mm, 2.7 µm. Gradient 20%-70% B in A over 10 minutes. A: 0.1% (v/v) TFA in mQ water; B: 0.1% (v/v) TFA in acetonitrile. MO_Ab4 Rt: 4.87.

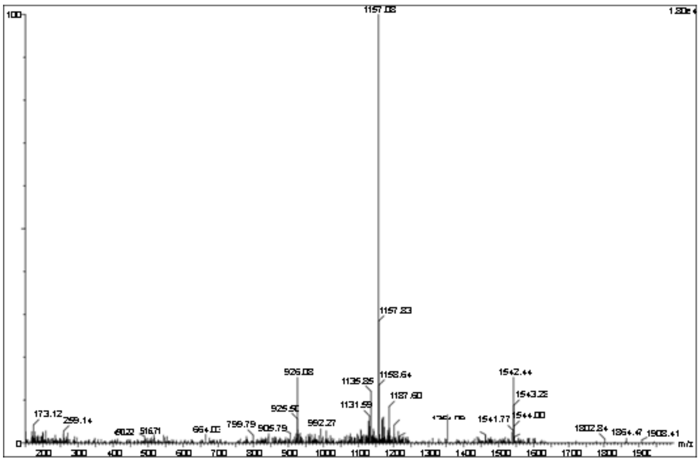


Figure 4.67 Mass spectrum of MO_Ab4 obtained using an ESI Waters ZQ instrument.

4.20 Evaluation of the Antibacterial Activity of Synthetic Peptides via Antibiogram Assay

The antimicrobial activity of the chemically synthesized peptides was evaluated against ten bacterial strains: *E. coli, M. flavus, P. aeruginosa, S. aureus, S. typhimurium,* enteroinvasive *E. coli* (EIEC), *P. mirabilis*, *S. epidermidis*, *E. faecalis*, and carbapenem-resistant *K. pneumoniae* (CRKP). Each peptide was initially resuspended in water at a concentration of 20 mM and then tested using an agar diffusion assay at three concentrations: 4 mM, 2 mM, and 0.2 mM.

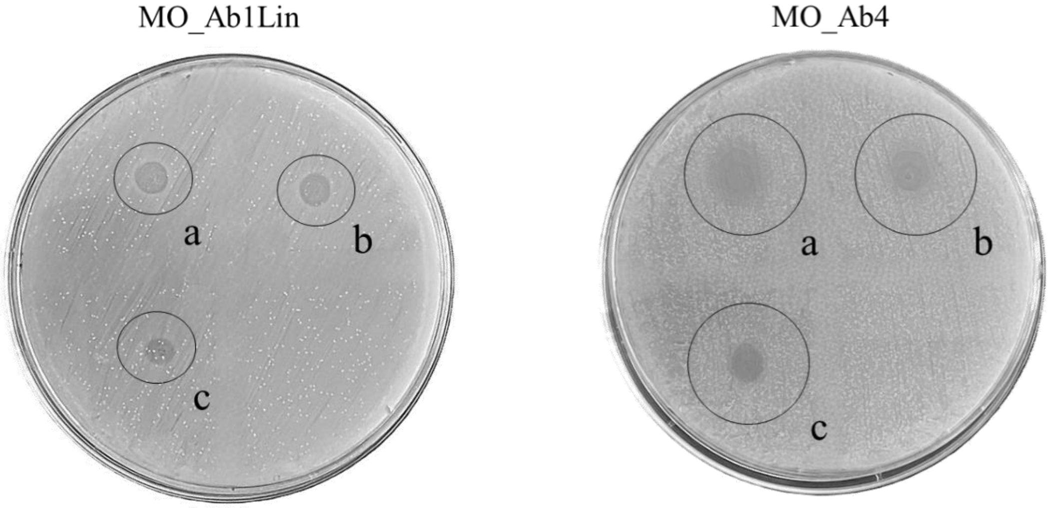


Figure 4.67. Agar diffusion test of synthetic peptide MO_Ab1Lin (on the right) and MO_Ab4 (on the left) performed against *E. coli*. (**a**) peptide at a concentration of 4 mM; (**b**) peptide at a concentration of 2 mM. The experiments were carried out in triplicate (three independent biological replicates).

The results of the agar diffusion assay against *E. coli* revealed the presence of inhibition zones for both peptides at all tested concentrations, indicating clear antimicrobial activity (Figure 4.67). Similarly, inhibition zones were observed for both peptides across all concentrations tested against *M. flavus*, with peptide MO_Ab4 producing significantly larger zones, suggesting a more potent antimicrobial effect (Figure 4.68).

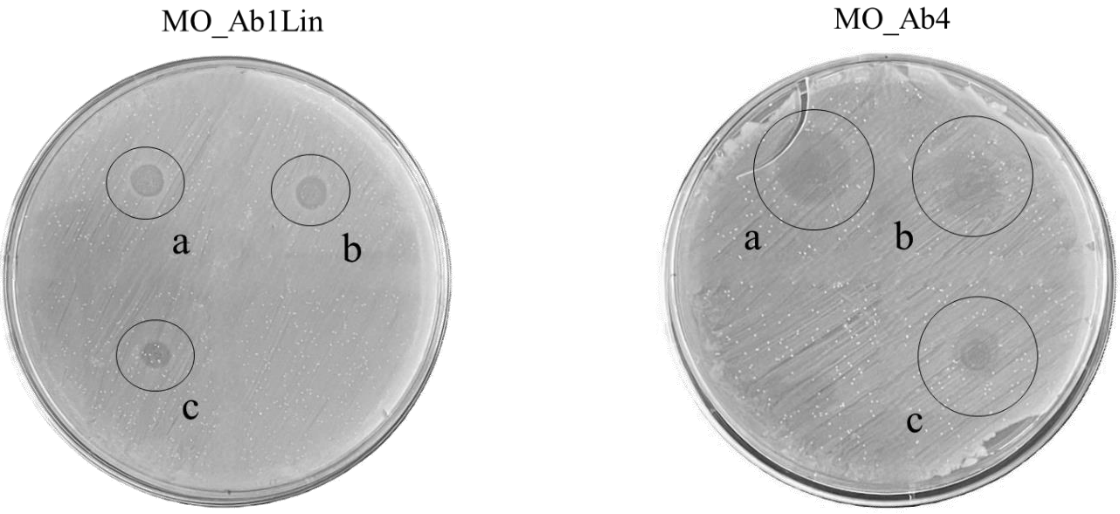


Figure 4.68 . Agar diffusion test of synthetic peptide MO_Ab1Lin (on the right) and MO_Ab4 (on the left) performed against *M. flavus*. (**a**) peptide at a concentration of 4 mM; (**b**) peptide at a concentration of 2 mM; (**c**) peptide at a concentration of 0.2 mM. The experiments were carried out in triplicate (three independent biological replicates).

Subsequent tests were conducted on a series of pathogenic strains, initially using a concentration of 0.2 mM. This concentration, however, did not exhibit antimicrobial activity against any of the strains. Therefore, higher concentrations of 4 mM and 2 mM were tested. The results showed that neither MO_Ab1Lin nor MO_Ab4 produced inhibition zones against *P. aeruginosa*, suggesting a lack of antimicrobial activity against this strain (Figure 4.69). Similarly, no inhibition zones were observed for MO_Ab1 against *S. aureus*, while inhibition zones were noted for MO_Ab4 at both concentrations, indicating selective activity of MO_Ab4 against this pathogen (Figure 4.70).

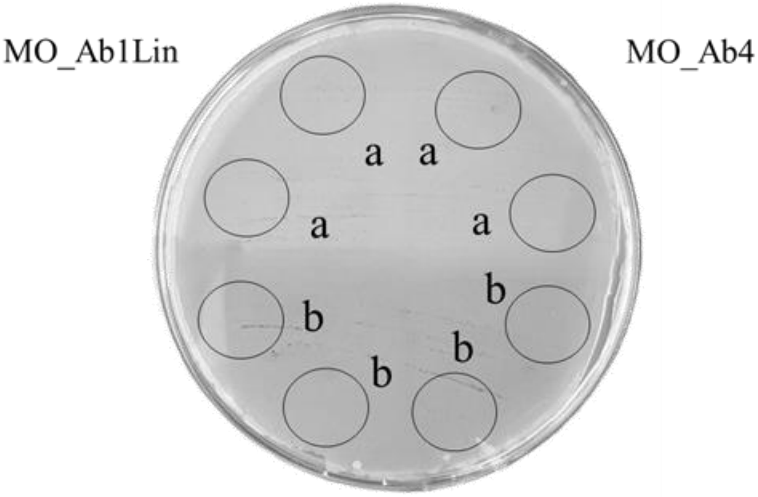


Figure 4.69. Agar diffusion test of synthetic peptide MO_Ab1Lin (on the right) and MO_Ab4 (on the left) performed against *P. aeruginosa.* (**a**) peptide at a concentration of 4 mM; (**b**) peptide at a concentration of 2 mM. The experiments were carried out in triplicate (three independent biological replicates).

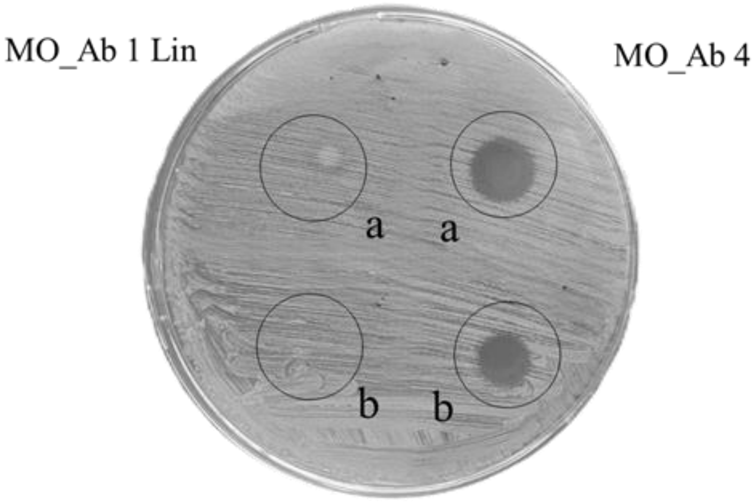


Figure 4.70. Agar diffusion test of synthetic peptide MO_Ab1Lin (on the right) and MO_Ab4 (on the left) performed against *S.aureus* (**a**) peptide at a concentration of 4 mM; (**b**) peptide at a concentration of 2 mM. The experiments were carried out in triplicate (three independent biological replicates).

The results against EIEC confirmed the absence of antimicrobial activity for both peptides, as no inhibition zones were detected (Figure 4.71). In contrast, when tested against *E. faecalis*, MO_Ab4 showed inhibition zones at both concentrations, while MO_Ab1Lin displayed no activity (Figure 4.72). Furthermore, neither peptide demonstrated antimicrobial activity against *S. typhimurium*, as no inhibition zones were observed (Figure 4.73).

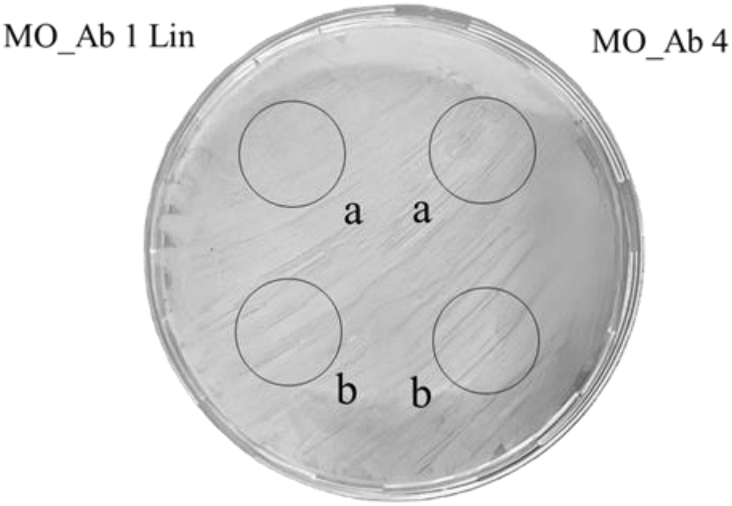


Figure 4.71. Agar diffusion test of synthetic peptide MO_Ab1Lin (on the right) and MO_Ab4 (on the left) performed against EIEC (**a**) peptide at a concentration of 4 mM; (**b**) peptide at a concentration of 2 mM. The experiments were carried out in triplicate (three independent biological replicates).

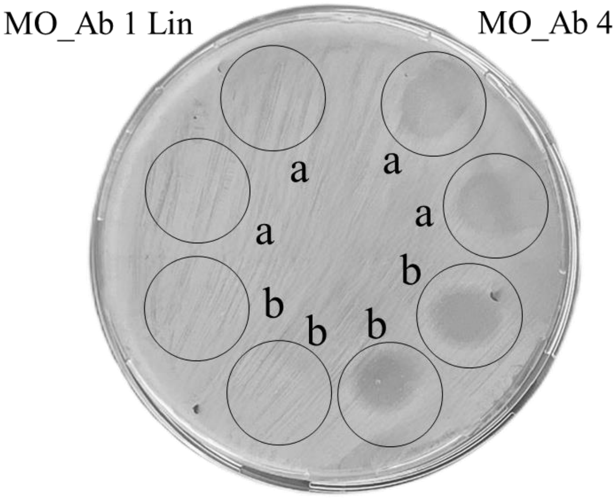


Figure 4.72. Agar diffusion test of synthetic peptide MO_Ab1Lin (on the right) and MO_Ab4 (on the left) performed against *E. faecalis* (**a**) peptide at a concentration of 4 mM; (**b**) peptide at a concentration of 2 mM. The experiments were carried out in triplicate (three independent biological replicates).

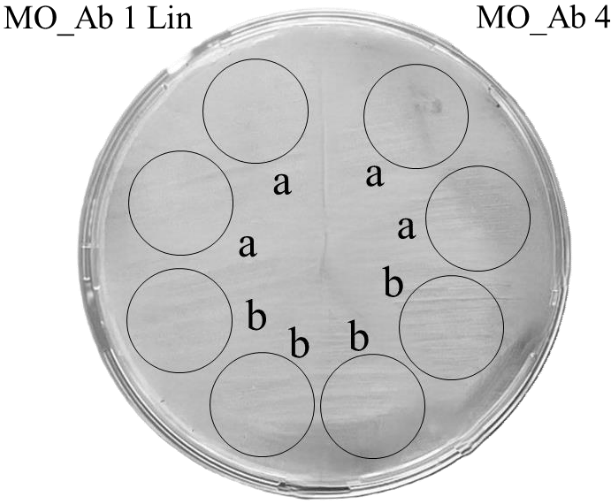


Figure4.73. Agar diffusion test of synthetic peptide MO_Ab1Lin (on the right) and MO_Ab4 (on the left) performed against *S. typhimurium* (**a**) peptide at a concentration of 4 mM; (**b**) peptide at a concentration of 2 mM. The experiments were carried out in triplicate (three independent biological replicates).

The tests conducted on *S. epidermidis* revealed that only the peptide MO_Ab4 consistently produced inhibition zones at both the 4 mM and 2 mM concentrations, indicating significant antimicrobial activity (Figure 4.74). In contrast, no inhibition zones were observed for either peptide when tested against *P. mirabilis*, suggesting a lack of antimicrobial activity against this strain (Figure 4.75). Finally, the peptide MO_Ab4 exhibited activity against CRKP only at the 4 mM concentration, while no activity was detected at the 2 mM concentration (Figure 4.76).

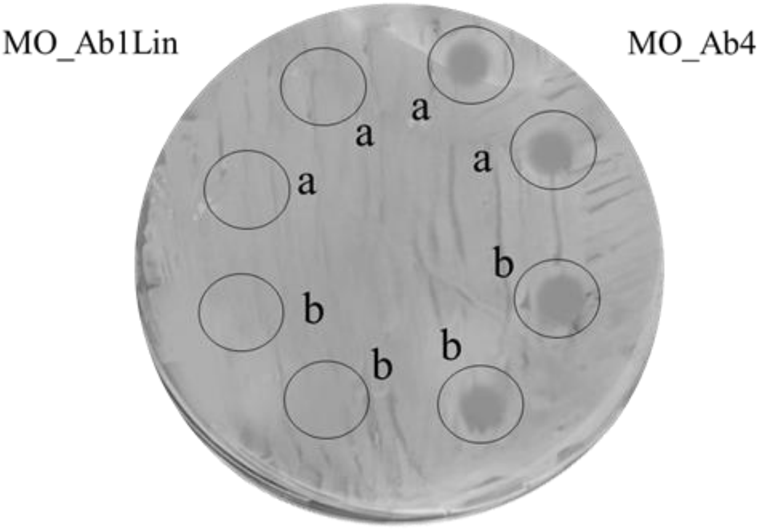


Figure 4.74. Agar diffusion test of synthetic peptide MO_Ab1Lin (on the right) and MO_Ab4 (on the left) performed against *S. epidermidis* (**a**) peptide at a concentration of 4 mM; (**b**) peptide at a concentration of 2 mM. The experiments were carried out in triplicate (three independent biological replicates).

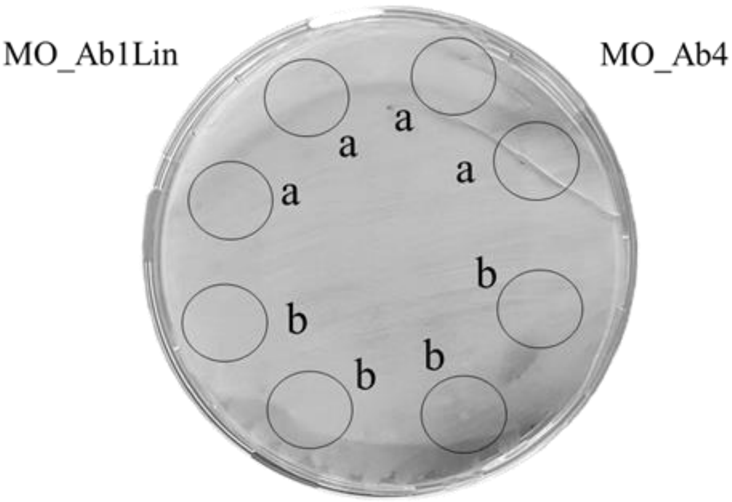


Figure 4.75. Agar diffusion test of synthetic peptide MO_Ab1Lin (on the right) and MO_Ab4 (on the left) performed against *P. mirabilis* (**a**) peptide at a concentration of 4 mM; (**b**) peptide at a concentration of 2 mM. The experiments were carried out in triplicate (three independent biological replicates).

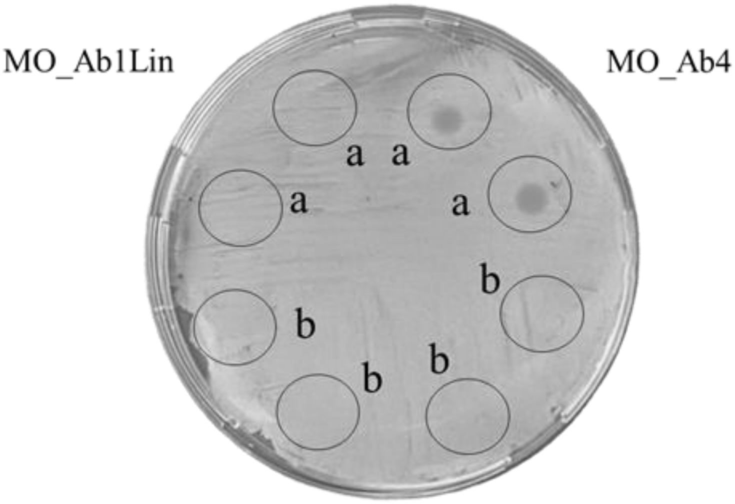


Figure 4.76. Agar diffusion test of synthetic peptide MO_Ab1Lin (on the right) and MO_Ab4 (on the left) performed against CRKP (**a**) peptide at a concentration of 4 mM; (**b**) peptide at a concentration of 2 mM. The experiments were carried out in triplicate (three independent biological replicates).

4.21 Molecular cloning of C-7081 and C-158 genes: Polymerase Chain Reaction products

To clone the gene of interest into heterologous systems, a PCR was performed using cDNA as a template, obtained from the reverse transcription of RNA extracted from *H. illucens* larvae. Primers were designed based on the sequence of the gene of interest. The amplification product was subjected to electrophoresis on an agarose gel at a concentration of 0.8% (Figure4.77). From the gel analysis, a band corresponding to the amplification product of 158 bp can be observed, which corresponds to the sequences of C-7081 and C-158, respectively.

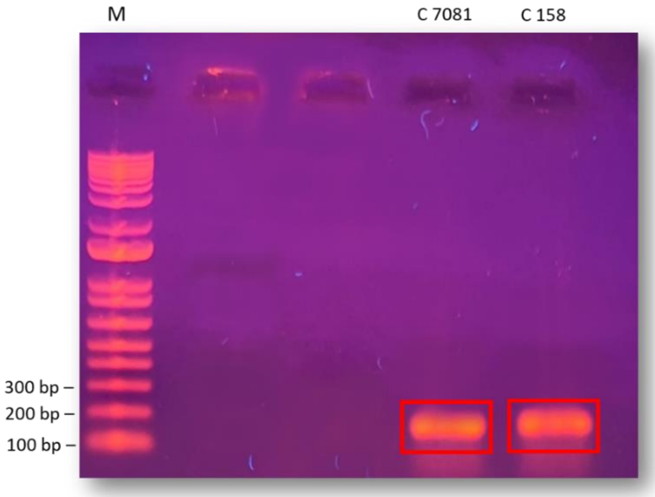


Figure 4.77. Agarose Gel Electrophoresis of inserts encoding for peptides C-7081 and C-158.

4.22 Cloning of C-7081 and C-158 into the pCR2.1-TOPO Vector

The PCR product of fragments C-7081 and C-158 was used for cloning into the pCR2.1-TOPO vector. The transformation was performed in calcium-competent *E. coli* TOP 10 cells, and the selection of transformed colonies was conducted on a culture medium containing ampicillin and X-Gal. The antibiotic prevented the growth of non-transformed bacteria, while X-Gal enabled the identification of white colonies, presumably containing the recombinant plasmid. To confirm successful transformation, a colony PCR was performed on 5 white colonies and 1 blue colony, using insert-specific primers. A negative control was included to verify the accuracy of the reaction. The amplification products were analyzed by electrophoresis on a 0.8% agarose gel. (Figure 4.78 and Figure 4.79).

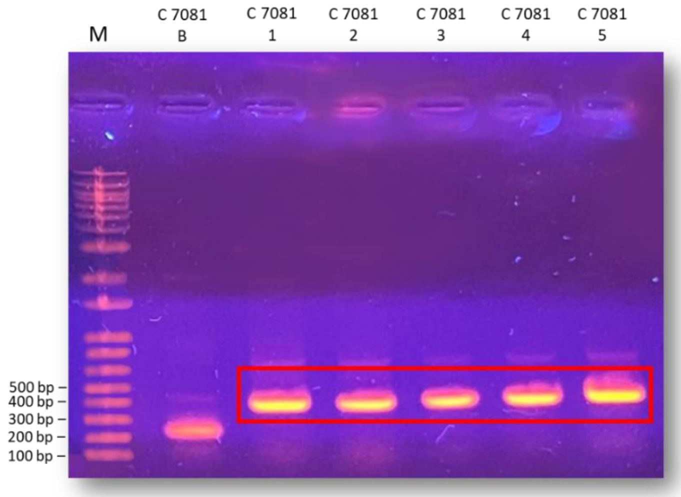


Figure 4.78. Agarose Gel Electrophoresis of Colony PCR: the band a 354 bp is given by the C-7081 trasforming colonies.

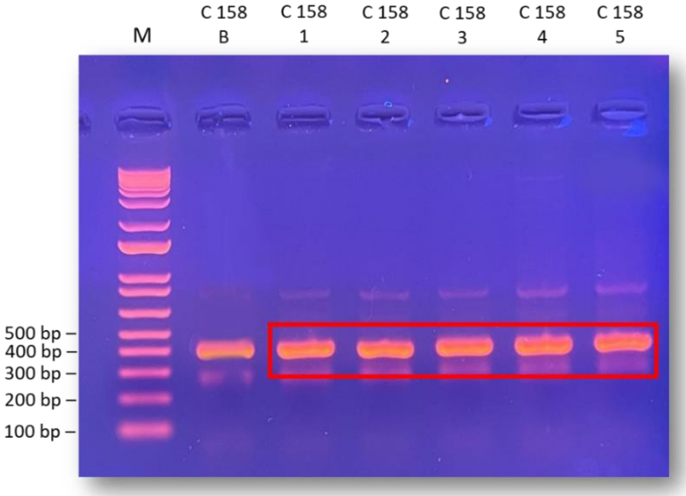


Figure 4.79 . Agarose Gel Electrophoresis of Colony PCR: the band a 354 bp is given by the C 158 transforming colonies.

The gel analysis shows a 354 bp amplification product in all five white colonies. This indicates that the insert is present within the vector.

4.23 Extraction of C-7081-pCR2.1-topo and C-158-pCR2.1-topo via Mini-Prep

Plasmid DNA extraction using a mini-prep was performed on the five colonies that tested positive following colony PCR verification, and one blue colony, it was used as a negative control. Plasmid DNA extracted was quantified using a NanoDrop™ OneC (Thermo Scientific, Waltham, Carlsbad CA, USA), yielding the following values (Table 4.8):

Table 4.7. Concentration of the extracted plasmid DNA

	Concentration (ng/μL)	A 260/280	A 260/230
C 7081 1	244.6	1.95	2.04
C 7081 2	119.7	1.93	1.61
C 7081 3	299.8	1.93	1.99
C 7081 4	54.0	2.08	1.58
C 7081 5	70.1	1.94	1.46
C 158 1	30.0	1.81	0.62
C 158 2	14.3	1.58	1.15
C 158 3	36.6	1.84	0.99
C 158 4	28.7	1.70	0.96

4.24 Analysis by Digestion of C-7081-pCR2.1-TOPO and C-158-pCR2.1-TOPO constructs

The plasmid DNA extracted via mini-prep was digested to isolate the C-7081 and C-158 fragments for ligation into the pPIC9K expression vector. The digestion was carried out using the restriction enzymes EcoRI and NotI (Biolabs, Ipswich, Massachusetts, United

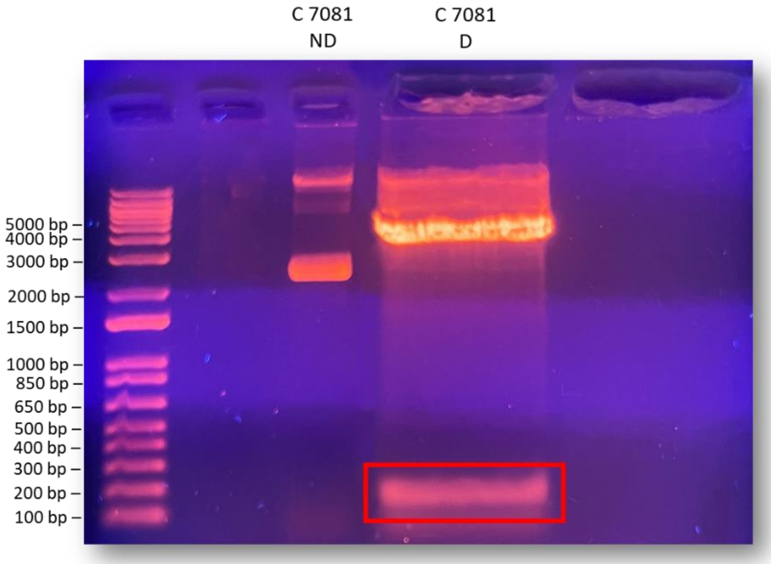


Figure 4.80. Agarose Gel electrophoresis of plasmid containing C7081 insert. Lane 1: not digested plasmid (ND). Lane 2: plasmid after digestion (D) with EcoRI and NotI restriction enzymes

States) and verified on a 0.8% agarose gel, confirming the presence of the inserts (152 bp band) and the vector (3900 bp band) (Figure 4.80, Figure 4.81).

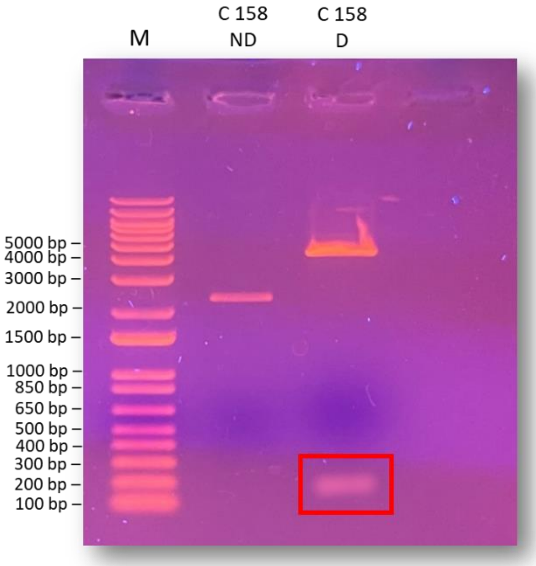
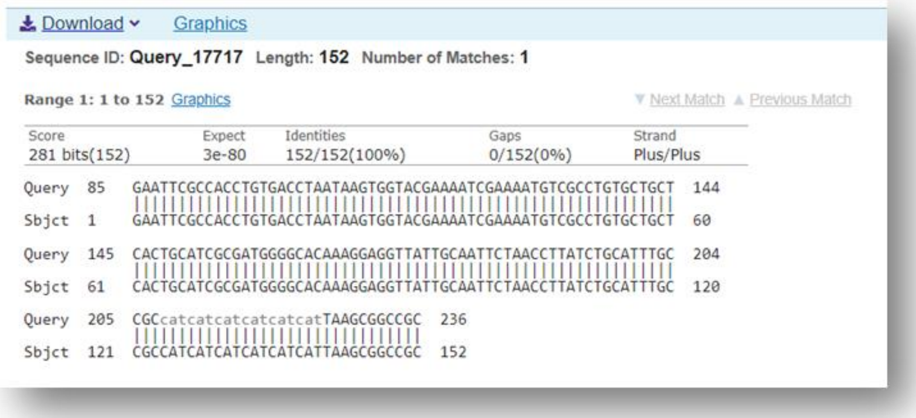


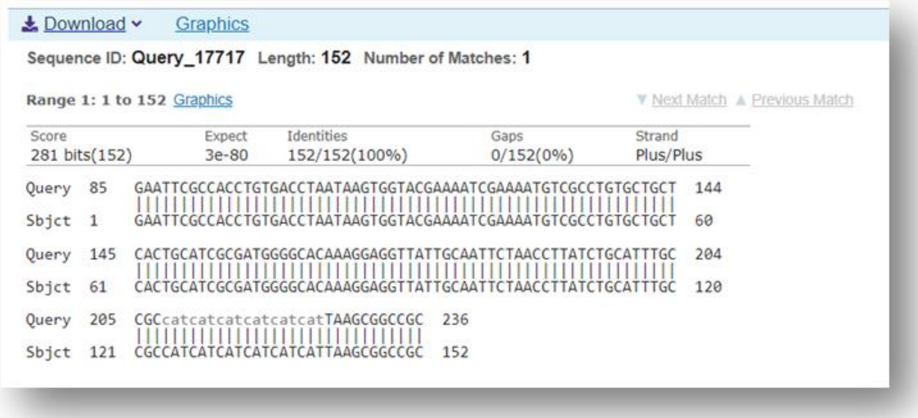
Figure 4.81. Agarose Gel electrophoresis of plasmid containing C158 insert. Lane 1: undigested plasmid (ND). Lane 2: plasmid after digestion (D) with EcoRI and NotI restriction enzymes.

4.25 Sequencing of C-7081-pCR2.1-TOPO and C-158-pCR2.1-TOPO

The constructs C7081-pCR2.1-TOPO and C158-pCR2.1-TOPO were sent to Macrogen Europe (Amsterdam, The Netherlands) for sequencing to evaluate the presence of mutations in the sequences. The alignment between the plasmid DNA nucleotide sequences provided by Macrogen Europe and the insert sequences obtained from the transcriptome showed 100% identity, confirming the absence of mutations in the sequences of interest (Figure 4.82).



```
Sequence ID: Query_17717  Length: 152  Number of Matches: 1

Range 1: 1 to 152 Graphics

Score           Expect  Identities       Gaps        Strand
281 bits(152)   3e-80   152/152(100%)    0/152(0%)   Plus/Plus

Query  85   GAATTCGCCACCTGTGACCTAATAAGTGGTACGAAAATCGAAAATGTCGCCTGTGCTGCT  144
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    GAATTCGCCACCTGTGACCTAATAAGTGGTACGAAAATCGAAAATGTCGCCTGTGCTGCT  60

Query  145  CACTGCATCGCGATGGGGCACAAAGGAGGTTATTGCAATTCTAACCTTATCTGCATTTGC  204
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   CACTGCATCGCGATGGGGCACAAAGGAGGTTATTGCAATTCTAACCTTATCTGCATTTGC  120

Query  205  CGCcatcatcatcatcatcatTAAGCGGCCGC  236
            ||||||||||||||||||||||||||||||||
Sbjct  121  CGCCATCATCATCATCATCATTAAGCGGCCGC  152
```



Sequence ID: **Query_17717** Length: **152** Number of Matches: **1**

Range 1: 1 to 152 Graphics

```
Score            Expect   Identities      Gaps         Strand
281 bits(152)    3e-80    152/152(100%)   0/152(0%)    Plus/Plus

Query  85   GAATTCGCCACCTGTGACCTAATAAGTGGTACGAAAATCGAAAATGTCGCCTGTGCTGCT  144
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    GAATTCGCCACCTGTGACCTAATAAGTGGTACGAAAATCGAAAATGTCGCCTGTGCTGCT  60

Query  145  CACTGCATCGCGATGGGGCACAAAGGAGGTTATTGCAATTCTAACCTTATCTGCATTTGC  204
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   CACTGCATCGCGATGGGGCACAAAGGAGGTTATTGCAATTCTAACCTTATCTGCATTTGC  120

Query  205  CGCcatcatcatcatcatcatTAAGCGGCCGC  236
            ||||||||||||||||||||||||||||||||
Sbjct  121  CGCCATCATCATCATCATCATTAAGCGGCCGC  152
```

Figure 4.82. BLASTn alignment between cloned sequences and sequences from C158 and C7081 sequencing

4.26 Digestion of C-7081-pCR2.1-TOPO and C-158-pCR2.1-TOPO and Purification from Agarose Gel

The C7081-pCR2.1-TOPO and C158-pCR2.1-TOPO constructs were digested to isolate the fragments for ligation into the pPIC9K expression vector. The restriction enzymes used were EcoRI and NotI (Biolabs, Ipswich, Massachusetts, United States). The digestion was verified on an agarose gel at a concentration of 0.8%, where the presence of the fragment (152 bp band) was observed (Figure 4.83).

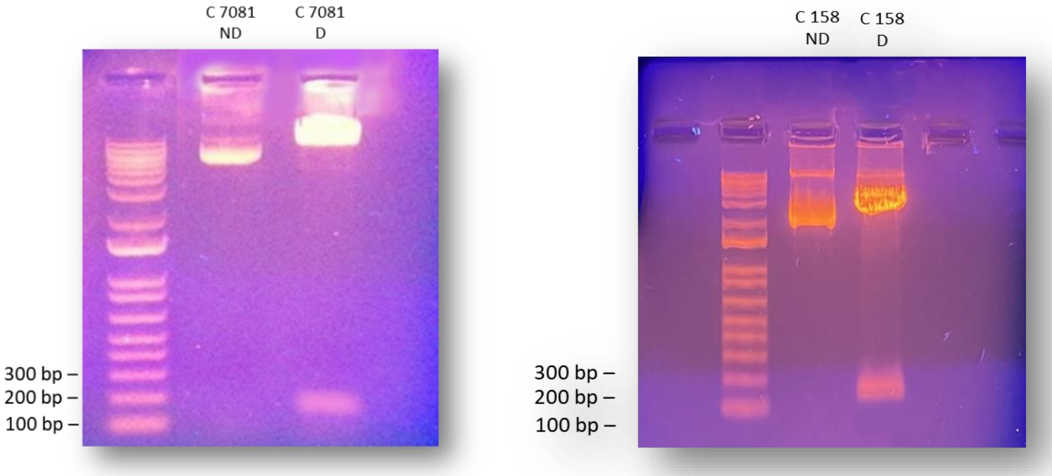


Figure 4.84. Agarose Gel electrophoresis of plasmid containing C-7081 and C-158 inserts. Lane 1: not digested plasmid (ND). Lane 2: plasmid after digestion (D) with EcoRI and NotI restriction enzymes

Subsequently, the inserts C7081 and C158 were purified from the agarose gel by excision of the corresponding band and quantified using a NanoDrop™ OneC (Thermo Scientific, Waltham, Carlsbad CA, USA), yielding the following values (Table 4.8):

Table 4.8. Concentration of the extracted inserts

	Concentration (ng/µL)	**A 260/280**	**A 260/230**
C-7081	19.5.	1.76	0.92
C-158	10.6	1.54	0.82

4.27 Cloning of C-7081 and C-158 into the Expression Vector pPIC9K

The genes encoding the peptides C-7081 and C-158 were subsequently cloned into the pPIC9K expression plasmid vector (Invitrogen). For this purpose, an overnight ligation reaction was set up. The following day, the product of this reaction was used to transform calcium-competent *Escherichia coli* TOP10 cells. To verify the presence of the recombinant vector in the transformed cells, a colony PCR was performed on colonies grown on a selective medium containing ampicillin. The colony PCR was carried out using specific primers for the pPIC9K vector, namely 5' AOX (forward) and α-factor (reverse).

The amplification products were analyzed by electrophoresis on a 0.8% agarose gel (Figure 4.84 and Figure 4.85). Visualization of the bands on the agarose gel confirmed successful transformation in only 4 out of the 19 colonies picked for C-7081 and only 1 out of the 9 colonies picked for C-158. Specifically, it was possible to observe the presence of a band of

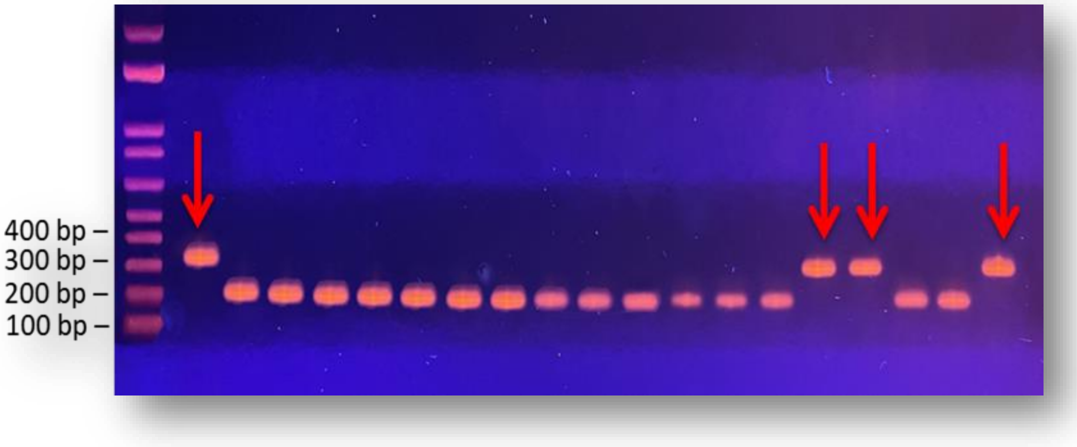


Figure 4.84. Agarose Gel Electrophoresis of Colony PCR: the band a 354 bp is given by the C-7081 transforming colonies.

the expected size of 354 bp, corresponding to the sum of the fragment (152 bp) and the amplified portion of the vector (193 bp).

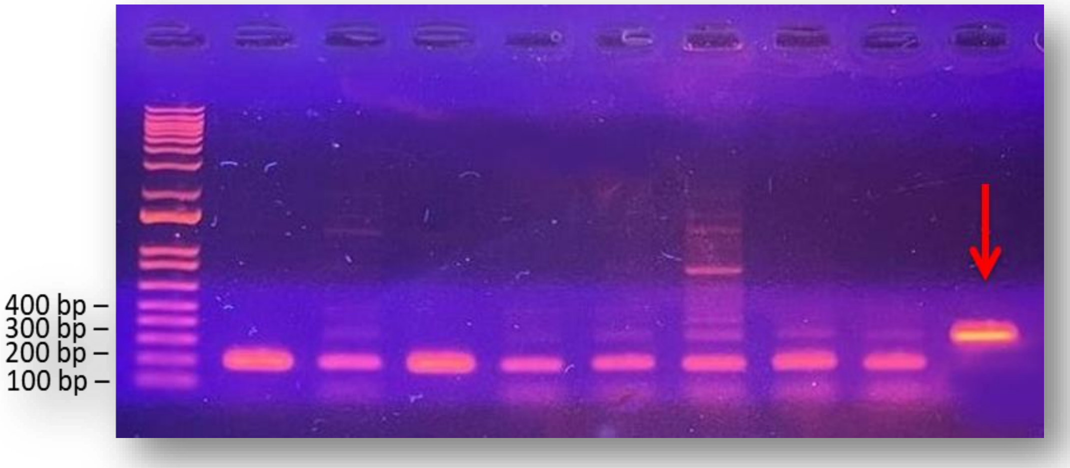


Figure 4.85. Agarose Gel Electrophoresis of Colony PCR: the band a 354 bp is given by the C-158 transforming colonies.

Plasmid DNA extraction using the midi-prep method was performed on the positively identified colonies. The extracted DNA was quantified using a Nanodrop spectrophotometer, and the obtained values are reported in the table below (Table 4.9):

Table 4.9. Concentration of the extracted plasmid

	Concentration (ng/µL)	**A 260/280**	**A 260/230**
C-7081	592.4	1.93	2.31
C-158	815.9	1.93	2.39

4.25 Linearization of C7081-pPIC9K and C158-pPIC9K constructs and transformation

The constructs C7081-pPIC9K and C158-pPIC9K were linearized to enable subsequent transformation into *Pichia pastoris* GS115 yeast cells via electroporation (Figure 4.87). The restriction enzyme used was SacI (Biolabs, Ipswich, Massachusetts, USA). The digestion was verified by electrophoresis on a 0.8% agarose gel, where a single band corresponding to the fully linearized constructs was observed (Figure 4.86).

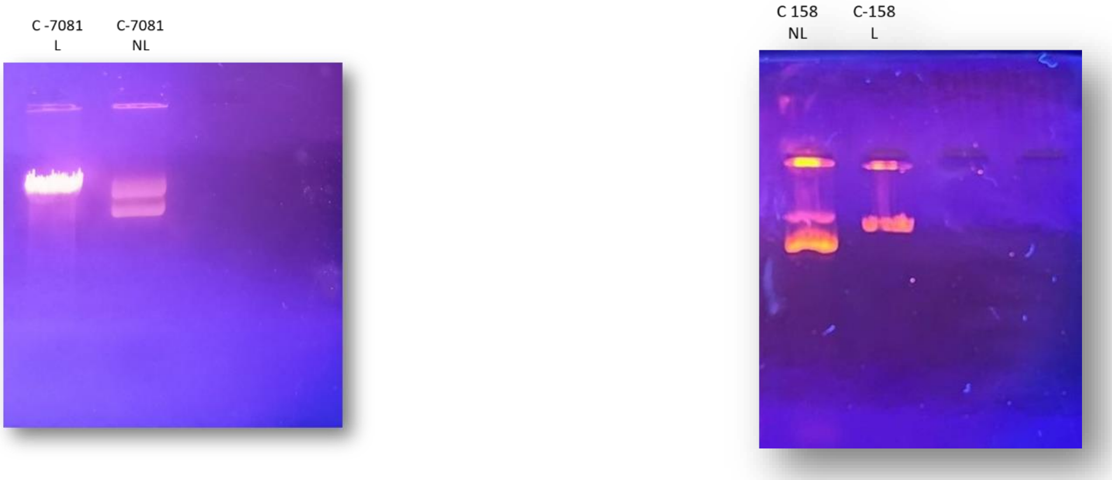


Figure 4.86. Agarose Gel electrophoresis of plasmid containing C-7081 and C-158 inserts. NL: not linearized plasmid. L: plasmid after digestion with SacI restriction enzyme.

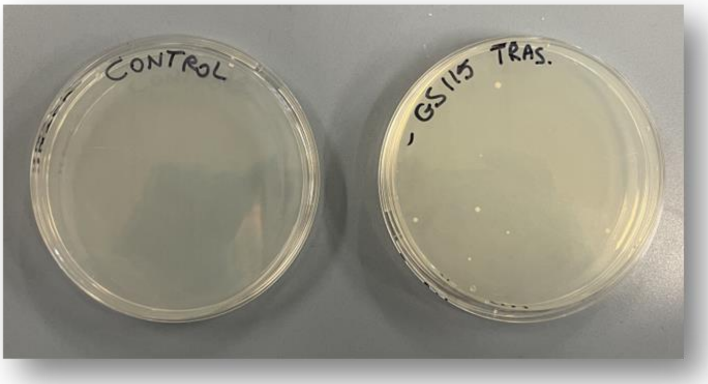


Figure 4.87. Colony screening: the colonies grown have the vector that gives them resistance to the antibiotic used for selection.

The transformed colonies were screened by performing colony PCR using primers specific to the insert and the vector (α-factor and AOX1) and by selecting media supplemented with geneticin (G418).

The colony PCR results show a band at 152 bp corresponding to the amplified insert region. Additionally, a band at 345 bp corresponding to the region amplified between the 5' AOX and α-factor primers and a band at 665 bp corresponding to the region amplified between the 5' AOX and 3' AOX primers are observed (Figure 4.88).

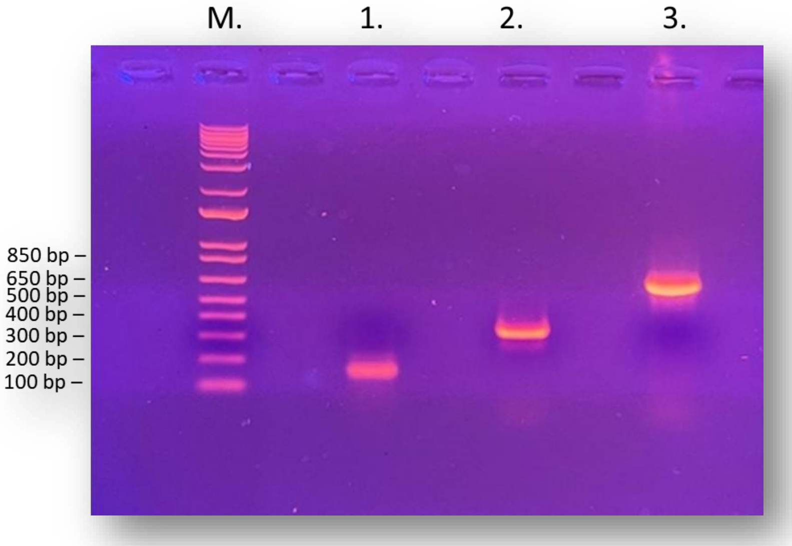


Figure 4.88. Agarose Gel Electrophoresis of Colony PCR: the band a 354 bp is given by the C-158 transforming colonies

5. Discussion

In recent decades, the excessive and inappropriate use of antibiotics in both human and veterinary medicine has accelerated the phenomenon of natural selection for resistant bacteria, resulting in a progressive reduction in the efficacy of many antimicrobial drugs (Calza, 2022; Schwarz & Chaslus-Dancla, 2001). Currently, only a few classes of antibiotics remain effective against certain multi-resistant pathogenic strains, and the global spread of resistance genes represents a significant public health emergency (Bassetti & Righi, 2013). As a result, the search for new molecules with antibacterial activity constitutes one of the most pressing challenges facing the scientific community. In this context, antimicrobial peptides (AMPs) have emerged as a promising alternative to conventional antibiotics (Gordon et al., 2005; Y. Li et al., 2012). AMPs are small cationic molecules that selectively interact with the negatively charged bacterial surface, destabilizing the membrane and inducing cell death (L. Zhang et al., 2016). Insects represent one of the most abundant and diverse sources of AMPs, offering a wide array of molecules and processes that are integral to their immune defense (Rocha et al., 2016). These organisms are highly adaptable, in part due to their innate immune system, which provides them with cellular and humoral responses against various pathogens. Moreover, the fact that insects often inhabit environments contaminated by different microorganisms has driven the evolution of potent defense mechanisms, including AMP synthesis, enabling them to survive under hostile conditions (Sultana et al., 2021).

Insect-derived AMPs thus hold significant potential for combating microorganisms that pose a threat to human health (Rocha et al., 2016; Sultana et al., 2021). Among the most promising insect species is the dipteran *Hermetia illucens*, which produces a significantly higher number of AMPs compared to other insects. Several studies have highlighted *H. illucens* as one of the insects with the largest number of genes coding for AMPs (Van Moll et al., 2022). Specifically, it has been demonstrated that *H. illucens* expresses over 50 genes encoding potential antimicrobial peptides, a number surpassed only by the beetle *Harmonia axyridis*. The wide spectrum of AMPs produced by *H. illucens* is directly related to the remarkable variety of substrates on which the larvae feed, often characterized by a high bacterial load. (Vogel et al., 2018).

In this study, we focused on the *in vitro* evaluation of the antimicrobial activity of peptide fractions extracted from the hemolymph of *H. illucens* following infection with the Gram-negative bacterium *Escherichia coli* or the Gram-positive bacterium *Micrococcus flavus*. Microbiological assays were conducted not only against *E. coli* and *M. flavus*, but also against a selection of human pathogenic strains, including multi-resistant strains such as methicillin-resistant *Staphylococcus aureus* (MRSA) and carbapenem-resistant *Klebsiella pneumoniae*. These strains pose a significant public health threat due to their resistance to traditional antibiotic treatments.

Following precipitation with organic solvents, the samples were subjected to bioautography, which identified a peptide fraction of approximately 10 kDa as responsible for the observed antimicrobial activity. This fraction was isolated from both control samples and those infected with *E. coli* and *M. flavus*, suggesting that the antimicrobial activity of *H. illucens* is not solely infection-dependent, but that the expression of certain AMPs can be induced by specific bacterial stimuli (Zdybicka-Barabas et al., 2017).

Initially, both *Escherichia coli* (Gram-negative) and *Micrococcus flavus* (Gram-positive) bacterial strains were used for the infection and antimicrobial activity analysis. *E. coli*, belonging to the Enterobacteriaceae family, is a bacterium commonly found in the human and animal intestines as a commensal but is also widely distributed in the environment and numerous food products (Kaper et al., 2004). *M. flavus*, on the other hand, belongs to the Micrococcaceae family and is ubiquitous in various environments, including soil, where it plays a key role in organic matter decomposition. This bacterium is also found on human and animal skin, acting as a commensal, but can also be detected in improperly stored food, where it contributes to spoilage (Kocur et al., 1972).

The results of the antimicrobial activity tests conducted against these bacteria demonstrated the efficacy of the AMPs produced by *H. illucens* larvae in response to infections. Data obtained from agar diffusion and microdilution assays conducted against *E. coli* confirmed that the peptide fractions derived from larvae infected with *E. coli* exhibited lower MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) values compared to those derived from larvae infected with *M. flavus*. Specifically, the peptide fraction from *E. coli*-infected larvae showed an MBC of 0.187 µg/µL, significantly lower than the peptide fractions derived from *M. flavus*-infected larvae, which required higher concentrations to achieve bactericidal effects. Similarly, the peptide fractions derived from larvae infected with *M. flavus*, tested against this Gram-positive bacterium, showed an MIC of 0.011 µg/µL and an MBC of 0.046 µg/µL, confirming the larvae's ability to produce specific AMPs in response to infections caused by Gram-positive bacteria.

Several studies in the literature have shown that exposure to Gram-negative bacteria such as *E. coli* can induce the production of specific AMPs that are particularly effective against other Gram-negative bacteria. Components of *E. coli* outer membrane, such as lipopolysaccharides (LPS), have been identified as key factors in triggering the immune response leading to AMP production. This process is crucial for destabilizing and disrupting the membranes of Gram-negative pathogens, thereby enhancing the organism's immune defence against such infections (Ebbensgaard et al., 2018; Ko et al., 2022). Likewise, infection by Gram-positive bacteria such as *M. flavus* has been observed to stimulate the expression of AMPs specifically targeted against other Gram-positive bacteria, suggesting that the innate immune system of organisms is capable of flexibly adapting to the type of bacterial infection, optimizing the defensive response (Mobley et al., 2024).

An interesting result of the present study is the observation that the peptide fraction obtained from the control larvae, which were not infected, still exhibited antimicrobial activity. Previous studies have already highlighted that *H. illucens* larvae produce AMPs as part of their innate immune response, even in the absence of bacterial infection. This constitutive production of AMPs confers a baseline antimicrobial activity to the hemolymph, which can become more pronounced and specific following infections, as demonstrated in our experiments (Azmiera et al., 2023).

The hemolymph samples precipitated with organic solvents, obtained from *H. illucens* larvae infected with *E. coli* and *M. flavus*, were subjected to antimicrobial activity tests against a wide range of pathogenic strains. Among these, *E. coli* enteroinvasive (EIEC), a Gram-negative bacterium known for its ability to penetrate the colonic mucosa, multiply within epithelial cells, and spread, causing inflammation and damage to the intestinal barrier, resulting in clinical manifestations of dysentery, was analyzed (Pasqua et al., 2017). The results showed that the peptide fraction obtained from larvae infected with *E. coli* exhibited greater antimicrobial efficacy against EIEC, as evidenced by a minimum inhibitory concentration (MIC) value of 0.023 µg/µL, which was lower than that of the control samples and those obtained from larvae infected with *M. flavus*. However, an increase in the minimum bactericidal concentration (MBC) was observed, likely due to the high virulence of the pathogenic strains used.

Further tests on *P. aeruginosa* demonstrated that the peptide fraction obtained from uninfected larvae exhibited greater antimicrobial activity compared to those derived from larvae infected with *E. coli* and *M. flavus*, with a MIC of 0.093 µg/µL for the control sample. *P. aeruginosa*, part of the ESKAPE group, is a Gram-negative bacterium known for its antibiotic resistance, and the WHO classifies it as a critical priority pathogen due to its ability to cause severe respiratory and systemic infections, particularly in immunocompromised patients (Moore & Flaws, 2011). Similarly, tests on *S. aureus* revealed consistent antimicrobial activity across all samples, regardless of bacterial stimulation, suggesting the intrinsic robustness of the peptide fraction. *S. aureus* is a Gram-positive bacterium responsible not only for skin infections, such as boils and abscesses, but also for more severe conditions like pneumonia, endocarditis, and sepsis (M. F. P. Lima et al., 2015).

Against *S. typhimurium*, a Gram-negative bacterium known for causing gastrointestinal infections (Galán, 2021), no significant differences were observed between the control and stimulated fractions, although MBC values were lower (0.187 µg/µL). Tests on *S. pyogenes*, a Gram-positive bacterium that causes infections such as pharyngitis and skin diseases (Efstratiou & Lamagni, 2022), demonstrated strong antimicrobial activity, with lower MIC values: 0.046 µg/µL for fractions derived from larvae infected with *M. flavus* and 0.023 µg/µL for those from control larvae and larvae infected with *E. coli*. However, only the highest concentration showed bactericidal activity (0.750 µg/µL for control and *M. flavus*-infected larvae), while no bactericidal activity was observed for the fractions infected with *E. coli*.

Further tests were conducted on *E. faecalis*, a Gram-positive bacterium that is a commensal of the gastrointestinal tract and responsible for urinary tract infections and other nosocomial infections, especially in immunocompromised individuals (Beganovic et al., 2018). Antimicrobial activity tests revealed MIC values of 0.093 µg/µL across all tested conditions and MBC values of 0.375 µg/µL.

Against *S. epidermidis*, a Gram-positive bacterium commonly associated with nosocomial infections related to medical devices (Beganovic et al., 2018), comparable antimicrobial activity was observed, with a MIC of 0.093 µg/µL. However, MBC values varied, with 0.375 µg/µL for control larvae and larvae infected with *E. coli*, and no bactericidal activity for larvae infected with *M. flavus*.

Subsequent tests were conducted on *P. mirabilis*, a Gram-negative bacterium known for causing gastrointestinal infections and also associated with urinary tract infections. This pathogen is frequently implicated in nosocomial infections, particularly in patients with urinary catheters, and is known for its ability to form biofilms and bladder stones (Armbruster et al., 2018). Antimicrobial activity tests revealed MIC values of 0.187 µg/µL, but no bactericidal activity was observed at any tested concentrations.

The antimicrobial tests conducted on these pathogenic strains revealed effective but variable antimicrobial activity in the peptide fractions obtained from both uninfected *H. illucens* larvae and those infected with *E. coli* and *M. flavus*. The lack of enhanced response against Gram-positive or Gram-negative bacteria following stimulation with bacteria of the same category can be attributed to the complexity of the insect immune response. Literature, as highlighted by Bulet et al. (1999) and Hoffmann and Reichhart (2002), indicates that AMP production in insects is not always specific to the type of bacteria that induce the response. Some AMPs exhibit a broad range of activity regardless of the stimulus, and their expression may be regulated by various factors within the innate immune system. These studies suggest that while stimulation with specific bacteria may induce AMP production, their effectiveness is not necessarily increased against the same group of bacteria (Gram-positive or Gram-negative) that initially triggered the response.

The antimicrobial activity of the peptide fraction precipitated with organic solvents obtained from *H. illucens* larvae was also tested against a series of resistant bacterial strains. Specifically, the activity was evaluated against five carbapenem-resistant *Klebsiella pneumoniae* strains, each selected for their distinct resistance profiles, and one methicillin-resistant *S. aureus* (MRSA) strain. These bacterial strains were isolated from the Microbiology Department of the University Hospital of Campania "Luigi Vanvitelli”. The World Health Organization (WHO) and other global health authorities have classified carbapenem-resistant *Klebsiella pneumoniae* (CRKP) and methicillin-resistant *Staphylococcus aureus* (MRSA), both part of the ESKAPE group, as high-priority pathogens due to their increasing resistance to antibiotics. The WHO has placed CRKP on the list of critical priority pathogens that urgently require the development of new antibiotics, as infections caused by CRKP are particularly difficult to treat and can lead to severe clinical outcomes. Similarly, MRSA is recognized as one of the major global public health threats, underscoring the need for effective new therapies to combat these resistant infections (WHO, 2024).

The results of the antimicrobial tests demonstrated that, although the peptide fractions tested were effective against resistant strains, there was significant variability in the susceptibility of the bacteria to the treatment. For example, the CRKP 70131 and CRKP 5 strains exhibited uniform MIC values of 0.187 µg/µL across all tested fractions. However, the MBC values varied: for CRKP 70131, the minimum bactericidal concentration was 0.375 µg/µL, while for CRKP 5, the MBC value increased to 0.750 µg/µL. Other *Klebsiella* strains tested showed a MIC of 0.093 µg/µL, but with highly variable MBC values. For CRKP 72895, for instance, the MBC value for the peptide fraction obtained from uninfected control larvae was 0.750 µg/µL, whereas it decreased to 0.187 µg/µL for the fraction derived from larvae infected with *E. coli*. The fraction obtained from larvae infected with *M. flavus* did not exhibit any bactericidal activity. The CRKP 70461 strain demonstrated MBC values of 0.187 µg/µL for the peptide fractions obtained from the hemolymph of both control larvae and larvae infected with *E. coli*, and 0.375 µg/µL for the fraction derived from larvae infected with *M. flavus*. Finally, for the CRKP 742 strain, none of the tested concentrations exhibited bactericidal activity in any of the samples analyzed. A single bacterial strain can respond differently to antimicrobial treatment due to phenotypic variability within the bacterial population. This phenotypic heterogeneity may arise from differing responses to slightly divergent tissue microenvironments or from genetic circuits where small endogenous fluctuations in a limited number of transcription factors drive gene expression, combined with a positive feedback loop (Weigel & Dersch, 2018). Additionally, bacterial persistence is an example of phenotypic variation, where genetically identical bacterial populations respond heterogeneously to antibiotic treatment. This phenomenon allows a small proportion of cells to survive the treatment even at very high drug concentrations (Gefen & Balaban, 2009; Stewart & Rozen, 2012). Persistence is not related to genetic resistance but rather to phenotypic variations that may be influenced by memory of past environments (Gefen & Balaban, 2009). Finally, bacteria can acquire resistance through *de novo* mutations or by acquiring resistance genes from other organisms. These mechanisms may enable the production of enzymes that degrade the drug, the expression of efflux systems that prevent the drug from reaching its intracellular target, the modification of the drug’s target site, or the production of an alternative metabolic pathway (Tenover, 2006).

The antimicrobial activity results against MRSA show a MIC value of 0.375 µg/µL, consistent across both the peptide fractions from control larvae and those from larvae infected with *E. coli* and *M. flavus*. The minimum bactericidal concentration (MBC) assay did not detect any bactericidal activity in the fractions from control larvae or those infected with *E. coli*, while a bactericidal effect was observed at 0.750 µg/µL in the fraction from larvae infected with *M. flavus*. This suggests that stimulation with Gram-positive strains may enhance the antimicrobial response against bacteria of the same group.

The peptide fractions obtained through organic solvent precipitation were tested for their potential antifungal activity against *C. albicans*. *C. albicans* is a common commensal fungus that can become an opportunistic pathogen, causing infections ranging from superficial to life-threatening systemic candidiasis (Mayer et al., 2013). It can affect various body sites, including the oral cavity, gastrointestinal tract, and vaginal tract (Talapko et al., 2021). The fungus can enter the bloodstream through epithelial damage or medical devices, leading to potential organ infections, particularly in immunocompromised individuals, who are at higher risk for invasive infections with high mortality rates (Sadik et al., 2018).

The results indicate strong antifungal activity against *C. albicans*. The MIC assay shows a minimum inhibitory concentration of 0.375 µg/µL for the peptide fractions from control larvae and larvae infected with *E. coli*, and 0.187 µg/µL for the fraction from larvae infected with *M. flavus*. The MBC values are 0.750 µg/µL for the fractions from control larvae and those infected with *E. coli*, and 0.375 µg/µL for the fraction from larvae infected with *M. flavus*. These findings suggest that stimulation with Gram-positive bacteria enhances the response against *C. albicans*. AMPs from insects, particularly *H. illucens*, show promise as alternatives to conventional antibiotics against various pathogens, including *C. albicans*. Studies have identified numerous AMPs in *H. illucens* with potential antibacterial and antifungal activities (Moretta et al., 2020). These peptides can disrupt cell membranes, inhibit filamentation, and combat biofilms of *C. albicans* (Di Somma, Moretta, et al., 2021; do Nascimento Dias et al., 2020). Synthetic peptides designed to mimic natural AMPs have also demonstrated potent anticandidal effects with low hemolytic activity (Lum et al., 2015; Nikawa et al., 2004). The efficacy of AMPs against *C. albicans* depends on factors such as amphipathicity, cationicity, and helical structure (Karlsson et al., 2006). Combining AMPs with conventional antifungals may enhance their therapeutic potential (Danesi et al., 2002;

Lum et al., 2015). These findings highlight the potential of AMPs, both natural and synthetic, as promising candidates for treating *C. albicans* infections.

Although the microbiological analyses, including antibiograms, bioautography, microdilution assays, and minimum bactericidal concentration assays, constituted the starting point of the experiments, a mass spectrometry analysis was also performed to identify constitutive and inducible AMPs and to analyze differential expression following stimulation with Gram-negative and Gram-positive bacteria. Using a combined transcriptomic and proteomic approach, 20 AMPs were identified as constitutively expressed, whose expression increased after bacterial infection, and 13 as inducible. Bacterial infection significantly stimulated the expression of specific antimicrobial peptides. Specifically, both *E. coli* and *M. flavus* induced the expression of six AMPs, including defensins, attacins, and cysteine-rich peptides, while one defensin was specifically expressed after infection with *E. coli*, and cecropins and defensins were induced by *M. flavus* infection. As expected, defensins were the most detected AMP group (Moretta et al., 2020).

Insect defensins generally exhibit higher activity against Gram-positive bacteria, such as *Staphylococcus aureus* (Di Somma, Moretta, et al., 2021) and *Bacillus subtilis* (Wei et al., 2015), although some also show antimicrobial activity against Gram-negative bacteria, particularly *E. coli* (Di Somma, Moretta, et al., 2021; Hwang et al., 2009). Defensin expression can be induced by both Gram-negative (Dimarcq et al., 1990; Y. Liu et al., 2020; Zhou et al., 2007) and Gram-positive bacteria (Cobo & Chadee, 2013; Dimarcq et al., 1990; Mandrioli et al., 2003), as confirmed by the experiments reported in this study.

Cecropins, α-helical AMPs, are active against both Gram-negative bacteria such as *E. coli*, *K. pneumoniae*, *S. typhimurium*, and *P. aeruginosa*, as well as Gram-positive bacteria such as *Staphylococcus* and *Bacillus* (Jayamani et al., 2015; Toro Segovia et al., 2017; Yang et al., 2011). Their expression can be induced by both Gram-positive and Gram-negative bacteria. For example, in Lepidoptera, different microbial infections result in distinct patterns of cecropin gene expression, suggesting that different signalling pathways may converge to induce the same immune response (Hong et al., 2008).

The results obtained in this study indicate that different AMPs can be induced depending on the bacteria used for infection, as previously reported in models such as *Drosophila melanogaster* (Kleino & Silverman, 2014; Silverman et al., 2009), *Diatraea saccharalis* (Rocha et al., 2016), *Galleria mellonella* (Mak et al., 2010), and *Rhynchophorus ferrugineus*

(Mastore et al., 2015). Rocha et al. demonstrated that infection of *D. saccharalis* larvae with *E. coli* and *B. subtilis* led to an increase in antibacterial activity, evaluated through antibiograms against *B. subtilis*, concomitant with the increased expression of two AMPs, a defensin and an attacin. In contrast, infection with the Gram-negative bacterium resulted in an exclusive increase in attacin levels (Rocha et al., 2016).

Similarly, Mak et al. showed that *G. mellonella* larvae infected with *E. coli* and *Micrococcus luteus* exhibited greater antimicrobial activity against *E. coli* when infected with the Gram-negative bacterium. HPLC analysis of the expressed peptides revealed that infection with *E. coli* led to a higher concentration of peptides, which was also observed in this study. The most stimulated peptides included a proline-rich peptide, a cecropin-d-like peptide, and an anionic peptide-3, the latter also stimulated by Gram-positive bacterial infection (Mak et al., 2010).

In a similar manner, Meghashree et al. demonstrated that infection with *E. coli* and *S. aureus* in *D. melanogaster* and *D. ananassae* larvae led to an increase in protein concentration in the hemolymph and stronger antimicrobial activity compared to uninfected larvae. HPLC and SDS-PAGE analyses of high molecular weight proteins revealed differential expression of induced peptides: three peptides were more expressed in *D. ananassae* and two in *D. melanogaster* after infection with *E. coli* and *S. aureus*. LC-MS/MS analysis identified a cecropin as the main peptide induced following infection with *E. coli* (Meghashree & Nagaraj, 2021). However, as reported by Meghashree et al. , the effectiveness of non-induced AMPs is not always easily detectable. In contrast to this study, experiments on *Periplaneta americana* showed that non-induced hemolymph exhibited no activity against either Gram-positive or Gram-negative bacteria, whereas induced hemolymph displayed strong activity against *Micrococcus luteus*, but less activity against *E. coli* (Basseri et al., 2016).

An analysis of the antimicrobial activity of two synthetic peptides was performed, selected based on their potential antimicrobial activity and water solubility. The peptides studied were MO_Ab1Lin, with a linear structure, and MO_Ab4, characterized by the presence of three disulfide bridges located between amino acids 3 and 34, 20 and 40, 24 and 42. Sequence analysis revealed a high degree of homology with the defensin family, suggesting that both peptides may share functional characteristics with these well-known antimicrobial peptides.

The antimicrobial activity of the peptides was assessed using an agar diffusion assay against a series of bacterial strains, employing three different concentrations: 4 mM, 2 mM, and 0.2 mM. The results showed that both peptides were active against *E. coli* and *M. flavus* at all three tested concentrations. However, when the peptides were tested against a broader range of pathogenic strains, it was observed that the 0.2 mM concentration did not exhibit any significant antimicrobial activity against any of the tested strains. At higher concentrations (4 mM and 2 mM), a clear difference between the two peptides emerged: while MO_Ab1Lin showed no antimicrobial activity, MO_Ab4 demonstrated effective, selective antimicrobial activity against Gram-positive bacteria. These findings suggest that the conformation stabilized by the disulfide bridges in the MO_Ab4 peptide may play a crucial role in its antimicrobial activity, conferring specificity towards Gram-positive bacteria and making it a potential candidate for the development of new antimicrobial agents.

Defensins are antimicrobial peptides that play a crucial role in innate immunity against various microorganisms, including bacteria, fungi, and viruses (Kübler et al., 2006; Lehrer et al., 1993). While initially thought to be primarily active against Gram-positive bacteria, recent research has revealed hidden anti-Gram-negative potential through strategic deletions: the results show that a 14-amino acid segment, derived from an AMP of insect origin, exhibits antimicrobial activity even against Gram-negative bacteria (Gao et al., 2024). While initially thought to act primarily through membrane disruption, recent studies have revealed more specific mechanisms of action (Sahl et al., 2005). For instance, oyster defensins selectively inhibit cell wall biosynthesis in Gram-positive bacteria by binding to lipid II (Schmitt et al., 2010). Human β-defensin-3 analogs show varying activities against different bacterial types, with structural elements influencing their selectivity (Sudheendra et al., 2015). Defensins can also interact with specific lipid receptors in microbial membranes, leading to targeted antimicrobial effects (Wilmes et al., 2011). The complex cell envelope of Gram-positive bacteria, including teichoic acids and peptidoglycan, influences defensin activity and specificity (Malanovic & Lohner, 2016).

Recent studies have enabled the identification of putative AMP sequences in *Hermetia illucens* through a combination of transcriptomic and proteomic approaches. This significant finding served as the foundation for the selection of two peptides, which were subsequently produced using recombinant DNA technology. The two peptides, designated C7081 and

C158, were identified as defensins and selected based on their pronounced antimicrobial activity, as revealed by bioinformatic analyses.

For the production of these peptides, a yeast expression system using *Pichia pastoris* was employed. This eukaryotic organism is particularly suitable for this purpose as it allows for post-translational modifications of the expressed proteins, such as the formation of disulfide bonds, which are characteristic of defensins. Although the two peptides were successfully cloned, they have not yet been expressed due to challenges encountered with the expression protocol. Specifically, issues with optimizing the expression conditions in *Pichia pastoris* prevented the successful expression of the peptides. As a result, one of the next steps in the project will focus on optimizing the expression protocol to ensure the proper expression and folding of the peptides.

Despite this limitation, these results represent an important step forward in the large-scale production of peptides with potential applications in the treatment of bacterial infections, including those caused by antibiotic-resistant strains. Once expressed, these peptides could constitute a new class of antimicrobial agents capable of effectively addressing infections caused by resistant pathogens, opening new avenues for antimicrobial therapy.

Further exploration of the AMP production in this species remains essential, especially in the context of expressing recombinant peptides like C-7081 and C-158.

Both natural and synthetic AMPs have demonstrated promising antibacterial activity against various pathogens while exhibiting minimal toxicity toward mammalian cells. This reduced cytotoxicity is a key factor in their potential development as therapeutic agents. Notably, the structure and composition of AMPs significantly influence their cytotoxic profile. Research indicates that the cationic nature of these peptides enhances their antimicrobial efficacy without proportionally increasing toxicity toward eukaryotic cells, likely due to the differences in membrane composition between bacterial cells and mammalian cells. Bacterial membranes are predominantly negatively charged, whereas human cells contain zwitterionic phospholipids and cholesterol, which may account for this selectivity (Monincová et al., 2014).

Several studies have demonstrated that certain AMPs exhibit minimal cytotoxicity while retaining potent antimicrobial properties. For example, Magainin II, a well-characterized AMP, has been shown to have low cytotoxic effects on murine and human fibroblast cell lines, highlighting its potential for therapeutic use with minimal risk to human cells (Jaradat & Al-Zeer, 2024). Similarly, peptides derived from the marine mollusk *Nerita versicolor* have been evaluated for their low toxicity in human cell lines, reinforcing the potential of these molecules as safe and effective antimicrobial agents (Rodriguez et al., 2023).

Insect-derived AMPs, in particular, have shown low cytotoxicity in studies involving human cell lines. Narayanan et al. (2014) demonstrated that peptides like pyrrhocoricin exhibit high selectivity and low toxicity, making them strong candidates for drug development. Further, Lee et al. (2021) highlighted that the insect-derived peptide poecilocorisin-1 did not exhibit toxicity in human epithelial cells while demonstrating significant anticancer activity in melanoma cell lines.

Recent findings on *H. illucens*-derived AMPs underscore their selective action against pathogenic bacteria without adversely affecting human cells. Moll et al. (2022) demonstrated that certain AMPs from *H. illucens* showed no hemolytic activity or cytotoxicity against lung fibroblasts and erythrocytes, suggesting a low risk of harm to normal human cells. This selectivity may be attributed to the unique structural configuration of *H. illucens* AMPs, which enhances their ability to target bacterial membranes more efficiently than mammalian ones (Van Moll et al., 2022).

6. Conclusion

This study explored the potential of AMPs derived from *H. illucens* hemolymph and two synthetic peptides as antimicrobial agents against a variety of pathogenic bacterial strains, including those resistant to antibiotics. The results confirmed the antimicrobial efficacy of *H. illucens* AMPs against both Gram-negative and Gram-positive bacteria in response to infections induced by *E. coli* and *M. flavus*. The analysis revealed significant differences in minimum inhibitory concentration and minimum bactericidal concentration values between peptide fractions derived from larvae infected with *E. coli* and those infected with *M. flavus*, demonstrating the ability of *H. illucens* to specifically respond to different bacterial infections.

A particularly interesting finding is the observation of antimicrobial activity even in the hemolymph of uninfected larvae, suggesting that the production of AMPs in *H. illucens* is not exclusively dependent on bacterial stimulation but constitutes part of the insect's constitutive immune response. However, bacterial infection significantly enhanced this activity, inducing higher expression of specific antimicrobial peptides. After microbiological assays, mass spectrometry analysis identified 20 AMPs constitutively expressed and 13 inducible by *M. flavus* and *E. coli* infection. The identification of these AMPs could represent the starting point for the discovery of alternative molecules to current antibiotics, helping to address the growing issue of antimicrobial resistance.

The peptide fractions precipitated with organic solvents also exhibited significant antimicrobial activity against multi-resistant pathogenic strains, including carbapenem-resistant *K. pneumoniae* (CRKP) and methicillin-resistant *S. aureus* (MRSA). However, the variability observed in MBC values across different CRKP strains highlight the complexity of bacterial responses to antimicrobial treatments and suggests the need for further studies to better understand the phenotypic dynamics influencing bacterial resistance.

In parallel, two synthetic peptides (MO_Ab1Lin and MO_Ab4) were examined and selected based on bioinformatic analyses predicting their antimicrobial potential. The antimicrobial activity of MO_Ab4, characterized by a structure stabilized by disulfide bridges, proved to be selective against Gram-positive bacteria, highlighting the crucial role of conformational structure in determining AMP specificity. Conversely, MO_Ab1Lin, in its linear form, did not exhibit significant activity against pathogenic strains at high concentrations, underscoring the importance of disulfide bonds for antimicrobial functionality.

Despite the progress made, the large-scale production of peptides C-7081 and C-158, identified as defensins through transcriptomic and proteomic approaches, has not been fully achieved due to challenges in optimizing the expression protocol in *Pichia pastoris*. Although both peptides were successfully cloned, their expression remains to be optimized, representing a technical challenge for future developments. Optimizing expression conditions and proper protein folding remains one of the key objectives for the future application of these peptides in antimicrobial therapy.

The evidence gathered in this study clearly demonstrates that AMPs derived from *H. illucens* represent a promising class of antimicrobial agents with significant potential for application against multi-resistant bacterial strains. However, further scientific investigation will be necessary to ensure their effective use in clinical settings. Specifically, optimizing the production of synthetic peptides and refining their molecular structure will be crucial to enhance their efficacy, stability and mechanism of action by which these peptides exert their antimicrobial activity. Understanding how they interact with pathogen cell membranes and related biological processes will be key to optimizing their effectiveness and developing novel therapeutic approaches. These efforts could pave the way for new therapeutic perspectives, especially in the fight against infections caused by pathogens resistant to conventional antibiotic. In parallel, to translate the *in vitro* findings into practical clinical applications, it will be essential to conduct further *in vivo* studies to confirm the clinical efficacy of these peptides. Another important aspect to consider is the long-term stability of these peptides and the risk of degradation in complex biological environments. Moreover, it will be necessary to deepen the understanding of the pharmacokinetics and pharmacodynamics of these peptides to ensure scalable and stable production.

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Identification of Multifunctional Putative Bioactive Peptides in the Insect Model Red Palm Weevil (*Rhynchophorus ferrugineus*)

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Abstract: Innate immunity, the body's initial defense against bacteria, fungi, and viruses, heavily depends on antimicrobial peptides (AMPs), which are small molecules produced by all living organisms. Insects, with their vast biodiversity, are one of the most abundant and innovative sources of AMPs. In this study, AMPs from the red palm weevil (RPW) *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae), a known invasive pest of palm species, were examined. The AMPs were identified in the transcriptomes from different body parts of male and female adults, under different experimental conditions, including specimens collected from the field and those reared in the laboratory. The RPW transcriptomes were examined to predict antimicrobial activity, and all sequences putatively encoding AMPs were analyzed using several machine learning algorithms available in the CAMP$_{R3}$ database. Additionally, anticancer, antiviral, and antifungal activity of the peptides were predicted using iACP, AVPpred, and Antifp server tools, respectively. Physicochemical parameters were assessed using the Antimicrobial Peptide Database Calculator and Predictor. From these analyses, 198 putatively active peptides were identified, which can be tested in future studies to validate the *in silico* predictions. Genome-wide analysis revealed that several AMPs have predominantly emerged through gene duplication. Noticeably, we detect a newly originated defensin allele from an ancestral defensin via the deletion of two amino acids following gene duplication in RPW, which may confer an enhanced resilience to microbial infection. Our study shed light on AMP gene families and shows that high duplication and deletion rates are essential to achieve a diversity of antimicrobial mechanisms; hence, we propose the RPW AMPs as a model for exploring gene duplication and functional variations against microbial infection.

Keywords: transcriptome; antimicrobial peptides; ACPs; AFPs; AVPs; bioinformatic tools; gene duplication

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Citation: Scieuzo, C.; Rinaldi, R.; Giglio, F.; Salvia, R.; Ali AlSaleh, M.; Jakše, J.; Pain, A.; Antony, B.; Falabella, P. Identification of Multifunctional Putative Bioactive Peptides in the Insect Model Red Palm Weevil (*Rhynchophorus ferrugineus*). *Biomolecules* **2024**, *14*, 1332. https://doi.org/10.3390/biom14101332

Academic Editor: Annarita Falanga

Received: 7 August 2024
Revised: 3 October 2024
Accepted: 15 October 2024
Published: 19 October 2024

1. Introduction

Antimicrobial peptides (AMPs) are small bioactive proteins naturally produced by all living organisms. They are essential components of the innate immune system, serving as the first line of defense against microbial infections in eukaryotes. In prokaryotes, AMPs are produced as a competitive strategy to inhibit the growth of other microorganisms [1–3].

The growing interest in AMPs as potential new drugs stems from pressing social and health emergencies, including rising antibiotic resistance, resistance to antineoplastic chemotherapy, and the emergence of infectious diseases, particularly zoonoses of viral and bacterial origin [4,5]. By 2050, indeed, antibiotic resistance could lead to the death of one person every three seconds, overcoming cancer as a leading cause of mortality [1,6]. The study and the exploration of alternative approaches to antibiotic use will greatly enhance quality of life, significantly improving public health and the healthcare system, while also providing substantial economic benefits [1].

AMPs are small/medium-sized molecules, generally ranging from 5–50 residues and play crucial roles in defense systems [7]. They have a low molecular weight, and most are cationic, showing amphipathic structural configuration characterized by the presence of both hydrophobic and hydrophilic regions [8]. These peptides primarily exert their antimicrobial effect by disrupting microbial membranes, making it difficult for microbes to develop drug resistance. Several models have been proposed to explain the membranolytic mechanism underlying the antimicrobial effects of AMPs [9]. The barrel-stave model describes how peptides laterally insert and diffuse through the lipid bilayer, arranging into helices and forming barrel-like channels that span the membrane [10]. The toroidal pore model involves peptide molecules rotating and inserting into the membrane bilayer, causing a rapid change in membrane conformation and creating a toroidal-shaped pore [11,12]. Finally, the carpet model describes peptides lying parallel to the membrane surface, forming a "carpet" that disrupts the membrane bilayer structure in a detergent-like manner at certain peptide concentrations, leading to micelle formation [6]. Although all living organisms produce AMPs, insects, with their extraordinary biodiversity, represent an almost inexhaustible source of these molecules [13,14]. They hold great potential to enhance the pharmacopeia, which is increasingly depleted of effective therapeutic agents [15,16].

The regulation and production of AMPs in insects are primarily due to the Toll pathway, which is activated by the extracellular cytokine-like polypeptide Spätzle. This pathway is involved in the detection of antigens such as fungi and Gram-positive bacteria, the induction of cellular immunity, and the release of AMPs by the fat body [17]. Specifically, peptidoglycan recognition proteins (PGRPs) mediate Toll activation, particularly GNBP 1 in the case of a Gram-positive bacterial infection and GNBP 3 when a fungal infection occurs. If Gram-negative bacteria provoke infection, the IMD signaling pathway is activated when specific receptors bind meso-diaminopimelic peptidoglycan-2 (DAP), resulting in a signaling activation cascade that triggers the transcription of specific AMPs [18,19]. Finally, if a viral infection takes place, the Janus kinase/signal transducer and activator of transcription (JAK-STAT) cascade is activated to induce AMP gene transcription [19].

Insect AMPs can be classified according to their structure. The three major structural classes are linear α-helical peptides without cysteine residues, peptides with a β-sheet globular structure stabilized by intramolecular disulfide bridges, and peptides that contain high numbers of specific amino acid residues, such as proline or glycine [13,20,21]. AMPs can be also classified in several structural families like abaecin, apidaecin, apisimin, attacins, bomanin, cecropins, cobatoxin, coleoptericin, defensins, diapausing, diptericins, drosocin, drosomycin, gambicin, gloverin, hymenoptericin, lebocins, metchnikowin, morocin, and ponericins, with new AMPs frequently being discovered [1,9,21,22]. This is easily understood when considering that insects, with around one million known species, represent the class of the animal kingdom with more species than the combined total of all other living organisms. This diversity contributes to their remarkable adaptability to environmental changes and tolerance to a wide range of infections, resulting in an incredible, almost inexhaustible biodiversity in terms of anatomical structures, physiological processes, and behavioral patterns [1,6,7,23].

Defensins are a small family of arginine-rich, cationic peptides. They are mostly stabilized by three disulfide bonds, and their fundamental structural feature is a β-hairpin. They bind directly to the cell membrane and create pore-like membrane defects that allow nutrients and vital ions to escape and are isolated from insect orders such as Diptera, Hymenoptera, Coleoptera, Trichoptera, Hemiptera, and Odonata [24,25]. Cecropins were first isolated from the hemolymph of the giant silk moth *Hyalophora cecropia* (Lepidoptera:

Saturniidae, L.), whence the term cecropin derived, and they are mostly isolated from various lepidopteran and dipteran species [26]. The main insect cecropins (A, B, and D), lacking cysteine, can lyse bacterial cellular membranes, inhibit proline uptake, and cause membrane leakage [27]. Attacins are glycine-rich proteins, which were initially discovered in the hemolymph of immunized pupae of *H. cecropia*. Attacins are categorized into two groups based on their amino acid composition: attacins with a basic group and attacins that contain acidic residues. The function of these proteins is to inhibit the synthesis of major outer membrane proteins, thereby compromising the integrity of the cell wall [28]. Lebocins were identified in the hemolymph of the silkworm *Bombyx mori* (Lepidoptera: Bombycidae, L.) after immunization with *Escherichia coli*, and these peptides are proline-rich and O-glycosylated [29]. Diptericins are a family of glycine-rich antibacterial peptides derived from Dipteran hemolymph proteins, primarily targeting the cytoplasmic membrane [30]. Ponericins, peptides extracted from the venom of the predatory ant *Pachycondyla goeldii*, adopt α-helical structures within cell membranes and exhibit hemolytic activity [31].

A comprehensive list of known AMPs from six kingdoms is reported in the Antimicrobial Peptide Database (APD) (http://aps.unmc.edu/AP, accessed on 7 September 2024). They comprise 383 isolated/predicted bacteriocins/peptide antibiotics from bacteria, 5 from archaea, 8 from protists, 29 from fungi, 250 from plants, and 2463 from animals, including genome-predicted and some synthetic peptides (190 predicted and 314 synthetic AMPs) (updated January 2024). The Coleopteran pest in the Curculionoidae family *Rhynchophorus ferrugineus*, also known as the Asian palm weevil or red palm weevil (RPW), is an insect whose larvae destroy almost 40 species of palm trees worldwide [32,33], belonging to 16 genera, and it has been classified as a serious pest on the A2 list according to the EPPO 2024 (https://www.eppo.int/ACTIVITIES/plant_quarantine/A2_list, accessed on 7 September 2024, European and Mediterranean Plant Protection Organization).

Throughout their development, lasting from 25 to 60 days, the larvae and adults of *R. ferrugineus* inhabit an environment composed of fermenting plant fibers, rich in microorganisms. Their survival in such a microbe-rich habitat is closely linked to having a robust and effective immune system [34]. The decision to focus the research on AMPs derived from *R. ferrugineus* is backed by recent studies demonstrating consistent antimicrobial activity of the larvae and adults of this Coleopteran species towards different microorganisms: *Bacillus thuringiensis*, *Bacillus subtilis*, *Enterococcus faecalis*, *Staphylococcus aureus* as Gram-positive bacteria [35,36], and *Escherichia coli* and *Klebsiella pneumoniae* as Gram-negative bacteria [36], as well as two species of nematodes, *Steinernema carpocapsae* and *Heterorhabditis bacteriophora* [32]. Moreover, antifungal activity has been detected in the larvae and adults of Coleoptera towards *Beauveria bassiana* and different species of *Penicillium* [35,36]. Advanced bioinformatic analyses allowed us to first identify putative AMPs in different transcriptomes through functional annotation by comparing them with known sequences deposited in the constantly updated protein databases [2,13]. The identified AMPs were analyzed using the CAMP_{R3} (Collection of Anti-Microbial Peptides) database (https://www.camp.bicnirrh.res.in/, accessed on 7 September 2024) to predict the putative antimicrobial activity, the iACP (a sequence-based tool for identifying anticancer peptides) online tool (https://lin.uestc.edu.cn/server/iACP, accessed on 7 January 2024) to predict the putative anticancer activity, the AVPpred (https://crdd.osdd.net/servers/avppred, accessed on 7 January 2024) server to predict the putative antiviral activity and the Antifp (antifungal peptide prediction) server (https://webs.iiitd.edu.in/raghava/antifp, accessed on 7 January 2024) to predict their putative antifungal activity. Their physicochemical properties were then evaluated with the Compute pI/Mw tool—Expasy (https://web.expasy.org/compute_pi/, accessed on 17 December 2023) and the Antimicrobial Peptide Database Calculator and Predictor (APD3) (https://aps.unmc.edu/prediction, accessed on 7 September 2024) [37,38].

2. Materials and Methods

2.1. Rearing of Rhynchophorus Ferrugineus and RNA Isolation

The RPWs were collected in 2009 with the direct permission of a cooperating landowner [Al-Kharj region (24.1500° N, 47.3000° E) of Saudi Arabia]. Since then, the laboratory colony has been maintained, as previously described [39,40]. Two weeks after pupation, the cocoons were harvested from the sugarcane stems, individually incubated in round 70 mm × 90 mm plastic jars with perforated screw caps, and checked daily for adult emergence. Ten-day-old unmated adult male and female RPWs were selected (male and female separately). Various body parts (antennae, snout, head, thorax, abdomen, gut, fat body, wings, and legs) were carefully dissected (male and female separately) under a light microscope, after insects were anesthetized using CO_2 for 1–2 min. Immediately after collection, the tissues were transferred in RNA later solution and then stored at −20 °C until total RNA extraction. The RPW male and female adults from the field were captured alive from the infested and removed date palm tree materials at Al Qassim (25.8275° N, 42.8638° E) in Saudi Arabia, and various body parts were collected by following the method described above.

2.2. Total RNA Extraction, cDNA Library, and Illumina Sequencing

For the subsequent RNA extraction, intended for the *de novo* construction of transcriptomes, specimens were collected separately for each sample, comprising different body parts from male and female adults reared in the laboratory, as well as those collected directly in the field. Total RNA was extracted from various tissues (~30 mg) of RPW male and female adults separately using a PureLink RNA Mini Kit (Thermo Fisher, Waltham, MA, USA). A NanoDrop spectrophotometer (Thermo Fisher, Waltham, MA, USA) was used to quantify and check the extracted RNA quality and synthesized cDNA quality. The quantity and quality of the total RNA were validated using a Qubit 2.0 Fluorometer (Invitrogen, Life Technologies, Carlsbad, CA, USA), and RNA integrity was confirmed using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). After ensuring the quality and the characteristic “hidden break” in the 28S RNA profile using 2100 Bioanalyzer, a paired-end cDNA library preparation using a TruSeq Stranded mRNA preparation Kit (Illumina Inc., San Diego, CA, USA) was prepared, following manufacturer’s protocols, which include the following steps: purification and fragmentation of total RNA, first- and second-strand cDNA synthesis, 3′ end adenylation, adapter ligation, and purification. Finally, the purified and PCR-enriched products were used for cDNA library preparation. The cDNA libraries were validated and quantified using a Qubit 2.0 Fluorometer. The HiSeq Illumina sequencing was performed at the core sequencing facility of the King Abdullah University of Science and Technology (KAUST), Jeddah, Saudi Arabia. Image deconvolution and quality value calculations were performed using Illumina GAPipeline1.3. Illumina adaptors were removed, and low-quality bases were trimmed off with the Trim Reads tool of the CLC Genomics Workbench/Server suite. Filtered paired-end reads were validated through a QC for Sequencing Reads visualization of the same suite. A reference *de novo* transcriptome assembly was constructed with the “*De Novo* Assembly” tool of the CLC Genomics Workbench/Server with default parameters (map reads to contigs function on and using paired read files). The resulting contigs files were functionally annotated using the previously described method [39–42]. RPW antennae, snout, head, thorax, abdomen, gut, fat body, wings, and legs (field and lab) were uploaded to the National Center for Biotechnology Information (NCBI) under Sequence Read Archive (SRA) and Transcriptome Shotgun Assembly (TSA) accession numbers.

2.3. Transcriptomes Assembly and Annotation

The transcriptome assembly and annotation were carried out following the method described in Antony et al. (2024), Gonzalez et al. (2021), and Antony et al. (2016) [39,41,43]. Contigs were annotated based on a local BLAST search using *R. ferrugineus* olfactory protein sequences using Geneious R7 v7.1.9 (http://www.geneious.com, accessed on 7 January 2023). Blast2Go analyses were performed on various tissue transcripts using the

BLAST2GO command line tool (*v*1.5) of the CLC. The top blast hit transcript clusters were extracted from the male and female (lab and field) assembled transcriptomes with an in-house command-line script. The reads per kilobase million (RPKM) values [44] and the gene and transcript level quantification were performed, and the transcripts per kilobase per million mapped reads (TPM) value of each gene was calculated manually based on the consensus length of each gene and total read counts. To assess transcriptome completeness, an Arthropoda BUSCO database, consisting of 1066 core genes that are highly conserved single-copy orthologues, was used to query the assembled FASTA files. For this process, the gVolante2 web server (https://gvolante.riken.jp/, accessed on 17 January 2023) was utilized with the following parameters: min_length_of_seq_stats: 1, assembly_type: trans, Program: BUSCO_v2/v3, selected reference_gene_set: Arthropoda.

2.4. Multiple Alignment

To generate alignments between amino acid residues of all sequences in each part of the body and identify identical AMPs, the Clustal Omega program (https://www.ebi.ac.uk/jdispatcher/msa/clustalo, accessed on 13 December 2023) was utilized. This tool enables the analysis of sequence patterns conserved through evolution, using the FASTA output format for multiple alignments of protein and nucleic acid sequences [45], and it also allows to detect duplicated sequences. According to physicochemical criteria, residues are color-coded differently to indicate conserved positions in the sequences, as shown in Table 1.

Table 1. Criteria of multiple alignments. The residues of the aligned sequences are coded according to the following criteria: AVFPMILW is shown in red, DE in blue, RHK in magenta, STYHCNGQ in green, and all other residues in grey. The residue range for each sequence is shown after the sequence name.

Residue	Color	Property
AVFPMILW	RED	Small (small + hydrophobic (including aromatic − Y))
DE	BLUE	Acidic
RHK	MAGENTA	Basic − H
STYHCNGQ	GREEN	Hydroxyl + sulfhydryl + amine + G
Others	GREY	Unusual amino/imino acids etc.

2.5. In Silico Analysis for the Antimicrobial, Anticancer, Antiviral and Antifungal Activity Prediction

The Blast2Go software [46] functionally categorized the sequences as antimicrobial peptides, which were translated into the corresponding amino acid sequences via the software Expasy translate tool [47]. Then, the amino acid sequences obtained by the Expasy translate tool (https://web.expasy.org/translate/, accessed on 17 December 2023) were analyzed to detect the possible presence of the signal peptide and pro-peptide, using the software Prop 1.0 (https://services.healthtech.dtu.dk/services/ProP-1.0/, accessed on 17 December 2023) [48] and SignalP 6.0 servers (https://services.healthtech.dtu.dk/services/SignalP-6.0/, accessed on 17 December 2023) [46]. CAMP$_{\mathrm{R3}}$ (http://www.camp3.bicnirrh.res.in/, accessed on 7 January 2024) is a database of sequences, structures, and family-specific signatures of prokaryotic and eukaryotic AMPs, and four machine-learning — support vector machine (SVM), discriminant analysis (DA), artificial neural network (ANN), and random forest (RF)— methods from the AMP prediction tool (http://www.camp3.bicnirrh.res.in/prediction.php, accessed on 7 January 2024) were used to *in silico* analyze the mature and active peptide regions of all the contigs annotated as antimicrobial [49]. The computed minimum threshold for a sequence to be regarded as antibacterial is 0.5. The algorithms were applied to all the sequences, and those with scores greater than 0.5 were automatically categorized by the software as putative antimicrobials. Antifp (https://webs.iiitd.edu.in/raghava/antifp, accessed on 7 January 2024) was developed to predict and design antifungal peptides; this server outputs the result as a numerical score, and 0.5 was used as the threshold criterion for this investigation [50]. The iACP web server (http://lin.uestc.edu.cn/server/iACP, accessed on 7 January 2024) has been used to identify the peptides with anticancer activity, based purely on its sequence information alone and via anticancer and non-anticancer scores [51]. The prediction of the antiviral activity was performed with the online server AVPpred (http://crdd.osdd.net/servers/avppred/, accessed on 7 January 2024), whose prediction is based on antiviral peptide motifs (with an output of YES or NO), sequence alignment, amino acid compositions, and physiochemical properties. It provides an overall prediction that is expressed with a cumulative prediction by 0, 1, 2, 3 or by all 4 methods [52].

2.6. Evaluation of the Physicochemical Properties

The Antimicrobial Peptide Database Calculator and Predictor tool (https://aps.unmc.edu/prediction, accessed on 7 January 2024) on the antimicrobial peptide database (APD3) [53,54] and the Compute pI/Mw tool—Expasy [47,55–57] were used to determine the corresponding physicochemical properties of the putatively identified active peptides after *in silico* analysis, such as peptide length, molecular weight, total hydrophobic ratio, total net charge, isoelectric point, and the Boman index.

2.7. Evolutionary Relationships of AMPs

The AMP sequences were correctly annotated and mapped to the *R. ferrugineus* genome [58] (GenBank accession numbers GCA_014462685.1 and GCA_014490705.1) using a BLASTn search against the *R. ferrugineus* genome, which was created using Geneious v7.1.9 (Biomatters) and correctly annotated. The exon-intron positions of the AMPs in the genome were mapped at the scaffold region in a different locus [58]. The mapped regions were extracted and manually aligned using the MAFFT program v7 38, which was used for gene structure illustrations. The NCBI graphical sequence viewer (v 3.50.0), available in the NCBI Genome Workbench, was used to graphically display the nucleotide and protein sequences at the scaffold region in a different locus. The amino acid sequence similarity of AMP genes was tested with the Psi-BLAST (NCBI) sequence alignment algorithm, based on e-value, bit-score, and percent identity. An attempt to infer duplication events through unrooted protein trees for the AMPs produced using BLAST pairwise alignment in the NCBI Tree Viewer (v 7.1.0.46) was performed. The AMP protein sequence similarity search using BLASTp e-value with a cutoff expectation of <2 and <10-e3 identified different AMP clades, shedding light on gene duplication events.

3. Results

3.1. De Novo Transcriptome Assembly and Gene Identification

To clearly identify the putative peptide candidates, next-generation sequencing (NGS) RNAseq was performed on the RNA isolated from the transcriptomes of male and female *R. ferrugineus* adults from different body parts: abdomen, antennae, fat body, gut, head, legs, snout, thorax, and wings. Specimens were collected from both laboratory and field environments. The raw data have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database, with the accession numbers for the different body parts of the red palm weevil detailed in Supplementary File S1.

NGS performed with RNA isolated from the abdomen, fat body, thorax, antennae, gut, head, legs, snout, and wings of *R. ferrugineus* male and female adults, bred in the lab or collected in the field, allowed us to generate a *de novo* transcriptome assembly (Table 2). All sequences were subjected to gene ontology (GO) analysis in Blast2GO for functional annotation (Table 2).

Table 2. List of analyzed transcriptomes. From left to right the name of the transcriptome, total contigs, minimum length, maximum length, % of contigs matching with assigned molecular functions in the GO database, and % of contigs non-matching with assigned molecular functions in the GO database are shown.

Transcriptome	Total Contig	Min Length (bp)	Max Length (bp)	% of Contigs Matching with Assigned Molecular Functions in the GO Database	% of Contigs Non-Matching with Assigned Molecular Functions in the GO Database
Abdomen Female Field	24.564	125	8.417	73	27
Abdomen Female Lab	27.961	106	8.383	68	32
Fat Body Male Field	38.109	107	17.994	56	44
Fat Body Male Lab	38.226	108	11.542	61	39
Thorax Female Field	24.289	97	11.037	74	26
Thorax Female Lab	28.064	69	5.700	69	31
Thorax Male Field	30.708	100	12.801	64	36
Thorax Male Lab	25.211	100	4.822	74	26
Antennae Female Lab	81.862	101	21.384	46	54
Antennae Female Field	59.627	104	25.370	47	53
Antennae Male Field	53.645	74	21.051	52	48
Antennae Male Lab	47.910	89	14.929	61	39
Gut Female Field	67.747	87	25.094	44	56
Gut Male Field	31.308	92	14.443	61	39
Head Female Field	28.195	125	22.558	72	28
Head Female Lab	35.232	107	16.119	66	34
Head Male Field	30.748	125	13.320	68	32
Head Male Lab	35.527	90	12.875	66	34
Legs Female Field	68.240	83	38.106	58	42
Legs Female Lab	70.207	115	12.319	58	42
Legs Male Field	75.700	101	23.454	57	43
Legs Male Lab	105.309	102	23.303	52	48
Snout Female Field	63.109	121	30.807	50	50
Snout Female Lab	120.977	72	21.750	93	7
Snout Male Field	126.195	104	34.191	52	48
Snout Male Lab	62.512	106	13.318	59	41
Wings Female Field	72.889	94	18.328	52	48
Wings Female Lab	46.395	115	17.855	58	42
Wings Male Field	73.685	93	19.966	53	47
Wings Male Lab	38.804	123	25.527	58	42

Annotation of the *de novo* assembly of the adult transcriptomes led to the identification of 827 contigs, which were annotated as putative antimicrobial sequences and subsequently analyzed.

3.2. Alignment of Sequences

This work used the coloring methods for multiple sequence alignment from the Clustal Omega tool. This approach allowed for the alignment of multiple sequences, highlighting areas of similarity that may be associated with specific features conserved more highly than in other regions (Supplementary File S2). Residues were colored according to their physicochemical properties, as reported in Table 1. The alignment was also useful for detecting duplicate contigs. In total, 457 duplicated sequences were detected in various body parts. Specifically, the field male duplicates were predominantly expressed in the fat body, the laboratory male duplicates in the head, the field female duplicates in the wings, and the laboratory female duplicates in the abdomen and legs.

Regarding duplicates (repeated sequences) in individual parts of the body, the following were identified: 5 duplicates in the abdomen, all of which were expressed in field females; 5 duplicates in the fat body, mostly identified in males in an equal number of field and laboratory males; 10 duplicates in the head, mostly expressed in laboratory males; 13 duplicates in the snout, mostly expressed in field males; 13 duplicates in the antennae, mostly expressed in field females; 5 in the gut, mostly expressed in field males; 14 in the legs in an equal number of field females and field males; 13 in the thorax, mostly in field females; and 18 in the wings, mostly in field females.

3.3. Expression Levels of Duplicate AMP Genes

Following alignment, the TPM (transcripts per million) values of duplicated sequences were compared (Supplementary File S3) in order to evaluate the gene expression levels of AMPs in each part of the body in different experimental conditions. The same sequences showed different expression levels, as reported in the heat maps (Figure 1).

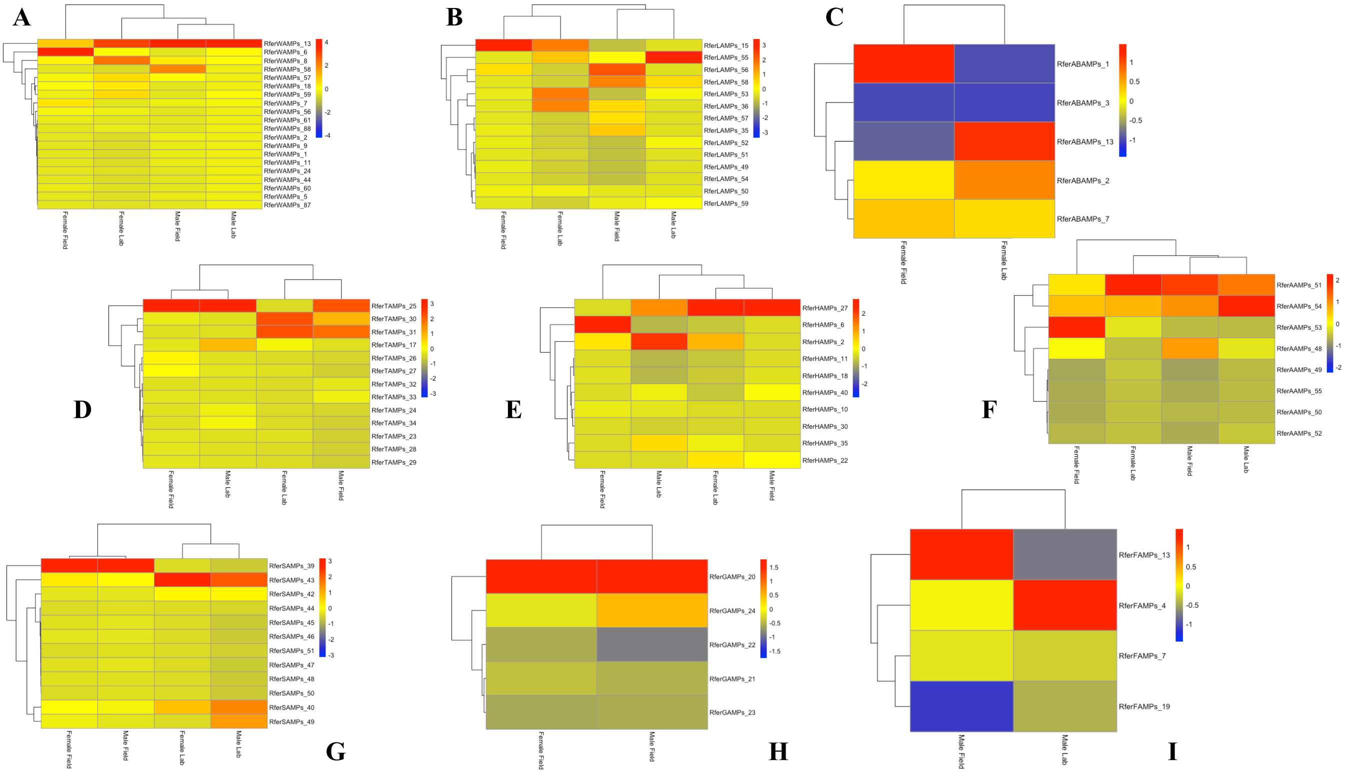

Figure 1. Heatmap showing the expression levels of duplicated genes in the different body parts of *Rhynchophorus ferrugineus* lab-reared and field-collected male and female adults. The heatmap colors represent transcript abundance in transcripts per million (TPM) from highest (red) to lowest (blue) expression levels. The data represented as log-transformed TPM values were tabulated and converted into heatmaps using R and R Studio software (version 2023.06.2+561). (**A**) Wings; (**B**) Legs; (**C**) Abdomen; (**D**) Thorax; (**E**) Head; (**F**) Antennae; (**G**) Snout; (**H**) Gut; (**I**) Fat Body.

3.4. Antimicrobial Activity Prediction

The sequences of interest detected in the adult transcriptome were analyzed using the free online database CAMP$_{R3}$ to predict their putative antimicrobial activity. According to the antimicrobial software analysis, 198 sequences showed putative antimicrobial activity.

Among the 198 genes encoding putative AMPs in the *R. ferrugineus* adult transcriptomes were identified 37 defensins, 6 cecropins, 62 hypothetical antimicrobial peptides, 1 knottin protein, 1 putative defense protein, and 91 between lysozymes and lysozyme-like proteins (Figure 2). Although lysozymes are not typically classified as AMPs due to their large size, they were taken into consideration because of their known antimicrobial properties.

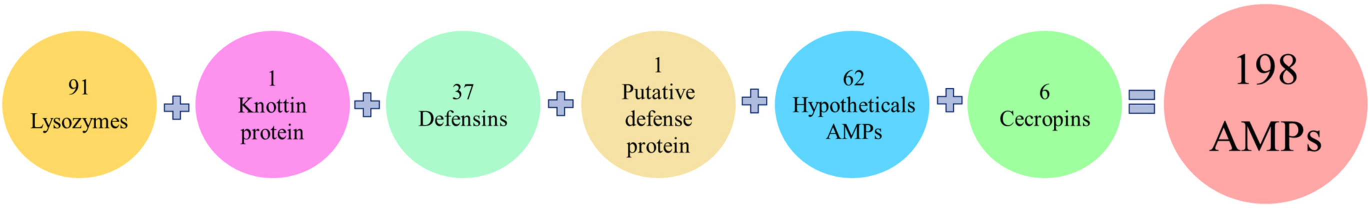

Figure 2. Classification of the 198 total AMPs detected in adult transcriptomes related to their family classification.

3.5. Antifungal, Anticancer, and Antiviral Activity Prediction

The 198 sequences were also analyzed to predict their antifungal, anticancer, and antiviral activity.

From Supplementary File S4, it is evident that five AMPs exhibit putative antifungal activity, while 101 contigs with putative anticancer activity were identified. However, none were found to have putative antiviral activity. Among the five AMPs with putative antifungal activity, two defensins and three lysozymes were identified. Among the 101 AMPs with putative anticancer activity, 23 are defensins, 54 are lysozymes or lysozyme-like, 20 are hypothetical antimicrobial peptides, 1 is a putative defense protein and 3 are cecropins (Supplementary File S4).

Specifically, these 198 AMPs displayed various combinations of antimicrobial, anticancer, and antifungal activity. Additionally, four peptides were found to possess all three activities: two defensins, and two lysozymes (Supplementary File S4).

3.6. Physicochemical Properties of the Identified Peptides

The physicochemical properties (length in amino acid residues, molecular weight (MW), total hydrophobic ratio in percentage, total net charge, isoelectric point (pI), and the Boman index in kcal/mol) of the putative peptides have been identified using the Antimicrobial Peptide Database Calculator and Predictor (APD3) and the Compute pI/Mw tool—Expasy and are reported in Supplementary File S4.

In particular, the length of amino acid residues in the sequences ranges from a minimum of 20 residues to a maximum of 165 residues in the adult transcriptomes (Supplementary File S4).

Most peptides with a low number of amino acid residues, a low hydrophobicity index, and a cationic nature are defensins. The two defensins with putative antifungal, antimicrobial, and anticancer activity have 44 and 43 amino acid residues, respectively, and very similar total hydrophobic ratios and positive total net charges. While there is no definitive criterion for correlating predicted physicochemical properties with corresponding bioactivities across all sequences, a notable association exists for most of them. Specifically, there is an interesting link between positive charge and putative antimicrobial activity, with 158 out of 198 sequences exhibiting this characteristic.

3.7. Evolutionary Relationships of AMPs

By using NCBI Acc. No. JAACXV010000404.1, we annotated and mapped all seven cecropins fragments in scaffold_405 onto the *R. ferrugineus* genome. The genomic organization revealed that the five distinct cecropins genes were distributed in locus_tag = "GWI33_008582, GWI33_008583, GWI33_008584, GWI33_008585, GWI33_008587, GWI33_008589 and GWI33_008590" and then across the same scaffold_405 in the *R. ferrugineus* genome, indicating possible emergence through tandem duplication (Figure 3). The functional cecropins gene length, CDS (coding region) length, and protein length mapped in scaffold_405 are shown in Figure 3. All cecropin genes contained two exons and an intron (Figure 3).

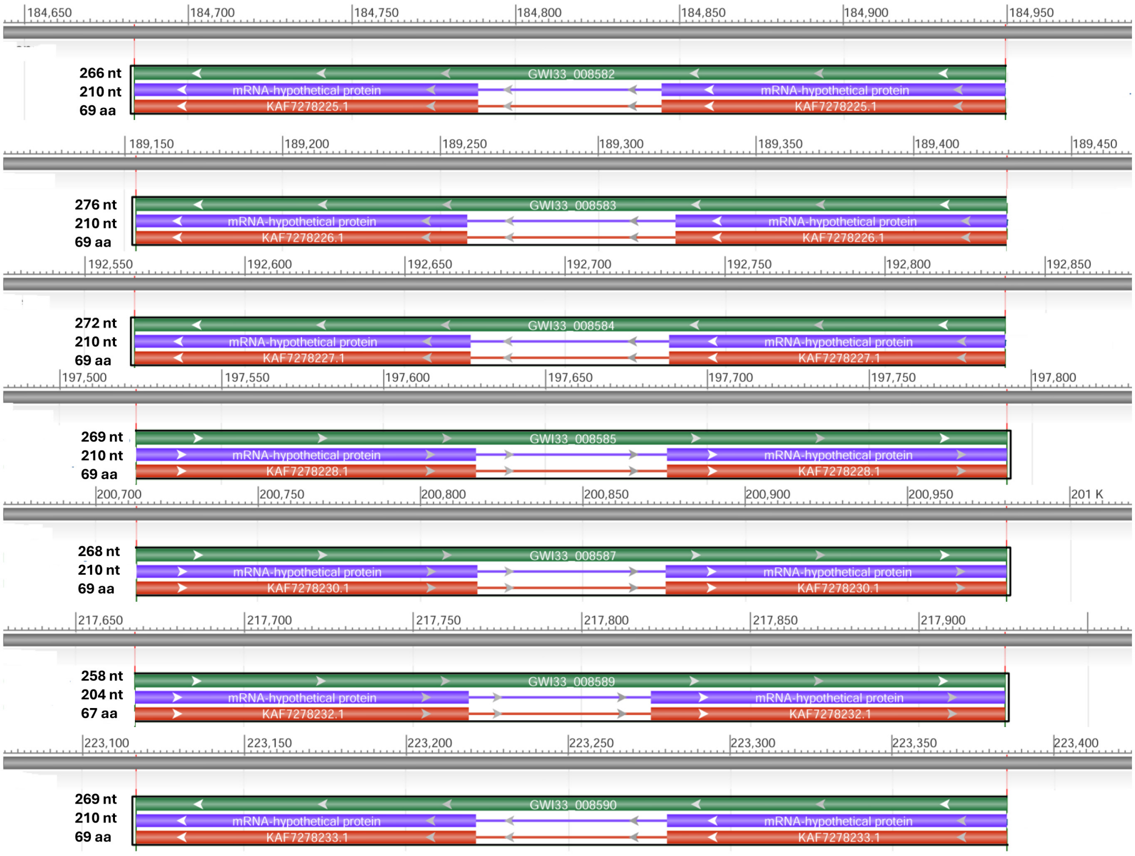

Figure 3. Graphical display for the cecropin genes mapped in scaffold_405 (NCBI acc no. JAACXV010000404.1) showing functional cecropins gene length, CDS (coding region) length, and protein length, which was generated using the NCBI graphical sequence viewer available in the Genome workbench. The visual code shows green, red, and purple, indicating gene, coding region, and mRNA, respectively. The line shows the introns and boxes for the exons.

Using NCBI DBSOURCE accessions JAACXV010014362.1, JAACXV010000413.1, JAACXV010014484.1, JAACXV010014200.1, JAACXV010014575.1, and JAACXV010014362.1, we annotated a deduced amino acid sequence of the six distinct defensin genes. Six scaffolds have been identified as carrying at least one, and often more than one, defensin gene. The genomic organization of defensins revealed that they were distributed across different scaffolds in the *R. ferrugineus* genome with an uneven distribution pattern (Figure 4). Among these, the first four major defensin genes were represented in the locus tags: GWI33_018859 (scaffold_66293), GWI33_009171 (scaffold_414), GWI33_020600 (scaffold_66394), and GWI33_018859 (scaffold_66154). Among the last two remaining defensins, there were four duplicates (locus_tag = "GWI33_019775, GWI33_019782, GWI33_019783, and GWI33_019784") distributed across the same scaffold_66293 (JAACXV010014484.1), and other two duplicates (locus_tag = "GWI33_017310 and GWI33_017312") in the same scaffold_65978 (JAACXV010014200.1) in the *R. ferrugineus* genome, indicating possible emergence through tandem duplication (Figure 4).

Figure 4. Graphical display for the defensin genes mapped in the six scaffolds (NCBI acc no. JAACXV010014362.1, JAACXV010000413.1, JAACXV010014484.1, JAACXV010014200.1, JAACXV010014575.1, and JAACXV010014362.1) showing functional gene length, CDS length, and protein length generated using the NCBI graphical sequence viewer available in the Genome workbench. The visual code shows green, red, and purple, indicating gene, coding region, and mRNA.

We identified two allelic variants in the locus tag "GWI33_019784" and the deduced amino acids predict 82-aa and 84-aa proteins (NCBI acc nos. KAF7266949 and KAF7266950), with a conserved DEFL_defensin-like domain at the C-terminal region (Figure 5). The amino acid sequence of both alleles has ≈ 98% identity, and two amino acids (Val32 and Ser33) were deleted in the defensin acc no. KAF7266949 (Figure 5). The functional defensin gene length, CDS length, and protein length mapped in scaffold_66293 and scaffold_65978 are shown in Figure 4.

```
                            10        20        30        40        50        60        70        80
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....
Defensin_KAF7266949 MFKFAFFGLLAVLVLSVHSAPVSEFEEELIQ--EHGRVKRATCDLLSFEAFGIKLNDSACAAHCLQLGRRGGHCNDEKVCVCRR
Defensin_KAF7266950 ...............................VS...................................................
```

Figure 5. Two allelic variants in the locus tag "GWI33_019784" and deduced amino acids predict 82aa and 84-aa proteins (NCBI acc nos. KAF7266949 and KAF7266950), with a conserved DEFL_defensin-like domain at the C-terminal region. Dots denote identical amino acid residues, and conserved DEFL_defensin-like domain at the C-terminal region are underlined.

We retrieved two scaffolds (66363 and 66088) containing potential hypothetical AMPs and, using NCBI DBSOURCE accessions JAACXV010014549.1 and JAACXV010014301.1, we annotated a deduced amino acid sequence for the two major hypothetical AMP families. The first group of hypothetical AMPs in the same scaffold_66363 (JAACXV010014549.1) contains two genes and was mapped in the locus tags GWI33_020371 and GWI33_020371 in the *R. ferrugineus* genome, indicating possible emergence through tandem duplication (Figure 6). The second group of hypothetical AMPs mapped in the scaffold_66088 (JAACXV010014301.1) has seven distinct tandem duplicates that were distributed in the locus tags = "GWI33_018093, GWI33_018094, GWI33_018095, GWI33_018096, GWI33_018097, GWI33_018098 and GWI33_018099" (Figure 6). The functional AMP gene length, CDS length, and protein length mapped in scaffold_66363 and scaffold_66088 are shown in Figure 6.

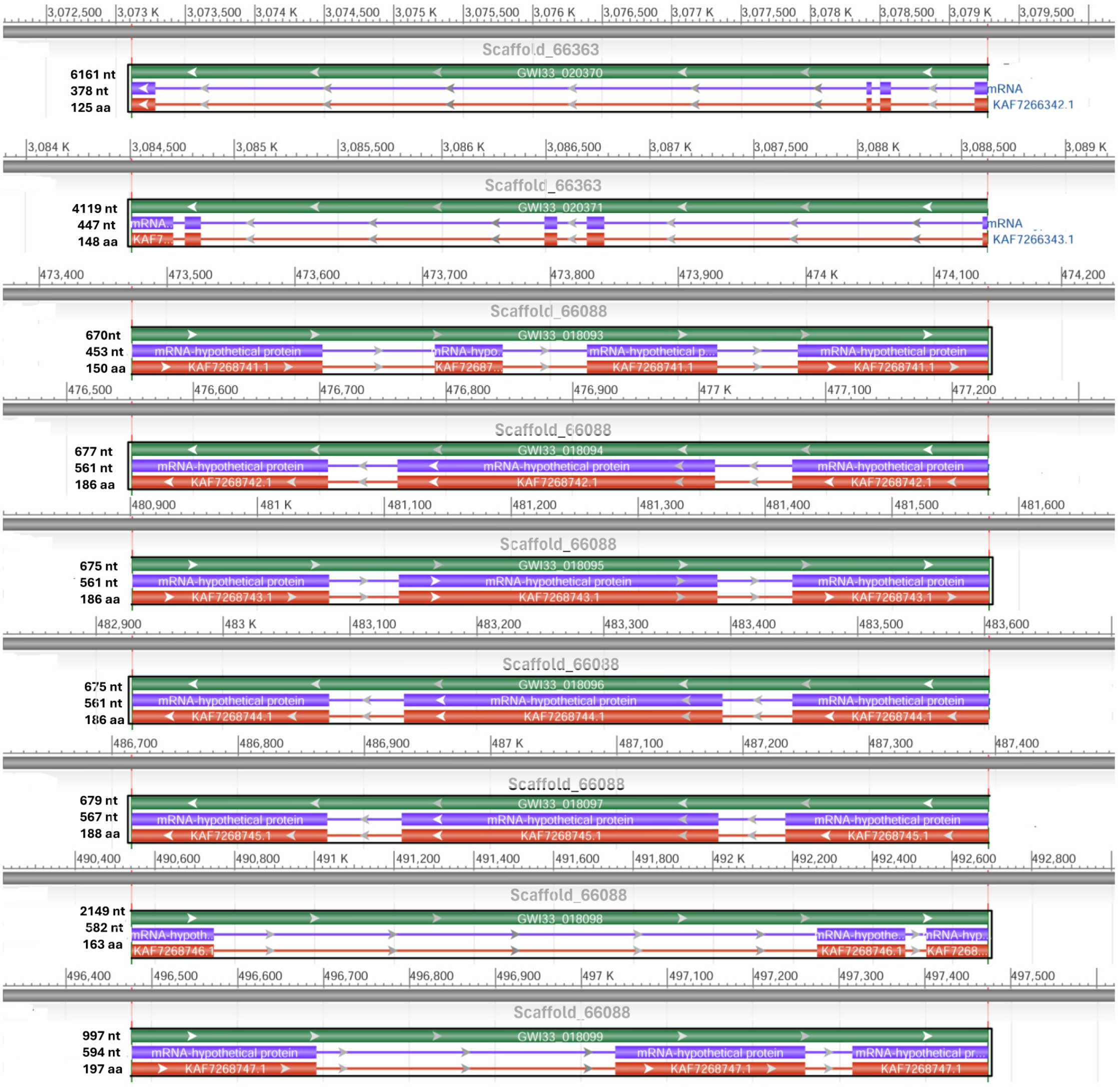

Figure 6. Graphical display for the hypothetical AMP genes mapped in the two scaffolds (66363 and 66088) (NCBI acc nos. JAACXV010014549.1 and JAACXV010014301.1) showing the functional AMP gene length, CDS length, and protein length generated using the NCBI graphical sequence viewer available in the Genome workbench. The visual code shows green, red, and purple, indicating gene, coding region, and mRNA, respectively. The line shows the introns and boxes for the exons.

We retrieved six scaffolds that have been identified as carrying at least one, and often more than one, lysozyme gene using NCBI DBSOURCE accessions JAACXV010014523.1, JAACXV010014472.1, JAACXV010000242.1, JAACXV010000047.1, JAACXV010013077.1, and JAACXV010013977.1. The first group of lysozymes in the same scaffold_66335 contained three distinct genes. It was mapped in the locus tags GWI33_020037, GWI33_020038, and GWI33_020039 (JAACXV010014523.1) in the *R. ferrugineus* genome, indicating possible emergence through tandem duplication (Figure 7). The second group of putative lysozymes mapped in the scaffold_66281 (JAACXV010014472.1) had two distinct tandem duplicates that were distributed in locus_tags = "GWI33_019655 and GWI33_019658. The functional AMP gene length, CDS length, and protein length mapped in scaffold_66335 and scaffold_66281 are shown in Figure 7. The remaining four lysozymes were distributed in the scaffold_243 (JAACXV010000242.1), scaffold_48 (JAACXV010000047.1), scaffold_64653 (JAACXV010013077.1), scaffold_65725 (JAACXV010013977.1), and mapped in the locus tags: GWI33_004805, GWI33_010182, GWI33_013206 and GWI33_015786 respectively. All these lysozymes were distinct genes with no evidence of duplication event.

Figure 7. Graphical display for the lysozyme genes mapped in the two scaffolds (66335 and 66281) (NCBI acc nos. JAACXV010014523.1 and JAACXV010014472.1) showing functional lysozyme gene length, CDS length, and protein length generated using the NCBI graphical sequence viewer available in the Genome workbench. The visual code shows green, red, and purple, indicating gene, coding region, and mRNA, respectively. The line shows the introns and boxes for the exons.

4. Discussion

The alarming spread of multidrug-resistant infections has prompted researchers to search for new antibacterial substances [59]. AMPs are promising candidates as alternatives to conventional antibiotics due to their low toxicity to eukaryotic cells and their broad spectrum of action against bacteria, mycobacteria, fungi, viruses, and cancer cells [60]. AMPs can kill bacteria in various ways, such as by disrupting membranes, concentrating on intracellular components, or interfering with metabolism [61,62]. Most AMPs are also cationic, which enhances their ability to interact electrostatically with negatively charged bacterial membranes [63,64]. Because of their enormous biodiversity and varied living conditions, insects are among the richest sources of AMPs among all living organisms [65,66].

In this study, we examined the putative genes encoding AMPs in the transcriptomes of *R. ferrugineus*, along with their expression levels and potential functions.

Multiple sequence alignments facilitated the detection of duplicate sequences. Alignments performed with Clustal Omega software were useful in identifying duplicate sequences within each body part and across different parts of the adult transcriptomes, resulting in the identification of 457 duplicated sequences in total.

The occurrence of duplicate transcripts may be attributed to gene duplication events. Specifically, the genome analysis of RPW revealed the existence of 837 duplicate genes, some of which are involved in pesticide resistance or detoxification processes [67]. Gene duplication is widely recognized as a primary mechanism contributing to the emergence of structural and functional diversity during genome development [68]. Moreover, it is a widespread phenomenon for AMPs. An adaptive model predicts that duplicated genes have distinct expression patterns [69], as it is possible to appreciate in this work.

After detecting the duplicate sequences, we evaluated the expression level of these sequences by comparing RPKM (reads per kilobase of exon per million mapped reads) values. This metric quantifies the relative abundance of a transcript within a population of sequenced transcripts, firstly in every individual part of the body and then between each part of the body of the adults.

The remaining sequences were analyzed *in silico* using the CAMP_{R3} database to evaluate their antimicrobial activity; a total of 198 sequences resulted in a putative antimicrobial activity. These sequences were analyzed by bioinformatic tools to detect and classify the putative activity of each peptide. It is possible to determine, therefore, whether a certain sequence corresponds to a peptide that acts, at least *in silico*, against viruses, bacteria, fungi and/or cancer cells [70–72].

Using databases to identify the similarities among the sequences with antimicrobial, antiviral, antifungal, and antitumor activity allows for the rapid identification of potentially valid sequences, facilitating a deeper exploration of these studies [73]. The bioinformatics approach, therefore, represents a valid starting point in the study of sequences present in transcriptomes, where there is a huge amount of data [74]. This allows a preliminary but efficient screening for selecting the most promising candidates that can then be progressively produced (through chemical synthesis or recombinant DNA technology) and functionally characterized [65,75,76].

A similar *in silico* approach was adopted in the study of Moretta et al. [56], where the larvae and the combined adult male and female *Hermetia illucens* transcriptomes were examined. This identified 57 putatively active peptides, 13 of which with antimicrobial activity, 22 with antimicrobial and anticancer activity, 8 with antimicrobial and antiviral activity, 2 with antimicrobial and antifungal activity, 7 with antimicrobial, anticancer and antiviral activity, 1 with antimicrobial, antiviral and antifungal activity, 2 peptides with antimicrobial, anticancer and antifungal activity, and another 2 positive to all activity predictions [65]. Five putative AMPs, which showed high antimicrobial scores with all prediction algorithms, were selected, chemically synthesized, or produced by recombinant DNA technology and positively screened against *E. coli*, *S. aureus,* and *Staphylococcus epidermidis*, confirming the antimicrobial activity evaluated *in silico* [7,65]. Data from *H. illucens* transcriptomes were also the starting point for a combined approach (transcriptomic and proteomic) to identify AMPs expressed in haemolymph under different experimental conditions [77].

In the work of Li et al. [76], defensins of the insect *Periplaneta americana* were identified by comparing the genome sequences of the insect with AMP sequences in databases. The authors identified five putative defensins and studied their physicochemical characteristics (length, amino acid composition, molecular weight, total hydrophobic ratio, total net charge, and isoelectric point), as well as their primary and secondary structures and the putative antimicrobial, antifungal and antiviral activity. Two AMPs demonstrated both antimicrobial and antiviral activity, while the other three defensins were predicted to have exclusively antiviral activity. The authors directed further experiments on AMPs with exclusive antiviral activity, conducting tests on *Drosophila melanogaster* Kc cells infected with the *Drosophila* C virus [76]. The viral DNA expression levels showed a reduced virus titer in cells treated individually with all three peptides. The most promising defensin was used in further experiments, showing a positive impact in *D. melanogaster* Kc cells by preventing apoptosis induced by viral infection, and in *D. melanogaster* larvae, limiting infection and increasing survival rate. These results confirmed the bioinformatics predictions.

The study of RPW transcripts, obtained under experimental conditions through *in silico* methods, has facilitated the identification of a significant number of AMPs, primarily falling into the defensins, cecropins, knottin proteins, and lysozymes classes. Defensins are used as a defense mechanism in various insect orders, including Diptera, Hymenoptera, Hemiptera, Coleoptera, and Lepidoptera [13]. Additionally, defensins have been identified in the ancestral order of Odonata, suggesting a potential evolutionary origin of insect defensins from a shared ancestral gene. Defensins are rich in cationic arginine residues;

they comprise 6–8 preserved cysteine residues and vary in size from 18 to 45 amino acids, and insect defenses are usually composed of 29–35 amino [78]. The fundamental structural feature of the defensin molecule is a β-hairpin, which is typically stabilized by three disulfide bonds [79]. Due to the outflow of vital and nutritious ions from the cell, the defensins bind to the cell membrane or create pore-like membrane defects [25]. Insect defensins primarily exhibit antimicrobial activity against Gram-positive bacteria, such as *Micrococcus luteus*, *Aerococcus viridians*, *Bacillus megaterium*, *Bacillus subtilis, B. thuringiensis*, and *S. aureus*. Certain insect defensins have also demonstrated activity against the Gram-negative *E. coli* bacteria and certain fungi [66]. The study conducted by Robles-Fort et al. demonstrates that defensins exhibit a significant antiproliferative effect on triple-negative breast cancer cells while showing negligible cytotoxicity toward normal cells [80].

Cecropins, which play a significant role in the cell-free immunity of insects [26], were initially extracted from the hemolymph of infected pupae of the moth species *H. cecropia*. They have since been identified in various lepidopteran and dipteran species. They are small proteins, with approximately 35 amino acid residues, that have efficacy against Gram-positive and Gram-negative bacteria due to their ability to lyse bacterial cellular membranes, impede proline uptake, and create leaky membranes [27,81]. A, B, and D are the three main insect cecropins comprising 5–37 residues [82]. Henao Arias et al. examined the cecropins derived from *O. curvicornis* and dung beetles [83]. The researchers observed a significant inhibitory effect of these cecropins against Gram-negative bacteria, specifically *E. coli* and *Pseudomonas aeruginosa* [83]. Research investigations on *Drosophila* specimens exhibiting gene deletions responsible for encoding cecropins have substantiated their involvement in the defense mechanism against Gram-negative bacteria, specifically *Enterobacter cloacae* and *Providencia heimbachae* [84]. One of the oldest and most well-known cecropins, Cecropin A, exhibited fungicidal properties against *Candida albicans*, *Malassezia obtuse*, and *Malassezia sloofiae*, which can cause a variety of pathological conditions of mammalian skin, and under certain conditions, antibacterial properties against multidrug-resistant bacteria, both Gram-positive and -negative [85,86]. *In vivo* investigations using C57BL/6 mice that evaluated the impact of cecropin A on induced inflammatory bowel disease (IBD) and alterations in gut microbiota in comparison to gentamicin have also been conducted [87]. In the study of Xu et al., the antitumor activity of *B. mori* AMPs, Cecropin A and Cecropin D, was shown against human esophageal cancer cells, with suppression of tumor cell proliferation, which induced apoptosis and inhibited migration and invasion, and no inhibitory effect on normal human embryonic kidney 293T cells [88].

Lysozymes, another component of the defense mechanism against bacteria, are bacteriolytic enzymes that hydrolyze b-1,4-glycosidic bonds between N-acetylglucosamine and N-acetylmuramic acid of the peptidoglycan layer in the bacterial cell walls [89]. They have been identified in the majority of animals and discovered in the salivary glands and guts of many insects [90–93]. As in vertebrates, lysozymes in insects are widely distributed as one of the components of bactericidal proteins and peptides found in the hemolymph, and synthesis is induced by infection with bacteria [1].

In this study, the analyses revealed variations in the number of AMPs across different developmental stages of *R. ferrugineus*, including the embryonic stage (E1-SRR926614, E2-SRR926615, E3-SRR926616, E4-SRR926617), larval stage (L1-SRR926618), pupal stage (SRR13297420), and adult stage. Adult transcripts showed a more significant number of contigs coding for putative AMPs compared to the larval transcripts of the same insect. The induction of immunity-related gene expression in RPW larvae is attributed to infection by pathogenic bacteria [94]. It is widely recognized that RPW larvae typically develop within the central region of palm trees, consuming tender tissues and sap, ultimately leading to the demise of the host plant by damaging the meristem [33]. Consequently, it is plausible to associate the reduced expression of AMPs in larvae with their decreased vulnerability to pathogens as a result of the aforementioned behavior. This finding could be supported by Wang et al., whose research revealed that the pupal stage had the greatest overall expression levels of the scale gene, compared to other life stages [37]. During the pupal stage, the morphology and mechanism of the RPW undergo significant alterations.

This concept was also supported by Manee et al. [95], who identified developmental stage-associated genes, differentially expressed genes, conserved signaling pathways, and constitutively expressed genes in RPW. Furthermore, in Yang et al. [96], the gene expression values of different developmental stages of RPW were analyzed, and the results indicated that pupae and male adults have an overall higher expression level. These findings supported the biological differences between the larval, pupal, and adult development stages of *R. ferrugineus* [96].

The higher presence of AMPs in pupae, compared to larvae, may be attributed to the need for protection during this transitional phase of life, characterized by tissue changes and reorganization [97–99]. In some instances, stronger immune activity has been noted during larval and adult stages [100]. However, comparisons between different species are challenging, as numerous factors can influence immune system response and AMP production.

Differences in antimicrobial response were experimentally tested in Cappa et al. [32], and Mazza et al. [101]. In these studies, larvae showed higher mortality compared to adults, and egg extracts could not stop the bacterial growth. Even though the eggs live in the same habitat as larvae and adults, they quickly hatch, and the chorion physical barrier may shield them from microbial attack [101].

The presence of AMPs in various body parts is also supported by Ma et al. [94], whose study showed significantly different expression levels of specific proteins in multiple tissues, such as the hemolymph, foregut, midgut and hindgut, fat body, head, and epidermis of RPW larvae. In particular, the gut and fat body are interesting due to their roles in the local immunocompetence of RPW larvae and the modulation of RPW immune homeostasis. Additionally, Muhammad et al. verified that the immune system of RPW plays a central role in sustaining and modulating the homeostasis of gut commensal microbiota [102]. Their study found that the presence of gut microbiota strongly upregulated the immunity-related genes of RPW larvae, which contain pathogen recognition receptors, nuclear factors, and in particular, AMPs. The study also revealed that the presence of gut bacteria could significantly improve the transcript abundance of several pattern recognition receptors (PRRs) and antimicrobial peptides also in the fat body [102]. These findings provide experimental evidence to support the conclusion that colonization of gut commensal microbiota has stimulatory effects on the immune system of RPW larvae, enhancing the host's immunocompetence through the upregulation of immune genes, which in turn stimulates the secretion of AMPs. Several studies have revealed that the gut microbiome can inhibit the development of *Plasmodium* and other human pathogens in mosquitos by upregulating important immune genes such as cecropins, defensins, and gambicins [103,104].

AMPs also play an important role in insect legs. According to Nomura et al. [105], regenectin, a C-type lectin found in hemolymph, appears transiently around developing muscle cells in the late stage of leg regeneration of *P. americana.* This suggests a systemic process that may involve the production of specific AMPs. In light of this, our analysis emphasizes the presence of repeated sequences in particular parts of the body, such as the legs and wings. This is probably because, in the adult, these peptides are crucial for properly supporting muscle development and regeneration of the legs and wing apparatus, which are fully sclerified and particularly robust in the adults of Coleoptera [105,106].

We manually annotated AMPs in the *R. ferrugineus* genome [58] and these annotations allowed us to correct the previous transcriptome annotation. Genome (GenBank: GCA_014462685.1) analyses revealed several AMP duplications from two to seven genes in clusters in the same scaffold. Of particular interest are seven cecropin genes in the cluster on scaffold_405, four defensin genes in the cluster on scaffold_66293, seven hypothetical AMP genes in the cluster in scaffold_66088, and three lysozyme genes in the cluster on the scaffold_66335, all representing potentially recent duplications. AMP gene duplications are of major interest since insect AMPs are known to evolve rapidly through gene gain and loss, followed by gene loss, divergence, and other processes, allowing each species to adapt to its specific ecological niche [107]. Elucidating the function of the different AMP duplicates would allow us to assess whether duplicates retained similar functions or evolved different functions. Interestingly, we identified two allelic variants of defensin in the locus tag GWI33_019784, which shared 99% identity with two amino acids (Val32 and Ser33) that were deleted in the defensin in one allele (Figure 5). As defensins exhibit a diversity of antimicrobial mechanisms, such as direct membrane disruption, neutralizing microbial toxins, and inhibition of microbial cell wall synthesis [108], the allelic variation in a defensin gene essential for these multifunctional roles accounts for the functional variations, which is an exciting area of further research.

5. Conclusions

This work analyzed *R. ferrugineus* transcriptomes data from adult females and males reared in laboratory and field conditions, using different bioinformatic tools to predict putative antimicrobial, antifungal, and anticancer activities.

In conclusion, although the results of the computational approach must be confirmed *in vitro* and *in vivo*, this strategy allows us to narrow the search field and select the most promising molecules from the growing amount of available data, thus supporting and expediting the discovery of new drugs.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/biom14101332/s1, Supplementary File S1: NCBI Accession numbers for different body parts of the Red Palm Weevil (*Rhynchophorus ferrugineus*). Data are collected from male and female specimens under both laboratory and field conditions, as reported in the SRA. Supplementary File S2: Multiple sequence alignments using coloring methods (Clustal Omega) of the peptide sequences from all parts of the body of male and female *R. ferrugineus* adult, both reared in laboratory and collected in field. Supplementary File S3: Expression Levels of Duplicate AMP Genes comparing the TPM (transcripts per million) values of duplicated sequences to evaluate the gene expression levels of putative antimicrobial sequences in each part of the body in different experimental conditions. Supplementary File S4: List of the 198 putative AMPs, identified in the transcriptome of the different body part of male and female *R. ferrugineus* adult, both reared in laboratory and collected in field. In the table are reported: sequence name, contig name and number, the adult condition (lab or field), the adult sex (male or female), the sequence description (annotation), the mature amino acid sequence, results of the antimicrobial (CAMP_{R3}), anticancer (iACP), antifungal (ANTIFP) antiviral (AVPpred), physicochemical parameter (APD3) (length, molecular weight, total hydrophobic ratio, total net charge, pI, Boman Index).

Author Contributions: Conceptualization, funding acquisition, and project administration: P.F., B.A. and A.P.; resources: M.A.A. and A.P.; bioinformatics—data assembly and annotation: C.S., R.R., P.F., J.J. and B.A.; data curation, P.F., R.S., C.S. and R.R.; methodology, P.F., C.S. and B.A.; supervision, P.F. and B.A.; writing—original draft, P.F., C.S., R.R. and B.A.; writing—review and editing, P.F., B.A., C.S., R.R., F.G., M.A.A., J.J. and A.P. All authors have read and agreed to the published version of the manuscript.

Funding: The authors extend their appreciation to the Deanship of Scientific Research, King Saud University, for funding through the Vice Deanship of Scientific Research Chairs and the Chair of Date Palm Research. The transcriptome data were generated using the funding received by B.A. and A.P. from the King Abdullah University of Science and Technology (KAUST) in Saudi Arabia (KAUST-OSR-2018-RPW-3816-1, and BAS/1/1020-01-01). This work was supported by funds received by J.J. from the Slovenian Research Agency (P4-0077). This work was supported by the University of Basilicata.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The datasets used and/or analyzed in this study are available from the corresponding author upon reasonable request. The RPW transcriptomes (male and female; laboratory and field) have been deposited in NCBI/DDBJ/EMBL SRA accession numbers BioProject PRJNA275430) and are reported in Supplementary File S1.

Acknowledgments: The authors also thank the date palm farmers in the Al-Qassim areas for their support in obtaining red palm weevils. We thank KAUST Bioscience Core laboratory personnel for their support in producing the raw sequence data.

Conflicts of Interest: The authors declare no conflicts of interest. P.F., B.A., and A.P. may have a financial interest in RPW AMP(s) patent applications related to drug development.

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A Glance into the Near Future: Cultivated Meat from Mammalian and Insect Cells

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The increasing global population and demand for meat have led to the need to find sustainable and viable alternatives to traditional production methods. One potential solution is cultivated meat (CM), which involves producing meat in vitro from animal stem cells to generate products with nutritional and sensory properties similar to conventional livestock-derived meat. This article examines current approaches to CM production and investigates how using insect cells could enhance the process. Cell sources are a critical issue in CM production, alongside advances in culture media, bioreactors for scalability, and scaffold development. Insect cells, compared to commonly used mammalian cells, may offer advantages in overcoming technological challenges that hinder cell culture development and expansion. The objective of this review is to emphasize how insects, as a cell source for CM production, could offer a more sustainable option. A crucial aspect for achieving this goal is a comprehensive understanding of the physiology of muscle and fat cells. In this work, the characteristics of insect and mammalian cells are compared, focusing particularly on muscle and fat cell development, regulatory pathways, hormonal regulation, and tissue composition. Insect cells are a promising source for CM, offering a sustainable and environmentally friendly alternative.

1. Introduction

Global meat production is anticipated to increase by almost 44 Mt by 2030, reaching 373 Mt because of rising production as meat prices resume following Covid-19, according to the publication "OECD-FAO Agricultural Outlook 2021–2030."[1] Meat consumption is influenced by a variety of factors, including prices, tradition, environmental concerns, animal welfare, and health. Population growth and economic improvement are primary drivers of rising meat consumption, with a projected 14% increase in global meat intake due to an anticipated 11% global rise in population by 2030. Specifically, this increase will be 12% in Latin America, 18% in Asia-Pacific, 30% in Africa, 0.4% in Europe, and 9% in North America. Economic growth and its structural changes encourage increased meat consumption. According to empirical studies about consumer behavior, higher income drives consumption of more high-value foods, such as animal proteins, and fewer low-value products, such as carbohydrates. All these factors have contributed to a dramatic increase in livestock production over the past decade, with growing demand for animal-based foods among a significant portion of the global population, represented by developing countries.[2]

This rising demand is problematic since current large-scale animal farming techniques (generating more than 50% of the world meat supply) are associated with public health risks, environmental degradation, and animal welfare concerns.[3] For example, 75% of new infectious diseases in humans are caused by animal sources (zoonotic), primarily as a result of increased human–animal interactions caused by animal husbandry, loss of natural habitats, and the increasing global population.[4,5] Animal husbandry, which accounts for 80% of antibiotics used in the United States and 73% of antibiotics sold globally, exacerbates

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The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/smsc.202400122.

DOI: 10.1002/smsc.202400122

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antibiotic resistance, causing increasing risk to human health. Based on the 2020 report by the United Nations Environment Programme on the prevention of future pandemics, the escalation in the worldwide requirement for animal protein products and the unsustainable intensification of agriculture, including the surge of intensive animal agriculture, are two of the seven significant anthropogenic factors that contribute to the emergence of zoonotic diseases. Beyond the dangers to human health, livestock are responsible for 14.5% of all anthropogenic greenhouse gas emissions measured in CO_2 equivalents. In addition, the production of animal feed has a substantial impact on the environment in terms of land and water use.[6] In response to growing concern about the sustainability of large-scale agriculture, new technologies are emerging for more efficient protein production. One such solution is cultivated meat (CM, also known as cell-based or cultured meat), which involves the production of meat through in vitro cultivation of animal stem cells, mimicking the natural process of cell growth and division in animals, resulting in a product like traditional meat in terms of nutrition and taste. This is intended to address environmental and animal welfare issues while meeting the needs of a growing global population. Research into CM dates to 2002, when it was observed that the utilization of cultured fish cells could potentially aid in the development of a goldfish muscle explant.[7] The first official tasting of CM was in 2013, when Dr. Mark Post's team created a highly publicized hamburger from bovine muscle cells. A growing number of organizations are currently commercializing and scaling CM (at least 70 were reported in mid-2021).[8] For these reasons, in November 2022, the Food and Drug Administration (FDA) assessed the safety of "Cultured chicken (*Gallus gallus*) cell material" provided by Upside Food, a producer of CM. The FDA determined that this was safe and found no evidence that its production process could introduce harmful substances or micro-organisms into the food.[9] Similarly, in March 2023, the FDA evaluated cultured *Gallus gallus* cell material from GOOD Meat,[10] a company that already sells CM in Singapore. Singapore was the first country to approve CM production in December 2020, specifically the cultivated chicken bites produced by the US start-up Eat Just consisting of cultivated chicken cells and plant-based components.[11,12] Food safety regulations vary between countries and regions. In the USA, the FDA oversees food safety, except for meat and poultry, which fall under the jurisdiction of the US Department of Agriculture Food Safety and Inspection Service (USDA–FSIS) under the Federal Meat Inspection Act (FMIA).[13] Regarding regulations for cell-based meat for human consumption, the USDA and FDA issued a joint statement in 2018.[14] Under this agreement, the FDA oversees early stages of cell-based meat development, including cell collection, development, differentiation, and proliferation processes.[15] This oversight applies to products derived from cell lines of USDA-amenable species and requires a USDA mark of inspection.[16] Once cells or tissues are ready for harvest, regulatory oversight shifts from the FDA to USDA–FSIS, which ensures the safety, labeling, and overall quality of cell-based meats. Both agencies inspect production facilities, with USDA–FSIS focusing on final production stages.[15] Unlike the United States, CM in Europe falls under either the EU Novel Foods Regulation, which pertains to foods and ingredients not significantly consumed in the EU before May 15, 1997,[16,17] or the genetically modified organism (GMO) Legislation (embodied by the GMO Directive[18] and GMO Regulation,[19] if the initial cell types used are induced pluripotent stem cells (iPSCs).[15,20] Member States conduct consultations to determine whether a particular food falls under which regulation, with safety assessments conducted by the European Food Safety Authority.

The main objective of this review is to assess the potential of insect cells as a sustainable and efficient source of CM. This involves a comprehensive comparison of their characteristics with those of mammalian cells, identifying their respective benefits and limitations. In exploring both muscle and adipose tissues, it is essential to recognize the distinct differences between mammals and insects in terms of cellular origins, molecular regulatory pathways, and physiological functions. These comparisons not only illuminate the biological complexity and diversity of these systems in both groups, but also highlight their potential biotechnological applications, including the cultivation of meat.

2. Production Process of CM

2.1. Structure of Muscle Tissue

In the production of CM, three fundamental elements play pivotal roles: cells, signals (present in the culture medium), and scaffolds. Cells are the key element, while the culture media provides essential nutrients and small molecules to support cell growth and functions. Scaffolds, made of biocompatible materials, serve as a support to which cells are anchored, facilitating their proliferation and differentiation (**Figure 1**). The aim of the in vitro CM process is to recreate the tissue structure of animals from different cell sources, primarily focusing on muscle and fat. Myoblastic cells, crucial for muscle tissue formation, can be obtained through various methods. The most common approach involves performing a tissue biopsy of the desired animal or utilizing postmortem tissues. The alternative approach utilizes a source of pluripotent stem cells (PSCs), such as embryonic stem cells (ESCs) or iPSCs. In the first scenario, primary cell cultures can be used directly. In the second, the cells undergo differentiation into mesodermal cells before becoming muscle progenitor cells.[21] Myoblast cells fuse naturally in a process known as myogenesis, that is the formation of muscle tissue that occurs particularly during embryonic development.

The architecture of skeletal muscle is a well-organized distribution of multinucleated contractile muscle cells (also known as muscle fibers) and related connective tissue.[22] Muscle development occurs in vivo during embryogenesis with the multiplication of mononucleated myoblasts, which eventually fuse and divide to produce muscle fibers.[23] Muscle fibers are functional units surrounded by connective tissue, intramuscular fat, blood vessels, and nerves. The muscle fibers are organized into bundles, and the surrounding connective tissue is composed of endomysium, perimysium, and epimysium. The vessels ensure the transfer of oxygen and nutrients.[24] The nutritional value of meat derives mostly from high-quality protein from muscle that contains all essential amino acids, essential fatty acids, and a variety of vitamins and minerals. Red muscle tissue

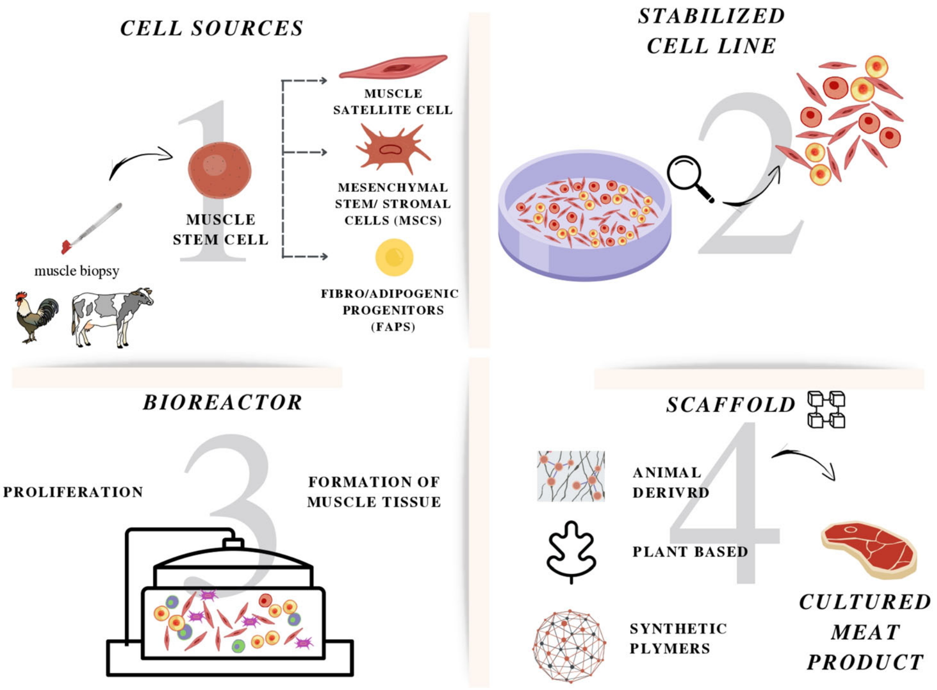

Figure 1. Overview of in vitro CM production. The first step involves harvesting cells from a live animal and obtaining stem cells. The primary cells are then cultivated in an appropriate, nutrient-rich medium and a cell line is established. Muscle and fat cells are cultured into a bioreactor, a sterile food production facility, to grow and differentiate until muscle tissue is formed. The cells will organize on scaffolds of various origins to form the edible product (Image Created with BioRender.com).

has more myoglobin and, consequently, more heme iron than white muscle tissue,[24] making it a more nutritious source of bioavailable iron. Intramuscular fats contribute to the texture, nutrition, and species-specific flavor of meat.[25] The predominant composition of intramuscular fat consists of adipocytes, which are situated within the interstitial spaces of muscle fibers and fascicles. Intramuscular adipose tissue is composed of various lipid components, including structural lipids, phospholipids, and intracellular lipid droplets located within muscle fibers. In addition, lipids found in fat contain crucial lipophilic vitamins, including A, D, K, and E, alongside essential omega-3 polyunsaturated fatty acids.[26]

2.2. Cell Sources

The composition of meat typically comprises ≈90% muscle fibers, 10% fat, and connective tissue,[24,27] although this varies depending on specific cut of meat and the species it is derived from. Skeletal myocytes are the most numerous cell types in meat, with adipocytes, fibroblasts, chondrocytes, and hematopoietic cells also present and often providing support (**Figure 2**A,B). Stem cells sourced from a living animal biopsy can be cultivated in vitro to yield substantial cell numbers. These versatile stem cells possess the capability to differentiate into either muscle or fat cells, determined by their specific type.[28]

Primary cell types for CM production must be capable of adequate self-renewal and differentiation into the mature cell types that characterize meat. Stem cells are the best option for use as a source of starting cells to satisfy these needs. Adult stem cells and PSCs are the two types of stem cells with the proliferative capacity and differentiation potential necessary for the generation of CM. Traditionally, tissue-specific stem cells have been the preferred cell source for CM production. They are undifferentiated progenitor cells present in the organs and tissues of animals. Tissue-specific stem cells are multipotent, meaning they may differentiate into several cell types, the majority of which are relevant to the organ or tissue in which they reside. Within the microenvironment of muscle tissue, the three most frequently encountered types of progenitor/stem cells are muscle satellite cells (MuSCs), mesenchymal stem/stromal cells (MSCs), and fibroadipogenic progenitors (FAPs). The progenitor cells possess the ability to undergo differentiation and give rise to various mature cell types, including but not limited to skeletal myocytes, adipocytes, chondrocytes, and fibroblasts. Muscle satellite cells are a type of stem cell that can be located beneath the basement membrane of muscle fibers. These cells can differentiate into myocytes, which then form multinucleated myotubes that are densely packed into myofibers. MuSCs are one of the most prevalent forms of tissue-resident adult stem cells,[29] and their extraction from animals and maintenance in vitro are well described.[30,31] MSCs are commonly found in the bone marrow, but they can also be found in other anatomical locations, such as skeletal muscles, and play a crucial role in muscle regeneration following damage.[32] MSCs possess the ability to undergo

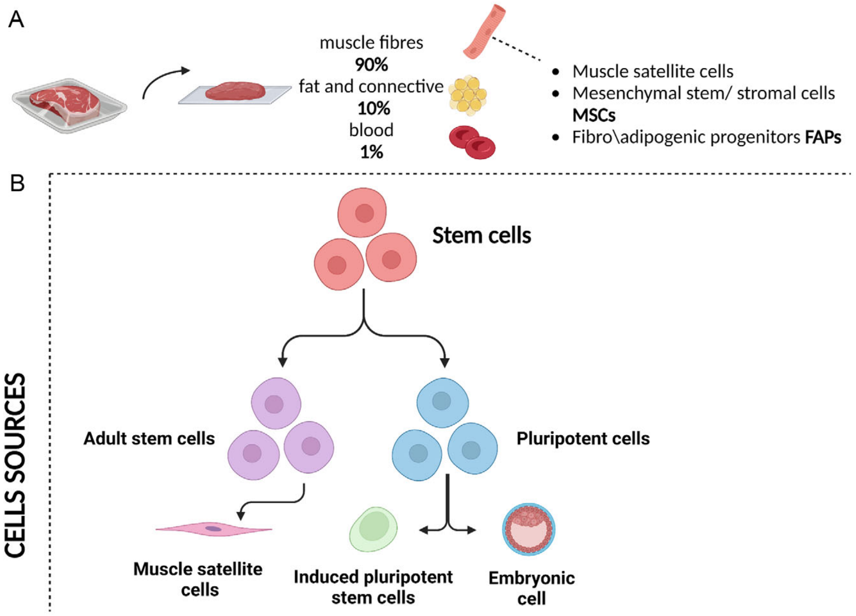

Figure 2. A) The main components of meat. In the microenvironment of muscle fibers, there are progenitor/stem cells such as muscle satellite cells, MSCs, and FAPs. B) Schematic representation of the main sources of stem cells capable of self-renewal and differentiation into the cells that characterize meat, that is, skeletal myocytes, adipocytes, chondrocytes, and fibroblasts (Image Created with BioRender.com).

differentiation into adipocytes, chondrocytes, and fibroblasts.[27,30] Mosa Meat, the pioneer of the first cultivated hamburger, has laid the groundwork for cleaner meat alternatives through the use of MSCs.[28,33] Various startups, such as Meatable[34] and BioTech Foods,[35] harness skeletal muscle cells from cattle and/or pigs to create CM products. These products range from minced meat alternatives to delectable and nutritious items like nuggets, hamburgers, and sausages. To achieve the ideal amplification level for processed CM, MuSCs must undergo cell fusion and transition into multinucleated, postmitotic muscle fibers. The differentiation process to myotubes in vitro begins upon MSC exposure to a differentiation medium, typically spanning 3–5 days.[36] Controlling MSC activity often involves manipulating extracellular signaling molecules present in the culture medium. Growth factors (GFs) such as insulin-like GFs (IGF-1 and -2), fibroblast GF (FGF), hepatocyte GF, and cytokines like Tumor Necrosis Factor-α and leukemia inhibitory factor play pivotal roles in driving MSC activation and proliferation.[37–39]

FAPs, a separate population of MSCs found in the interstitial space of skeletal muscle, are another important cell type for skeletal muscle development and regeneration. FAPs can differentiate into both fibroblasts and adipocytes, which are the connective and fatty tissues found in meat and play a crucial role in myogenic development and organization.[31,40] Dedifferentiated fat cells (DFAT) have been identified as a plausible cellular source for the cultivation of adipose tissue. These cells are obtained through the process of dedifferentiation of mature adipocytes. Several commercially accessible immortalized preadipose cell lines exist, such as 3T3-L1, 3T3-F442A, and OP9. These exhibit distinct attributes, conducive to the generation of cell culture fat for human consumption. These include a notable capacity for cellular proliferation, resilient differentiation into adipocytes, uniformity in cell populations, uncomplicated maintenance procedures, and comprehensive characterization. Nonetheless, most of these cellular lineages are derived from murine origins, thereby restricting their efficacy in the context of investigating and advancing the production of CM.[26]

When satellite cells, MSCs, and FAPs are combined, they have the potential to generate all the cell types present in meat. While tissue-specific stem cells are readily available and capable of differentiating into the necessary mature cell types found in meat, their proliferation and maintenance in vitro are restricted. PSCs have potential as a second cell source for CM production, even though primary tissue-specific stem cells are a popular cell source. PSCs, such as ESCs and iPSCs, are highly proliferative in culture and can differentiate into every cell type seen in the three primary germ layers (i.e., mesoderm, endoderm, ectoderm). ESCs are sourced from the inner cell mass of the blastocyst, a developmental stage that takes place during the initial phases of mammalian growth. iPSCs are generated by triggering pluripotency genes in somatic cells (Figure 2B).[24,41] PSCs derived from non-muscle sources can be isolated from various domestic animals and harnessed as myogenic cell reservoirs for CM production. Recent advancements include the chemical and genetic modification of pig PSCs to prompt their differentiation into myogenic cells capable of forming embryonic muscle fibers.[42,43] Gourmey utilized PSCs to craft cultured foie gras.[44,45] However, it is crucial to note that any CM derived from PSCs necessitates clear labeling as genetically modified and must undergo comprehensive safety assessments due to regulatory requirements.[43]

2.3. Cell Immortalization and Differentiation

2.3.1. Rationale for Immortalization of Cultivated Meat Cells

Although primary cell cultures have the benefit of being able to be employed relatively quickly for meat production, they have the disadvantage of being limited in the number of cell divisions they can undergo before senescence or cell cycle arrest. This makes long-term and commercial production difficult. The use of primary cell cultures necessitates repeated biopsies from live animals, as well as the testing and approval of these biopsies for use in food production.[40] Unlike primary cell cultures, immortalized cell lines are not susceptible to senescence and can undergo an endless number of cell divisions. The production of immortalized cell lines is a crucial requirement in the field of cell culture. Even though work on cell lines began more than 50 years ago, there are few cell lines suitable for the cultivation of meat. Indeed, they must conform to particular requirements such as the capacity to proliferate and differentiate (e.g., form mature muscle from muscle cells and accumulate lipids as fat cells) efficiently on an industrial scale, be authorized as safe for ingestion as food, and have the desired properties in terms of flavor, consistency, and nutrition.[44] The primary culinary components of animal meat consist of skeletal muscle and adipose tissue. Pertinent cellular entities include satellite cells and stem cells derived from fat tissue, alongside MSCs, versatile fibroblasts, and various types of stem cells.[46,47] Myoblast cell lines from model animals are the closest existing cell lines. In addition to consumer impressions, current cell lines lack the flavor, nutrients, and texture associated with meat.[48,49] Only recently attempts have been made to establish banks for collecting cell lines appropriate to the development of CM. For example, the Good Food Institute and Kerafast (Boston, Massachusetts) are working together to maintain a bank of terrestrial and aquatic cell lines that can be used for research on CM; however, the number of useful cells remains relatively low.[50]

2.3.2. Methods to Immortalize Cultivated Meat Cells

Currently, there are three methods to obtain immortalized cell lines: spontaneous immortalization, the development of the telomerase catalytic subunit (TERT), and stimulation via viral genes that inactivate the p53/p14/Rb pathway. Each technique employs telomerase expression, cell cycle inactivation/bypass, or both.[40,51] Mammalian cells rarely spontaneously immortalize, and spontaneous immortalization is typically associated with malignancy. This was the first approach used to produce cell lines from the first immortal cell lines recovered from mouse fibroblasts in the 1940s, as well as the *Hela* cell line isolated from cervical cancer cells extracted from Henrietta Lacks. Immortalization can also be triggered by mutagenesis via radiation or chemical carcinogens.[52,53] It is also possible to identify clones with immortalization markers, significant TERT expression, or low p15/p16/Rb expression by serial passage of a cell line. The biotechnological company "Future Meat Technologies" has generated a cell line that has spontaneously been immortalized. The present cell line was obtained through the cultivation of fibroblasts that were extracted from a chick embryo, followed by the isolation, concentration, and expansion of colonies of cells that exhibited superior growth characteristics, also known as foci. The colonies underwent expansion to attain a uniform morphology culture, which exhibited the ability to sustain itself beyond 20–30 divisions, exceeding the growth potential of unaltered somatic cells. Each time a non-immortal cell divides, the telomeres become shorter, up to a point called the Hayflick limit. This makes the telomeres vulnerable to damage and causes senescence. Infection of human fibroblasts and keratinocytes with a retrovirus-encoding human TERT results in the immortalization of the cell lines. Ectopic expression of TERT in human endothelial cells was also immortalized using plasmid transfection.[51,54]

Activating the p53/p16/Rb pathways, which bypass the stress response system, is an additional strategy for immortalizing cells. Under normal conditions, p53 is activated in response to DNA damage or other stresses, resulting in cell cycle arrest and apoptosis.[55] Rb and p16 activation inhibit the activation of DNA replication by other proteins, resulting in cellular senescence.[55,56] Because p16 and Rb are blocked or altered, DNA replication can continue, resulting in cell division.[40] TERT expression or p15/p16/Rb inactivation alone is frequently inadequate to immortalize a cell line, indicating that both telomere shortening and the p53/p16 stress response must be avoided. As previously demonstrated, myoblasts must avoid both senescence-triggering events to attain immortality.[57] Upside Foods submitted a patent application in 2016 to immortalize cell lines by overexpressing TERT and utilizing CRISPR-Cas to suppress the expression of p15 and p16 in chicken skeletal muscle cells.[58] TERT overexpression by an ectopic TERT gene enhanced cell proliferative capacity indefinitely, but the deletion of p15 and p16 alone increased cell proliferative capacity. Myogenic cell lines can also be made immortal by expressing genes in a way that skips the shortening of the telomeres and the p16 stress pathway. Other approaches to immortalizing myogenic cell lines can avoid both telomere shortening and stress pathway p16 by ectopically expressing TERT and inhibitors of Rb kinase 4 cyclin-dependent (CDK4) and cyclin D1.[51,59,60]

2.3.3. Differentiation in the Context of Cultivated Meat

Muscle cell differentiation occurs in vivo when MuSCs transition from a quiescent to a proliferative state, culminating in myoblast formation.[23] From the multiplication of myoblasts, adequate quantities are produced for muscle regeneration, and a portion of these cells also revert to a quiescent state. Because proliferation and differentiation are mutually incompatible processes in in vitro cell growth, cells are typically expanded first and then triggered to differentiate. Differentiation is often achieved by eliminating GFs or introducing differentiation-promoting proteins. For example, eliminating serum from the culture medium stimulates in vitro differentiation of muscle stem cells, and further maturation can be induced by mechanical and electrical stimulation. Their combined effects especially enhance the early stages of cell proliferation in the absence of a support structure. By acting as a support for propagation and differentiation, scaffolds play a crucial function in terms of mechanical stimulation; their application for cell differentiation mimics the extracellular matrix (ECM)–cell interactions, generally found in vivo via activation of integrin receptors. The effects of electrical stimulation on rat L6 myoblasts were demonstrated using a commercial cell culture stimulation device.[61] Electrical stimulation controls myogenic differentiation by reducing the expression of small GTPases[62] (**Figure 3**). When generating CM products, it is likely that muscle cells will be differentiated by the simplest method possible to recapitulate the texture and nutrition of animal-derived meat.

Adipose tissue is responsible for the regulation and homeostasis of energy metabolism. It is mostly composed of adipocytes surrounded by fibroblasts, fibroblast–preadipocyte cells, endothelial cells, nerve cells, and immune cells.[63] For CM production, effective differentiation of adipocytes (i.e., lipid accumulation) is essential. More research is required to create expandable adipogenic stem/progenitor cell lines from meat animal species, food-grade culture conditions for mature adipocytes, and scalable protocols for creating edible fat tissue, even though the molecular functions and mechanisms of adipocytes have been relatively well studied.

Several approaches have been developed for distinguishing preadipocytes from cells capable of lipid droplet accumulation and exhibiting the morphological and biochemical characteristics of mature white adipocytes.[64] Differentiation strategies for preadipose cell lines and primary cultures of fat precursor cells have been created, and the responsiveness of preadipocytes to inducing stimuli can vary greatly. In the presence of fetal bovine serum, preadipocytes spontaneously differentiate into fat cell groups to some extent. The quantity of lipid production may be dose dependently regulated by altering the serum concentration in the growth medium. Inducing substances such as dexamethasone, used to activate the glucocorticoid receptor and 3-isobutyl-1-methylxanthine receptor pathways (IBMX) (or 1-methyl-3-isobutylxanthine, MIX), which is used to stimulate the cAMP-protein-dependent kinase, can enhance differentiation.[65] In addition, high quantities of insulin have been combined with these inductors. It has been established that insulin/IGF-1, glucocorticoid, and field-signaling pathways are involved in the adipocyte differentiation process.[66]

2.4. Culture Media for Cultivated Meat

How to develop and maintain concurrently muscle satellite cells (or, more broadly, muscle precursor cells [MPC]), myoblasts, myocytes (also known as myotubes or myofibers), adipose-derived stem cells, adipocytes, and fibroblasts is a significant unresolved technical challenge to produce CM.[67]

Cell proliferation and maintenance depend on a variety of components, such as hormones and GFs. These signals, which are produced in vivo by endocrine glands, bind to specific receptors on the cell membrane or in the cytoplasm and activate the pathways that control cell division and proliferation. Cell culture media contain all the components necessary for cellular survival as well as to stimulate responses such as adhesion, proliferation, and differentiation. In general, basal media consist of carbon and nitrogen sources like glucose, glutamine, and other amino acids; inorganic vitamins and salts; and signaling molecules like GFs.[53,67] Formulas for culture media vary according to the application. Recently, several commercially available basal media have become available and usually contain most, but not all, of the ingredients needed for cells to grow, such as glucose, amino acids, and vitamins. Conventionally, basal media formulations are supplemented with complex components of animal origin (i.e., serum) that supply additional nutrients and signaling

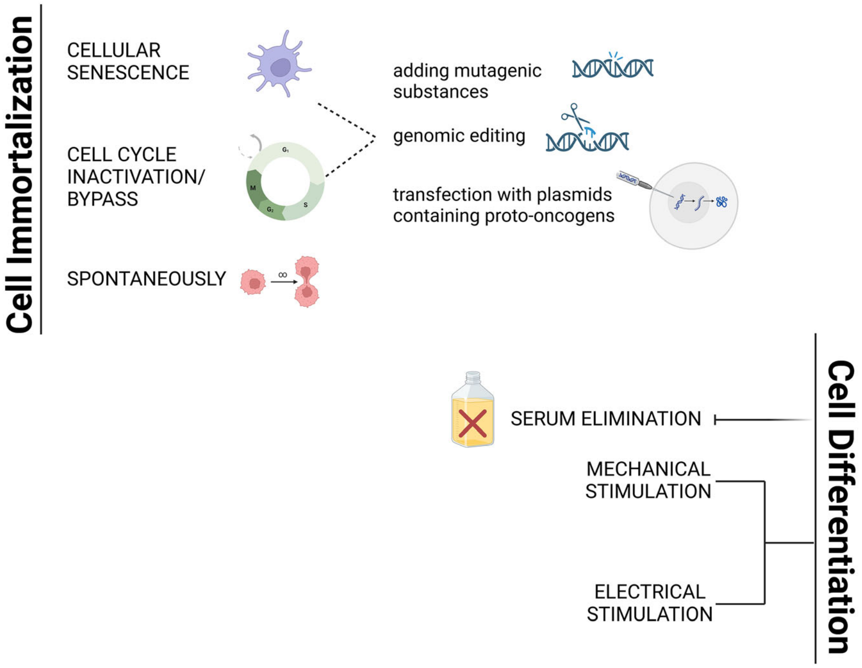

Figure 3. Three methods to obtain immortal cell lines: development of the TERT to avoid cellular senescence, inactivation, or loss of cell cycle checkpoint control by acting on the p53/p14/Rb pathway or spontaneous immortalization (Image Created with BioRender.com).

molecules.[53] Eagle minimal essential medium (MEM),[68] Dulbecco-modified Eagle medium (DMEM),[69] and Ham F-12[70] are some examples. Serum is the blood fluid collected following blood coagulation; it is rich in proteins, nutrients, GFs, and hormones. Although serum is traditionally used for muscle cell culture,[71] it is antithetical to CM production because it is derived from animals. Indeed, a food-grade medium that is inexpensive, can control large-scale cell proliferation and differentiation, has acceptable sensory properties, and contains no animal products, is necessary for the effective production of CM.[67] Recently, serum-free media have been created and treated with GFs (particularly insulin, FGF2, and TGF) extracted from animal serum or recombinantly produced.[72,73] Essential 8 medium and FGF are two serum-free media formulations that are commonly used in research to promote stem cell growth. These two formulations have recently shown promise in CM applications, as they support the proliferation of primary bovine myoblasts for at least 6 days.[74,75] Many ingredients, including hormones and GFs, are essential for the proliferation and maintenance of cells. In vivo, these signals are generated by endocrine glands and bind to specific receptors on the cell membrane or in the cytoplasm, activating the pathways involved in cell proliferation and differentiation. Insulin and IGFs can promote the development of pluripotent, or adipose-derived, stem cells into adipocytes when required in a CM application.[76] FGF2 is a GF commonly added to muscle cell proliferation media that has a trophic impact on myoblasts by suppressing differentiation.[77] Insulin is a commonly used hormone for in vitro maintenance of stem cells. In rat cell cultures, insulin addition stimulated the development of myoblasts.[78] The control of catabolism by glucocorticoids influences the proliferation of distinct cell types. In vitro, dexamethasone reduces myoblast doubling time of muscle stem cells, increasing their proliferation potential.[79] In addition, dexamethasone treatment promotes satellite cell myogenic differentiation, as evidenced by increased sarcomere formation and enhanced contraction in the resulting myotubes.[80] The traditional adipogenic differentiation cocktail includes insulin, dexamethasone, indomethacin, isobutylmethylxanthine, and rosiglitazone.[81] Moreover, it has been shown that only two inducers, rosiglitazone and insulin, are needed for serum-free adipogenesis.[82] Tissue development requires many GFs, including TGF-β, differentiation factors, and bone morphogenetic proteins. In vitro-cultivated myoblasts treated with TGF-β are inhibited from developing into myotubes, but their diminished differentiation capacity may be restored once TGF-β treatment is stopped.[83]

In vitro-cultivated animal cells may become contaminated with bacteria, fungi, and yeast. Penicillin, streptomycin, amphotericin B, and gentamicin are some of the antibiotics and antimycotic drugs that have historically been used to treat these contaminations.[84] However, antibiotics should generally be avoided during CM production due to their negative side effects on consumers' sensitivities and they may also contribute to the spread of antibiotic-resistant bacteria. Both issues could make it more challenging for consumers to accept CM products. Thus, the procedure for producing CM prohibits the use of antibiotic media supplements,[67] which are otherwise crucial in the initial phases of primary cell culture development.

For in vitro cell cultivation, it is essential to replicate the animals in an in vivo environment. Incubators are primarily used to maintain a constant temperature, humidity, and pH for cellular homeostasis. The inclusion of sodium bicarbonate buffer in the cell culture medium enables the carbon dioxide (CO_2) content in the incubator to be regulated, hence preserving the medium pH. The normal incubator settings for culturing stem cells, including muscle cells, are 36.5–37.5 °C, like normal physiological temperature, and 5%–10% CO_2 for pH regulation.[53] Cellular characteristics such as proliferation and differentiation are influenced by variations in cellular respiration and mitochondrial activity due to differences between physiological (1%–6%) and atmospheric (20%) oxygen concentrations. In addition, several recent studies have shown that hypoxia impacts the stemness of muscle stem cells.[85] In contrast to atmospheric oxygen concentrations, mouse satellite cells can grow under hypoxic conditions (2% O_2), proliferating twice as rapidly. When transplanted into cardiotoxin-damaged muscle, cells grown under hypoxia generated more new muscular fibers than cells cultivated in normoxia.[86] Similarly, the myogenic potential of pig satellite cells was improved at low oxygen levels.[87]

To achieve successful CM production, several key factors must be considered: access to cost-effective food-grade media-component, the capacity to manage cell growth and differentiation on a large scale, desirable sensory characteristics, and the exclusion of animal-derived components. Valuable insights for developing suitable culture media can be gained from understanding traditional applications of culture media and the metabolic pathways involved in muscle development and protein synthesis. Additionally, strategies employed to enhance media for large-scale microbial fermentation processes, which yield fundamental chemicals and less valuable food components, can offer valuable guidance.

It is crucial to acknowledge that the culture media used can significantly impact the sensory properties of harvested muscle or meat. Residues from the media present within or on cells could influence flavor, texture, or color. For example, certain amino acids like glutamic acid and asparagine, which contribute to the umami taste in meat, are found in some cell culture media, introducing an additional layer of complexity to media formulation considerations. Recent studies, including preliminary sensory assessments, suggest that laboratory-scale CM prototypes demonstrate acceptable organoleptic qualities. The process of developing new formulations for CM media begins by identifying key components through experimental or theoretical analysis, followed by optimizing their concentrations. This task is complex due to the large number of components involved, exemplified by DMEM containing between 30 and 52 components, which can lead to intricate interaction effects among them.[88] Furthermore, physiological variability of cell lines necessitates reoptimization as processes evolve and new[89] components are identified, a common scenario in an industry working with diverse cell lines.[90] Therefore, efficient methods for identifying and adjusting concentrations are crucial. Traditionally, media design begins with a one-factor-at-a-time approach, where each component is assessed individually for its effect on cell response. However, this method overlooks interaction effects, potentially resulting in suboptimal media designs.[91] To overcome this limitation, design of experiment (DOE) techniques like factorial, Plackett–Burman, and central composite designs are employed. These methods involve simultaneously changing multiple nutrient concentrations, enabling faster optimization, and have been successfully applied in various industries to characterize and optimize production processes.[92–96] DOE experiments are conducted at the extreme ends of the design space to estimate first-order effects of each media component without interference from others. These experiments are complemented by response surface models (e.g., linear or polynomial models) to predict optimal concentrations and sequentially improve mixtures.[97] While effective, these methods can still be experimentally intensive, particularly when optimizing formulations with numerous variables. Alternatively, stochastic optimization methods like genetic algorithms treat media combinations as evolving chromosomes under selection pressures, aiming to maximize fitness (e.g., biomass) of nutrient combinations.[98] Modern stochastic methods integrate mathematical surrogate models (e.g., neural networks) to aid in prediction and store information about component effects and interactions, enhancing optimization efficiency over time.[99] These advanced optimization techniques have been successful in designing complex microbial media with fewer experiments compared to traditional DOE, demonstrating promising efficiency improvements in media formulation. Continued advancements aim to make these methods more accessible to practitioners, reducing reliance on specialized expertise in artificial intelligence and numerical optimization methodologies.[100]

2.5. Scaffolding

In most cell culture applications for biotechnology, tissue culture flasks and Petri plates are used to establish 2D cell cultures. 2D cell culture is the most common approach for studying cell morphology and the effects of prospective therapies on cell functions. When cells are transplanted from their original tissue into a 2D environment, they typically lose their normal shape, resulting in alterations of the metabolism and gene expression.[101] 2D cell culture techniques do not adequately recreate the in vivo environment of the native tissue complex of skeletal muscle, with the absence of cellular connections and communication between cells resulting in slower cell proliferation, less differentiation, and an inability to create epithelial tissue characteristics such as tubular and cystic structures.[101] To control the in vitro formation of muscle tissue, scaffolds are often used to simulate the ECM generated by cells and support cell adherence, proliferation, and differentiation. A suitable scaffold for the growth of muscle cells must be edible and cytocompatible and, if utilized in a 3D format must facilitate the exchange of gases, nutrients, and waste to avoid necrosis of the cells. Mimicking the rigidity and protein composition of native ECM helps to replicate the natural microenvironment. This mimicry promotes cell–cell and cell–matrix communication, facilitating cell proliferation and differentiation.[102] The scaffolds for CM will likely be modeled in line with tissue engineering (TE) scaffolds, based on biocompatibility, biodegradability, and mechanical properties, while pore size, architecture, and manufacturing methodologies must also be considered.[103] Scaffold architecture usually should be porous in order to allow continuous media perfusion, mimicking natural vascularized tissue. Given that muscle tissue (myocytes) constitutes the predominant component of meat, the goal of TE for CM is to produce muscle tissue using cell culture and proper scaffolding.

Scaffold requirements for fat cells are less stringent compared with muscle cell culture, but growth of fat cells within a 3D matrix will likely improve mouthfeel of final products. However, they must still serve as viable substitutes for the typical role played by the ECM. Currently, a variety of scaffolds, including those of synthetic and natural origin, are extensively employed. Polyglycolic acid (PGA) and poly lactic glycolic acid (PLGA) are synthetic materials that are utilized as scaffolds for muscle cells. It is noteworthy that within the category of natural scaffolds, various types exist, such as collagen, collagen-chitosan hydrogels crosslinked with glutaraldehyde, fibrin, and HYAFF, which is a polymer derived from hyaluronic acid (HA).[104]

Such scaffolds can be constructed with both synthetic or natural polymers, including those obtained from plants and animals, depending on the origin of the material.[105]

2.5.1. Natural Polymers

Frequently used scaffolds for skeletal TE are composed of three main groups of natural polymers including proteins (silk, collagen, gelatin, fibrinogen, elastin, keratin, actin, myosin), polysaccharides (cellulose, amylose, dextran, chitin/chitosan, glycosaminoglycans), and polynucleotides (DNA, RNA).[106] Natural edible and food safe polymers are commonly used in the production of CM, with plant protein-based scaffolds being particularly desirable because of the high volume availability, low cost, nutritional value, and cytocompatibility.[107,108]

2.5.2. Animal-Derived

Avoiding the use of animal-derived scaffolds in CM production is an ethical choice, which aims to minimize negative impacts on the lives of animals and promote sustainable and compassionate practices in the food industry. However, it is important to discuss their characteristics by presenting some examples. Collagen is considered an optimal material for scaffolds, that resemble the ECM for human skeletal muscle engineering, and the majority of bioartificial muscles (BAM) are grown on collagen scaffolds.[109–112] Gelatin, a natural component of meat generated when collagen is denatured by processing and heating, has been used to manufacture CM, although it is generally preferred to avoid animal-derived materials in the process.[113] Cultured bovine aorta smooth muscle cells and rabbit skeletal muscle myoblasts replicated several morphological and mechanical properties of natural meat, but lacked the contractile architecture because the gelatin fibers used as the substrate was crosslinked to prevent deterioration.[114] Fibrin scaffolds, a naturally occurring fibrous protein that forms blood clots at injury sites, have been used to maximize BAM vascularization. According to the findings, fibrin gel is sufficient for the generation of vascularized BAMs.[113,115,116] HA is commonly used in TE because it promotes rapid wound healing and regulates adipogenesis, angiogenesis, and tissue organization in cells. In addition, attempts have been made to replace animal-sourced HA with endotoxin-free microorganism-generated HA via genetic engineering.[117,118] Another biopolymer of animal origin used in skeletal muscle scaffolds is chitosan. Chitosan is the main derivative of chitin, a biopolymer found in the exoskeleton of crustaceans and insects and in the cell walls of fungi.[119] Currently, the main source of chitin is from crustaceans, but because of limitations linked to seasonality and the poor sustainability of crustacean farming, alternatives are needed. Insects, particularly bioconverters, represent a new alternative and more sustainable source of chitin and chitosan. Indeed, bioconverter insect farms, aimed at the production of animal feed and organic byproduct management (using as insect feed), have spread worldwide, generating huge amounts of insect chitin, mainly derived from pupal exuviae and dead adults. The first characterization of insect chitin and chitosan showed a high degree of similarity with crustacean counterparts, providing a good starting point to use insect biopolymers in the same applications already tested using crustacean sources, including scaffolds.[120,121]

2.5.3. Plant-Derived

Plant-derived scaffolds (e.g., zein, soy protein, wheat gluten) are of interest for CM researchers because of their biodegradability and edibility. Occasionally, these scaffolds may also impart nutritional value and texture to CM.[122] One limitation of plant-derived scaffolds is their lack of mechanical properties; however, this can be remedied by crosslinking. Physical crosslinking, such as UV or thermal processes, are commonly employed. Otherwise, ingestible or FDA-approved enzymatic or chemical crosslinkers like citric acid, sodium hydroxide, sodium phosphates, or transglutaminase can be utilized to alter the properties of plant-derived scaffolds, enhancing their mechanical strength to support cell growth. The choice of crosslinkers depends on many factors, such as the base polymeric material, the scaffold architecture, the synthetic process, and cell culture conditions.[123,124]

Proteins derived from plants can be converted into fibers, films, and hydrogels. In addition, they are readily accessible and reasonably priced. Soy and zein-derived proteins are commonly used in the production of scaffolds. Soy protein is beneficial for TE since it is biocompatible and shares biochemical properties with the ECM.[125] Textured soy protein is favorable for cell adhesion, proliferation, and differentiation of bovine cells and has been used as an edible scaffold to generate cow muscle tissue.[126] Soy protein has also been combined with other natural polymers such as chitosan and cellulose and demonstrated favorable adherence and proliferation of multiple cell types (L929, Schwann cells, and human MSCs).[127–129] Corn zein protein is being investigated for medical applications due to its adaptability and biocompatibility.[130,131] It is soluble in ethanol, which facilitates electrospinning and the formation of nanofibers, and nontoxic crosslinking enables fibroblast cell adhesion and growth on electrospun scaffolds.[132] Zein scaffolds have also been shown to increase the adhesion, proliferation, and differentiation of human MSCs.[133]

2.5.4. Polysaccharides

In TE applications, biocompatible polysaccharides derived from plants, such as cellulose and starch, have been used. Cellulose, a linear polysaccharide, is considered as the most sustainable material due to the inexhaustible supply from plant cell walls. Cell culture research has employed a range of cellulose fibers.[134,135] However, cellulose is non-degradable in the human body. Pectin, a natural polysaccharide derived from plant cell walls, provides useful properties as an artificial ECM.[136] Pectin/carboxymethyl cellulose/microfibrillated cellulose (pectin/CMC/MFC) scaffolds with different concentrations of MFC (0–0.4%) support NIHT3 fibroblast cell survival.[137] Starch is typically blended with synthetic polymers to increase its mechanical and structural qualities as a scaffolding material.[136] Alginate, derived from brown seaweeds like *Laminaria hyperborea* and Lessonia, is widely present in coastal waters globally and holds promise as a scaffold material. It is a biocompatible, nontoxic, nonimmunogenic, and biodegradable biopolymer that is economically viable and easily manufacturable. Alginate can be converted into a hydrogel by crosslinking with bivalent cations such as calcium ions (Ca^{2+}), making it suitable for applications in various fields including food due to its safety profile. However, its negative charge impedes natural cell adhesion, limiting its use in specific applications. To address this, RGD-modified alginate gels are commonly used as in vitro cell culture platforms, allowing control over myoblast phenotypes. Myoblast adhesion and proliferation on RGD-modified alginate gels surpass those on unmodified gels. Moreover, the delivery of VEGF and IGF-1 from alginate gels regulates angiogenesis and myogenesis, facilitating muscle regeneration. Despite its suitability for cell–CM scaffolds, alginate poor cell adhesion remains a challenge, limiting its use to specific applications. Addressing this, researchers achieved 82% cell adhesion coverage by controlling the structure during alginate ionic crosslinking. After an 11-day culture period, they evaluated cell adhesion, differentiation, and network formation, observing a 12.7% increase in cell growth. Finally, a hybrid cell–CM product was created by blending mycelium-derived single-cell protein with cell–CM, yielding an edible, cost-effective product with desirable texture.[138]

Agarose, derived from marine red algae, is a natural polysaccharide highly valued in biomedical applications, due to its unique ability to form thermoreversible gels. However, unmodified agarose lacks the optimal cell adhesion properties required for effective TE and cell culture applications.[139] To overcome this limitation, researchers have implemented chemical modifications, such as carboxylation via TEMPO-mediated oxidation under alkaline conditions.[140] This approach introduces carboxyl groups onto the agarose backbone, significantly enhancing cell adhesion and bioactivity. The introduction of carboxyl groups transforms the surface properties of agarose, promoting cell adhesion, proliferation, and differentiation through improved protein absorption. Additionally, the conjugation of dopamine to carboxylated agarose further enhances cell adhesion, leveraging dopamine adhesive characteristics inspired by marine mussel proteins.

The chemical modifications of agarose were meticulously characterized using advanced analytical techniques including FT-IR, 13C NMR, and gel permeation chromatography, confirming the successful integration of carboxyl and dopamine functionalities. In vitro cell culture experiments have demonstrated that these modifications substantially enhance the bioactivity of agarose, making it a promising scaffold material specifically tailored for CM production.[141]

In summary, the strategic modification of agarose with carboxyl and dopamine functionalities represents a significant advancement in scaffold design, particularly crucial for CM production. This innovative approach supports the development of TE scaffolds that facilitate robust cellular adhesion, growth, and differentiation, thus advancing the field of alternative protein production.

2.5.5. Decellularized Plant Scaffolds

As an alternative to synthetic polymers or animal-derived scaffolds, the cellulose skeleton of plant tissue can be employed as an affordable scaffold for mammalian cells following decellularization.[142] Cellular content is removed from the natural plant material to create an acellular, 3D scaffold that preserves its structural, chemical, and mechanical cues via chemical, physical, or enzymatic methods (trypsin, nucleases, hypo/hypertonic solutions, detergents, solvents). After that, this scaffold may be repopulated with animal cells to create tissue-engineered constructions for a variety of uses.[143] Natural topographies in decellularized plant tissue scaffolds are capable of simulating some of the in vivo features of matrices. However, decellularized plant scaffolds lack a variety of metabolic signals, found in the natural environment, that are necessary to mammalian development. In order to customize these scaffolds for certain cell types, biofunctionalization or coating with functional surface proteins may be required.[144] Decellularized plant scaffolds, such as those comprising jackfruit, spinach leaves, and broccoli, have been examined as prospective scaffolds for the regeneration of vascularized tissue mass, utilizing the existing structure to provide perfusion during cell culture.[145,146] If the decellularization procedure is nontoxic, it could be used to manufacture CM with structure and lend texture to the final product.[122]

2.5.6. Synthetic Polymers

To develop a scaffold for CM, the components must either be edible or biodegradable without creating toxic byproducts.[122] Otherwise, the cells must be separated from the scaffold. Synthetic polymers most often used in TE are copolymers of polylactic acid (PLA), polyglycolic acid (PGA), and polylactic glycolic acid (PLGA). These are polymers that can be absorbed by living organisms or break down hydrolytically.[147,148] PLA has emerged as a crucial material in TE due to its ability to replicate the physical characteristics of the human ECM. PLA nanofiber nonwovens, particularly those created through electrospinning, have garnered significant interest for their potential as TE scaffolds. However, recent studies have shifted focus toward melt-blown PLA fabrics as alternative scaffolds. These fabrics can be tailored with varying crystallinities, tensile moduli, and pore diameters to mimic specific tissue properties. In a recent study, melt-blown PLA nonwovens were engineered to resemble human dermis, showing promising outcomes when tested with human dermal fibroblasts over various time frames. Results demonstrated robust cellular attachment, proliferation, and migration, along with cellular penetration through the scaffold thickness. These findings suggest that melt-blown nanofiber nonwovens hold substantial promise as TE scaffolds, potentially opening new avenues for research in this dynamic field. In another advancement, 3D printing technology has been employed to create patient-specific scaffolds using PLA-based materials like PLA-Baghdadite (Bgh). Following fabrication, these scaffolds were treated and coated with chitosan (Cs)-vascular endothelial GF (VEGF) or lyophilized Cs-VEGF to enhance their properties. The coated scaffolds exhibited superior porosity, compressive strength, and elastic modulus compared to traditional PLA samples. Importantly, these scaffolds were found to promote osteogenic differentiation when cultured with rat bone marrow-derived MSCs, showcasing their potential for bone healing applications. Moreover, innovative scaffold design strategies have been explored using PLA, such as directional porous structures fabricated via ice templating and phase inversion techniques. These scaffolds were engineered to accelerate bone repair by facilitating the growth and proliferation of bone cells. The study showcased the scaffold biocompatibility, mechanical properties, and efficacy in promoting bone regeneration in animal models with large-sized defects. Previously, edible films formed of PLA have been obtained from dairy waste (via use in fermentation to generate lactic acid that is subsequently polymerized).[149,150]

However, these materials should have a minimal environmental effect in line with the objectives of CM, and, above all, it could not be a good model for CM scaffold due to its animal origin.[151,152] Synthetic polymers often lack biological activity when compared to natural polymers. Hybrid natural–synthetic scaffolds may be useful to satisfy the criteria for CM scaffolding.[153]

If the scaffold is included in the final food product, the fabrication process and outcomes must be safe for ingestion. The texture, digestion, cooking, water-binding capacity, and flavor of scaffolds for CM must be considered, particularly in ways that are different than for medical scaffold designs. Ensuring suitability for human consumption as a food ingredient involves a comprehensive approach. This includes not only nutritional analysis, but also mechanical testing to assess texture (such as Warner–Bratzler shear force, water-holding capacity, and cooking loss). The morphology of a 3D scaffold may be optimized, including fiber size, surface topology, porosity, and pore alignment.[154]

2.6. Bioreactors

Bioreactors are critical for cell expansion and provide stimulation and capacity to scale up cell sources to produce CM. A bioreactor is a container that provides a controlled environment for the growth and development of its cellular contents. A bioreactor maintains the proper biological conditions for cells and culture media, including aiding nutrient transport and cell expansion and differentiation by stirring or stimulating the cells. The classification of bioreactor types is based on the method of medium input into the bioreactor main vessel: batch, fed batch, and continuous.[155] A batch bioreactor is a chamber that contains a predetermined volume of growth medium and operates by cultivating cells until they reach their maximum density, at which point they are harvested for utilization or transferred to a larger bioreactor.[156] A fed-batch bioreactor, also known as a semicontinuous bioreactor, has an inlet channel for providing fresh media to the cells at predetermined time intervals chosen to maximize growth. In the absence of a connector to remove conditioned media and cellular products that collect during culture, a fed-batch bioreactor can also increase volume over time.[157] This distinguishes fed-batch bioreactors from the last major category, continuous. In the production of CM, the preference is often given to fed batch or continuous medium introduction. This approach supports the handling of substantial media volumes, is amenable to automation, and facilitates the recycling of conditioned medium.[158] In addition to classification based on medium intake and removal, bioreactors may also be classified based on mixing of internal contents. The bioreactor mixes contents to promote growth and development of the cells. Mechanical bioreactors achieve mixing by agitators or impellers, and these are the most frequently used bioreactors for bioprocess development. The most common mechanical bioreactors are stirred tank bioreactors, which employ an impeller to induce convective flow and facilitate nutrient circulation and diffusion inside the vessel. For bioprocess scaling, stirred tank bioreactors have been the most widely used and since they are well established and scalable, they may be the most suitable bioreactor type for scaling the production of CM.[159] Spinner flasks may create turbulent flow that is not favorable to cell multiplication, and the direct contact of cells with the propeller may cause damage. For mammalian cell growth, a continuous stirred tank reactor, which combines continuous medium input with a stirred tank bioreactor system, has been extensively used. Another example of mechanical bioreactor is the rotating-wall vessel bioreactor that rotates the bioreactor primary vessel around its central axis to dynamically cultivate the vessel contents in suspension.[160] Rotating-wall vessel systems have the advantage of minimum shear stress and may facilitate the formation of 3D aggregates. Nonetheless, several cell types have elevated apoptotic rates early in culture using these systems. Rotating-wall vessel systems employ batch culture, but perfusion can be incorporated to automate the operation.[161] A mechanically active bioreactor system is the last prevalent mechanical bioreactor type. The bioreactor employs a regulated application of mechanical force to cells or tissue scaffolds, specifically using dynamic compression. This stimulation promotes cellular growth by simulating the natural developmental environment and can strengthen and align cells or scaffold structures.[162] This form of agitation may be advantageous for the development of CM, as myofiber alignment and mechanical strength are essential characteristics.

A hollow fiber bioreactor has been used to promote the proliferation of skeletal muscle cells.[163,164] The classification of hollow fiber bioreactors as hydraulic bioreactors indicates that mixing is accomplished by liquid flow rather than mechanical mixing. This entails seeding the cells in a matrix of porous hollow fibers so that they adhere to the surface of the fibers, where the medium can also circulate. A hollow fiber system has the advantage of producing minimal shear stress and allowing for a greater variety of nutrients to be carried, making it excellent for highly metabolic cell types. Another bioreactor type is air lift, which achieves mixing using gas purging and may be useful for meat production.[165] However, it lacks the record of accomplishment of other bioreactor designs that have been improved for several large-scale bioprocesses.

Several cellular parameters must be evaluated when designing a bioreactor system to produce CM. Several kinds of meat cells, including myocytes, are anchorage dependent and must attach to a surface to proliferate and differentiate appropriately. Before differentiating into specialized cell types that require anchoring, it may be possible to grow the initial cell source in suspension. Alternately, growth methods employing nonadherent free-floating spherical aggregates may be beneficial to avoid the potential requirement for a substrate during bioreactor development.[165] This culture method would be more applicable to sources of PSCs that can be cultivated as free-floating aggregates. Other adult stem cell sources, such as MSCs and muscle satellite cells, necessitate attachment substrates. There is also a risk of necrotic core development if the aggregates become too large and limit nutrition and oxygen passage. Considerations should also be given to the idea of co-ccultivating muscle and fat cells to obtain CM. Today, however, it is still challenging to perfect the growth media that can support both cell types; it is likely that the two cell types will be cultivated separately soon. Perfusion bioreactors, which combine continuous medium input with perfusion flow, are a method for producing meat products of a certain size.[166,167] This is due to perfusion flow rate in these bioreactors that can be adjusted to the shape and size of the cultivated tissue. However, it should be noted that an increase in perfusion flow rate in proportion to the size and scale of the scaffold may lead to elevated shear stress and reduced pressure, potentially causing cellular harm. Certain bioreactor systems may be excellent for producing one form of CM, but they may not be suited for the development of other types and sizes of meat. As the field expands and seeks to meet a wide variety of CM products, bioreactor systems for large-scale production will need to be continually optimized.

3. Insect Cells as a Source of Cells for Cultivated Meat

Cells sourced from a variety of species, principally bovine, porcine, and avian, have been targeted for the development of CM. Cells derived from less common species may be useful in overcoming current technological challenges that prevent the development and extension of cell cultures, such as the need for adherent cells and the high cost of media. Mammalian cells require a set of specific growth conditions and tight process control to maintain their functions: pH range of ≈ 6.8–7.8, temperature range of 30–39 °C, CO_2, specific antibiotics, expensive GFs, animal-derived serum, and adhesion for growth. Although some media formulations exclude serum, mammalian cells are difficult to adapt or thrive in serum-free conditions.[168,169] Furthermore, most CM-relevant cell types require adherent cultures, constraining growth by surface area. These limitations render large-scale production of mammalian-based cell culture systems difficult and inefficient.

In contrast, insect cells have properties that indicate suitability for large-scale production in a more cost-efficient manner. The use of insect cell culture for food applications has been summarized recently[170] and will be briefly discussed here. Insect cells can tolerate a wide range of environmental variables, including pH (6.0–7.0) and temperature (20–32 °C), and are typically grown without CO_2. The immortalization process can also occur spontaneously, as demonstrated by several insect cell lines in the Cellosaurus database and one cell line derived from *Manduca sexta* have been explored for food purposes.[171,172] Insect cells have the flexibility to grow either in suspension or attached to surfaces because they are not affected by contact inhibition. This means they can be cultivated in suspension bioreactors, where factors like surface-to-volume ratio and cellular biomass concentration can be finely tuned within a confined space.[123,124] A further difficulty with mammalian cell culture is the amount of culture medium required to support cells because of the high glucose consumption rates and toxic byproduct accumulation during cell expansion. In the context of cellular agriculture, this is a focal point of ongoing research as cost-effectiveness is crucial for consumer adoption of CM products, and media is a large contributor to production costs. In contrast, insect cells produce fewer toxic byproducts like lactic acid, due to their metabolic processes, they exhibit lower sensitivity to toxic compounds (e.g., ammonia, a byproduct of catabolism), and they require less glucose for growth, thereby reducing the cost of materials and the volume of culture media required. These characteristics result in reduced material costs and lower volumes of culture media necessary, simplifying and making production scalability more economical. Rubio et al. (2019) investigated the differences between mammalian and insect cell cultures, focusing on cost, maintenance, and adaptability. These characteristics are crucial for advancements in TE, particularly in applications such as biofabrication, biobots, and CM. **Table 1** provides a detailed comparison between these two types of cultures, highlighting their respective advantages and limitations.[173]

Currently, finding a reliable and scalable source of insect muscle and fat cells is a significant obstacle to the production of insect tissues for food. To appreciate how insect cell cultures may be utilized to make CM, it is essential to understand the physiology of the cell types of interest. The mechanisms described are advantageous because they provide a method to explore in vitro the key biological properties of insect muscle and fat cells. Nonetheless, establishing stable lines of muscle and adipogenic progenitor cells capable of protracted proliferation and possible differentiation are essential for the manufacturing process and its scalability.[174]

3.1. Muscle Cells

Key differences between mammalian and insect muscle development include the origin and types of muscle cells, the molecular pathways that regulate them, and the function of muscle in the body. **Table 2** offers an intricate comparative analysis concerning cell types, development, and molecular pathways. For an extensive review of primary myogenic insect cell culture attempts, readers are referred to Rubio et al. 2019.[175] It has been suggested that CM production can help with environmental and animal welfare issues. While establishing bioproduction methods from mammals has been the focus of academic research on cell-CM, it would be preferable to begin with relevant animal species like insects. More study is required to determine whether the pattern observed in mammalian cell types, in which cell multiplication decreases as animals age, is also reflected in the relationship between the age of insects and their proliferative ability.

The demands that have given rise to the establishment of this new frontier may be addressed through understanding of the features of muscle and adipogenic growth. Additionally, controlling the creation of muscle or fat in vitro would require knowledge of the signals that govern these processes in vivo, and mammalian indications are not suitable for insect cells.

3.1.1. Muscle Types

Several cell types that are all specialized for contraction are referred to as "muscle." Despite their other differences, all

Table 1. Comparative analysis of mammalian and insect cell cultures for CM. The table outlines key differences between mammalian and insect cell cultures, focusing on growth conditions, cost-effectiveness, and adaptability, while highlighting the advantages and limitations of both sources.

	Mammalian cell cultures	Insect cell cultures
Advantages	–Widely used in medical and biotechnological research –Better consumer acceptance	–More adaptable to serum-free media –Growth in the absence of CO_2 –Withstanding adverse environmental conditions –Growth near at room temperature (20–32 °C) –Less nutrients requirement –Less frequent replacement of medium –Lower costs –Easier transition between adherent and suspension cultures
Limitations	–More glucose consumption –More lactic acid production and need for replacement of acidified culture medium –Not easily adaptable in serum-free media –Growth in adhesion, due to contact inhibition, therefore needing large spaces –More susceptibility to environmental conditions –Necessity of controlled levels of CO_2 –Need to maintain a temperature of 37 °C –Higher costs –Complexity of scalability	–Further studies are needed –Complexity of scalability –Low consumer acceptance

Table 2. Comparison of muscle cells in mammals and insects. The table compares the characteristics of muscle cells in mammals and insects, highlighting differences in cell types, development, and molecular pathways and analyze the unique structural and functional distinctions in their muscle systems.

	Types of Muscle	
Characteristics	Mammals	Insects
Classification	Divided into skeletal, cardiac, and smooth muscle.	Divided into skeletal and visceral; primarily striated muscle, only a small part lining the internal organs is smooth type.
Cell structure	Skeletal and cardiac muscles have striations, while smooth muscle does not. Skeletal muscles can be large and multinucleated.	Similar striated structure, with skeletal muscles attached to the exoskeleton; visceral muscle is less numerous.
Contractile units	Actin and myosin in sarcomeres.	Like mammals, actin and myosin are organized in sarcomeres.
Number of nuclei	From mononucleate to multinucleate depending on the type of muscle.	From binucleate to multinucleate, often fewer nuclei are compared to mammals.
	Muscle Development	
Development process	Mammals	Insects
Differentiation	Differentiation from satellite stem cells to myofibers, using GFs such as FGF.	Formation from myoblasts (skeletal muscle precursors), which divide into founder cells and fusion-competent myoblasts.
Regeneration	Presence of satellite cells for the regeneration of skeletal muscles.	Limited regeneration; some insects regenerate muscles during metamorphosis.
Interaction with other tissues	Interactions with the nervous system for innervation and regulation.	Essential interactions with neurons for complete development.
	Molecular Pathways	
Molecular regulation	Mammals	Insects
Regulatory factors	MyoD, Myf5, myogenin, MRF4, CTNNB1, and GSK3B are crucial for muscle development. FGF, IGFs, myostatin, TGF regulate proliferation and differentiation.	Twist, a class of bHLH factors, regulates the distinction of myoblasts. Notch and Ras/MAPK pathways for the selection of muscle precursors.
Hormonal regulation	Hormones such as IGF, GH, and sex hormones are important for muscle growth.	Ecdysone, induced by PTTH, and juvenile hormones regulate growth and metamorphosis.

muscles share the same metabolic processes that drive contractions. This process involves the interaction of actin and myosin proteins within the muscle fibers, with ATP serving as the primary energy source.[176]

In vertebrates, the broadest classification of muscle is based on the presence or absence of regular cross striations. In mammalian systems, there are three main types of muscle: skeletal, cardiac, and smooth, which has no striations. Skeletal muscle cells can be enormous (up to half a meter long with a diameter of 100 μm in adult humans) and are often called muscle fibers because of their elongated shape. Each cell constitutes a syncytium containing many nuclei immersed in the same cytoplasm. Other types of muscle cells are more conventional in that they possess a single nucleus.[176]

The walls of numerous organs and tubes in the body are lined with layers of smooth muscle cells, and smooth muscle does not contract voluntarily. The smooth muscle cells shorten when forced to contract, driving the organ's luminal contents, or the cell shortening changes the diameter of a tube to control the flow of its contents. Smooth muscle cells lack the striated banding pattern present in cardiac and skeletal muscle and are neurally innervated by the autonomic nervous system. In addition, hormones, autocrine/paracrine substances, and other regional chemical signals regulate the contractile state of smooth muscle.[177] The thick central layer of the heart is made up of cardiac muscle (also known as myocardium). The individual cells that make up the heart muscle are known as cardiomyocytes. Cardiomyocyte main function is to contract in order to create the pressure required to pump blood through the circulatory system.[178] Each cardiac muscle cell, or cardiomyocyte, is a tubular structure made up of chains of myofibrils, which are rod-like components inside the cell. Sarcomeres, the primary contractile units of muscle cells, are repeated in sections to form the myofibrils. Long proteins that form myofilaments, or thick and thin filaments, make up sarcomeres. Actin is a protein found in thin myofilaments, whereas myosin is a protein found in thick myofilaments. As the muscle contracts and relaxes, the myofilaments move past one another. When seen under a microscope, the arrangement of thin and thick myofilaments overlapping within the cell' sarcomere gives the illusion of being striated, similar to that of skeletal muscle.[179] All insect muscles follow a similar structure, with elongated cells holding the contractile components and frequently inserting into the integument at each end. But various muscles have diverse internal arrangements of the muscle cells, and wing muscles frequently have distinctive shapes.[180,181]

Insect muscles are almost all striated and are divided into two groups: skeletal muscles and visceral muscles, only a small part of which are of the smooth type, lining the walls of internal organs. Skeletal muscles can be attached to the exoskeleton internal surface area in significantly greater numbers than can fit on the skeletal framework of vertebrates. Elongated contractile fibers that are parallel to or converge at the point of insertion form the skeletal muscles. A consistent network of longitudinal and circular fibers can develop in visceral muscles.[182] Somatic muscles (or of the body wall) of insects like *Drosophila melanogaster* do not have many muscular fibers like those found in mammals. Additionally, the 30 segmentally repeated muscle fibers that make up somatic muscles are arranged in a clear pattern. The body wall muscles of many insects have from 4 to 24 nuclei, compared to up to 1000 nuclei in mammalian muscles. The visceral muscles that surround the gut, in addition to the somatic muscles, are syncytial. Circular binucleate muscles with partial fusion and multinucleate longitudinal muscles make up the larval midgut muscles. In the flight muscles, quiescent satellite cells have been identified that, as in mammals, can be activated by injury.[183]

3.1.2. Muscle Development

As already mentioned, prototypes of CM products have focused on the differentiation of stem cells, such as muscle satellite cells, to produce skeletal muscle, which is the main component of traditional meat. Consequently, our focus will be on the development of skeletal muscle. Myogenesis, the process of muscle formation, is substantially conserved in both invertebrate and vertebrate species. The embryogenic myogenesis of *D. melanogaster* is a well-recognized model for studying the genes and mechanisms that drive muscle development.[76]

Myoblasts, precursors of skeletal muscle cells, fuse to form multinucleated cells after a proliferation period. Like somatic musculature, the muscles of the visceral mesoderm (circular and longitudinal muscles) of many insects are composed of founder cells and fusion-competent myoblasts. The founder cells responsible for the development of the circular and longitudinal muscles originate from[176] distinct regions within the mesoderm. The founder cells of the visceral circular muscles (cFC) and the myoblasts competent for fusion (cFCM) originate from the mesoderm of the visceral trunk, abbreviated as TVM.

Founder cells and fusion-competent myoblasts are the two groups of myoblasts that fuse in a variety of insects. Founder cells express a particular combination of identity transcription factors that facilitate the identification of muscle fibers and define their orientation, shape, size, and attachment site. Fusion-competent myoblasts, on the other hand, have a generic identity. They express the transcription factor Lame Duck (Lmd), but it is not yet clear how reprogramming occurs.[183]

During fusion, they undergo a profound change in phenotype that depends on the coordinated activation of a set of specific genes (see Section "Molecular Pathway"). The regulatory protein is myoD1, normally expressed only in myoblasts and muscle cells. The experimental induction of myoD1 is also capable of transforming a fibroblast into a myoblast. The skeletal muscle cell, once formed, is generally preserved throughout the animal's life.

Some myoblasts persist in the adult muscle and appear as small, flattened, quiescent cells in close contact with mature cells within their basal lamina envelope. These satellite cells are activated to proliferate when the tissue is damaged or, for example, by artificially treating them with FGF. Myoblasts maintained in culture for up to 2 years retain the ability to differentiate and fuse to form muscle cells in response to appropriate changes in culture conditions. FGF is essential in keeping myoblasts in a proliferative state and preventing them from differentiating.[176] Since there is evidence that insect myoblasts require direct interaction with neurons to fully develop, the absence of support cell types in the initial cultures may be the source of restricted differentiation in these isolated insect muscle cells that have been immortalized. For CM large-scale production, the ability to control the proliferation and differentiation of cultured muscle cells is critical.

3.1.3. Molecular Pathway

In mammalian systems, myoblasts are actively growing MPCs that are produced once a quiescent satellite cell is activated. Its proliferation is fueled by the myogenic regulatory factors (MRFs) MyoD and Myf5. Proliferation is aided by FGF, inhibited by myostatin and transformed GF (TGF). IGFs, or insulin-like GFs, promote both proliferation and differentiation. Myoblast fusion into primary myofibers is fueled by IGFs and the MRF myogenin. IGFs and MRF4 encourage further fusion and differentiation, resulting in secondary fibers that eventually mature into myotubes with associated quiescent satellite cells. A second pair of MRFs, MRF4 and Myogenin, are increased during differentiation, promoting differentiation and fusion as well as assisting in maintaining the mature muscle structure.[67] It has been demonstrated that CTNNB1 (-catenin) and GSK3B (glycogen synthase kinase-3) control the direction of skeletal myogenesis in animals like pigs from the earliest stages of embryonic development through terminal differentiation.[61] The two types of myoblasts found in insects originate from mesodermal regions that exhibit elevated levels of the bHLH transcription factor Twist. The high Twist domain exhibits a distinct mechanism whereby a muscle progenitor cell is selectively identified through a crosstalk between the Notch and Ras/MAPK signaling pathway. The progenitor myoblasts undergo asymmetric division, resulting in the emergence of either two founder cells or a founder cell and an adult precursor cell (AMP). The latter, while in a state of quiescence and undifferentiation during embryonic development, undergoes reactivation in the second larval instar, ultimately leading to the generation of adult fly muscles. The myogenic cluster residual cells that exhibit Notch expression undergo differentiation into myoblasts that are capable of fusion.[67]

3.1.4. Hormonal Regulation

In *D. melanogaster*, as in many other holometabolous insects, muscle formation occurs twice: during embryogenesis and metamorphosis. These insects show critical morphological differences between larval and adult stages. As a result, the muscles that develop during the embryonic stage of an organism are eliminated during the pupal phase of metamorphosis and substituted with adult muscles.[183]

Without the insertion of additional myoblasts or nuclear division within the muscle syncytium, muscles in the larval stage enter a degree of hypertrophy. The arrangement of adult muscles differs significantly from that of larval muscles. The embryonic mesoderm engages the precursors of the adult muscles and delays their differentiation. The myoblasts for the head and thorax muscles are retained in the imaginal discs until pupal development, when the muscles develop. The imaginal disc is one of the components of a holometabolous insect larva that will change during pupal metamorphosis into a section of the adult insect's exterior. There are disc pairs that may be used to create various structures, including wings, legs, antennae, and other parts.[184] Most larval muscles are histolyzed to create adult muscles during metamorphosis, while adult muscles are de novo formed through the migration and fusion of adult MPCs.[185]

Insect muscle cells undergo profound changes during the animal's lifecycle. In this dynamism during growth, molting, pupation, and metamorphosis, certain hormones play an important role. In several species, the molt is stimulated by a hormone called ecdysone (also known as molting hormone). This hormone is secreted by two prothoracic glands, situated in the insect's thorax, and it is responsible for the growth and differentiation of adult structures. The production of ecdysone is in turn stimulated by a brain hormone, namely the prothoracicotropic hormone (PTTH).[186]

In holometabolous insects, complex signals control both the timing and developmental stage as the animal undergoes metamorphosis. The endocrine function that controls these stages has been extensively studied in several moths, including the silk moth (*Bombyx mori*) and the tobacco hornworm (*Manduca sexta*). Whether a molt leads to larva, pupa, or an adult depends on the presence or absence of juvenile hormone. The juvenile hormone is present in the earlier larval stages, and the larval molt leads to a bigger larva. In the last larval instar, the level of juvenile hormone falls sharply, and pupa is formed. The final molt, when the pupa develops into an adult, depends on the absence of juvenile hormone.[186] Despite a less comprehensive understanding of hormonal pathways in insect muscle development compared to humans and other animals, significant advancements have been achieved in differentiation and proliferation. Low doses of 20-hydroxyecdysone stimulate myoblast proliferation in *M. sexta*, but concentrations beyond the critical threshold inhibit myoblast growth. Methoprene, an analogue of juvenile hormone, inhibits the capacity of high doses of ecdysteroid (Ecd) to induce proliferative arrest and differentiation.[187] The hormonal regulators of the differentiation process can be employed to regulate cell growth throughout production.[188]

In mammals, primary muscle-resident progenitor cells isolated from skeletal muscle differentiate into smooth and skeletal muscle, whereas satellite cells only differentiate into skeletal muscle. Differentiation in culture is based on using biological or chemical substances in cell culture media.[189] Proliferation, differentiation, and fusion processes are associated with the activity of several known hormones, GFs, and transcription factors. The pathways in which they are involved play a crucial role in controlling muscle growth, energy metabolism, and repair of damaged muscle tissue. Some of the most important hormones involved in muscle development include testosterone, insulin, growth hormone (GH), IGF-1, cortisol, and other thyroid hormones. The molecular control mechanisms that direct skeletal muscle development have significant implications for medicine, agriculture, and food technology.[61] In addition to their biological functions, the genes involved serve as important markers for monitoring and optimization in CM applications.[67]

3.2. Fat Cells

In cellular agriculture, fat is essential for flavor and nutrition. To provide healthy and tasty food, both muscle and adipose tissue are needed. Many differences can be detected between the adipose tissue of mammals and that of insects, which vary in terms of their cellular composition, regulation, function, and anatomical location. **Table 3** summarizes numerous distinctions observed.

3.2.1. Mammalian Fat Tissue Composition

In mammals, adipose tissue is a connective tissue that primarily serves as a lipid storage of food and energy, as well as providing a significant amount of heat, water, and thermal insulation. Adipose tissue derives from MSCs that form during embryonic development from the mesoderm.[190] Adipose tissue also plays a role in the body's metabolism through the production of hormones, cytokines, proteins, and peptides. In mammals, adipose tissue is composed of white adipocytes (the primary site for energy storage) and brown adipocytes (specialized in thermogenesis). The predominant lipid-containing tissue in mammals is white adipose tissue (WAT), also known as unilocular adipose tissue. The location of WAT tissue is predominantly in the subcutaneous and abdominal region, and prominent deposits are also observed in skin and bone.[191] WAT is predominantly found in the mesentery and intraperitoneal, with a lesser presence in the bone marrow and surrounding the visceral organs. The subcutaneous adipose tissue, apart from serving as a reservoir of energy, functions as a thermal insulator against low temperatures.

WAT adipocytes are circular in shape and possess a significant size, measuring over 100 μm in diameter. They are characterized by a substantial lipid droplet that occupies most of their internal space. Adipocytes that are unilocular in nature are segregated by the slender strata of lax connective tissue, which are replete with reticular fibers that are secreted by the adipocytes themselves. Furthermore, it should be noted that every adipocyte is enveloped by a slender coating of ECM, which is situated near the plasma membrane. The outer lamina, which bears resemblance to the basal lamina of the epithelium, and is distinct from the adjacent connective tissue, is referred to as the sheath. In addition to mature white adipocytes and septa, various other cell types, including mast cells, macrophages, leukocytes, dispersed fibroblasts, and undifferentiated adipocytes, can also be observed. The dermal deposit and subcutaneous deposit are distinct entities that are physically segregated. Brown adipose tissue (BAT), also known as multilocular adipose tissue, is due to the presence of adipocytes that possess numerous small lipid droplets within their cytoplasm. It is prevalent in hibernating species, developing

Table 3. Comparison of mammalian and insect fat cells. The table delineates a comparative analysis of fat cells in mammals and insects, elucidating their unique characteristics, functional roles, and potential applications in cellular agriculture for food production.

Composition and Development

Aspect	Mammalian fat cells	Insect fat cells
Origin	Derived from MSCs in the mesoderm during embryonic development.	Located in the hemocoel, with various origins specific to insect physiology.
Types and functions	White adipocytes for energy storage, primarily in subcutaneous and abdominal areas; brown adipocytes are specialized for thermogenesis.	Trophocytes for nutrient storage and metabolism; enocytes for carbohydrate synthesis; mycetocytes, symbiotic prokaryotic microorganisms for nutrient synthesis; chromatocytes for lipid storage to support metamorphosis; urocytes for storing urate granules.
Appearance and Features	WAT has a large, circular shape, adipocytes unilocular; BAT, adipocytes multilocular, rich in mitochondria and with a high concentration of blood vessels, which contribute to the brown hue.	Cells are versatile in function, not specifically color coded but distinguished by their specific roles and content (such as lipid, protein, and carbohydrate).

Function and Regulation

Aspect	Mammalian fat cells	Insect fat cells
Regulatory mechanisms	Governed by hormones such as insulin (energy storage), leptin (appetite control), and adiponectin (glucose and lipid metabolism). Bioactive lipids from BAT promote glucose uptake and thermogenesis.	Regulated by a range of hormones like AKH (energy mobilization), Ecd (metamorphosis), juvenile hormone (growth and development), which impact a wide array of physiological processes from growth to systemic immunity.

Research and Applications

Aspect	Mammalian Fat Cells	Insect Fat Cells
Cultivation for food	Studies focus on deriving adipocytes from pluripotent and MSCs and DFATs for CM applications. Commonly used cell lines include 3T3-L1 and other murine lines for research and food production. Continued exploration into efficient and scalable methods to cultivate mammalian adipose tissue in vitro, aiming at texture and taste that mimic natural meat.	Emerging research into cultivating insect fat cells, notably for their roles in nutrient storage and release which can enhance muscle cell co-culture systems. Specific culture conditions are being developed to optimize lipid accumulation and usage in sustainable food production systems.

fetuses, and mammals during the perinatal period. BAT emerges prior to WAT during the developmental process. Brown adipocytes are smaller than white adipocytes and possess a rounded nucleus that is situated in the central regions of the cytoplasm. The abundant presence of cytochrome oxidase within the mitochondria of adipocytes is responsible for the brown hue exhibited by fresh BAT. The high concentration of blood vessels within the tissue is also a contributing factor to the brown hue. BAT adipocytes are distinguished from WAT adipocytes by the presence of the UCP1 protein, which serves to disengage the chain of electron transporters from ATP synthesis. This results in the utilization of the proton gradient energy for the purpose of heat generation.[191]

3.2.2. Insect Fat Body Composition

Numerous insect species have high quantities of essential fatty acids such as omega-3 and omega-6.[192] In addition to lipids, the fat body tissue of insects contains proteins and carbohydrates.[193] The insect fat body is essential to metabolic processes. It is situated in the hemocoel, where its constituent cells are near the haemolymph, allowing the exchange of metabolites. The fat layer just below the body wall is typically peripheral or parietal, while the layer that surrounds the feeding channel is frequently perivisceral. Although parts of the fat body also extend into the chest and head, the majority is in the abdomen. Fat body shape can vary widely between orders and species. In hemimetabolous insects, the larval fat body remains mostly unchanged in the adult form. In holometabolous insects, the fat body goes through a remarkable metamorphosis in which the tissue separates into individual cells. The adult adipose cells in the majority of holometabolous insects come from the larval adipose cells, but the adult fat cells in the Hymenoptera and higher Dipterans are created entirely de novo.[194] The storage of body fat plays a fundamental role in the lifecycle of holometabolous insects. Throughout the larval feeding stages, energy stores accumulate to facilitate the metamorphic process and create reserves for the emerging adult organism.[195] Furthermore, the quantity of nutrients accumulated in the larvae has significant implications for their adult life, as diminished larval fat body size leads to decreased reproductive capacity.[196] Mature arthropods that exhibit a nonfeeding lifestyle depend on these endogenous reserves to sustain their vital functions and reproductive activities. The process of egg development necessitates a significant transfer of resources from the adipose tissue to the ovaries. The significance of fat body reserves transferred from larval stages for oogenesis is evident in autogenic mosquitoes. In these mosquitoes, the activation of the target of the rapamycin signaling pathway and the subsequent maturation of eggs following a blood meal depend on the accumulation of sufficient nutritional reserves during larval development.[197]

In insects, the fat body is composed of five distinct cellular subtypes, exhibiting heterogeneity in their composition, dimensions, and functions and physiological roles across various developmental phases. Trophocytes are the most abundant cells. These cells are primarily responsible for the retention, excretion, and elimination of organic compounds. The cells exhibit variability in both size and quantity. Alterations in size are attributed to the accumulation of numbers and the expansion of the vacuoles. Four distinct types of vacuoles can be identified: digestive vacuoles, which facilitate metabolism and nutrient release during energetic or diapause expenditure; and storage vacuoles, which are responsible for the storage of reserve substances. Condensation vacuoles are associated with the Golgi apparatus and lysosomes and typically harbor proteins and surface vacuoles that arise from the fusion of vesicles (as observed in cellular specimens). The quantity of trophocytes is subject to variation, in addition to variations in their structural composition. Male insects exhibit a lower count of trophocytes in comparison to their female counterparts. Additional cells are also observed during the process of molting.[198]

Enocytes are a type of cell that exhibits a circular or oval shape and are commonly found in association with the epidermal layer of the cuticle. These cells may also be present alongside the predominantly parietal adipose body. Enocytes possess a nucleus that is situated centrally, along with mitochondria, smooth endoplasmic reticulum, and vacuoles that contain lipid, protein, and glycogen droplets and granules. These cells are capable of synthesizing carbohydrates that are transported between the hemolymph and body fat.[199]

Mycetocytes are cellular entities primarily composed of symbiotic prokaryotic microorganisms. They co-exist in a perpetual state of symbiosis with insects in a certain quantity. Mycetocytes are observed at the level of cytoplasmic fat and glycogen granules. Mycetocytes are present in nutrient-deficient and imbalanced environments and are responsible for the biosynthesis of certain essential nutrients, including amino acids and B-group vitamins.[200]

Chromatocytes are thin cells that show a central nucleus and a clear cuticle. These cells are situated in the thinnest layers of the fat body and accumulate lipids to support metamorphosis. Chromatocytes are present in select species of aquatic insects. Finally, urocytes exhibit distinctive features such as a diminished endoplasmic reticulum and a vacuole containing urate granules. Urate is derived from either the metabolic breakdown of nucleic acids or the degradation of proteins. The primary function of these cells is to accumulate and retain urate granules.[201] The fat body was one of the earliest forms of insect tissue to be cultivated in vitro for the study of protein production.[202] Important proteins, such as vitellogenin, the precursor protein of the egg yolk, and growth hormones that bind to proteins, are generated by fat body cells.[203–205] Fat–tissue-specific cells grow slowly at first but can be formed into continuous lines.[206] Other insect cells can be grown in vitro using fat body cells: the accumulation and release of nutrients by fat cells can extend the survival and contraction of muscle cells in vitro for months without altering the medium.[207] Similarly, the development of embryos in vitro can be improved through the fat cells.

3.2.3. Fat Tissue Function

Adipose tissue in mammals serves various functions, including acting as a crucial mediator of metabolic control and communication, regulating thermoregulation, providing protection against cold and trauma, and controlling reproduction and satiety.[190]

The insect's fat body, instead, is a versatile organ that performs a variety of physiological functions, including metabolic regulation, signal integration, regulation of molting and metamorphosis, and synthesis of hormones that modulate systemic function and immune protein synthesis. The substrates and products of numerous pathways in fat cells include lipids, carbohydrates, and proteins, which can serve as sources of energy production, reserves, and mobilization during various stages of life such as diapause, metamorphosis, and flight. The adipose tissue also acts as the primary site for the integration of innate and adaptive humoral immune responses, as it is primarily responsible for the synthesis of antimicrobial peptides.

Throughout the insect's life cycle, the adipose tissue undergoes a sequence of modifications, including development, expansion, and restructuring in the embryonic, larval, and pupal stages, while also governing reproductive processes in the adult stage. These alterations and regulatory mechanisms are regulated by hormonal and nutritional signals.[201]

3.2.4. Fat Tissue Regulation

In mammals, the regulation of metabolism in adipose tissue is primarily governed by insulin, which serves as the catalyst for the absorption and storage of energy.[208] Leptin is an additional hormone that doesn't cause feelings of satiety. However, decreased levels of leptin serve as an indicator of reduced energy reserves, leading to an increase in appetite and the desire to consume food.[209] The regulation of glucose and lipid metabolism within mammalian fat tissue is attributed to Adiponectin, which also facilitates a metabolic profile that exhibits antiatherogenic, anti-inflammatory, and insulin sensitizing properties.[210] The identification of molecules that have effects has contributed to the progress in comprehending adipose tissue as an endocrine organ. Bioactive lipids, including 12,13-dihydroxy9Z-octadecenoic acid (12,13-diHOME) and 12-Hydroxyeicosapentaenoic acid (12-HEPE), are secreted by BAT and promote the uptake of glucose and fatty acids in both BAT and skeletal muscles, thereby facilitating sustained thermogenesis.[211]

The metabolism of the fat body in insects is governed by a multitude of compounds, enzymes, and substances, primarily through the influence of hormones that modulate the activity of metabolic processes within the adipose tissue. Hormonal activity plays a crucial role in the process of insect metamorphosis, including the development and timing of molting. The hormones that commonly regulate various processes include adiponectin AKH, Ecd), juvenile hormone (JH), neuropeptide activating the diapause-pheromone hormone biosynthesis (DH-PBAN), corazonin (crz), leucochine (Lk), CCHa2, allanostatin-A (Ast -A), tachykinin (Tk), limostatin (Lst), cytokines, short neuropeptide F (sNPF), and neuropeptide F (NPF). The neurosecretory cells in the heart bodies create the peptide known as AKH.[212] Additionally, AKH is expressed in the middle intestine, muscle, body fat, and ovaries. It is comparable to glucagon and has 8–10 amino acids. Numerous insects have several AKHs, and migrating locusts have three variations with distinct bioactivities.[213] The hormone is originally present as a prohormone that splits off as AKH from the peptide associated with the adipokinetic hormone precursor (APRP) when it is activated. Due to the management of energy stores and their mobilization in insects' bodies during mutation and metamorphosis, the activity of AKH is present at the most crucial developmental phases.[214] It largely affects how enzymes such as glycogen phosphorylase, which converts glucose into sugars, and triglyceride lipase, which is involved in lipid metabolism. A common reaction to abrupt changes in lipid levels is the formation of AKH. The transduction signal AKHR (i.e., AKHR) activates the hormone. After that, phospholipase C, which converts membrane lipids into inositol 1,4,5-trisphosphate and diacylglycerol, is activated by AKHR. AKHR influences inositol triphosphate (IP_3) concurrently, which elevates calcium ions in the endoplasmic reticulum and transfers them to the cytoplasm. In addition, the activation of the hormone impacts the commencement of the activity of adenylate cyclase and hence the generation of cAMP. As a result, AKH activation controls the amount of TG in the fat body.[215] The hormone raises heart rate, motility, and neural signaling; it improves muscular tone; and it protects against oxidative stress. It also affects CREB, calcium homeostasis, and the expression of genes related to fat degradation.[215,216]

JH regulates various processes that affect the larva's growth and appearance and promotes the production of vitellogenin, a crucial precursor to the bull protein that is delivered into the ovocytes. It has been demonstrated that JH regulates protein granule existence and that its absence signals metamorphosis by causing the cytolysis of the larval body fat and the synthesis of a new one. The counterpart JH-1 also results in the vacuolization of aged trophocytes. This hormone low content induces the formation of vitellogenin in the fat body.[217,218]

Ecd functions in opposition to and concurrently with JH. By promoting tissue dissociation (the metamorphosing stage), tissue remodeling, and the emergence of autophagic structures. Ecdysone controls the timing of metamorphosis. JH prevents premature aging and transformation. Serious deformities, issues with mutation, and a lack of transformation are brought on by a deficiency of any of these hormones.[218]

The intricate nature of hormonal regulation in insects suggests that hormonal signals may have an impact on the storage of lipids in fat cells, and therefore, it is recommended that in vitro fat body culture includes supplementation with such signals. While the application of these systems in mammals is well understood and can be readily applied in the CM industry, the understanding of these systems in insects is comparatively limited, necessitating further research.

Currently, most of the experimental research on cellular agriculture has prioritized the production of muscle cells, given their prominence in the biomass of meat products. Nevertheless, it is widely recognized that fat content plays a crucial role in determining the taste, consistency, nutritional value, and consumer acceptability of cellular meat. The optimal source to produce cultivated fat remains uncertain. However, in mammals, several cell types exhibit the ability to undergo adipogenic differentiation in vitro.[219] PSCs represent a potential source for the generation of mature adipocytes through successive differentiation. PSCs have been derived exclusively from ESCs originating from blastocysts of various animal species, including pigs and cows.[220,221] Adipocytes may be derived from MSCs, which are typically extracted from adipose tissue and bone marrow. Several studies have demonstrated the feasibility of extracting adipocytes from the larval phases of various insects. This involves dissecting the fat body from the abdomen and subsequently mincing it in a suitable culture medium.[170] However, further research is necessary into methods for large-scale production and control of lipid accumulation in insect fat body cells.

Several studies have demonstrated the possibility of co-culturing muscle cells and insect fat cells. Particularly, the functions performed by fat cells, such as nutrient storage and release, have been found to enhance the survival and contraction of muscle cells. Additionally, it has been observed that both cell types can be cultivated using the same culture medium, which is a challenging task for mammalian cells due to the distinct media formulations required by the two cell types.[207,222] To create edible and nourishing food items, it will be crucial to cultivate both fat cells and muscle cells.[170]

4. Economic, Environmental, and Nutrition Sustainability of CM

Using TE techniques, in vitro CM allows meat production without the use of animals. In vitro CM may be more advantageous than traditional meat production in terms of costs, health, animal welfare, and the environment impact.[223,224] On August 5, 2013, in London, an in vitro beef burger was first publicized and tasted. Since then, the media has presented cell-based meat as a novel approach to generate meat with enormous opportunity.[221] Insects, as a potential alternative source of protein, also play a role in discussions about sustainable nutrition. It was possible to make a comparison between the nutritional profile of edible insects and mammalian meat. The Orkusz study[225] compares the nutritional value of insects with that of meat from slaughtered animals, highlighting that both are rich in proteins, essential amino acids, unsaturated fatty acids, vitamins, and minerals. Although it is not possible to definitively state that insects are nutritionally superior to meat, some insect species show higher energy and specific nutrient contents, such as proteins and polyunsaturated fatty acids, compared to meat. Insects also have higher levels of certain minerals and vitamins and are a source of vitamin C and fiber, which are not present in meat. These nutritional characteristics make insects a potentially valuable resource for CM production to enrich diets and improve health, contributing to the fight against global malnutrition. A significant benefit of CM production is improved control over flavor, fatty acid composition, fat content, and the ratio of saturated to polyunsaturated fatty acids, by modifying the culture medium composition or coculturing with other cell types. In vitro CM does not require killing animals, and animal suffering as well as the number of animals used in meat production are projected to decrease because of in vitro CM; in theory, the supply of meat for the entire planet could be produced by a small farm that provides occasional biopsies.[226] Ten stem cells could produce 50 000 metric tons of beef, if they divide and differentiate continuously for 2 months. Although in practice much optimization is needed to get to this level of efficiency,[227] furthermore, strict quality control regulations, which are impossible to implement in contemporary animal husbandry, slaughterhouses, or meat packing plants, could significantly reduce the likelihood of meat contamination and the incidence of zoonotic diseases in large-scale CM facilities. Additionally, traditional meat exposure to hazards like pesticides, arsenic, dioxins, and hormones should be significantly reduced.[182] The in vitro cultivation of meat allows faster production of the final product compared to livestock, focusing on key meat components such as muscles while avoiding the production of unnecessary tissues like bones, respiratory organs, digestive organs, skin, and nervous system. In traditional meat production systems, a significant portion of the food consumed by animals does not effectively transform into meat due to metabolism and the formation of inedible parts like bones and brain tissue. In contrast, lab-grown meat is time and energy efficient, taking only a few weeks instead of months (for chickens) or years (for pigs and cows) to be ready. Moreover, producing meat in vitro from insect cells has many environmental advantages linked to the differences from mammalian cells.[170] In vitro CM will also significantly minimize land needs. The carbon footprint of meat products should also be reduced through in vitro manufacturing, which can also reduce greenhouse gas emissions from raising livestock for meat by up to 90% and land and water resources for raising meat by up to 80%, although further research and development is necessary to support these estimates during large-scale CM production.[228] The substantial decrease in land use projected creates opportunities for other uses of the land, such as reforestation, which may help in the recovery of many endangered species. The scientific, environmental, and animal rights sectors are also supportive of in vitro meat production because it is a more environmentally friendly method of manufacturing meat with fewer negative impacts on human health. By cultivating cells from rare or endangered animals held in captivity, or even cells retrieved from samples of extinct creatures, it would be possible to create new types of meat and meat-based products for future markets, effectively enabling their consumption without impacting current populations. In many instances, such as those involving space missions, polar stations, troop encampments in remote theatres of war, and bunkers intended for long-term personnel survival after nuclear or biological attacks, it may be more effective to produce food on-site. In vitro meat production is a potential solution in these circumstances. In particular, the European Space Agency (ESA) is looking for ideas into how cellular agriculture could be used to grow food on long-term space missions. This will reduce the quantity of perishable food that must be transported, give an alternate source of nourishment, and provide fresh food. Such a novel food production system for space should be included in a closed-loop arrangement so that resources, especially the growth medium, may be recycled or regenerated, thereby minimizing dependence on supplies from Earth.[229]

In vitro meat production has many supporters, but also raises concerns. The unnatural nature of in vitro meat is an issue with adoption by the public and appears to be a factor in opposition to new food technology, at least in Europe.[230] Novel foods are supposed to be essential to the shift to sustainable food systems. Nevertheless, whether and how much they are adopted into the diets of the public will determine their success. Frameworks for the production and distribution of CM are starting to be developed by governments and regulatory agencies. Having clear guidelines can help to increase consumer confidence and trust. According to research in the literature, the main obstacles to the adoption of CM include contextual issues like price,[231] emotions like fear[232] and disgust, and cognitive problems like lack of familiarity.[233] In response, several tactics have been put out to boost consumer acceptance of CM. These tactics include educating customers about the production method and the advantages of CM, as well as facilitating production scale up[234] point to present the product at a lower price. This is going to be a major motivator for consumer adoption.[235,236] CM advocacy will be greatly aided by influencer collaborations and educational efforts, and it should become a common choice for people looking for ethical, sustainable, and healthy food options as the sector develops.

5. Conclusion

CM has the potential to provide consumers with the nutrition they need while significantly reducing the animal suffering, environmental, and human health issues associated with conventional meat farming.

Due to the differences between insect and mammalian cells, in vitro CM from insects has considerable advantages for the environment and for large-scale production with a more cost-efficient approach. These include 1) the adaptability of insect cells to both adherent and suspension growth; 2) cost reduction of culture media. Mammalian cells require larger amounts of culture medium and components due to the high rates of glucose consumption and accumulation of toxic byproducts during cell expansion. Insect cells consume less glucose during growth, accumulate less lactic acid due to slower cell metabolism, and are less sensitive to toxic compounds. 3) tolerance of a wide range of environmental variations, including pH (6.0–7.0) and temperature (20–32 °C); and 4) they typically grow in the absence of CO_2, and the immortalization process can occur spontaneously.

Acknowledgements

The authors thank TotalEnergies EP Italia S.p.a., for supporting this work. This work was supported by University of Basilicata, TotalEnergies EP Italia S.p.a., European Union Next Generation EU within the Ph.D. Program founded by "D.M. 352/2022 PNRR-Ricerca".

Conflict of Interest

The authors declare no conflict of interest.

Authors Contributions

F.G. and S.O. contributed equally to this work. Conceptualization was taken care of by P.F. Writing and the original draft preparation were taken

care of by P.F., F.G., C.S., and S.O. Writing, review, and editing were taken care of by P.F., F.G., C.S., S.O., V.P., D.I., S.L., R.S., A.L.G., and D.K. Supervision was taken care of by PF.

Keywords

cellular agriculture, cultivated meat, insect cell cultures, scaffolds, sustainable foods

Received: April 28, 2024
Revised: June 6, 2024
Published online: July 8, 2024

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Exosomes and Signaling Nanovesicles from the Nanofiltration of Preconditioned Adipose Tissue with Skin-B® in Tissue Regeneration and Antiaging: A Clinical Study and Case Report

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Citation: Svolacchia, F.; Svolacchia, L.; Falabella, P.; Scieuzo, C.; Salvia, R.; Giglio, F.; Catalano, A.; Saturnino, C.; Di Lascio, P.; Guarro, G.; et al. Exosomes and Signaling Nanovesicles from the Nanofiltration of Preconditioned Adipose Tissue with Skin-B® in Tissue Regeneration and Antiaging: A Clinical Study and Case Report. *Medicina* **2024**, *60*, 670. https://doi.org/10.3390/medicina60040670

Academic Editor: Salvatore Di Somma

Received: 27 February 2024
Revised: 29 March 2024
Accepted: 13 April 2024
Published: 21 April 2024

Abstract: *Background and Objectives*: This three-year clinical trial aimed to demonstrate that only the signaling vesicles produced by ADSCa, containing mRNA, microRNA, growth factors (GFs), and bioactive peptides, provide an advantage over classical therapy with adipose disaggregate to make the tissue regeneration technique safer due to the absence of interfering materials and cells, while being extremely minimally invasive. The infiltration of disaggregated adipose nanofat, defined by the Tonnard method, for the regeneration of the dermis and epidermis during physiological or pathological aging continues to be successfully used for the presence of numerous adult stem cells in suspension (ADSCa). An improvement in this method is the exclusion of fibrous shots and cellular debris from the nanofat to avoid inflammatory phenomena by microfiltration. *Materials and Methods*: A small amount of adipose tissue was extracted after surface anesthesia and disaggregated according to the Tonnard method. An initial microfiltration at 20/40 microns was performed to remove fibrous shots and cellular debris. The microfiltration was stabilized with a sterile solution containing hyaluronic acid and immediately ultrafiltered to a final size of 0.20 microns to exclude the cellular component and hyaluronic acid chains of different molecular weights. The suspension was then injected into the dermis using a mesotherapy technique with microinjections. *Results*: This study found that it is possible to extract signaling microvesicles using a simple ultrafiltration system. The Berardesca Scale, Numeric Rating Scale (NRS), and Modified Vancouver Scale (MVS) showed that it is possible to obtain excellent results with this technique. The ultrafiltrate can validly be used in a therapy involving injection into target tissues affected by chronic and photoaging with excellent results. *Conclusions*: This retrospective clinical evaluation study allowed us to consider the results obtained with this method for the treatment of dermal wrinkles and facial tissue furrows as excellent. The method is safe and an innovative regenerative therapy as a powerful and viable alternative to skin regeneration therapies, antiaging therapies, and chronic inflammatory diseases because it lacks the inflammatory component produced by cellular debris and fibrous sprouts and because it can exclude the mesenchymal cellular component by reducing multiple inflammatory cytokine levels.

Keywords: tissue regeneration; nanovesicles; exosomes; micro RNA; Jaluexos

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1. Introduction

Skin tissue harvested and processed at different sizes between 50 and 100 microns can be used effectively on scars due to the presence of tissue progenitors [1]. Adipose tissue can provide more viable tissue progenitors for micrografts between 50 and 100 microns through a simple procedure [2]. From 1 mL of lipoaspirate, approximately 2.0 to 6.0 × 10^7 cells can be obtained with a cell viability of 90% [3], and, from one gram of the same tissue, 5 × 10^4 progenitor cells can be isolated, although with debris in the suspension [4]. The uniqueness of adipose tissue is that it is possessed by every individual and is easily accessible for sampling, even with simple instruments such as a syringe and a needle of adequate size. The procedure for obtaining progenitors, which is the fraction of adult mesenchymal stem cells from adipose tissue without vital adipocytes, involves extraction and disaggregation according to the method of Tonnard et al. [5]. However, to obtain progenitor cells in a suspension without inflammatory components such as fibrous shots and cellular debris, which are responsible for the activation of the Toll-like (TL) system [4], the adipose tissue must be subjected to microfiltration after disaggregation [6,7]. The progenitor cells have to undergo cytofluorometry [8] to acquire the characteristics of adult stem cells. Through the phenomenon of plasticity, progenitor cells can induce the formation of new tissues via the formation of new cells that improve the characteristics of the receiving tissue [7].

The phases of tissue regeneration take place through a series of interactions between progenitors and newly formed cells immersed in the extracellular matrix, blood vessels, signals mediated by signaling proteins, and some regenerative microRNAs produced by the progenitors [9]. The method used in this study makes it possible to induce the same tissue regeneration mechanisms, with greater biological safety, using only the signaling of microvesicles produced by the progenitors, since these are the cells that transmit the signaling proteins [10]. These microvesicles, called exosomes, are capable of transmitting information to cells, having therapeutic effects through proteins and mRNAs and the microRNAs they contain [10].

Exosomes, by definition, are spherical or elliptical vesicles with a size varying between 50 and 200 nanometers (0.05–0.2 µm). They are mediators of all the cellular activities that produce them [10], which is achieved by releasing their information inside the target cells, as reported in the ARVO conference [11], with therapeutic activity on the cells of tissues different from them [12]. When exosomes are released from adult stem cells, they remain active even in the absence of the cells that produced them, activating the tissue regeneration process [10]. Exosomes can maintain the functional therapeutic phenotype of the adult stem cells that produced them by influencing tissue responses to lesions and positively interacting with cell metabolism [13].

When derived from healthy tissues, they induce risk-free regeneration [14], where bioactive lipids, nucleic acids, mRNAs, and microRNAs induce a regenerative biological response in the recipient cells. Exosomes can induce and activate biological functions even in senescent or dysfunctional cells, limiting the acquired expression of the senescent phenotype and preventing the expression of MMPs [15]. They can inactivate the TL4/NF-κB inflammatory cascade by reducing TLR4 levels [16]; they can reduce IFN-γ, TNF-α, and IL-1β levels, reducing local inflammatory phenomena [17]; and they can increase the expressions of TGF-β1 and IL-10 [18]. The exosomes derived from viable precursors of adipose micrografts induce a noninflammatory phenotype in macrophages toward the M2 phenotype [19], and they regulate autophagy [20]. In lesions of the dermis and epidermis, they allow more rapid physiological healing through the transfer of their microRNAs [21]. They promote endothelial formation, reduce oxidative stress damage, and improve nitric oxide synthesis [22]. The viable precursors of adipose tissue micrografts can release exosomes with the presence of microRNA-126. This microRNA can protect cells from acute events typical of hypoxia–reperfusion pathology by regulating neo-angiogenesis and endothelial cells [22,23]. They can restore the efficiency of the connection of the membrane potential of superoxide dismutase (SOD1) [24] and can inhibit elastase through the release of alpha-1-antitrypsin (AAT) [25] in the tissues, which results in a wide limitation of tissue aging phenomena. Exosomes induce plasticity in dermal fibroblasts [26] and allow physiological neo-collagenogenesis [27].

The exclusive use of exosomes makes it possible to design therapy that excludes the cells that have secreted them, thus allowing low immunogenicity [28]. Exosomes express the Alix protein on their surface [29], which is an adaptor protein associated with the cytoskeleton that regulates the function of tyrosine kinase (TK) and CD63, which play fundamental roles in cells by regulating development, activation, growth, and motility. Endothelial cell lines defined as HUVECs are activated by exosomes, suggesting that they promote angiogenesis in vitro and in vivo [30,31]. They reduce tissue degeneration by reducing apoptosis [32]. They improve the outcome of wounds and scars by increasing fibroblast proliferation and migration [33] and Wnt/β-catenin signaling [34] and by up-regulating gene expression in the recipient tissues [35]. They allow over-regulation in the cells of proliferative markers such as cyclin D1, cyclin D2, cyclin A1, and cyclin A2; and growth factors such as VEGFA, PDGFA, EGF, and FGF2; and they stimulate and activate the AKT and ERK pathways, leading to a significant increase in re-epithelialization, physiological collagen deposition, and neovascularization in dermal lesions [36]. Adipose-derived adult stem cells are capable of producing a significant amount of exosomes [37], and this phenomenon occurs in both normoxic and hypoxic environments [33,38], with a positive functional impact on the receiving cells [38]. By using ADSCa-derived exosomes, it is possible to transfer a large amount of information into tissues [38], but ADSCa-derived exosomes must be separated from interfering components such as cellular debris and fibrous processes [39].

Exosomes from ADSCs can be obtained by extraction using filters of the appropriate size [40,41]. Specifically, exosomes derived from hypoxic ADSCs have a size that can vary from 20 to 300 nanometers (0.02–0.3 microns), with an average size of 90 nanometers (0.09 microns) [42]. ADSCa can be preconditioned without any manipulation to modulate the composition of the exosomes they secrete [43,44], from which the profiles of 148 microRNAs have been isolated [45]. Proteomic analysis has identified 1466 proteins that positively interfere with cellular functions [46]. The exosomes released by the previously conditioned adult stem cells allow a greater therapeutic effect [47], and preconditioning without manipulation is emerging as a key strategy to improve the functions of ADSCa to obtain exosomes that improve their efficacy in regenerative medicine [48,49]. This three-year clinical trial aimed to demonstrate that the signaling vesicles produced by ADSCa, containing mRNAs, microRNAs, GFs, and bioactive peptides in the phenomena of chrono- and photoaging of facial tissues, have an advantage over therapy with disaggregated adipose defined nanofat to make the tissue regeneration technique safer and minimally invasive. This study's objective was to apply and evaluate the feasibility of a specific protocol rather than comparing control groups undergoing different procedures. This approach utilized established and validated methods to assess, through cytofluorometry, the presence, quality, and quantity of signaling vesicles released by adipose-derived adult stem cells.

2. Material and Methods

A total of 72 female patients aged between 34 and 68 years (mean age 48 years) were studied. They signed an informed consent for the use of lipoaspirate for the procedures described. This study was approved by the local ethics committee under protocol number

367/2021 and was conducted in accordance with the tenets of the Declaration of Helsinki. Skin-B® 5 mL sterile solution containing amino acids and nonviscoelastic macromolecular hyaluronic acid was from Italfarmacia (Rome, Italy).

None of the patients had inflammatory pathologies of the dermis or epidermis, except for the presence of age-dependent phenomena and photoaging. No unapproved substances such as proprietary products or drugs were used in this study under conditions other than those approved. The presence of nanovescicles was determined by positive selection using an EV Isolation Kit Pan, Human of Milteniy Biotec Company, Bergisch Gladbach, North Rhine-Westphalia, Germany. The EV Isolation Kit Pan for humans facilitates the isolation of intact exosomes or extracellular vesicles (EVs) from cell culture supernatant, plasma, urine, or ascites. The isolation is performed by positive selection using MicroBeads recognizing the tetraspanin proteins CD9, CD63, and CD81. The isolation protocol is based on the renowned MACS technology, which enables fast isolation of high-purity and high-yield EV. The Visual Analogue Scale (VAS), NRS, and Berardesca Scale were used for data collection [50]. In addition, the MVS was used to document changes in scarring outcomes over time; in our study, it was used to assess the overall improvement in skin appearance, taking into account the three parameters mentioned above (stability, softness, and hydration).

3. Results

After identification of the donor area, adipose tissue extraction and local infiltration with Klein's solution were carried out as a method of anesthesia, and, after waiting for the whitening of the area induced by the presence of adrenaline in the solution contained therein, a total of 3.5 mL of adipose tissue was extracted using a 10 mL luer-lock syringe and a 16 G needle or with a multiport small-hole cannula (Figure 1). The tissue sample was decanted for 15 min to remove the anesthetic fluids, yielding 3 mL of adipose tissue, which was immediately disaggregated using the classic Tonnard method [5].

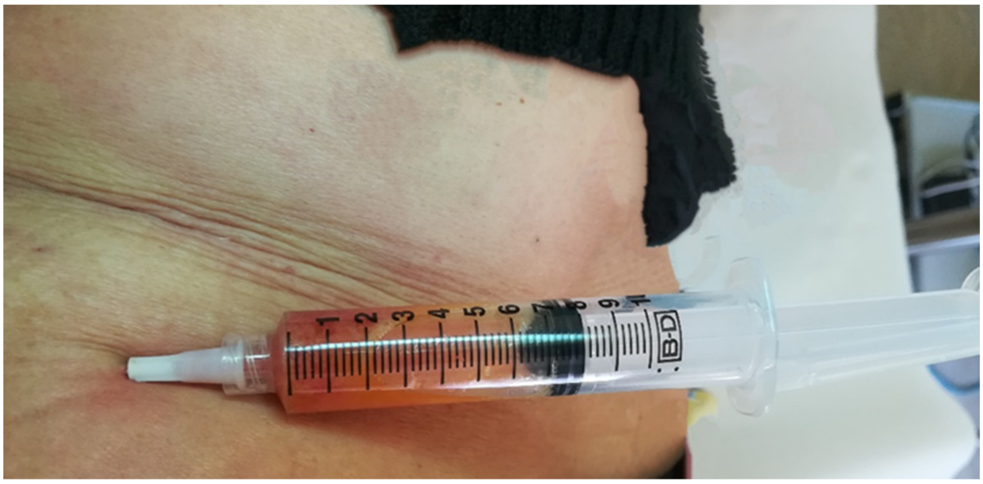

Figure 1. Extraction of adipose tissue.

During the disaggregation between the two syringes and employing a three-way tap, simultaneous filtration was carried out through a filter at 20/40 microns connected to one end (Figure 2). The filtration at 20/40 microns during the disaggregation of the tissues made it possible to eliminate the fibrous shots and the larger cellular debris protecting the side population in the harvesting syringe [5].

Figure 2. Fragmentation and simultaneous filtration at 20/40 microns.

A vial of Skin-B® 5 mL sterile solution containing amino acids and nonviscoelastic macromolecular hyaluronic acid was added to the tissue thus obtained to condition the ADSCa and then was ultrafiltered to the final dimensions of 0.20 microns (200 nanometers) with an appropriately sized filter (Figure 3). The 200-nanometer ultrafiltration also guaranteed the exclusion of hyaluronic acid chains of different molecular weights from the final suspension to avoid influencing the clinical results of hyaluronic acid on the skin. The exosomes were isolated using only a size-based ultrafiltration technique [42,43].

Figure 3. The 0.20-micron filtration.

This ultrafiltration technique made it possible to obtain microvesicles that did not contain almost any of the components of the interfering adipose disaggregate [27,38]. The large number of exosomes that may be lost during the ultrafiltration process is compensated for by an extremely fast, reproducible, painless, and minimally invasive technique since it involves the extraction of only 3.5 mL of adipose tissue, which can provide approximately 6.0×10^7 cells with a cell viability of 90% [3,4]. Confirmation of the presence of exosomes in the microfiltrate was obtained by testing the procedure using an EV Isolation Kit, which allows the specific isolation of intact exosomes or EVs from cell culture supernatant, plasma, urine, or ascites. Isolation is performed by positive selection using microbeads that recognize tetraspanin proteins. The isolation protocol is based on MACS technology, which enables the rapid isolation of high-purity and high-yield EVs. Through this procedure, it was verified that, despite the final filtration procedure at 0.2 microns (200 nanometers), there were still particles covering part of the characterization signal, but we could see the presence of numerous vesicles using exosomal marker CD81, typical of regenerative functions [51], and the mesenchymal/endothelial marker CD146, specific for ADSCa after stabilization [52] (Figure 4).

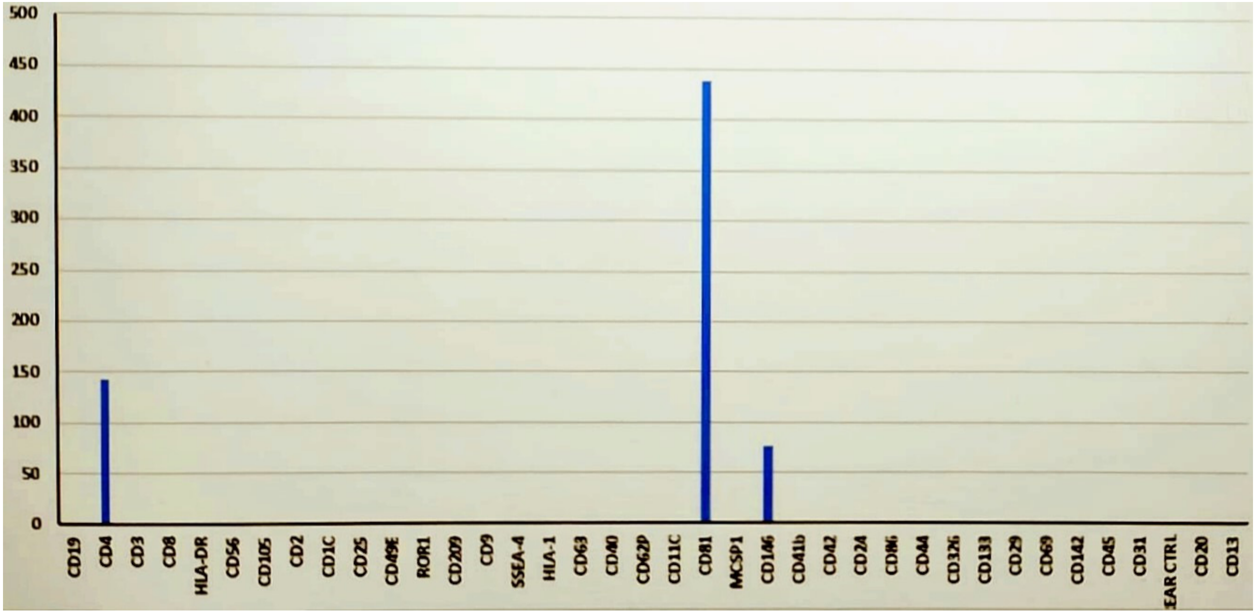

Figure 4. Flow cytometry of the suspension obtained after ultrafiltration.

However, the dilution of a fat disaggregate is always necessary because of the possible contraindications that a final filter of 0.20 microns may have for lipid emulsions. The final suspension containing the microinjections was, by convention, sterile since it was obtained at values around 0.2 microns. This procedure resulted in a final suspension of 3 mL (Figure 5). Once the suspension was obtained, it was injected with the same syringe and a 30 G 6 mm needle using the mesotherapy microinjection technique over the whole face, with the needle inclined at 45°, releasing a minimum amount of suspension when the needle was withdrawn until the formation of a visible wheal. However, this is not considered a drug, so we did not know the dosage or quantification. Additionally, it was not possible to know a priori the number of exosomes produced by the adult mesenchymal cells contained in each adipose tissue sample before treatment. We can state that, in the standardized sampling from each patient and examined by flow cytometry, we highlighted 450 million secretory vesicles with the CD81 marker. The procedure lasted about 30/40 min for each patient.

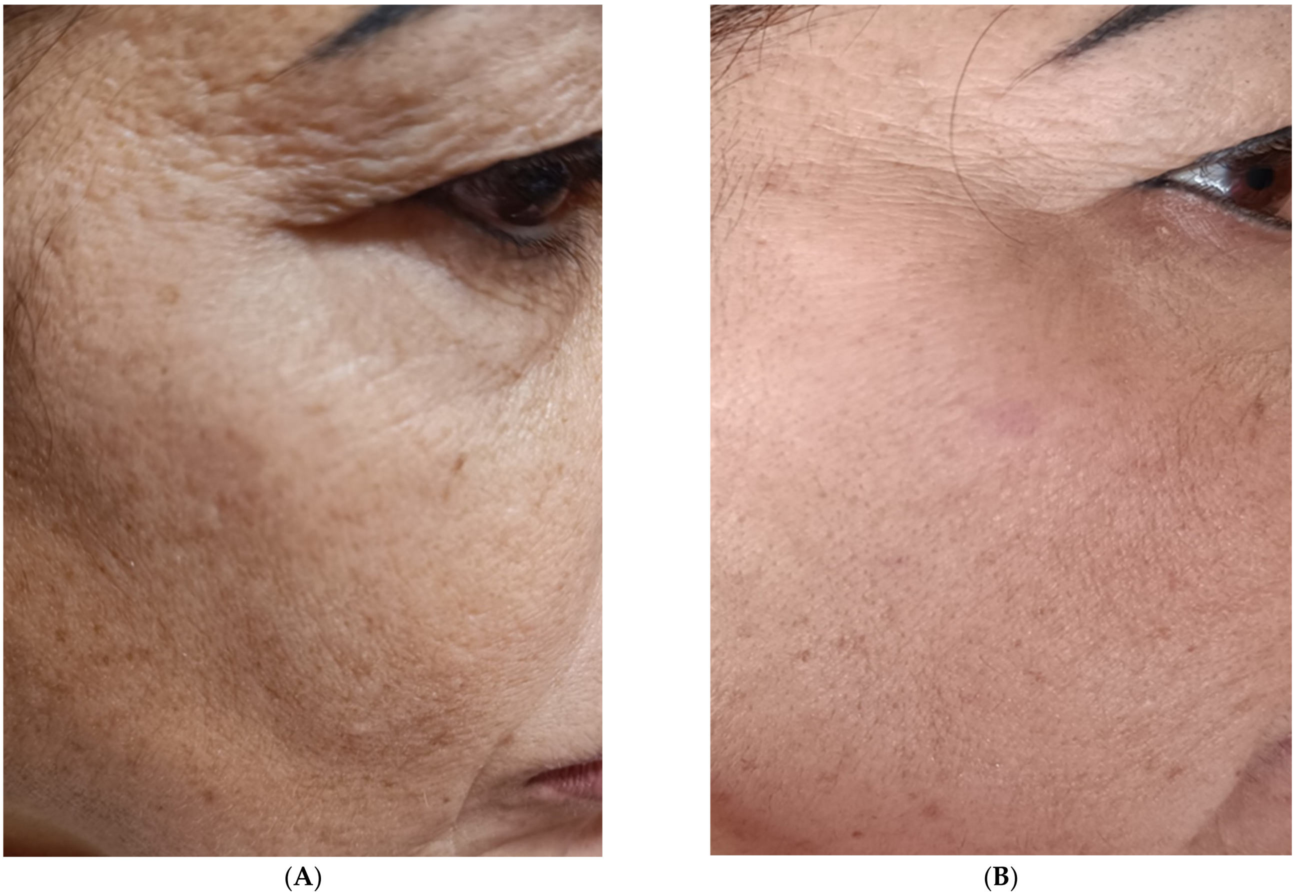
(**A**) (**B**)

Figure 5. (**A**) Before the infiltration treatment; (**B**) 30 days after treatment.

After the first clinical results on the use of 0.20-micron ultrafiltration [41] using only a physiological saline solution as mechanical support for the 0.2-micron filters, additional patients were enrolled and subjected to a clinical study and using an even higher-purity ultrafiltrate with a solution containing a vial of Skin-B® 5 mL sterile solution containing amino acids and nonviscoelastic macromolecular hyaluronic acid. This procedure was called Jaluexos, both as filter support and as preconditioning, to modulate the composition of microvesicles produced by ADSCa [43,44] and the CD44 expressed on them to have a greater number of mRNAs and microRNAs to address regenerative simulation using a solution of Dulbecco's modified Eagle medium [8].

A significant improvement in skin parameters was observed using this method. Compared to D0 (pretreatment), at a follow-up of 15 and 30 days after a single treatment, patients assessed their satisfaction with the treatment received by assigning scores for fine lines and wrinkles using a scale of 0 to 4 for each criterion (0 = unsatisfactory; 4 = satisfactory), as suggested by Berardesca et al. [53]. In addition, the individual signs of wrinkles and defect severity obtained for each treatment and each area were objectively assessed using a 10–0 NRS with separate scores for each area (10 = signs of high wrinkle severity or signs of high defect severity; 5 = signs of medium wrinkle severity or average defect severity; 0 = signs of low wrinkle severity or average defect severity) This scale provided a numerical measure of the severity of a general facial defect and, more specifically, the severity of wrinkles before the start of treatment (D0) (Figure 5A) and during follow-up (D30 in Figure 5B).

The results presented in Figures 6 and 7 show that treatment with exosomes induced a reduction in the signs of tissue aging in all patients.

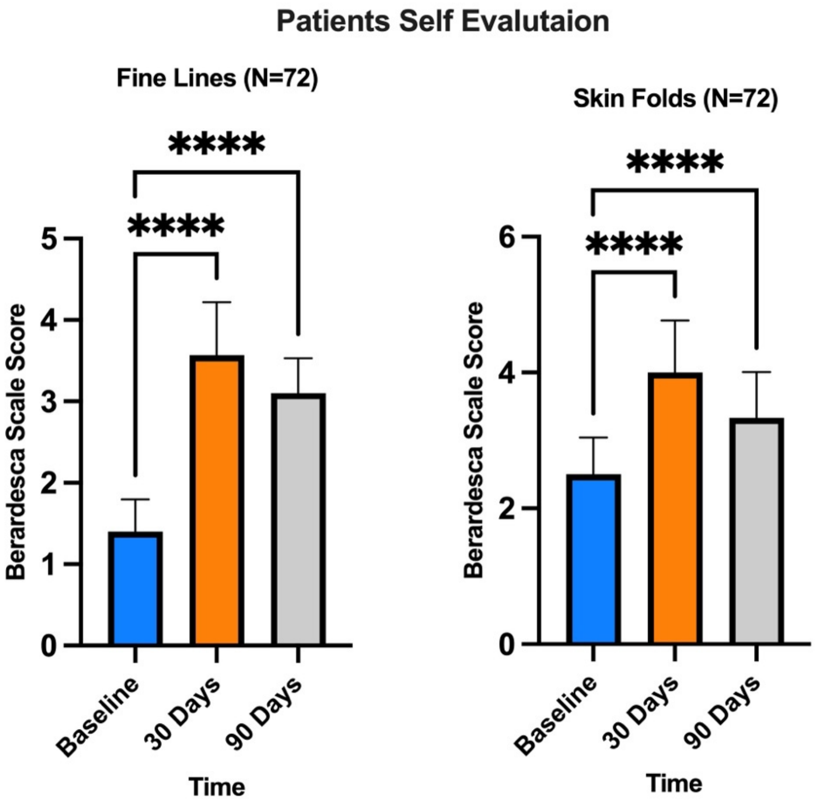

Figure 6. Berardesca Scale for patient satisfaction evaluation. Subjects evaluated their satisfaction in comparison to D0 (before treatment), 30 days and 90 days after treatment, by giving scores on firmness and cutaneous relief. Scale of 0–4 for each criterion (0 = unsatisfactory; 4 satisfactory). **** $p < 0.0001$ (one-way ANOVA).

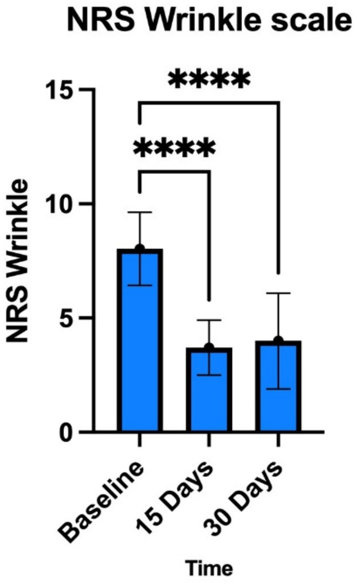

Figure 7. Numeric Rating Scale (NRS) evaluating defect severity and wrinkles: 10–0 scale with separate scores for each site (10 = wrinkle or defect severity; 5 = medium wrinkle signs or medium defect severity; 0 = low wrinkle signs or medium defect severity); $p < 0.05$. **** $p < 0.0001$.

The MVS was also used for follow-up evaluation, and the parameters analyzed were stability, softness, and hydration, as shown in Figure 8. The treatment was found to be extremely safe with the method used, and no adverse effects were recorded.

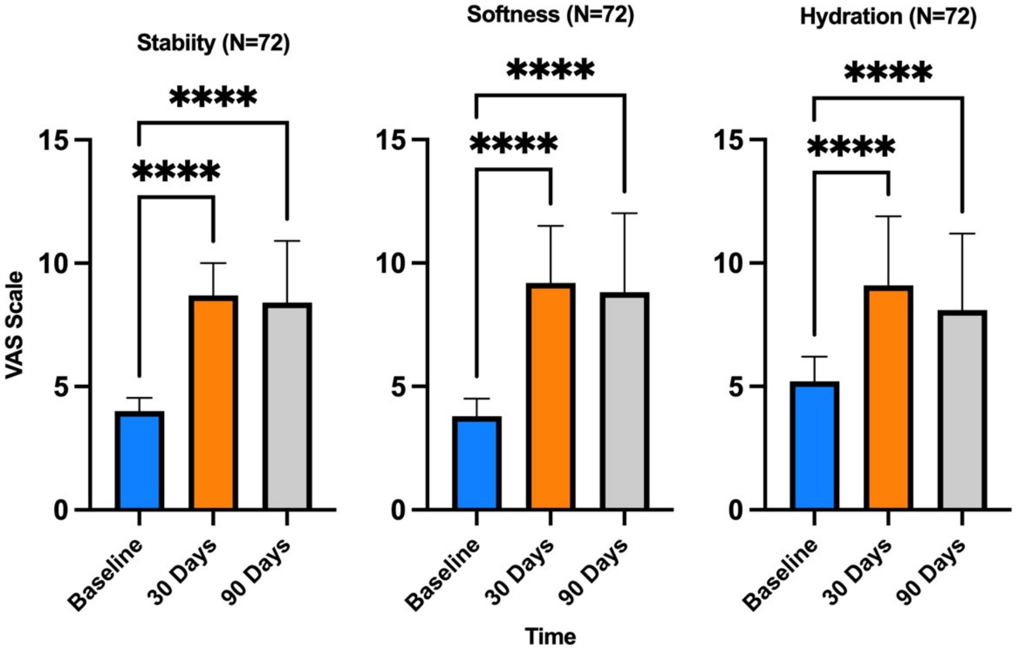

Figure 8. Modified Vancouver Scale used for the evaluation of stability, softness, and hydration. The Modified Vancouver Scale was used to estimate the improvement in skin appearance 15 days and 30 days after treatment in comparison to D0 (before treatment). The parameters considered were stability, softness, and hydration. **** $p < 0.0001$ (one-way ANOVA).

4. Discussion

It was hypothesized that ADSCa-derived signaling vesicles and exosomes could be extracted from adipose tissue disaggregated according to the method described by Tonnard et al. [5], microfiltered at 20/40 microns, conditioned with Skin-B®, and nanofiltrated. The stages of tissue regeneration take place through a series of interactions between newly formed cells immersed in the extracellular matrix, blood vessels, signals mediated by signaling proteins, and the microRNAs produced by them. It was hypothesized that tissue-regeneration mechanisms could be induced using only signaling microvesicles produced by tissue progenitors with greater biological safety and that microvesicles would be able to transfer information employing proteins, mRNAs, and the microRNAs contained in them to cells, having a therapeutic effect. We aimed to clinically demonstrate that there was an improvement in the skin and to verify the presence of exosomes in the 200-nanometer nanofiltrate by flow cytometry. We used the Berardesca Scale, NRS and VAS. All three scales are valid, reliable, and appropriate for use in clinical practice, although the VAS is more difficult to use than the others. For general purposes, the NRS has good sensitivity and generates data that can be analyzed for various purposes. The exosomes from ADSCs can be obtained by extraction using filters of the appropriate size, and those derived from normoxic ADSCs and those derived from hypoxic ADSCs have a size that can vary from 20 to 300 nanometers (0.02–0.3 microns), with an average size of 90 nanometers (0.09 microns). ADSCs can be preconditioned without any manipulation to modulate the composition of the exosomes they secrete. Preconditioning facilitates the hypoxia of ADSCa, and the secretome of hypoxia-preconditioned ADSCa plays an important role in promoting cell proliferation and migration, improving angiogenesis, and inhibiting apoptosis and inflammation. The exosomes released by the previously conditioned adult stem cells produce a greater therapeutic effect, and preconditioning without manipulation is emerging as a key strategy for improving the functions of ADSCa to obtain exosomes with improved efficacy in regenerative medicine. All patients were satisfied with the treatment. The physical examination that the patients underwent during the follow-up was in line with their self-assessment.

5. Conclusions

It is now known that cell-based communication, even at a distance, occurs through signaling microvesicles, defined as exosomes. There are no other clinical studies on skin chrono- and photoaging using signaling microvesicles obtained by extraction from adipose tissue using a simple nanofiltration technique from preconditioned ADSCa, diluted in suspension but without any manipulation. Stabilization by the binding of hyaluronic acid to the CD44 of ADSCa positively affects the quality and number of microvesicles in the suspension. This clinical study showed that it is possible to extract signaling microvesicles with the typical markers CD81 and CD146 using a simple ultrafiltration system. The extraction of exosomes by ultrafiltration through a 0.20-micron filter eliminated the cellular components as well as sterilized the solution [54]. The method proved to be safe and fits into the field of innovative regenerative therapies as a powerful and viable alternative to epidermal regeneration therapies.

Author Contributions: Conceptualization, F.S.; Methodology, A.C., C.S. (Carmela Saturnino); Writing—Original Draft Preparation, G.G. and G.C.I.; Investigation, L.S.; Data Curation, F.G. and G.F.; Visualization, P.F., C.S. (Carmen Scieuzo), R.S.; Formal Analysis, P.D.L., C.S. (Carmen Scieuzo) and F.G.; Validation, C.S. (Carmen Scieuzo). All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: This study was approved by the local ethics committee of University of Rome "La Sapienza " under protocol number 367/2021 and was conducted in accordance with the tenets of the Declaration of Helsinki.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The stored data is currently covered by privacy.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

AAT	Alpha-1-antitrypsin
ADSCa	Adult mesenchymal stem cells (MSCa) derived from adipose tissue
EGF	Epidermal growth factor
EVs	Extracellular vesicles
FGF2	Fibroblast growth factor
GF	Growth factor
IFN-γ	Interferon-γ
IL-1β	Interleukin-1β
IL-1017	Interleukin-1017
MMPs	Matrix metalloproteinases
NF-κB	Nuclear factor kappa B
NRS	Numeric Rating Scale
PDGFA	Platelet-derived growth factor A
SOD1	Superoxide dismutase
TGF-β1	Transforming growth factor β1
TK	Tyrosine kinase
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor-α
VAS	Visual Analogue Scale
VEGFA	Vascular endothelial growth factor A

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Entomologia Generalis, Vol. 44 (2024), Issue 4, 833–851

A comprehensive review of entomophagy under legal, historical, safety, and nutritional profile

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With 5 figures

Abstract: In the last decade the use of insects has grown globally in relation to all sectors of the food chain. In about 100 nations around the world, insects are traditionally part of the human diet; this practice is termed entomophagy. Although many studies have been conducted on the importance of edible insects as alternative sources of protein, most of them have focused on consumer acceptance or aversion/neophobia. As human food, insects have various advantages. They are rich in proteins, vitamins, and minerals and can be used in nutrition. Additionally, insects are efficient in bioconverting organic matter into biomass for food, adding the advantage of disposing of some by-products. Insects as foods of the future offer great promise, and their impact is expected to grow as new insights are pursued into quality, cultivation, nutrition, taste and safety. This review aims to be an overview of the laws regulating entomophagy, the history of insect consumption worldwide, safety considerations, and nutrient composition.

Keywords: entomophagy; novel food; Tenebrionidae; Gryllidae; legislation; *Hermetia illucens*

1 Introduction

In approximately 100 nations worldwide, insects have traditionally been an important part of the human diet (Durst 2010) and, recently, entomophagy has gained great interest from both industry and consumers (Nešić et al. 2023). Several international authorities and organizations, such as IPIFF (International Platform of Insects as Food and Feed), NACIA (North America Coalition for Insect Agriculture), AFFIA (Asian Food and Feed Insect Association), and IPAA (Insect Protein Association of Australia), have highlighted insects as future players in the commercial food and feed chain (van Huis 2013; Moruzzo et al. 2021a). Indeed, according to Rabobank, demand for insect protein would rise from 120,000 metric tons to 500,000 metric tons by 2030 (FEEDINFO 2021).

In China and Thailand, industrial-scale farms produce insects for human consumption. In the United States, Canada, and Europe, major companies such as Beta-Hatch (USA), Enterra Feed Corporation (Canada), Oberland Agriscience (Canada), Protix (Netherlands), Agriprotein (Germany), and Ÿnsect (France) raise insects for livestock feed and as a fishmeal replacement. More than 460 patent applications for *Hermetia illucens* (Linnaeus 1758) were published between 2017, and 2022 (https://www.lens.org/), covering rearing procedures, cosmetic and medical applications of its derivatives and agronomic purposes (Franco et al. 2021; Triunfo et al. 2022). Additionally, around 200 patents have been filed for insects as edible sources, with notable growth since the mid-2010 (Kim et al. 2019). The edible insect sector is relatively new, having only been around for about ten years, and its success will require a favourable combination of agricultural research/education, industry, and government support worldwide (van Huis 2022). In development projects, industry and academia frequently collaborate, as in the EU project SUStainable INsect CHAIN (SUSINCHAIN) (Veldkamp et al. 2020) or in the United States, where a Center for Environmental Sustainability through Insect Farming (CEIF) was established (Tomberlin et al. 2022). Meanwhile, significant investments are needed due to the technology used in this sector, that differs greatly from those utilized in the livestock industry (https://www.lens.org/).

The aim of this review is to provide a comprehensive overview of edible insect consumption, addressing the his-

DOI: 10.1127/entomologia/2024/2524
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torical, administrative, legislative, scientific, and psychological aspects related to insect consumption.

2 Worldwide insect consumption history

One third of the world's population is accustomed to eating edible insects (Raheem et al. 2019). Insects have been a regular part of human diets for millennia (FAO 2013). Although some Western cultures are reluctant to consume insects, they have played an important role in the history of human nutrition in Africa, Asia, and Latin America (Bodenheimer 1951). According to Wageningen University "List of the World's Edible Insects", there are 2,111 species of insects consumed worldwide. These include 679 species consumed in the Americas, 524 in Africa, 349 in Asia, 152 in Australia, and 41 in Europe. Traditional insect consumption remains common in underdeveloped nations, as it provides nutritional, economic, and ecological benefits to rural communities (DeFoliart 1999). Many people live below the international poverty line and are unable to access high quality food, which results in undernutrition, especially protein-energy malnutrition, in Africa, Latin America, and Asia. In addition, trends toward 2050 predict a steady population increase to over 9 billion people, which will drive increased food/feed production from available agroecosystems and even greater pressure on the environment (FAO 2017). Despite improvements, alternative foods, especially new sources of protein, must be found to ensure responsive and suitable future food systems.

Globally, the most commonly eaten insects are beetles (Coleoptera) (31%) (Fig. 1), which make up about a third of the total (Costa-Neto 2015). Eating caterpillars (Lepidoptera) is particularly popular in sub-Saharan Africa and is estimated at 18%. Bees, wasps and ants (Hymenoptera) rank third with 14%, most being common in Latin America, followed by grasshoppers, locusts and crickets (Orthoptera) (13%). Next are cicadas, leafhoppers, planthoppers, scale insects, and true bugs (Hemiptera) (10%), termites (Isoptera) (3%), dragonflies (Odonata) (3%), flies (Diptera) (2%), and finally other insects (5%) (Costa-Neto 2015). Hymenoptera are gathered and eaten largely in larval or pupal phases, while Lepidoptera are consumed as caterpillars. Both adults and larvae of coleopterans are eaten, while the Orthoptera, Isoptera, and Hemiptera orders are mostly consumed as mature adults (Raheem et al. 2019).

2.1 Africa

African diets feature a range of wild items, including edible insects (Fig. 2). In Africa, almost 1,500 species of insects are classified as edible (Raheem et al. 2019). The resale value of *Gonimbrasia belina* (Westwood 1849) (mopane worm) in Southern Africa is around $85 million. These caterpillars are found in mopane woodlands in Angola, Botswana, Mozambique, Namibia, South Africa, Zambia, and Zimbabwe (FAO 2003), with Southern Africa harvesting an estimated 9.5 billion mopane caterpillars annually (Raheem et al. 2019). In many African countries, the larval stages of Lepidoptera and winged termites are the most often eaten insects (Illgner & Nel 2000). In Uganda, winged termites and grasshoppers are the most commonly eaten insects (Owen 1973). In Malawi, edible insect larvae are available from mid-October to December when food supplies are running low, whereas in Zambia, caterpillars are a crucial nutrient source from November to February, accounting for 40% of the diet (DeFoliart 1999). Grubs of the palm weevil, *Rhynchophorus phoenicis* (Fabricius 1801), are consumed after frying in western Nigeria and are widely

Fig. 1. Three orders of edible insects most consumed in the World (Jongema 2017).

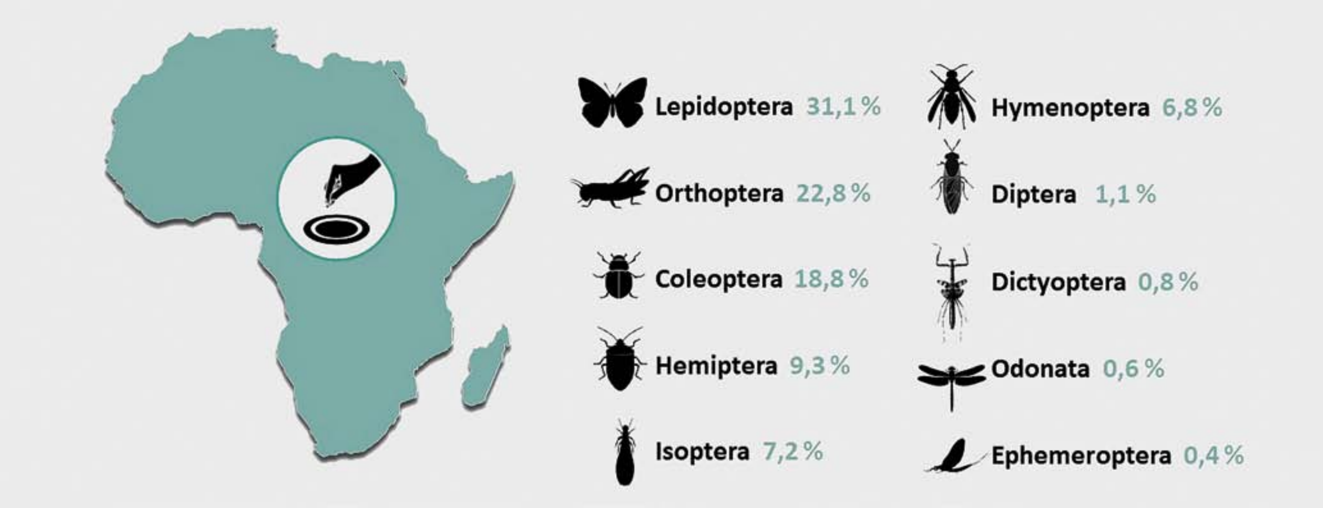

Fig. 2. List of recorded orders of edible insects in Africa. The image shows the percentage frequencies of insect orders considered edible (Jongema 2017).

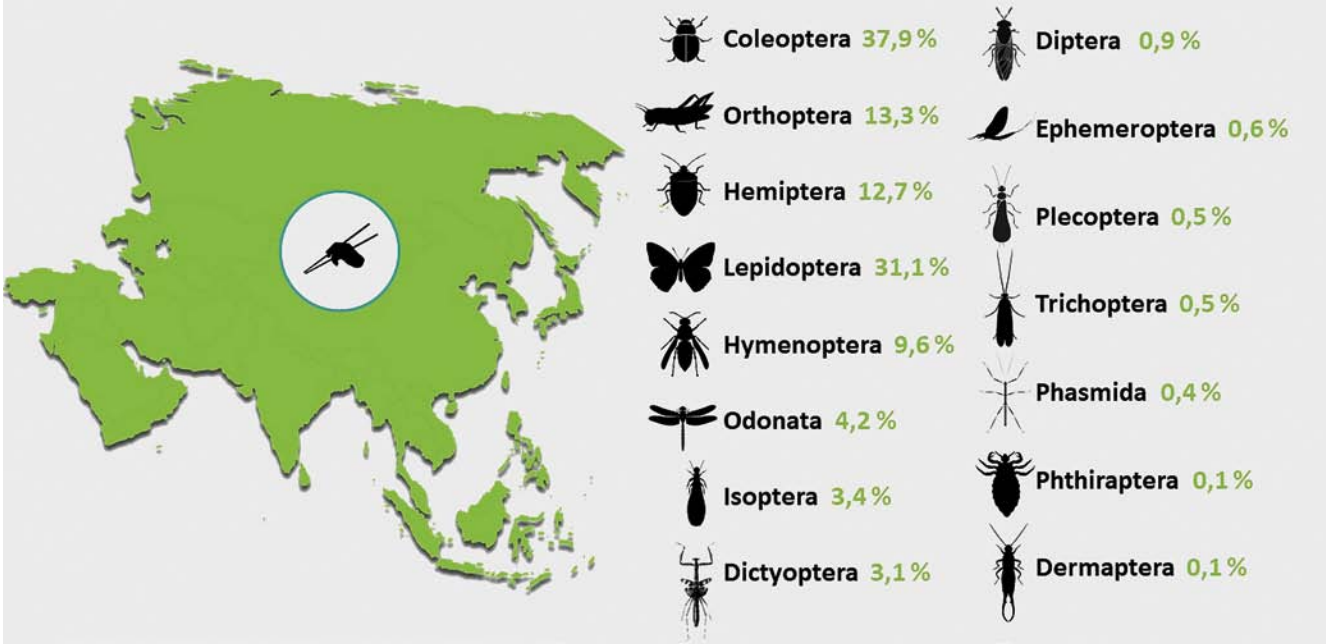

Fig. 3. List of recorded orders of edible insects in Asia. The image shows the percentage frequencies of insect orders considered edible (Jongema 2017).

available in local markets (DeFoliart 1999). In South Africa, insect consumption is common in Mpumalanga, Northwest, Limpopo and Gauteng provinces. Grasshoppers, flying termites, mopane worms, bugs, jewel beetles and white grubs are among the most commonly consumed insects. *Arytaina mopane* (Uhler 1896), *Monomotapa insignis* (Butler 1895), *Sternocera orissa* (Buquet 1835), *Cyrtacanthacris tatarica* (Linnaeus 1758), and *G. belina*, constitute a few examples of the insects that are consumed in Africa (Nonaka & Ethnoentomology 1996).

2.2 Asia

In Asia edible insects are widely consumed. The most regularly eaten categories include beetles, caterpillars, bees, wasps, ants, grasshoppers, locusts, crickets, cicadas, leafhoppers and plant hoppers, scale insects, true bugs, termites, dragonflies, and flies (Fig. 3). In Bangkok, Thailand, 164 species are harvested and sold in marketplaces and supermarkets (Yhoung-Aree & Viwatpanich 2005). Over 20,000 insect farming companies, mostly small local enterprises, are registered in Thailand. Despite a decline in insect consumption globally, it remains prevalent and growing in Thailand, where insects are a “food of choice”, often priced higher than chicken, pork, or beef (Yen et al. 2010).

In Japan, at least 117 different species of insects have been used as food throughout history. The orders Orthoptera (22 species), Hymenoptera (17 species), and Lepidoptera (23 species) were the three most important groupings of edible insects in the traditional Japanese diet (Payne 2015). In Japan, traditional meals include insects such as *Oxya yezoensis* (Matsumura 1906), *Oxya japonica* (Thunberg 1815), larvae and pupae of *Vespula lewisi* (Spradbery 1973), and pupae and adults of *Bombyx mori* (Linnaeus 1758). After being cooked with soy sauce and sugar, these insects are packaged and sold (Mitsuhashi 1997). Eating edible insects has a long tradition in China, with 178 insect species widely consumed. In rural China, insects are generally produced and bred for human diet, medical reasons, and animal feed (Zhi-Yi 1997). Grasshoppers, silkworm pupae, wasps, bamboo insects, and stink bugs are among the 20–30 species frequently consumed in restaurants year-round. China also has a robust insect supply chain, including commercial farms and trade routes (Chen et al. 2009). In Korea, insect foods have long been a staple of traditional diets and the government continues to promote their use (Ghosh et al. 2017).

2.3 America

Insects have never been considered part of the traditional American diet, although many species are consumed (Fig. 4). Prior to European colonization, several indigenous tribes in America consumed edible insects as an important part of their diet. Some caribou hunters eat a byproduct of their hunting: the warble fly larvae, large parasitic flies belonging to the genus *Hypoderma* (Latreille 1818) (Schrader et al. 2016). Native Americans in the Great Basin are the most documented consumers of insects (Madsen & Schmitt 1998). Other examples of insect species consumed in North America include *Coloradia pandora* (Blake 1863) (Aldrich 1921), *Cotinis nitida* (Linnaeus 1764) (Sutton 1995), *Anabrus simplex* (Haldeman 1852), and *Camponotus punctulatus* (Mayr 1868). Archaeological finds have revealed evidence of entomophagy in the Great Basin and adjacent regions of California, Nevada, and Arizona (Brickell 1737). Bits of insects were preserved in mummified remains in the Ozark Mountains and Kentucky’s Salts Cave (Yarnell 1974). Periodical cicadas of the genus *Magicicada*, which appear in large numbers every 17 years, were eaten in the eastern United States and are still consumed today. The use of this seasonal resource is a unique example of an edible insect that crosses cultural boundaries between indigenous and colonial civilizations (Schrader et al. 2016).

Latin America has a population of over 590 million people, with over 50 million indigenous peoples (Costa-Neto 2015). Insects play an important role in food security and subsistence of the majority of these indigenous people (van Huis 2013). In the northwest Amazon, insects account for about 5% to 7% of total protein consumption throughout

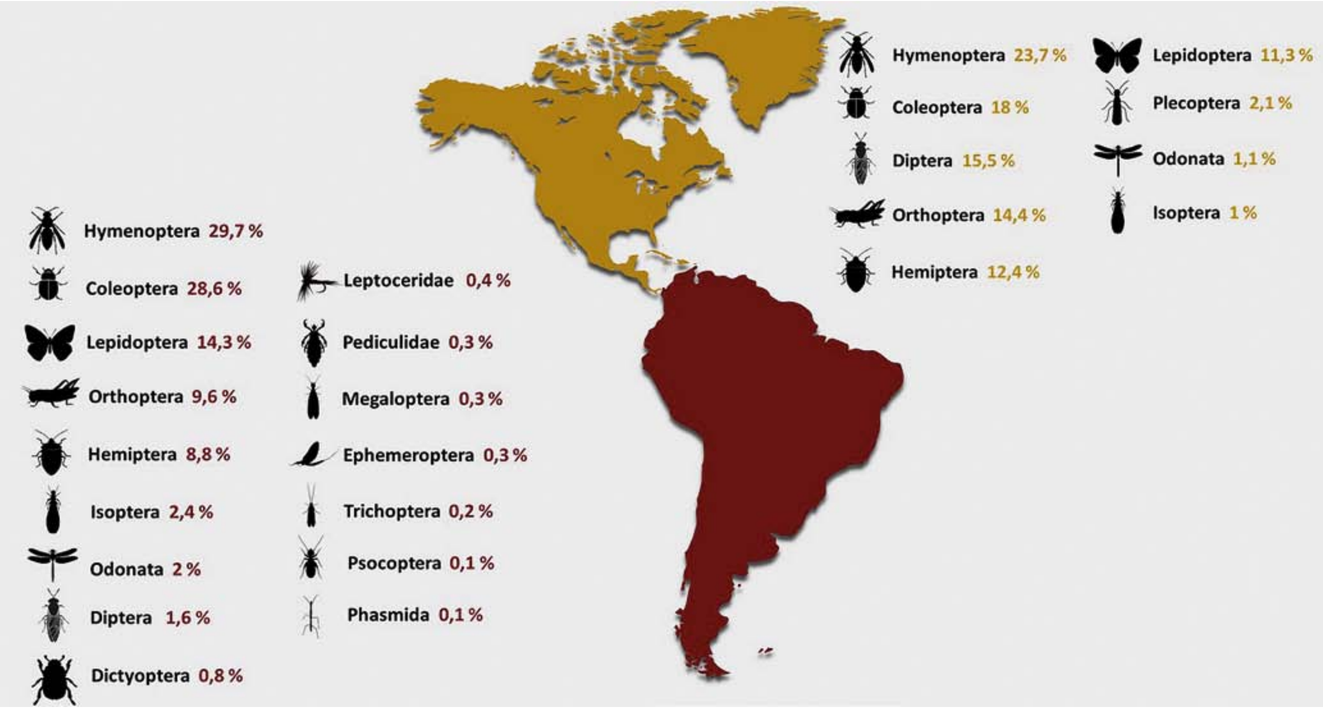

Fig. 4. List of recorded orders of edible insects in America. The image shows the percentage frequencies of insect orders considered edible (Jongema 2017).

the year. During the months of May and June, when availability is at its greatest, their contribution rises to 12–26% (Dufour 1987). The "*escamoles*," along with "*gusanos blanco y rojo del agave*," "*botija*," "*xamues*," "*ahuahutle*", "*axayacatl*," "*vinitos*," and "*avispa negra*", have been a resource for more than 500 years in Mexico (Ramos-Elorduy 2006). The "escamoles" (Dolichoderinae ants of the genus *Liometopum* (Mayr 1861)) were part of the Aztec Emperor Moctezuma's tributes, and their exploitation has continued to this day. The current price for these insects is considerable ($200.00 USD/Kg) due to their exquisite taste and market demand. However, research by inexperienced people, lured by the market value of these insects, has led to a reduction in their renewability, endangering the survival of the species (Ramos-Elorduy 2006).

2.4 Oceania

According to ethnographic studies, insects were once a key element of the diet of Australian Aborigines. More than 60 Australian endemic insect species have been documented as being historically consumed by First Nations people. They included *Melophorus* spp. (Mayr 1862), *Endoxyla leucomochla* (Meyrick 1886), Cerambycidae (Latreille 1802), and *Agrotis infusa* (Donovan 1805) (Fig. 5) (Bodenheimer 1951). During the *A. infusa* harvest season, up to 700 First Nations people gathered in the mountain ranges of the Capital Territory (Brindabella Ranges), New South Wales (Mt Kosciuszko, Bogong Peaks and Tinderry Mountains), and the Victorian Alps. The moths were collected, roasted on hot ash and consumed, allowing for corroborees, feasts, weddings, meetings, and intertribal exchanges (Warrant et al. 2016). The increasing adoption of Western diets by First Nations tribes has led to the decline of historically practiced entomophagy, which is sometimes considered archaic by younger generations (Ponce-Reyes, & Lessard 2021). Insects are considered a growing sector in Australia and, at present, they are a real opportunity to become a major participant in the international edible insect market (Reyes & Lessard 2023). According to AgriFutures Australia, the Australian insect business is an emerging industry with the potential to reach or exceed A$10 million per year in the next five years.

2.5 Europe

Growing or consuming insects is generally seen as taboo in Europe and the Western world in general. In the UK, a Food Standards Agency poll found that only 26% of respondents were interested in trying insect-based food, with men and younger people showing more openness. However, the fact that one-third of the world's population consumes insects, has aroused the interest of industry in Europe and North America. For customers in countries without a tradition of eating insects, the idea is often met with revulsion due to cultural, sociological, and psychological factors. Concerns about safety, potential health risks, and disgust are significant barriers (Skotnicka et al. 2021). A study of a heterogeneous population of Italian respondents found that, although interest in insect meals has grown, consumers still see them as unnecessary and repulsive. The propensity for consumption, based on the various criteria used, appears to be very low (neophobia: 8–24%, insect phobia: 4–21%) (Moruzzo et al. 2021b). However, there are some European countries with an advanced cultural background in this sector, which has made the acceptance of insects easier. Research on the acceptance of which has entomophagy conducted in European countries, such as the Netherlands, has found that people who have eaten insects before have a more positive attitude toward entomophagy (Lensvelt & Steenbekkers 2014).

In Finland, where insect food was authorized in 2018, a study conducted among 212 people aged between 14 and 69,

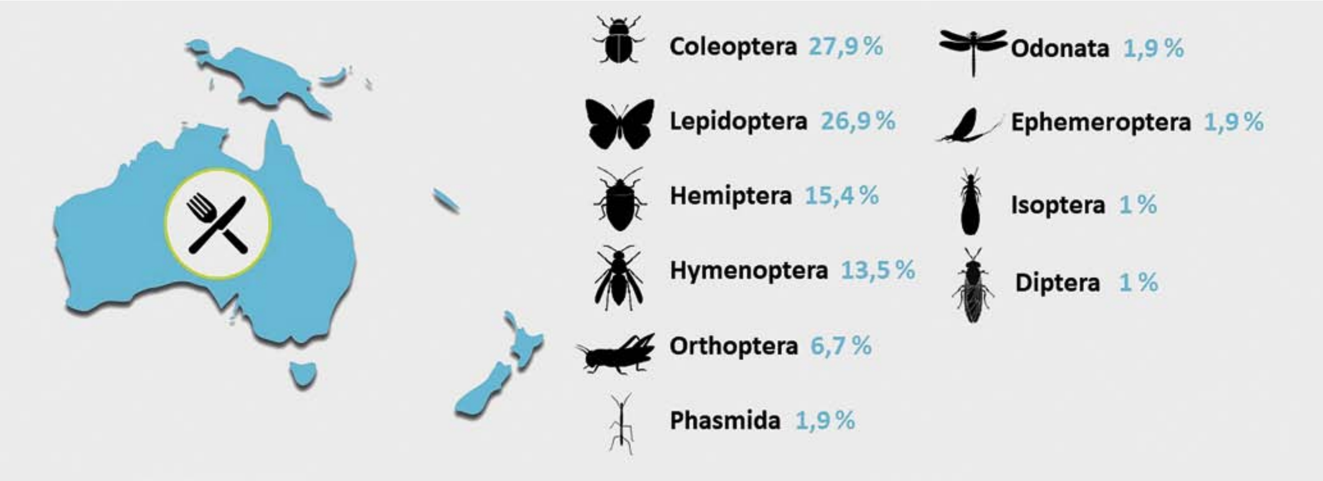

Fig. 5. List of recorded orders of edible insects in Oceania. The image shows the percentage frequencies of insect orders considered edible (Jongema 2017).

showed that younger people (under 30) were more positive about eating insects (71%) compared to older age groups. Acceptability increased when insect cuisine was prepared by a third party, such as a restaurant, or when positioned as a sustainable alternative to high-emission animal proteins. Key factors for consumers included taste, affordability, and perceived sustainability (Halonen et al. 2022). In Hungary, nearly 60% of 400 adult meat consumers were aware of eating insects and those looking to reduce their meat consumption were open to replacing it through the consumption of insects (Gere et al. 2017). In a study conducted on 718 students in Germany, 17.8% of respondents have eaten insects once, 5.3% more than once, and only 0.1% habitually ate insects. *Tenebrio molitor* (Linnaeus 1758), grasshoppers, various insects (flies, maggots), and *Alphitobius diaperinus* (Panzer 1797) were the most consumed insects (Dupont & Fiebelkorn 2020).

3 Legislative point of view

As insect farming gains popularity, concerns about the safety and regulation of their consumption and sale are increasing. In countries where entomophagy is common, there are often no governmental regulations, and they rely on guidance from the World Health Organization (WHO) and the Food and Agriculture Organization (FAO). Conversely, countries without a history of entomophagy but with stringent food safety policies impose stricter regulations on the use of insects as “novel food and feed ingredients”. For food purposes, whole insects and insect food products must adhere to basic food legislation principles, including hygiene procedures, traceability, notification, labelling, and a self-control system based on Hazard Analysis and Critical Control Points (HACCP) principles (Halloran et al. 2020).

3.1 Africa

In continents like Africa, where insect consumption is traditionally part of the culinary culture, there are few or no insect laws and regulations. The legal landscape for edible insects in Africa is complex. Nationally, most countries tolerate edible insects without specific legislation. Food law in African countries is shaped by both international and national regulations, with key influences from the African Union and various regional trade blocs. Some countries, such as Benin and Namibia, are interested in establishing legal frameworks for edible insects, while others, like Cape Verde, have explicitly excluded insects from the definition of legal food. Interest in formal regulations exists in countries like Congo-Kinshasa and Niger. Overall, the regulation of edible insects in Africa is still in its early stages (Grabowski et al. 2020).

3.2 Asia

In Japan, novel foods do not require pre-market approval, and the Ministry of Health, Safety and Welfare is responsible for food safety. In China, the Ministry of Health must approve innovative food raw materials. Proteins obtained from insects and silkworm pupae have been accepted as a novel food (Belluco et al. 2013). Thailand, the world’s largest producer of crickets, has introduced guidelines for cricket farming (Good Agricultural Practices for cricket farming, Thai Agricultural Standard 8202-2017) to ensure product quality and safety. The standard specifies requirements for farming equipment, feed, water, animal health, environment and record keeping. Feed must be unspoiled, water uncontaminated, clean and sanitized equipment, and all chemicals must be used according to the manufacturer’s recommendations (Lähteenmäki-Uutela et al. 2021).

3.3 America

In the United States, the Food and Drug Administration (FDA) regulates insect foods, and in 2013 it stated that insects are considered as food in the Food, Drug and Cosmetic Act (Title 21 of the U.S. Code). Products containing insects must meet FDA regulations, which include bacteriological testing and certification of good manufacturing practices. Wild-collected insects cannot be sold; they must be properly reared. Labels must include the common and scientific names of the insects and any relevant allergy hazards (Lähteenmäki-Uutela et al. 2021). In Canada, novel foods must be approved by Health Canada, to verify their safety and nutritional adequacy before sale (*Canada Gazette Gazette Du Canada Part II* 1999). If a product has been a staple in the diet, a food is not considered novel and can, therefore, be normally consumed. In Mexico, a wide variety of insects are used both as food and for medicinal purposes (Viesca Gonzalez et al. 2009). Food safety is controlled by two government secretariats, SSA (Secretariat of Health) and SAGARPA (Secretariat of Agriculture, Livestock, Rural Development, Fisheries and Food). All sanitary products and services are governed by Normas Oficiales Mexicanas (NOMs). In Mexico, there are no insect-specific NOMs yet, but general NOMs apply, such as Norma Oficial Mexicana NOM-251-SSA1-2009 on hygiene standards for food, beverages, and dietary supplements. Moreover, insect foods must indicate the species and life stages involved (Lähteenmäki-Uutela et al. 2021).

3.4 Oceania

Except for indigenous Australians, insect consumption has traditionally been rare in Australia. However, a culture of adapting to insect ingestion has recently emerged (Durst 2010). In Australia, insects for food use are classified as a “non-traditional food,” rather than a “novel food”, allowing their breeding and sale without specific government legislation or regulation. The Australian Insect Protein Association (IPAA) has developed rules to ensure quality and safety for its members, which are steadily increasing (https://insectproteinassoc.com/). Insect-based foods are classified under Standard 1.5.1 of the Food Standard Code, which covers non-traditional foods whose safety requires further investigation. Before new food can be sold in Australia and New Zealand, they undergo a detailed assessment by Food Standards Australia New Zealand (FSANZ), including evaluation of risk, nutritional value, chemistry and consumption patterns. FSANZ can also grant exclusive authorization for new food or ingredients for 15 months upon request (https://www.foodstandards.gov.au/).

3.5 Europe

In the EU, in the absence of any evidence of significant historical consumption within the European Union prior to 15th May 1997, all insect products are considered "novel foods" under the EU Novel Food Regulation (EU) 2015/2283, and they must be approved by the EFSA (European Food Safety Authority) before being marketed (Regulation – 2015/2283 – EN – EUR-Lex). As a result, before insects for human consumption can be sold in the EU, a license for a "new food" must be secured at the European level. On 1st June 2021, the European Union authorized the marketing of dried *T. molitor* larvae for food use: the first insect to pioneer licensing entomophagy in Europe (Implementing regulation – 2021/882 – EN – EUR-Lex). The second edible insect approved, on 12th November 2021, was *Locusta migratoria* (Linnaeus 1758) (Implementing regulation – 2021/1975 – EN – EUR-Lex). On 10th February 2022, the European Commission authorized the marketing for food use of a third insect, *Acheta domesticus* (Linnaeus 1758) (Implementing regulation – 2022/188 – EN – EUR-Lex). Finally, on 5th January 2023, frozen, paste, dried and powdered forms of *A. diaperinus* were authorised for marketing in the European Union (Implementing regulation – 2023/58 – EN – EUR-Lex). The approval of these four insects, in a relatively short period of time, is a clear signal of change and openness to this new, readily available, high-nutrient food source, which, albeit slowly, is also gaining a foothold in Europe. However, it will be necessary to wait and see the response of consumers. In addition to domestic consumption and production, the EU has enacted legislation regulating the importing of edible insects from countries outside the European Union. Indeed, EU Regulation 2019/626 has established a list of countries authorized to export approved insects in the European Union (Canada, Switzerland, UK, South Korea, Thailand and Vietnam) (Implementing regulation – 2021/1327 – EN – EUR-Lex). Belgium is one of the most advanced countries in Europe in terms of entomophagy. Due to the lack of legal certainty on the scope of the "old" Novel Food Regulation (Regulation (EC) No. 258/97, repealed on 1st January, 2018 by Regulation (EU) 2015/2283), the Federal Agency for Food Chain Safety created a specific law for edible insects, and the Belgian authorities have accepted the marketing of some whole insects for human consumption. This acceptance applied only to ten insect species: *A. domesticus, Achroia grisella* (Fabricius 1794), *A. diaperinus, B. mori, Galleria mellonella* (Linnaeus 1758), *Gryllodes sigillatus* (Walker 1869), *L. migratoria, Schistocerca americana* (Drury 1773), *T. molitor*, and *Zophobas morio* (Fabricius 1775) whose food safety had been assessed in a "shared guidance" statement by the FASFC Scientific Committee and the Board of Health (Common Advice SciCom 14-2014 and SHC No. 9160). On 1st October, 2020, the Court of Justice of the European Union ruled that whole insects are not covered by Regulation (EC) No. 258/97 (Case C-526/19), and, as a result, Belgium' tolerance policy was phased out and replaced with the implementation of transitional measures described in Article 35 of Regulation (EU) No. 2015/2283. Whole insects are not covered by Regulation (EC) No. 258/97, but are now considered "novel foods" under Regulation (EU) 2015/2283. The Dutch Centre for Nutrition has a web page on human consumption of insects (https://www.voedingscentrum.nl/nl.aspx). In Denmark, four Danish universities have set up the consortium INFOOFEE (Danish Consortium on Insects as Food and Feed), composed of researchers and innovators studying the possibilities of using insects as a sustainable source of food and feed from a variety of perspectives. The production and distribution of insects in Denmark is regulated by the Danish Veterinary and Food Administration (DVFA) (https://en.foedevarestyrelsen.dk/). According to Danish law, the use of insects as food for personal use is exempt from the EU Novel Foods Regulation and is, thus, the sole responsibility of the individual consumer. Some insects in the form of whole animals were legally marketed in Denmark, prior to 1st January 2018, with transitional procedures. If an insect was legally placed on the Danish market as food before 1st January 2018, the bug may remain on the market until a decision is made on the application submitted. Furthermore, under the transitional provisions of the Novel Foods Regulation, the following insects that are in the process of being approved in the EU can be marketed as food in Denmark: *L. migratoria, G. sigillatus, A. domesticus, A. diaperinus*, and *H. illucens* (DVFA). In Germany, several edible insect products have also been available on market for some time despite EU regulations. In December 2016, the Swiss Council passed the Edible Insects Act, which allows the sale and consumption of three species: *A. domesticus, L. migratoria*, and *T. molitor*. The requirements of the food agency, the Federal Food Safety and Veterinary Office (FSVO), are strict and complicated. The insects must have been grown for human consumption and must be handled according to food safety guidelines after slaughter. After Brexit, it is unclear whether edible insects will be allowed in the UK. However, the approach of the UK Food Standards Agency (FSA) appears to be challenging. Norway is not a member of the European Union, but it is part of the European Economic Area and, therefore, follows a set of European regulations. Other European nations, many of which are still hostile to welcoming edible insects into their culture, are following the guidelines laid down by the European Union.

4 Nutritional composition of edible insects

Insects have a very high nutritional value, rich in protein, amino acids, fat, carbohydrates, vitamins and minerals. Edible insects, considered as a food source for humans, have a very small carbon footprint, producing fewer greenhouse gases and requiring much less land than conventional livestock like chickens, pigs and cattle (Li et al. 2020). Furthermore, compared with traditional livestock, insect breeding has a higher feed conversion efficiency, or feed conversion rate (FCR), meaning how efficiently feed is converted into biomass, and can be defined as the feed requirement per unit of body weight gain. The high conversion efficiency of insects is due in part to their poikilothermic nature, which means they do not use a significant portion of their energy intake to regulate body temperature (Ramos-Elorduy 2008). Furthermore, up to 80% of a cricket body is edible and digested, compared to 55% and 40% for chicken and pigs, respectively (Nakagaki & DeFoliart 1991); e.g., the gastrointestinal tract, blood, some internal organs, tendons, skin, and bones are some of the by-products encountered in a slaughterhouse (Irshad & Sharma 2015). Taking this into consideration, FCR of *A. domesticus* is twice that of poultry, 4 times higher than in pigs, and more than 12 times higher than in cattle (van Huis 2013).

4.1 Proteins

Dietary proteins are important because they provide the body with the nitrogen and amino acids necessary for synthesizing and maintaining the approximately 25,000 proteins encoded in the human genome, as well as other metabolically active nitrogenous compounds (‘Scientific Opinion on Dietary Reference Values for Protein’, 2012).

A parameter when assessing protein quality is the ratio between essential (E) and not essential (N) amino acids. According to FAO/WHO criteria, the E/(E+N) ratio should reach about 40% with E/N = 0.6 (Belluco et al. 2013). Protein content of insects is high, with many species ranging above 60% (Kouřimská & Adámková 2016), and is influenced by the substrate on which they grow (i.e., vegetables, grains, different kinds of waste). E.g., grasshoppers in Nigeria fed on bran have almost double the protein content of those fed on maize (van Huis 2013), while *H. illucens* larvae grown on spent grain diets had 15% or more protein than those reared on fruit only diets (Scala et al. 2020). The metamorphosis stage can also influence protein content; e.g., *Zonocerus variegatus* (Linnaeus 1758) protein content is about 14.4 and 21.38% for second larval stage and adult, respectively (Ademolu et al. 2010). Studies on protein content of almost 100 edible insects at different developmental stages found that raw protein content ranges between 20% and 70% (Hu Cui 1996; Yang 1998). Orthoptera order (crickets, grasshoppers, locusts) represent the group with the highest protein content: *Melanoplus femurrubrum* (De Geer 1773), *Sphenarium histrio* (Gerstaecker 1884), and *Melanoplus mexicanus* (Saussure 1861) have 77.00%, 71.15–77.00%, and 58.90–77.13% of proteins, respectively (Ramos-Elorduy Blásquez et al. 2012; Rumpold & Schlüter 2013).

Compared to commercial consumed meat, edible insects usually contain more protein; adults of *T. molitor* (24.13 g/100 g) and larvae of *B. mori* (23.1 g/100 g), *G. belina* (35.2 g/100 g) and *T. molitor* (25.0 g/100 g) contain a higher protein content than poultry breast muscle, beef sirloin, and horse meat, all having a protein content ranging from 19.2 g and 21.5g/100g. Concerning amino acid levels, edible insects are a source of complete animal protein, containing all essential amino acids (Orkusz 2021), although most insects have limited levels of either tryptophan or lysine (Bukkens 2005). According to researchers *G. belina, R. phoenicis, Oryctes rhinoceros* (Linnaeus 1758), *Macrotermes bellicosus* (Smeathman 1781), *A. domesticus, Gryllus bimaculatus* (De Geer 1773), *T. molitor, Z. morio*, and *B. mori* show the presence of all the essential amino acids, with levels ranged from 60 mg/100 g for tryptophan to 440 mg/100 g for lysine (Finke 2002; Ekpo 2011; FAO 2013; Siulapwa et al. 2014; Payne et al. 2016; Ritvanen et al. 2020; Orkusz 2021). Insect proteins are also highly digestible (between 77% and 98%), even if, when eating whole insects including exoskeleton, could be less absorbable, due to the presence of chitin (Belluco et al. 2013). The digestibility of insect protein is promising but varies with species and can be improved by chitin removal.

4.2 Fats

Fat is the most energetic macronutrient and facilitates the absorption of lipid-soluble dietary components such as vitamins. Dietary fats include fatty acids, triacylglycerols, phosphatidylcholine and cholesterol, all of which are important for many vital processes in the body. Fatty acids can be classified according to the number of C=C double bonds in the carbon chain: if there are no double bonds they are classified as Saturated Fatty Acids (SFA), if there is just one double bond then they are termed Monounsaturated Fatty Acids (MUFA), and, finally, Polyunsaturated Fatty Acids (PUFA) if there are two or more double bonds (EFSA 2010a).

Fatty acids can also be divided into essential, those that cannot be synthesized by humans and must be obtained in the diet, and those that are not essential. Essential fatty acids include omega-3 (i.e., α-linolenic acid) and omega-6 (i.e., linoleic acid) fatty acids.

Insect fatty acid composition is influenced by the substrate on which they feed (Bukkens 2005). Fat content of edible insects is usually between 10 and 60% of dry matter. Caterpillars are among insects with the highest fat content, ranging from 8.6 to 15.2 g/100 g of fresh insect (Tzompa-Sosa et al. 2014), but also larvae of coleopteran can have high fat content, as reported for *R. phoenicis*, whose total lipid content ranged from 13.9 to 30.5g/100 g of fresh larvae (Fogang Mba et al. 2018); while insects from the Orthoptera order have a relatively low-fat content (3.8 to 5.3g/100 g of fresh insect), with species like *Oxya chinensis* (Thunberg 1815) with only 2.2% of fat on dry matter (Durst 2010). Most edible insects have SFA contents that are usually low (less than 40% of the total fatty acids) (DeFoliart 1991), while they tend to have a higher content of "good fat" like MUFA and PUFA (Kouřimská & Adámková 2016). The average amount of SFA in insect orders ranges from 31% for Hymenoptera to 42% for Isoptera and the two main components of the SFA are generally palmitic acid and stearic acid (Bukkens 1997). In contrast, the average amount of MUFA ranges between 22% for Isoptera and 49% for Hymenoptera (with the oleic acid being usually the predominant MUFA), while PUFA ranges between 16% for Diptera and 40% for Lepidoptera (Rumpold & Schlüter 2013).

Most of the fatty acids in the *H. illucens* larval fatty acid profile are saturated fats, with palmitic acid and lauric acid being the most abundant (Franco et al. 2024).

Oils extracted from *R. phoenicis, Ruspolia differens* (Serville 1838), *Z. variegatus, Macrotermes spp.* (Holmgren 1910) and *Imbrasia spp.* (Hübner 1819), were analyzed and found to be rich in PUFA and MUFA, usually containing essential fatty acids such as linoleic and α-linolenic acids (Womeni et al. 2009). *T. molitor* provides considerable amounts of PUFA, between 21 and 62% of total lipids (Nowak et al. 2016), while the mean PUFA content of the insect order Coleoptera was 27% of total lipids (Rumpold & Schlüter 2013). When compared to traditional meat, insect fatty acids are similar to those of poultry and fish in their degree of unsaturation, while beef and pork contain lower amounts of PUFA (DeFoliart 1991). Furthermore, the presence of linoleic acid is considerably higher in insects, such as *A. domesticus, T. molitor, Z. morio* and *G. belina*, compared to commonly consumed meat (Orkusz 2021).

After triglycerides, phospholipids are the second most abundant group in insect fat, with a content usually less than 20%, parameter that varies according to the stage of life and species (Tzompa-Sosa et al. 2014). Cholesterol generally represents the most abundant sterol in insects, although they vary from low (none in the edible leaf-cutter ant, *Atta cephalotes* (Linnaeus 1758)) to a level similar to animal derived foods (1 mg sterol/g tissue), depending on the species and diet (Ritter 1990). In a study conducted on seven different insects, *A. domesticus* (98 mg/100 g) and *G. bimaculatus* (195 mg/100 g) showed the highest cholesterol content, while the lowest was in *T. molitor* larvae (45 mg/100 g) and adults (51.3 mg/100 g). These values are similar or even higher when compared with traditional meat: a chicken drumstick has a cholesterol content of 84 mg/100 g, while goose carcass contains 32.8 mg/100 g.

In *M. bellicosus* and *G. belina* the average content of cholesterol in the lipid fraction was 3.6% (Ekpo et al. 2009). In addition to cholesterol, other sterols found in edible insects include campesterol, stigmasterol, and β-sitosterol (Sabolová et al. 2016). Insects have a good nutritional fat value and, due to their content of omega-3 and omega-6, could play an important role in supplementing these essential fatty acids in diets.

4.3 Carbohydrates

Carbohydrates represent the primary source of energy in most human diets. Chemically, carbohydrates include dietary fibres, which are nondigestible carbohydrates such as cellulose, hemicellulose, and pectin, that help to maintain normal bowel/colonic function, promote bowel regularity, and ensures a healthy digestive system/function (EFSA 2010b). The chemical classification of carbohydrates is usually based on molecular size and monomeric composition, with three principal groups of sugars (1–2 monomers), oligosaccharides (3–9 monomers) and polysaccharides (10 or more monomers) (EFSA 2010b). While edible insects are rich in protein and fat, they are poor in carbohydrates; however, insects represent a special animal group regarding dietary fibre (Nowak et al. 2016). Indeed, insects contain significant amounts of chitin, an insoluble polysaccharide derived from the exoskeleton of insects (van Huis 2013). The average fibre content ranges from 5.06% for Isoptera to 13.56% for Hemiptera, with maximum yields for *Polyrhachis vicina* (Roger 1863)and *Latebraria amphipyroides* (Guenée 1852) (~29%) (Ramos-Elorduy et al. 1997; Oranut et al. 2010). Among the species with the lowest fibre contents, there are *Aegiale hesperiaris* (Walker 1856) larvae with 0.12% (Melo et al. 2011), *Apis mellifera* (Linnaeus 1758) larvae with 1.00–1.33% (Ramos-Elorduy et al. 1997), and *Brachytrupes* spp. (Serville 1839) with 1.01–11.61% (Banjo et al. 2006; Ramos-Elorduy Blásquez et al. 2012).

4.4 Minerals and vitamins

Vitamins and minerals are essential micronutrients for the normal functioning of the human body and need to be obtained from the diet (EFSA 2022). Depending on the species, edible insects contain different amounts of micronutrients (Orkusz 2021). Edible insects are a good source of iron; e.g., both *G. belina* and *L. migratoria* have a high iron content (respectively, 31–77 mg and 8–20 mg/100 g of dry matter) (Oonincx et al. 2010), and adult insects of *A. domesticus* and *G. bimaculatus* are rich in iron too (Orkusz 2021).

Zinc is also found in edible insects, with an average content comparable with meat: *R. phoenicis* contains zinc at a concentration of 26.5 mg/100 g, more than double the zinc concentration found in beef (Bukkens 2005), while *G. belina* contains 14 mg/100 g of dry matter (Kouřimská & Adámková 2016). Most insects have a similar or higher content of these minerals than in other animal-based foods, thus, offering insect sources of food as potentially useful to alleviate mineral deficiencies (Mwangi et al. 2018). Edible insects also generally contain sufficient amounts of manganese, copper, and potassium (Rumpold & Schlüter 2013). Edible insects are usually low in sodium and can be utilized in low-sodium diets (Rumpold & Schlüter 2013).

Insects contain a variety of water-soluble or lipophilic vitamins (Finke 2002; Oonincx & Dierenfeld 2012), and can be a primary source of water-soluble vitamins of the B group. Dried insects, especially coleopteran like *T. molitor* and *Z. morio*, are rich in riboflavin, pantothenic acid, and biotin, while insects like grasshoppers, crickets, locusts, and beetles are also rich in folic acid (Rumpold & Schlüter 2013). *A. domesticus* have significant B12 vitamin content: from 5.4 µg per 100 g in adults to 8.7 µg per 100 g in nymphs (Kouřimská & Adámková 2016). However, vitamin B12 content is significant in all insect species (Finke 2002; Bukkens 2005). Vitamin C is present in some edible insects, in contrast to meat, where it is totally absent (Orkusz 2021). Anyhow, insects are not a good source of this micronutrient, as it is present only in trace amounts (Kouřimská & Adámková 2016). *Drosophila melanogaster* and *Microcentrum rhombifolium* (Saussure 1859) have significant vitamin E content, with levels of around 11 mg per 100 g of dry matter (Oonincx & Dierenfeld 2012). Significant vitamin E content has been found in other insects, such as larvae of *Rhynchophorus ferrugineus* (Olivier 1790) (35 mg of α-tocopherol and 9 mg of tocopherols β + γ per 100 g of dry matter) (Bukkens 2005), in *B. mori* (9.65 mg of tocopherols per 100 g of dry matter) (Tong et al. 2011), and in *R. phoenicis* (4.76 mg of total tocopherols per 100 g of fresh larvae) (Fogang Mba et al. 2018). *Imbrasia oyemensis* (Rougeot 1955), *Imbrasia truncate* (Aurivillius 1908), and *Imbrasia epimethea* (Drury 1822) contain retinol and β-carotene (vitamin A), with levels ranging from 32 to 48 µg per 100 g of dry matter for retinol and 6.8 to 8.2 µg per 100 g of dry matter for β-carotene (van Huis 2013), while 100 g of *R. phoenicis* fresh larvae can supply up to 67 µg of vitamin A (Fogang Mba et al. 2018).

4.5 Antinutrients

Antinutrients (i.e., oxalates, tannins, alkaloids, phytates and saponins) are natural or synthetic substances that, when taken in high amounts and over a long period of time, impair the bioavailability and/or utilization of nutrients in the human body (Asanga et al. 2015). It is well documented that deficiencies in vitamin B1 (thiamine) can be caused by the heat-resistant thiaminase present in *Anaphe venata* (Butler 1878) larvae, which are ingested as supplementary protein in Africa (Okonji et al. 2012). A 2014 study on *Henicus whellani* (Chopard 1950) reported a level of 9.3, 1.7, 5.3, and 5.2 mg/100 g of oxalates, tannins, saponins, and alkaloids, respectively; so, the presence of these compounds could be a limitation in diets and requires further studies (Musundire et al. 2014). The antinutrient content of the edible termite *M. bellicosus* was quite low (Ntukuyoh et al. 2012), while a recent study conducted on *O. rhinoceros* and *Z. variegatus*, found that antinutrient values are within acceptable limits, indicating that they are good suppliers of macro and micronutrients (Anaduaka et al. 2021). Unfortunately, most studies on the bioavailability and digestibility of edible insects did not include antinutrient compounds, so the overall picture of the interplay between nutrients and antinutrients is unclear and future research should provide more balanced data for a better assessment of edible insect nutritional quality, and, thus, any limitations to consider in terms of consumption.

5 Safety

As the consumption of edible insects is growing around the world, food safety concerns remain. According to the EFSA, the production method, insect species, stage of harvest, and substrates employed in the rearing process influence the concentration of contaminants in insects and insect-derived meals (EFSA 2015). Chemical, biological and allergy considerations are the three types of potential food safety hazards linked with edible insects that need to be more fully documented (Gere et al. 2017).

5.1 Chemical hazards

Insect-derived food products, like those derived from other animals, may include dangerous chemicals. Feed substrates can contain chemical hazards capable of bioaccumulating in insects, including heavy metals and chemicals such as selenium, dioxins, and polybrominated diphenyl ethers (Belluco et al. 2018). Other chemicals, such as biocides to clean facilities and equipment or veterinary medications to treat certain diseases, may also contaminate the insect cultures during rearing and need to be monitored. Contaminants can build up in insects raised for food, posing a risk to human health (EFSA 2015). With respect to the insect species raised on the feed, the substrate may have the largest impact; so, the presence of most chemical contaminants in insects can be controlled by assessments of feeds used to propagate the insects (EFSA 2015).

5.2 Heavy metals

Metals like arsenic, cadmium, lead, and mercury occur naturally and can be present as residues in food due to their presence in the environment and due to human activities. Their accumulation in the body can lead to harmful effects over time (EFSA 2015). Heavy metal concentrations in insects are affected by the feed substrates, the insect species and stage of development (EFSA 2015).

Lead (39.1–455.6 ppm), chromium (38.1–190.5 ppm), and low concentrations of cadmium (0.02–0.21 ppm) were found in *R. differens* from Uganda and were attributed to forages grown in close proximity to significant industrial discharges (Kasozi et al. 2019). High concentrations of lead were also found in dried grasshoppers associated with insect feed in highly polluted mines in Mexico (Handley et al. 2007). Low concentrations (< 0.03 mg/kg) of lead, cadmium, and arsenic were found in a study conducted on *L. migratoria*, bought from various shops in Belgium (Poma et al. 2017). Low levels of arsenic (0.12 mg/kg), cadmium (0.02 mg/kg), and lead (0.73 mg/kg) were reported in edible grasshoppers *O. chinensis formosana* in Korea (Hyun et al. 2012). Mercury, lead, cadmium, and arsenic were found in low concentrations in *B. mori, Holotrichia spp.* (Hope 1837), *A. domesticus*, and *Patanga succincta* (Johannson 1763) in Thailand. In detail, *Holotrichia spp.* had the highest levels of arsenic and mercury, with 0.576 mg/kg and 0.08 mg/kg, respectively, *A. domesticus* had the highest lead level of 0.155 mg/kg, while all the insect samples had cadmium levels less than 0.05 mg/kg. Anyhow, all these species contained heavy metals at concentrations well below maximum legal values (Köhler et al. 2019). The amounts of three different heavy metals (mercury, cadmium, and lead) in grass and four insects (*Calliptamus italicus* (Linnaeus 1758), *Oedipoda caerulescens* (Linnaeus 1758), *Oedipoda germanica* (Latreille 1804), and *Chorthippus* (*Glyptobothrus*) *crassiceps* (Ramme 1926)) that feed on grassland were studied (Devkota & Schmidt 2000). Heavy metal concentrations in both the grass and insect samples were in the order: lead > cadmium > mercury. Cadmium concentrations were 2–4 times greater in all four grasshopper species studied than in the grass, and the amount of lead in the grass was lower than in the four grasshopper species. Mercury levels were two times greater in *C. crassiceps* than in grass, but mercury levels were lower than cadmium and lead levels (Devkota & Schmidt 2000). This study demonstrates the possibility of heavy metal bioaccumulation in insects that fed on contaminated substrates. In the study of Kolakowski et al., nineteen samples of edible insects (fifteen from cricket, four from silkworm) contained detectable arsenic, with varied values from 0.030 mg/kg (whole silkworm pupae) to 0.34 mg/kg (cricket powder), with an average of 0.094 mg/kg. Furthermore, cadmium was absent in all silkworm samples, but detected in all cricket samples with levels ranging from 0.031 to 0.23 mg/kg, with an average of 0.083 mg/kg; lead was present in 52% of the samples with concentrations from 0.019 to 0.059 mg/kg, with an average of 0.034 mg/kg; mercury in 93% of cricket samples but in none of the silkworm samples, with levels from 0.00094 to 0.028 mg/kg, with an average concentration of 0.0062 mg/kg (Kolakowski et al. 2021).

5.3 Veterinary drugs and hormones

Veterinary medication residues (antibiotics, hormones) in substrates such as manure may end up in insects and their products. Moreover, antimicrobials or other veterinary medications can be used in insect breeding (EFSA 2015). Furthermore, veterinary medications may be sprayed or mixed with substrates to battle insect diseases, and this use may leave residues in insects. Data on veterinary drug residue and hormones in edible insects are scarce. Several veterinary drugs including salicylic acid, metoprolol, paracetamol, and nicarbazin were detected in *T. molitor, L. migratoria, A. domesticus*, and *H. illucens* (de Paepe et al. 2019). Paracetamol and structural analogues are widely distributed and accumulated in aquatic environments (Wu et al. 2012) and were detected in various insect samples below the Screening Detection Limit (SDL), while metoprolol tartrate, a commonly detected drug in drinking water (van den Brandhof & Montforts 2010) and nicarbazin, a commonly used antibiotic frequently found in manure were found in concentrations higher than the SDL (de Paepe et al. 2019).

5.4 Pesticide residues

Pesticides are plant protection products that contain at least one active substance. According to EFSA, pesticide residues may pose a risk to public health (EFSA 2015). Edible insects absorb pesticides from plants growing in herbicide-treated areas (Saeed et al. 1993). Edible locusts in Kuwait contained chlorinated pesticides and unusually large quantities of the phosphorus-containing pesticides sumithion (740 µg/kg) and malathion (49.2 µg/kg) (Saeed et al. 1993). The presence of 511 pesticides in edible crickets and silkworm was investigated and detected in 89% of the crickets and 50% of silkworms-based products, with most of the positive samples containing just one pesticide residue, and glyphosate being the most frequently detected (Kolakowski et al. 2021). Edible insects in Togo were assessed for pesticide residues, and they were not found in *Brachytrupes membranaceus* (Drury 1770), *Rhabdotis sobrina* (Gory & Percheron 1833), *Gnathocera impressa* (Olivier 1789), *Gnathocera trivittata* (Swederus 1787), or *Cirina forda* (Westwood 1849), while desethylatrazine was found in *Acanthacris ruficornis* (Fabricius 1787) (0.320 µg/kg), *Oryctes monoceros* (Olivier 1789) (0.217 µg/kg), and *M. bellicosus* (0.532 µg/kg) (Badanaro & Dué 2022). Diuron was found in the latter species, while the insecticide permethrin was exclusively found in the species *R. phoenicis* (0.556 µg/kg). Multiple pesticide residues have not been found in the investigated species, and, therefore, the studied species have a low tendency to bioaccumulate pesticides (Badanaro & Dué 2022). The accumulation of pesticides varies depending on the insect species, their developmental stage, the pesticide dosage in the feed or environment, as well as whether the insects were caught in the wild or farmed. Overall, pesticides in edible insects do not appear to constitute a significant human health risk, while monitoring for safety is still the key (Kolakowski et al. 2021).

5.5 Toxins produced by insects

Plant poisons can be sequestered by some insect species, making them less appealing to predators, such as for glucosinolates in *Murgantia histrionica* (Hahn 1834) (Aliabadi et al. 2002). Other insect species are able to produce toxins themselves, such as Tenebrionidae family insects that can produce (benzo)quinones and alkenes (Crespo et al. 2011) or moths of the *Zygaena* genus, which can produce cyanogenic glucosides that release cyanide when degraded (Zagrobelny et al. 2009). Venomous insects are categorized into two groups: phanerotoxics and cryptotoxics. As with bees and ants, phanerotoxics have organs for the synthesis and delivery of venoms. In most cases, these chemicals are inactivated in the digestive tract. Cryptotoxic insects lack an external secretary mechanism and become toxic only after being swallowed. Toxins may be present in cryptotoxic insects, and these compounds might be concentrated in certain structures or distributed throughout the body (EFSA 2015). According to EFSA, for *Musca domestica* (Linnaeus 1758), *H. illucens, T. molitor, Z. morio, A. diaperinus, G. mellonella, A. grisella, B. mori, A. domesticus, G. sigillatus, L. migratora migratoriodes*, and *S. americana* (insect species identified as having significant potential for use in food and feed in the EU), there are no immediate indications that they excrete reactive, irritating, or toxic substances (EFSA 2015).

Some researchers used repeated dose toxicity experiments, ranging from 250 to 3,000 mg/kg body weight per day, in rats to assess the safety of edible insects such as *Allomyrina dichotoma* (Linnaeus 1771) larvae (Noh et al. 2015) and *T. molitor* (Han et al. 2016). A complete toxicological assessment, including hypersensitivity assays, was performed and no treatment-related side effects were found. Furthermore, the freeze-dried powder of *T. molitor* showed no genotoxic effect in vitro and in vivo (Han et al. 2016). The lack of genotoxicity in vitro was also confirmed for water soluble extracts of *Z. variegatus* and *Oryctes boas* (Fabricius 1775), even though both extracts indicated some oxidative stress in vitro (Memiş et al. 2013). It is also reported that traditional edible insects consumed in China are safe, nontoxic, and have potential as novel food resources (Gao et al. 2018).

5.6 Polycyclic aromatic hydrocarbons (PAHs)

PAHs (Polycyclic Aromatic Hydrocarbons) are a wide group of chemical molecules that include two or more fused aromatic rings, typically produced by the incomplete combustion or pyrolysis of organic matter, or through industrial processes. Some of them can cause cancer and be genotoxic (EFSA 2008). Data available for PAHs contamination in edible insects are scarce. PAH concentrations in *M. domestica, H. illucens*, and *Calliphora vomitoria* (Linnaeus 1758) ranged from 0.28 to 9.82 µg/kg for PAH4 and < 0.05 to 2.2 µg/kg for benzo(a)pyrene, all of which are carcinogenic or potentially carcinogenic (Charlton et al. 2015). The presence of PAHs in *M. bellicosus* and *C. forda* from Togo was assessed for both raw and processed samples (Badanaro & Dué 2022). *M. bellicosus* did not contain any PAHs. Raw *C. forda* was also PAH free, while processed *C. forda* contained only fluoranthene (classified as substance of very high concern due to its persistence, bioaccumulation and toxicity by the European Chemicals Agency) among the eight PAHs investigated, but at a concentration (0.31 µg/kg) well below the legal limit allowed in food (1 µg/kg) (Badanaro & Dué 2022). In conclusion, the consumption of edible insects studied by the researchers in Togo does not present a risk of contamination with PAHs (Badanaro & Dué 2022), while, according to other researchers, some insect-cooking techniques adopted in Africa may pose a carcinogenic danger due to the presence of toxic substances (like PAHs) that may be generated by chemical interactions between the insects and other ingredients, such as acrylamide, furans, chloropropanols, and heterocyclic aromatic amines (van der Spiegel et al. 2013).

5.7 Biological hazards

Since there is a significant evolutionary gap between humans and insects, insect pathogens are often exclusive to invertebrates and do not affect vertebrates (Eilenberg et al. 2015).

According to EFSA, there are two types of microbiota associated with the consumption of edible insects to be considered as potential hazards: those intrinsically linked to insects as part of their lifecycle and those introduced during farming and processing. Moreover, the risk of infection posed by human ingestion of insects and their products is influenced by the feed and the processing methods adopted during farming, storing, and consumption (EFSA 2015).

5.8 Bacteria

Arthropods are well known vectors for bacteria, such as *Salmonella*, and insects, such as flies, can acquire and spread *Salmonella* (Holt et al. 2007). Also, *Campylobacter* has been isolated from insects such as beetles and flies in contact with infected poultry; however, based on experimental conditions, it has been reported that *Campylobacter* can only survive for a limited time in insects (EFSA 2015). The Belgian Federal Agency for the Safety of the Food Chain's Scientific Committee published a report on the microbiological state of insects raised especially for food production, including *T. molitor, L. migratoria* and *Z. morio*: high values of 10^7 cfu/g were reported for total aerobic bacterial, anaerobic count, and Enterobacteriaceae. For *B. mori*, lower levels (< 10 cfu/g) for Enterobacteriaceae were measured (FASFC 2014). A 2011 study in Africa found harmful microbes in the larva of *Bunaea alcinoe* (Stoll 1780), including *Staphylococcus aureus, Bacillus cereus, Proteus*, and *Escherichia coli* (Braide et al. 2011). Other human pathogenic bacteria such as *Vibrio, Streptococcus, Staphylococcus, Clostridium* and *Bacillus* were found in a study assessing the microbiota diversity present in edible insects processed and sold in Thailand (Osimani et al. 2017). A recent study that characterized microbes in *R. differens* collected in the wild in Uganda also suggested that edible insects could harbour potentially harmful bacteria genera; *Campylobacter, Bacillus, Staphylococcus, Neisseria, Pseudomonas*, and *Clostridium* bacteria were identified (Ssepuuya et al. 2019). According to a survey on the microbiological status of 55 freeze-dried insect-based products conducted in Europe, more than half (59%) were above the process hygiene standard of 10^6 cfu/g for aerobic bacteria in raw materials used in meat preparation (NVWA 2014). The threshold of 10^3 cfu/g for Enterobacteriaceae in raw materials used in meat preparations was also exceeded in 65% of cases. *Clostridium perfringens, Salmonella*, and *Vibrio* were not found in the samples, while *B. cereus* was less than 100 cfu/g in 93% of the samples (NVWA 2014). EFSA states that microorganisms may be present in non-processed insects depending on the feed and conditions for production. Active reproduction of pathogens in the digestive tract of insects does not appear to be a concern, thus incidence of contamination will likely be lower than in other non-processed sources of animal protein and any risk of transmission can be reduced by processing (EFSA 2015).

5.9 Viruses

Most insect viruses are species or family specific, meaning they are only harmful to invertebrates and not to human or other vertebrates (EFSA 2015). However, insects can act as replicative vectors for viruses that infect vertebrates. Arboviruses are arthropod-borne viruses that cause disease in humans (such as dengue fever, West Nile fever, Rift Valley fever) (King et al. 2012). They also reproduce successfully in mosquitoes and midges. As a result, these viruses can successfully proliferate in both vertebrates and invertebrates crossing species barriers. However, there is no evidence that such viruses occur in food and feed insects (EFSA 2015). Insects can also be passive vectors of human and livestock viral diseases (Sawabe et al. 2006). However, insect pathogenic viruses found in food and feed insects are peculiar to insects and are, therefore, not considered a threat to vertebrate animals or humans. Viruses of vertebrates can persist in substrates and be taken up by insects produced for food or fed via the substrate. The risk of transmission can be reduced by selecting the appropriate feed and proper processing (EFSA 2015).

5.10 Parasites

The risks from parasites depend on environmental conditions and feed substrates (EFSA 2015). Parasites belonging to the families Lecithodendridae and Plagiorchidae could be transmitted orally (Chai et al. 2009). A parasite that could be transmitted to humans by edible insects (such as ants) is *Dicrocoelium dendriticum* (Boye et al. 2012). Other parasites found in edible insects include protozoa, such as *Entamoeba histolytica* and *Giardia lamblia*; they are found in cockroaches as possible foodborne and waterborne diseases, *Toxoplasma* spp. in *Periplaneta americana* (Linnaeus 1758) and *Blattella germanica* (Linnaeus 1767), and *Sarcocystis* spp. (Graczyk et al. 2005). Another example of how insects can act as vectors is trypanosomiasis. According to the World Health Organization (2010), around 10 million people in the Americas are infected with Chagas disease (also known as American trypanosomiasis), and over 10,000 people die annually. Infection has been linked to the inadvertent eating of insects or the consumption of contaminated food (Pereira et al. 2010). Although there are parasites in insects linked to sporadic human parasitic disease, there is little data on parasites found in farmed insects. A properly maintained closed farm setting would lack the hosts required for parasite life cycles to be completed and adequate management prior to consumption, such as freezing and boiling, can further reduce any possible hazards (EFSA 2015).

5.11 Fungi

Insects can carry entomopathogenic fungi, that generate insect-specific toxins but are generally safe for vertebrate animals, including humans (EFSA 2013). In rare cases, diseases caused by entomopathogenic fungi are encountered in immunocompromised people (Roberts & st. Leger 2004). Yeasts and fungi were found in fresh, freeze-dried, and frozen *T. molitor* and *L. migratoria* (FASFC 2014). Mycotoxins, harmful secondary metabolites produced by fungi, are resistant at ordinary food preparation temperatures and can be present in insect feed (Imathiu 2020). High levels of aflatoxins were found in several commercial lots of *G. belina* highlighting the importance of proper processing and storage in order to avoid contamination (Durst 2010). Mycotoxin-forming fungus from *Aspergillus* spp. and *Penicillium* spp. were identified in the substrate and insect body of *G. sigillatus* industrially farmed for human consumption (Vandeweyer et al. 2018). A 2017 study showed that in *Gynanisa maja* (Klug 1836) (11 µg/kg), *Gonimbrasia zambesina* (Walker 1865) (12 µg/kg), and *Macrotermes falciger* (Gerstäcker 1891) (24 µg/kg), average aflatoxin concentrations were above regulatory limits (10 µg/kg) (Kachapulula et al. 2018). These findings clearly suggest that edible insects can contribute to aflatoxicosis, but, in general, EFSA stated that these risks can be managed by proper hygienic measures in the production chain (EFSA 2015).

5.12 Prions

Prions are infectious agents causing fatal conditions affecting the brain and the nervous system, such as bovine spongiform encephalitis, scrapie, and Creutzfeld-Jakob disease (Mézes & Erdélyi 2020). EFSA identifies three potential prion-related risks with insects: insect-specific prions, insects as mechanical vectors of animal/human prions, and insects as biological vectors of prions (EFSA 2015). Since insects lack prion-encoding genes, prion proteins cannot be naturally expressed in these hosts. Prions from mammals cannot multiply in insects, and, as a result, insects are not thought to be biological vectors or amplifiers of prions (EFSA 2015). In conclusion, based on the available information, insects farmed on a feed or in an environment containing infectious prions could act as mechanical vectors of infection, posing a risk of prion disease transmission through food, but, since mammalian prions cannot replicate in insects, the quantity of infectivity contained in the feed will determine the overall prion infectivity transported by insects (EFSA 2015).

5.13 Allergy hazards

Food allergies represent an adverse immune response to food allergens and can lead to severe consequences (Imathiu 2020). Although solid epidemiological data on food allergies to edible insects is lacking, cross-reactivity, particularly with crustaceans, has been identified as a significant concern. This is due to the presence of shared allergens such as tropomyosin, arginine kinase (AK), aspartic protease, hemocyanin, glutathione S-transferase, troponin C, myosin light chain, serine protease, and a-amylase (Ribeiro et al. 2021). Primary sensitisation (either through environmental or occupational exposure) has also been linked to allergic reactions to edible insects (Pomés et al. 2017). The intake of *G. belina* has been connected to a few cases of anaphylactic shock (Okezie et al. 2010). In a Belgian study, 19% of participants showed sensitization to the tested insects (Francis et al. 2019). In China it was estimated that up to 18% of fatal reactions to foods were linked with insect consumption (Ji et al. 2009), while in Laos 7.6% of insect consumers experienced allergic reactions (Barennes et al. 2015). In Asia, AK was identified as the most common allergen in *B. mori* pupa (Liu et al. 2009), and, during 2010–2019, allergens were responsible for 25% of the identified hazards in edible insects. According to EFSA a potential approach might be to list the insect protein as well as the potential allergenicity or cross reactivity on the product label. Allergens in edible insects appear to be resistant to thermal treatment and enzyme digestion, which is similar to the behaviour of crustacean allergens (Ribeiro et al. 2021). In conclusion, allergies to crustaceans (due to cross-reactivity) and people who are regularly exposed to edible insects are the two largest risk categories for developing food allergies to insects (Ribeiro et al. 2021).

6 Conclusions

Although the consumption of insects is part of the history of many of the world's populations, some cultures remain strongly reluctant to adopt this practice. However, edible insects can be a promising resource to address global challenges of food security and sustainability. To make insect consumption more common and safe, in addition to researching sustainable farming practices and scalable production methods, strong safety standards and regulations need to be established to ensure the health and safety of consumers. Additionally, a comprehensive nutritional profile of various insect species needs to be developed to fully understand the benefits and potential risks related to consumption. Finally, cultural acceptance and consumer perceptions need to be studied to develop effective strategies for the promotion of insect-based foods. Addressing these challenges will pave the way for the integration of edible insects into mainstream diets around the world.

Acknowledgements: This study was carried out within the Ecosystem of Innovation "Tech4You – Technologies for climate change adaptation and quality of life improvement" and received funding from the European Union Next-GenerationEU (PIANO NAZIONALE DI RIPRESA E RESILIENZA (PNRR) – MISSIONE 4 COMPONENTE 2, INVESTIMENTO 1.4 – DD 1049/23.06.22 – ECS_00000009). This manuscript reflects only the authors' views and opinions; neither the European Union nor the European Commission can be considered responsible for them. This work was supported by Basilicata Region within the framework of FSC "Fondo per lo Sviluppo e la Coesione" ("Gestione del ciclo di scarti e sottoprodotti della filiera agroalimentare attraverso la loro bio-conversione in prodotti di valore" D.G.R. n. 652 30/09/2022)

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Manuscript received: February 6, 2024
Revisions requested: June 4, 2024
Revised version received: June 18, 2024
Manuscript accepted: June 27, 2024

Entomologia Generalis, Vol. 44 (2024), Issue 4, 813–831

Edible insects: an overview on farming, from processing procedures to environmental impact, with a glimpse to traditional recipes and to future cultured meat

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With 3 figures and 2 tables

Abstract: In the last decade, the use of insects has grown globally in relation to all sectors of the food chain. Insect farming offers a sustainable alternative to conventional livestock production, with lower environmental impacts and efficient resource use, as shown by Life Cycle Assessments (LCA). However, challenges in scaling production, standardizing processes, and addressing regulatory gaps remain. Continued research and collaboration are essential to fully realize the potential of insects as a sustainable protein source for human consumption. This review analyses some elements related to insect consumption, from the aspects of the hygiene, the different breeding and environmental impact to the description of the processing techniques, also providing the reader with some practical examples related to some world-famous recipes to offer an idea of how insects are perceived as food. The methods used in the processing of edible insects are critical to improving their nutritional content, safety, and palatability. The acceptance of insect-based cuisine can be increased by incorporating insects into traditional foods, thereby minimizing reactions of disgust. In addition, a look to the near future examines the possibilities of cultured meat made from insect cells, which presents encouraging paths toward the creation of sustainable protein, offering a revolutionary strategy that will transform future food production systems toward efficiency and sustainability. This is an absolutely innovative aspect for the production of edible proteins.

Keywords: Entomophagy; food; nutrition; insect industry; insect marketing; hygiene; *Hermetia illucens*

1 Introduction

In recent years, entomophagy, the insect eating by humans, has worldwide attracted great interest from business companies and consumers (van Huis & Tomberlin 2017). Insects can have several benefits as human food, including their high protein, vitamin, and mineral content, and they are also extraordinarily efficient at converting the foodstuffs that they consume into biomass that humans can eat (Hanboonsong et al. 2013). Producers have focused their efforts on creating new products based on both simpler formulation (e.g., whole dried insects, powder forms) and more elaborate insect-based foods (e.g., bars, bread, crackers) (Pippinato et al. 2020). More than 80% of all related published articles in the last five years used the terms "edible insects". However, despite these many studies conducted on the importance of edible insects as alternative protein sources, the majority of these have mostly focused on consumer behavior and attitudes (Payne et al. 2016). Over the last decade, there has been an increase of public and private interest in using insects for food and feed (Veldkamp & Gasco 2023). This growing activity has resulted in the creation of several startups throughout the world, as well as the rise of major industrial insect-raising companies capable of generating tons of insects every day (Rivero 2023).

Specialized production processes were created to raise mealworms, crickets, and locusts for human consumption as the concept of using insects for food gained hold (Melgar-Lalanne et al. 2019). Insect rearing companies voluntarily adopted stringent cleanliness standards and tracking systems after realizing the necessity of food safety measures. These procedures agree with the standard guidelines established by the food industry, such as Hazard Analysis and Critical Control Points (HACCP) in the food and feed processing

DOI: 10.1127/entomologia/2024/2651
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(Żuk-Gołaszewska et al. 2022). Ensuring the development of this industry in Europe is largely dependent on legislation that addresses sanitary requirements throughout the production process and marketing operations (European Commission 2022). The International Platform of Insects for Food and Feed (IPIFF) published a thorough Guide on Good Hygiene Practices in June 2022, updated in February 2024, in response to the increased demand (IPIFF 2024).

This Guide aims to help companies produce safe insect-based foods while adhering to European Union (EU) standards. Additionally, some insect species like the yellow mealworm (*Tenebrio molitor* L. (Coleoptera: Tenebrionidae)) and the house cricket (*Acheta domesticus* L. (Orthoptera: Gryllidae)) make excellent options for food and feed production due to their high feed conversion ratio and nutritious composition. However, to reduce any possible hazards related to eating insects, it is essential to maintain hygienic and safety requirements during the breeding and processing phases. For example, little literature exists on the fungal toxins, and their role has to be checked in further studies, both for the consumers and people employed in production. Overall, using insects as a sustainable source of protein offers encouraging prospects for tackling issues related to global food security and reducing environmental impact (IPIFF 2024). Nonetheless, the viability and acceptability of insect-based products in the food and feed sectors depend on sustained study and strict respect to hygienic standards (Okaiyeto et al. 2024). In order to completely realize the prospect of insect breeding for sustainable food source, processing methods for edible insects are essential because they enable the development of raw materials that may be used in a variety of culinary applications (Li et al. 2023). These processes fulfill the growing need for sustainable food choices by allowing the transformation of whole insects or their processed forms to meet safety and nutritional criteria (Hassoun et al. 2022). Processing involves using mechanical or chemical methods to alter or preserve insect products so they may satisfy the strict safety and nutritional standards imposed by industry, consumers, and regulatory organizations (Lähteenmäki-Uutela et al. 2021). Insects may be prepared in a variety of ways, including whole, smashed or paste, and protein or fat extracts, by using methods including freeze-drying, sun-drying, boiling, and frying; this allows the insects to be used in a broad variety of food and feed products (Ojha et al. 2021). Processing methods not only increase the shelf life and safety of edible insects, but they also improve their sensory attributes, increasing customer acceptability and palatability (Okaiyeto et al. 2024). Edible insects are high in nutrients, but their incorporation into Western diets is difficult because of their flavor and visual appeal. If, however, insects are included into well-known foods like pasta, bread, and snacks, acceptability and consumption may rise (Liceaga 2022). Because they can be prepared in a variety of ways, including drying, frying, steaming, and roasting, edible insects are used in many different cuisines and culinary traditions across the world (Aguilar-Toalá et al. 2022). Insects, from the toasted grasshoppers of Mexico to the curry insects of Thailand, are considered valuable for their distinct tastes and healthful properties in many cultures (Krongdang et al. 2023). In terms of future prospects, the ability to create cultured meat from insect cells is a frontier in the production of sustainable nutrients (Kumar et al. 2021). A potential way to satisfy the increasing demand for meat worldwide while reducing the negative effects on the environment and the suffering of animals in traditional livestock farming is to produce meat that is made using insect cells (Treich 2021). Insect cell cultures, which make use of tissue engineering techniques, provide prospects for the productive and economical creation of nutrient-rich food items, opening the door for creative solutions to upcoming food-related issues (van Huis 2020).

2 Hygiene

Over the last 10 years there has been a boom in public and private interest in using insects as food and feed. This activity is currently being pursued by hundreds of start-ups throughout the world. Some large industrial insect raising firms capable of generating tons of insects per day have developed too. The academic community interest in this area has also grown rapidly, particularly in the last three years. Public media frequently emphasizes the environmental benefits of employing insects as food and feed. When the idea of utilizing insects for these purposes became popular, several insect rearing enterprises established specialized production lines to raise mealworms, crickets and locusts for human use. These specialized production lines were required since it was recognized that precautions were essential for food safety. Strict hygiene standards, as well as tracking and tracing mechanisms for these food sources, were self-imposed (IPIFF 2024). In general, human and animal meals should be treated for safety, to eliminate possible risks from raw materials during primary production (such as breeding, feeding and harvesting) and ensure safety and for this reason, Hazard Analysis and Critical Control Points (HACCP) are applied during food and feed processing, as a basic requirement of most food industries (Gates 2012; EFSA 2017). HACCP provides for the identification, analysis, and control of physical, chemical, and biological risks (Arévalo et al. 2022). In Europe, the proceedings of legislation and rules addressing hygiene throughout the production process and marketing activities will contribute to the future success of this sector (Hubert 2019). In June 2022, with an update in February 2024, IPIFF provided a detailed Guide called "IPIFF – Guide on Good Hygiene Practices" (Figure 1); the primary goal of the Guide is to assist operators producing insects for food and/or animal feed in achieving consumer and animal health protection through the manufacture of safe goods. The Guide advises insect producers on how to

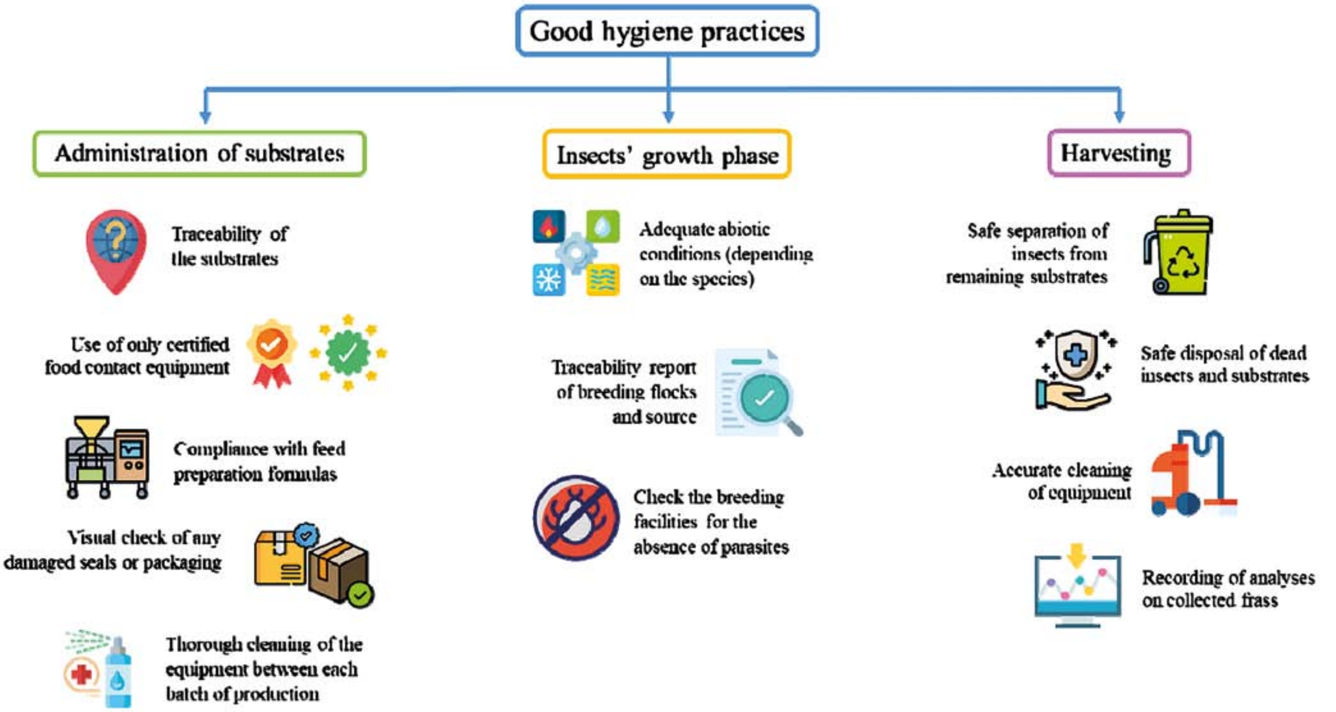

Fig. 1. Summary of 'recommended practices' or 'warning points' associated with insect rearing activities. The image is an infographic illustrating different aspects and considerations in a process or system, organized into three main categories and highlights the importance of thorough planning, quality assurance, regulatory compliance, and continuous monitoring in the lifecycle of a process or system, emphasizing safety, cleanliness, and efficiency at each stage.

apply EU food and feed safety legislation and other related EU requirements (i.e., food and feed labelling, EU animal-by-products, and legislation on transmissible spongiform encephalopathies (TSE)), while also incentivizing them to create a robust food and feed safety management system. To support the knowledge, during the production of the Guide, IPIFF engaged many European organizations of food and feed business sectors, as well as other interested parties, such as the Members of the Advisory Group on the Food Chain and Animal and Plant Health. The Guide covers the production of insects for human consumption or animal feed (including feed for food producing animals, pet food and fur animals) and includes production steps from feeding insects, breeding, killing, and other processing steps, storage, transport, or retail activities, to final delivery of the product to consumers, feed manufacturers, or farmers. However, the Guide does not address the precise processes and/or precautions that operators must take when handling insects/insect products and/or their by-products (i.e. insect frass) intended for 'technical applications' (i.e. non-food and/or feed usage and use of insect fat as biofuel, valorisation of insect frass as organic fertiliser). The Guide identifies elements that require special attention from insect producers, as previously identified by the European Food Safety Authority (i.e. 'the specific production methods, substrates used, harvest stage, insect species and development stage and methods for further processing, environmental effects'), in order to achieve compliance with food and feed safety objectives, as defined in applicable EU regulations. Furthermore, the Guide is not intended as a substitute for the current European or national Regulations; consequently, operators should always consult appropriate regulatory requirements for legal compliance. The general health hazards connected with insect intake have previously been addressed in various published risk profiles and scientific opinions (Finke 2015; Schäfer et al. 2016). Because of the enormous diversity within the insect world, there is a need to explicitly target species relevant to European consumers. For example, because of the appealing nutritional composition and efficient feed conversion ratio when compared to other animals, *A. domesticus* is a promising insect. Understanding microbial communities in raised crickets under environmentally controlled circumstances is vital for recognizing future hazard analysis requirements as a key control point for these crickets (Fernandez-Cassi et al. 2020). This species is frequently imported outside the EU, with unclear feeding regimes, transit, packing or manufacturing circumstances, all of which may influence microbial loads and populations. To avoid microbial contamination, it is crucial to use endogenous species in controlled environments. For crickets, the control of such contamination is generally achieved for the following reason: 1) the reared crickets do not appear to carry pathogens with detrimental effects on farming (i.e. cricket densovirus); 2) any insects that escape from rearing facilities would have a negligible ecological impact on local ecosystems and biodiversity; 3) the crickets can be sourced locally and sustainably, for both ecological and economic benefits.

Tenebrio molitor meal has promise in the food sector as a component in several products due to its excellent nutritional content and extended shelf life (Arévalo et al. 2022). Furthermore, compared to entire insects, its moderate flavor, texture, scent, and color increase acceptance. However, the meal has various potential physical, chemical, and biological concerns including allergies which must be addressed by food safety systems such as HACCP. For the producer of *T. molitor* meal, the majority of concerns occur when they enter in the process as a raw material. Rearing the larvae by themselves, using adequate substrates, can help to control these concerns. However, insect producers should also utilize preventative maintenance to reduce concerns linked with utensils and other equipment, as well as effectively train people to follow Good Manufacturing Practice (GMP) compliant production processes. Also, the establishment of a CCP during *T. molitor* meal manufacturing enables monitoring of factors such as drying time and temperature, particle size, fasting time, packing, and storage. Controlling these factors at the right time guarantees that the defined limits are not exceeded, and that the meal produced is safe for customer consumption (Arévalo et al. 2022).

3 Breeding

The most common method of collecting insects is by gathering them from local natural settings. In line with the customs and cultures of small-scale producers, a wide variety of species at various life stages can be harvested (van Huis & Oonincx 2017). Insects are mostly harvested from fields for domestic use, a process that has no negative influence on the environment and aids in preserving insect supplies over time (Melgar-Lalanne et al. 2019). These farmers have the knowledge and experience needed to identify the host vegetation and timing for growing a particular edible species without harming the ecosystem (Durst & Hanboonsong 2015). Since pre-Hispanic times, traditional insect harvesting has been practiced in Mexico. Interestingly, more than 400 insect species have been collected from the wild, primarily from terrestrial environments, and they were not considered pests but rather as important food sources (Durst & Hanboonsong 2015). Every species is harvested differently depending on its developmental stage (eggs, pupae, larvae, or adult), season (rainy or dry), and location (forest, desert, or agricultural fields) (Melgar-Lalanne, et al. 2019). Due to trophic chain disruptions, unregulated overharvesting is harming the environment at present (Gahukar 2016; van Huis 2022). Efforts should be made to outlaw or control harvesting during breeding seasons and utilizing less damaging harvesting methods (Durst & Hanboonsong 2015). Some edible wild species have been semi-domesticated where warm, rainy weather is suitable for cultivation (Varelas & Langton 2017). As a worldwide mission, at least for member countries, the Food and Agriculture Organization (FAO) took the initiative to develop a policy and recommended a program to feed people using alternative sources of protein, such as insects (Dust & FAO 2010). The goal is to produce more edible insects in a way that is economically effective, safe, and sustainable considering the rising demand and need for these sources. This document emphasizes the necessity to transition from outside harvesting to indoor farming in terms of technology (Gahukar 2011).

Most insects can be reared in small spaces or containers, they have a short life cycle, can eat agricultural waste instead of grains, can be raised in both urban and rural areas, and can produce short-term financial returns. These factors make it relatively easy and affordable to domesticate edible insects. Due to the economic success, house crickets and yellow mealworms are the two most commonly farmed insects worldwide (Melgar-Lalanne et al. 2019). They have high production densities, low technological needs, and certain life stages do not require sunshine (Hanboonsong et al. 2013). Moreover, the majority of farmed insects may be readily reared in compact, vented plastic containers at high ambient temperatures (up to 30 °C) and relative humidity (up to 70%), while being fed on organic waste. Edible insects are categorized as livestock according to the European Regulation (EC) No. 1069/2009, thus, it is important to abide by edible insect welfare requirements.

Mealworm breeding is fast and inexpensive from a financial perspective (Wang et al. 2011). The egg stage lasts 3 to 9 days, the larval stage is 26 to 76 days, and the pupal stage is 5 to 17 days (Li et al. 2013). Individual growth rate and size are influenced by a variety of parameters, including temperature, humidity, light intensity, feed content, and species density within breeding tanks (Wu et al. 2009). A proper diet is required to maintain good health (Dussutour et al. 2016). *T. molitor* larvae are commonly fed on bran or wheat flour, oat or corn, and protein sources like powdered milk. Fruits and vegetables (carrots, potatoes, lettuce, and chayote) can also be used to supplement the diet with moisture and grain residues (Nguyen et al. 2015). The diet should also contain 5–10% yeast, 80–85% carbohydrates, and vitamin B1 from cereals. The insect can also utilize energy from fatty tissue, absorb minerals from its harder tissues, more efficiently excrete certain components to ease food shortages (nutritional stress) (Adámková et al. 2017). Cannibalism is also a cause of nutritional stress or a lack of breeding space (Wu et al. 2009), which reduces the breeding production. The most popular methods of killing insects include freezing, direct grinding, or cooling followed by boiling. However, it is important to use strategies that minimize animal suffering (van Huis 2013).

Among the mealworms, *Alphitobius diaperinus* P. (Coleoptera: Tenebrionidae) (lesser mealworm) is another species used as food. Currently found everywhere, *A. diaperinus* is thought to have originated in sub-Saharan Africa (Sammarco et al. 2023) and the tropical east African region (Lambkin 2001). This kind of beetle is typically regarded as a pest, especially of grain goods (Spilman 1991; Hosen et al. 2004). Furthermore, because of the favorable humid and warm conditions for their growth, both larvae and adults are frequently found in chicken farms and henhouses, without harming the health of the chickens themselves (Francisco & do Prado 2001). The novel food should be produced in compliance with ISO 22000 regulations, using GMP and hazard analysis critical control point (HACCP) principles. There are three separate stages to the production process: farming, harvesting, and post-harvest processing. Insect breeding and larval rearing are both parts of farming. Depending on the temperature and the availability of food, larvae emerge four to seven days after the eggs are produced and grow through six to eleven larval instars in 40 to 100 days. According to several studies (Strother & Steelman 2001; Renault et al. 2003, 2004; Hosen et al. 2004; Calibeo-Hayes et al. 2005), *A. diaperinus* develops on various diets and in various ambient temperatures and microhabitats. The larvae eat waste, spilled feed, occasionally dead birds, and cracked eggs. For these reasons, lesser mealworms are significant carriers of several parasites and pathogens that affect poultry, including the Newcastle disease, avian influenza, Gumboro disease, the leucosis or Marek's disease virus (Falomo 1986), and the turkey coronavirus (Watson et al. 2000). The formation of particular prion illnesses as a result of consuming food derived by this larval stage is not expected given the vegetable origin of the feed substrates and the absence of prion or prion-related encoding genes in insects (EFSA 2015).

Crickets may be easily raised inexpensively, and they have been regularly used as food for ornamental fish (Taufek et al. 2018). Cricket species, *A. domesticus* and *Gryllus bimaculatus* D. G. (Orthoptera: Gryllidae), are resilient and nutritional (Straub et al. 2019; Bawa et al. 2020). Additionally, both species are routinely raised in large-scale production alongside a variety of other insects (Cortes Ortiz et al. 2016). Crickets adapt rapidly, thus if enough food (grains and vegetables) and water are supplied for a few days after arrival, it is possible to rear healthy insects (Roe et al. 1980). Moreover, this insect is an excellent species for breeding because of its short life cycle (from 60 to 70 days), high reproductive capacity (range of 200–1,500 eggs/female) (Patton 1978), resistance to illness, and ready availability stock from cricket farms. The life cycle of *A. domesticus* includes an incomplete transformation, as happens for insects known as "hemimetabolous," which implies that when the nymph emerges from the egg, its physiological make-up resembles that of the adult (von Hackewitz 2020). If the correct circumstances are present, the eggs will hatch in 8 days and the juvenile crickets reach adulthood after 6–8 weeks (FAO 2013), and the mating activity begins 24 to 72 hours after maturation. The harvesting day can often range from day 30 to day 55 on farms (von Hackewitz 2020). The time of development is temperature dependent, and this is the first parameter that should be kept under control because *A. domesticus* is an ectotherm; these animals are not able to adjust their body temperature over time, and, thus, temperature control is essential (Eckert et al. 1988). Temperature also influences the quantity and fertility of eggs laid by crickets. Crickets must have a balanced diet, with high protein content but also with vegetables, grains, carbohydrates and vitamins. Small amounts of fresh fruit and vegetables are needed as sources of hydration. Because of the high protein demand of the house cricket, the price of the feed rises, thus, one of the main challenges in the farming of crickets is the feed (van Huis & Oonincx 2017). Some farmers are utilizing high-protein feed only until the crickets are 20-days old in order to save money. After that, until harvest, lesser protein feed is utilized (von Hackewitz 2020).

Currently, *Locusta migratoria* L. (Orthoptera: Acrididae), also known as migratory locust, is found in Australia, Asia, Africa, and Europe (GBIF Secretariat 2019). Due to density-dependent phase polyphenism, there are two distinct phenotypes of this locust: solitary and gregarious. The adult insect population is mated during farming, and the nymphs are raised. The adult insects are separated from the eggs so that the nymphs can develop independently. The nymphs develop under carefully controlled temperature and humidity in stainless steel containers (Turck et al. 2021). Since these insects are specialized grass-feeders (Raubenheimer & Simpson 2003), they can be fed on plant-derived material. After removal from the substrate and excretions, adults (aged 3 to 5 weeks) are harvested. Following harvest, a minimum 24-hour fast is imposed to give adults time to empty their bowels. The adults are killed during the post-harvest processing by freezing and storage at −18 °C (Turck et al. 2021).

Hermetia illucens L. (Diptera: Stratiomyidae) (also known as the "Black Soldier Fly", BSF) is a Diptera whose larvae have the extraordinary ability to feed on organic substrates, including manure (Franco et al. 2022), animal waste, fruits and vegetables (Scala et al. 2020; Scieuzo et al. 2022), and bioconvert them into larval biomass rich in proteins, lipids, chitin and its derived chitosan (Triunfo et al. 2021), and AMPs (antimicrobial peptides) (Moretta et al. 2020; Scieuzo et al. 2023). BSF is not a pest and can be reared and harvested without special infrastructure. Although, also without a clear legislation about, the BSF farms produce larvae advised for use as animal feed, there are local regulatory constraints on how this can be accomplished. Larvae might theoretically be processed and transformed into a textured protein with a strong flavour for commercial usage in human diets. Their capacity to turn waste and by-products into food gives them a significant advantage over other insects, creating value and completing nutrient loops while lowering costs and pollution (Wang et al. 2019). It is challenging to locate evidence of BSF ingestion by humans (Mitsuhashi 2016). As ethnographers are not often entomologists and that locals are not always inclined to use the scientific name of an insect when eating it, this prevents precise identification of the species being consumed (Ramos-Elorduy et al. 1997; Mitsuhashi 2016). BSF larvae are suitable for human eating in terms of nutrition (protein mainly) but, based on our current notions of which fatty acids are deemed healthy, BSF larvae have one of the least healthy fatty acid profiles when compared to other insects (Franco et al. 2024). Larval amino acid and fatty acid profiles can vary according to feeding substrates (Spranghers et al. 2018; Ushakova et al. 2019), so it is possible to calibrate the nutritional needs of the consumers. According to Chia et al. (2018), temperature and diet, the two most important environmental factors, can affect not only the rate of insect development and seasonal and daily cycles (Logan et al. 1976), but also various aspects of insect biology, such as immature survival, adult life span, growth, fecundity, fertility, sex ratio, and population growth parameters (Gabre et al. 2005; Schneider 2009). Development, for example, might take two weeks to several months depending on the substrate type, and the resultant larvae can have a protein concentration varying from 10 to 40% of body weight (Oonincx et al. 2015). Optimal temperature and humidity conditions par insect species are summarized in Table 1.

4 Environmental impact

Livestock provides 25% of the total protein content eaten by humans, making animal-based food items an essential source of protein in diets as a whole. Livestock also has a large negative influence on the environment, contributing to 10–12% of all anthropogenic CO_2 emissions. Moreover, one of the human activities with the greatest environmental impact is food production. Agriculture alone is responsible for 30% of the world's greenhouse gas emissions and 70–85% of its water impact (2.5 times more than global transport). Alternative solutions, that meet the demand for food through sustainable production systems, are also increasing due to the expanding worldwide need for protein sources. The projection of a 70% increase in food production by 2050 emphasizes the significance of environmental effects associated with meat production (Tomlinson 2013). The creation of numerous meat alternatives, including plant-based, dairy-based, and animal-based options, strives to reduce the negative effects of livestock on the environment (Smetana et al. 2019). In this perspective, insect farms are an efficient alternative. In traditional farming, insects are much more effective at bioconverting organic matter into animal protein and nutritional energy than their vertebrate competitors. Using insects as food and feed has a lot of potential as a sustainable option in future food systems. Indeed, in terms of amino acids, lipids, minerals, and vitamins, the high nutritional quality of edible insects is on equal level with that of animal products. According to several studies (Oonincx & de Boer 2012; van Huis 2013; Siegrist & Hartmann 2019), meat substitutes have a lower environmental impact than traditional meat. LCA (Life Cycle Assessment) is a tool for assessing the environmental effects of products and services while taking into account the entire life cycle of the pertinent system or product. Environmental effects on food items can be traced from the production of agricultural inputs to consumption in homes and restaurants, as well as trash disposal. Although there are several other environmental impact assessment techniques, LCA is the most comprehensive and it is utilized for most food items and supply chains and has been embraced as the methodological foundation for environmental declaration schemes. The products must perform the same function, which is specified and quantified in the functional unit, in order to be comparable. The amount of edible portions (e.g., 1 kg of the edible fraction) or animal protein could serve as the functional unit for insect production systems (e.g., 1 kg of protein). For human toxicity, the subcategories classified are global warming, human health, human carcinogenic toxicity, human noncarcinogenic toxicity, ionizing radiation, ozone formation, stratospheric ozone depletion, water consumption and fine particulate matter formation. The impact of mealworm production on resources is also better; mealworm protein production has a lower resource impact than edible pig protein, with a resource impact value of 7.53 USD per kg of mealworm protein and 59.04 USD per kg of pork protein (Table 1). This is because the inputs used in the mealworm industry are designed to minimize the impact of depletion of resources. Moreover, according to estimates, compared to the 2–5 hectares needed for pig farming to provide the same quantity of protein, insect farms only need one hectare of land (Alexander et al. 2017). Finding substitute feed sources using surpluses and waste from crop production, while also keeping in mind that high-protein feeds produce the most effective conversion ratios, is another strategy to reduce the effects associated with feed production. Similar data were obtained by Halloran et al.

Table 1. Optimal temperature and humidity regime conditions for the rearing of the insect species addressed in section *Breeding*. TM: *T. molitor*, ADi: A. *Diapernus*, AD: *A. domesticus*, LM: *L. migratoria*, HI: *H. illucens*.

Insect species	Optimal temperature (°C)	Optimal RH (%)	Reference
TM	25–28	70	Soares Araújo et al. 2019
ADi	30–33	90	Dunford & Kaufman 2006
AD	30–32	60–70	von Hackewitz 2020
LM	34–42	70	Hamilton 1950
HI	30	70	Chia et al. 2018

(2015) in their comparison of the production of broilers and cricket; with the exception of ozone depletion and resource depletion, broiler production had the worst effects across the board when using 1 kg of edible mass as the functional unit (Halloran & Flore 2018). Particularly, broiler production increased the potential for acidification from 0.12 Mole of H-eq to 0.08 Mole of H-eq on the current cricket farm, the global warming potential from 3.90 kg of CO_2-eq to 2.57 kg of CO_2-eq, the freshwater ecotoxicity from 35.45 CTUe (comparative toxic unit) to 26.41 CTUe, and the potential for terrestrial eutrophication from 0.49 Mole of N eq to 0.40 Mole of N eq. The Global Warming Potential (GWP) of the current cricket farm scenario is comparable to two LCAs of mealworm production, with an equivalent of 2.7 kg CO_2-eq. per kg of fresh weight (Oonincx & de Boer 2012), 2.84 and 3.02 CO_2-eq. per kg of fresh weight (Oonincx & de Boer 2012; Smetana et al. 2015a). The phase of feed production was likewise identified as one of the main hotspots in these two investigations. In accordance with the abovementioned studies, Salomone et al. (2017) investigated the various environmental impacts for different phases of a BSF breeding in Italy. Observing the single impact categories, the results shown that, considering the total impact related to GWP (30.2 kg CO_2-eq – 100% of total contribution), the contributions of transport of input materials phase and the larval frass and dried larvae production phase are 5.4 kg CO_2-eq (18% of total contribution) and 22.5 kg CO_2-eq (75% of total contribution), respectively, while the phases of substratum production and egg and larvae production contribute 2.1 kg CO_2-eq (7% of total contribution) and 0.2 kg CO_2-eq (0,7% of total contribution), respectively.

Fossil depletion was the second-largest impact noted by Smetana et al. (2015b) and it is comparable to the significant energy use as determined by Oonincx & de Boer (2012). According to research by Miglietta et al. (2015), mealworms need 4.3 m^3 of water per kilogram of edible mass, which is roughly ten times more water than the present cricket farm in Thailand. The feed production is mostly responsible for mealworms' water impact. Another important function that the bioconverter insects, particularly the BSF larval stage, can contribute to improve is the disposal of food waste, of any organic matter. The primary goal of food waste management methods is to reduce waste, but finding innovative ways to value waste is also a valid and possibly beneficial option. The bioconversion process through BSF larvae is a very appealing option, given that it could be a valuable solution to different issues: food waste management, the rising demand for feed, and the competition between land use for food or energy crops (Zheng et al. 2012; Salomone et al. 2017). Organic waste from the agrifood chain is used to produce a valuable insect biomass in the form of protein and fat sources to be used in the animal feed industry. The use of the LCA approach in biorefinery solutions is quickly becoming essential (Olofsson & Börjesson 2018).

Studies on the quantification of the emissions that insects create are currently insufficient in number. Indeed, there is a lack of experimental data on emissions from insect production, and what little data there is only applies to a relatively small subset of insect species. Indeed, only two studies on the Greenhouse Gas emissions from insect species have been published (Hackstein & Stumm 1994; Oonincx et al. 2010). Compared to meat products, insects are thought to be a less harmful source of protein for the environment. The impact of insect rearing is greatly influenced by the species, production method, and nutrition because some of these factors result in more emissions than others (Oonincx et al. 2015). Despite extensive coverage of insect-related topics in literature, there is still a scarcity of data on an industrial scale. For accurate comparisons with conventional protein sources and the creation of industrial recommendations, industrial scale is crucial. According to Halloran et al. (2015), Salomone et al. (2017), and Thévenot et al. (2018), most analyses of economic viability and environmental impact are carried out for small pilot or small industrial scale of production with a rate of 0.02–1 ton of dry insect biomass processed per day. Additionally, the majority of research depends on incomplete and aggregated data rather than a consequential LCA method, which has the ability to detect changes in the market system brought on by new technologies and goods (van Zanten et al. 2018; Larrea-Gallegos et al. 2019).

Compared to the production of fish protein, the environmental impact of insect protein is significantly lower. Fish farming, or aquaculture, can also have substantial negative environmental effects, including habitat destruction, water pollution, and high feed conversion ratios. For instance, the production of farmed fish such as salmon requires substantial inputs of feed derived from wild fish and agricultural crops, which intensifies overfishing and deforestation issues (Naylor et al. 2000; Hall et al. 2015). Based on Pelletier et al. (2009), Henriksson et al. (2014) and Parker et al. (2021), insect protein production (mealworms and crickets) generally has a lower or comparable GWP per kg of protein compared to fish protein production (salmon, tilapia, and catfish). This suggests that insects could be a more environmentally sustainable source of protein. Furthermore, the water footprint of insect protein production is substantially lower than that of fish protein production. The efficient use of water in insect farming is crucial in a time when water scarcity is becoming an increasingly critical global issue (Miglietta et al. 2015).

5 Processing procedures for edible insects

Utilizing insect breeding for the production of raw materials opens avenues also for sustainable food sourcing, enabling the processing of whole insects or their processed forms to serve various culinary purposes and applications, thus fostering innovative solutions for future food production, addressing the growing demand for sustainable food options.

Processing means “to modify or preserve something by performing a series of mechanical or chemical processes on it (Dossey et al. 2016)”, and each process must be examined by the consumer, industry, and regulatory body to see if it meets its nutritional and safety criteria. In addition, processing is necessary for most foods in general to improve quality, palatability, and to improve food safety. Cooking, for example, is a form of processing (Dossey et al. 2016). In 2024, the global edible insect market for human consumption is expected to reach a value of US$ 653.7 million and is forecasted to reach a valuation of US$ 1.1 billion by the 2034 (Global Market Insights 2024).

One important aspect in the processing of insects is to remove the insect dejections which is typically achieved using a commonly used mechanical sieve, in particular with a mesh size 3–5 mm, which is specifically chosen to allow the smaller dejections to pass through while retaining the larger insects. The insects are fit for subsequent procedures for human food production since this separating procedure guarantees that they are cleaner and devoid of trash. Because it strikes a compromise between removing dejections efficiently, easily and preserving the insects themselves, the mesh size is essential (Peng et al. 2022).

Insects are killed by freeze-drying, sun-drying, or boiling after being harvested in the wild or grown in a domesticated context. They can be processed and ingested in a variety of ways, such as whole insects, ground or paste, and protein, fat, or chitin extracts for supplementing food and feed products. Insects can also be cooked alive and eaten (FAO 2017). In particular, frying improves the sensory quality of food by forming aromatic compounds, appealing colors, crust, and texture. Cooking insects may also improve their safety, acceptability, palatability, and digestibility (Caparros Megido et al. 2018). Although nutritional values are important, the product must also be palatable from a sensory standpoint in order to be consumed consistently. Because the consumption of insects as food is uncommon in the Western world, consumers must be persuaded not only of the nutritional benefits, but also of taste and overall sensory appeal (The World Bank 2017). Depending on the species, life stages of insects, and customary cuisines of different countries, insects can be cooked in a variety of ways, including hot air drying, oven broiling, roasting, pan frying, deep frying, boiling, steaming, and microwaving (Melgar-Lalanne et al. 2019). Oven broiling, in particular, gives insects the desirable aroma of steamed corn. Flavor, taste, and texture of edible insects change from one product to another. For this reason, some books on recipes with edible insects describe these features: for example mealworms, crickets and grasshoppers have an intense aroma of cereal, nuttiness, and wood, a less pronounced aroma of broth and an intense flavor of nut, cereal, and umami and slightly less intense flavor of vegetables; ants have an intense sourness like lemon; termites have a pronounced aroma of nuttiness and broth, with notes of cereal, wood, and soy sauce (Evans et al. 2017; van Huis & Dicke 2014). Because each bug has its own distinct flavor, they are suitable for inclusion in various types of foods (Carcea 2020).

6 Dried whole insects

In tropical nations, insects are commonly eaten whole; however, some insects, such as grasshoppers and locusts, require portions of the body to be removed, i.e., wings and legs, to reduce the risk of intestinal constipation that could be possibly caused by ingestion of the large spines on the insect tibia (Food and Agriculture Organization of the United Nations 2013). Fluidized bed drying, freeze-drying, hot air oven drying, microwave drying, smoke drying, torrefaction, and solar drying are all techniques used to dry intact edible insects and extend shelf life (Melgar-Lalanne et al. 2019). The freeze-drying method involves extracting ice from a sample and drying it via sublimation (Grabowski & Klein 2017). The product is frozen, then the pressure is reduced and heat is added to allow the frozen water in the substance to sublimate. One advantage of freeze drying is that it preserves heat-sensitive components, because it is carried out at a low temperature (Klunder et al. 2012). However, compared to most other drying processes, this approach has low productivity and high cost. Solar drying, often known as sun drying, is one of the most traditional drying procedures used for intact edible insects (Kröncke et al. 2019). Because of the minimal energy input and ease of implementation, it is typically used at the household level. The theory behind sun drying is that solar radiation heats the sample as well as the surrounding air, increasing the rate at which water evaporates from the insects. One problem related to the application of this method is that it can vary depending on weather and sunlight conditions (Mutungi et al. 2019). Smoke drying of whole edible insects is another traditional method. The insects are exposed to smoke generated by pyrolysis of wood. Typically, this procedure is combined with salting, and the entire process consists of a smoke chamber that incorporates the steps of salting, drying, heating, and smoking. Whole edible insects are also dried using microwaves. Microwaves penetrate the insects during microwave drying and are converted into heat, which evaporates the contained water. Microwave drying has the advantage of taking less time to dry insects than freeze-drying or oven drying. Microwave drying, like oven drying, can denature proteins and impact the functional qualities of the resulting components (Kröncke et al. 2019).

7 Granular forms with grinding/milling

The most difficult challenge in the expansion of acceptability of edible insects is reducing reactions of disgust by people. Thus, the acceptance of insect-based cuisine can be increased by 'hiding' insects in traditional foods. Researchers have employed cereal-based meals like bread, bakery items and pasta, which are popular around the world and widely accepted by the public, as a carrier to introduce varied percentages of insect flours to increase nutrition (Carcea 2020). In these preparations, the amount of insects varies and is largely tied to the type of product. The insect content as an ingredient is classified as follows: > 90%, between 90 and 10%, and less than 10% (Pippinato et al. 2020). Soybeans, for example, are frequently converted into tofu and other meat substitutes: meat is processed into hamburgers and hot dogs, and fish is processed into famous meals like fish fingers (Food and Agriculture Organization of the United Nations 2013). Edible insects can, likewise, be processed into more palatable forms in the same way: they are frequently formulated into a paste or powder and added to foods. Drying, grinding, or milling the insects is a simple approach to obtain powder (Choi et al. 2017). Chili paste made from crushed and pulverized giant bugs (*Lethocerus indicus* L. (Hemiptera: Belostomatidae)) is a common main ingredient in Thailand and the Lao People's Democratic Republic (known locally as "nam phik" and "jaew maeng da", respectively): this as a practical example that granular or paste version of the insects are more acceptable in societies where customers are not accustomed to consuming whole insects (FAO 2021). Dehydrating or roasting whole insects, then grinding them into a fine powder called flour, is how most insect flours are made (Liceaga 2021). The potential use of insect powders as a novel protein source for gluten-free products is an intriguing feature of insect powders in relation to bakery products (Nissen et al. 2020). A fine particle size dry powder would be the best insect-based component format for most products (Bußler et al. 2016). Powders: 1) offer longer shelf life (typically more than a year, depending on production/processing method, packaging, and storage environment), 2) can be blended effectively with a variety of other ingredients without compromising texture or structural integrity of the product, 3) can have the mildest flavor and scent, as well as the lightest color, depending on the processing procedure, 4) are the easiest technique to put bug into a product without "noticing the insect", which is great for market acceptability, and 5) are appropriate for most food equipment (Dossey et al. 2016).

8 Extracted insect nutrients

Insects have nutrients that can be extracted and added to diets (Hajj et al. 2022). Insect proteins, in particular worm proteins, for example, are isolated with the aim of adding them to food products to increase nutritional value (Zhao et al. 2016). Since insects have not been significant in Western food culture, consumers may be hesitant to accept insects as a viable protein source. Extracting insect proteins for human food products could be a good way to gain customer acceptance (Yi et al. 2013). However, supplementing meals with insects requires a thorough knowledge of the characteristics of the extracted proteins: amino acid profile, thermal stability, solubility, gelling, foaming and emulsifying ability (Lock et al. 2016). By separating the extracted protein groups according to solubility, they can be used for specific foods (Smetana et al. 2015b). Enzymatic procedures to obtain proteins with particular chain lengths are another option. Fluidized bed chromatography and ultrafiltration are protein separation technologies (Dong et al. 2020). These innovative food processing methods and others like microwave, pulsed-electric field, ultrasound and high hydrostatic pressure alone or coupled with enzymatic hydrolysis can reduce protein allergenicity of food proteins, compared to bioactive peptides extracted using conventional heating, including edible insect products, in order to increase product quality, reducing the molecular weight and enhancing the bioavailability and solubility of nutrients (Dong et al. 2020). The extracted insect proteins could be used in animal feed, depending on economic feasibility (Smetana et al. 2015a; van Huis et al. 2017). Protein extraction is demanding (Smetana et al. 2015b), with high costs, legislation, limited production capacity and initial plant costs as significant hurdles to overcome (Ojha et al. 2021). Thus, insect-based proteins remain expensive and need process optimization for large-scale production (Fortune Insects Insights 2024). Moreover, according to Chen et al. (2010), unsaturated fatty acids promote healthy human growth, prevent the skin, and decrease the production of thrombi and blood platelet clotting. Conversely, consuming dietary saturated fat has been connected to an increase in low-density lipoprotein (LDL) cholesterol, which is linked to a higher risk of cardiovascular disease. Randomized clinical trials and epidemiological research have shown that replacing saturated fat with polyunsaturated fat is advantageous for coronary heart disease (Siri-Tarino et al. 2010). Another potential issue regards the amount of chitin in the exoskeleton of insects; indeed, chitin, an insoluble polysaccharide, is the most prevalent type of fiber found in insects (van Huis 2013). Edible insects, particularly those with hard exoskeletons, have a high fiber content because of chitin, although having the potential to reduce protein digestion, can also have a function as dietetic fiber (Muzzarelli et al. 2001; Bukkens & Paoletti 2005). Nutritional value of edible insects is shown in Table 2.

9 Drying pastes, slurries, and liquids forms

Liquids, as well as slurries or pastes, are the next most suitable insect-based ingredient formats. Liquids, especially if the insect material is finely powdered, mix well with other ingredients and products, and can be used in a variety of food processing equipment (Dossey et al. 2016). They can be pasteurized and even exported as an ingredient, albeit liquid shelf life is always significantly shorter than a dry

Table 2. Nutritional values of the most consumed edible insects (Giaccone 2005; Bukkens & Paoletti 2005). ZM: *Zophas morio*, GM: *Galleria mellonella*, TM: *T. molitor*, AD: *A. domesticus*. Data for proteins, fibers and lipids are reported as percentage of dry matter. Data for saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA) are reported as percentage from total lipids.

	ZM	GM	TM	AD
Proteins	44.0%	40.0%	57.0%	69.0%
Fibers	4.7%	3.4%	5.9%	8.0%
Lipids	45.0%	54.0%	30.0%	21.0%
SFA	39.5%	30.2%	28.8%	33.8%
MUFA	39.7%	48.6%	39.7%	25.8%
PUFA	16.5%	19.6%	28.8%	35.2%

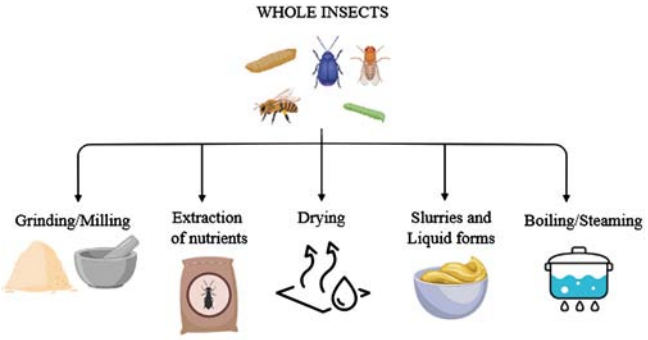

Fig. 2. Scheme on processing procedures for edible insects. The figure represents various processing methods for whole insects, including grinding/milling, nutrient extraction, drying, creating slurries and liquid forms, and boiling/steaming.

product (Dossey et al. 2016). Insects can be dried in whole form or as pastes, slurries or liquids, but, in the latter case, the drying process becomes less efficient, more expensive, and time-consuming when the product contains more water (Noyens et al. 2021). Slurries, liquids, and pastes also have a more uniform composition, resulting in a more consistent dry product (Mermelstein 2015). Drying liquids, pastes, and slurries into powders or meals can be done in a variety of ways: drum drying, spray drying, fluid bed drying, tray drying, freeze-drying, boiling, roasting, vacuum drying, and dehydrating are some options. A product (liquid, paste, or slurry) is produced and propelled into the air as a spray or mist using either spray atomization (with a high-pressure spray nozzle) or rotary atomization (where material is spun or "flun" into the air at a rapid velocity to create fine droplets of spray) (Nasr et al. 2002). As the water is rapidly removed, the spray or mist is suspended and cycled in warm dry air and falls to the bottom of the spray drying device (Dossey et al. 2016). The water is removed from the droplets/particles at the end of the procedure, leaving just the solid nonvolatile (and non-water) components (Dossey et al. 2016). As a result, fine solid particles are formed. Freeze-drying insects in their complete, unground form, as well as paste, slurry, or liquid forms, is another possibility (Dossey et al. 2016). The best approach for retaining the chemical and other qualities of any substance, biological material, or food is freeze-drying (Dossey et al. 2016). Freeze-drying, however, is costly, takes time, is inefficient, and necessitates access to highly specialized and energy-intensive (electricity-intensive) equipment that is not available in many parts of the world (Tarkan 2015).

10 Boiling and steaming

When insects are cooked by boiling or vacuuming, they become soft and juicy (Caparros Megido et al. 2018). Boiling and steaming mealworms keep the larvae looking and feeling fresh, with steamed maize, and boiled mushroom flavors define larvae cooked by boiling and steaming (Baek et al. 2019).

Blanching is also useful for insects. It involves placing a food in boiling water for a short time, removing it, and then plunging it into ice water or placing it under cold running water to terminate the thermal process. This process is useful to lower bacteria load and inactivate degradative enzymes that cause food spoilage in most commercialized edible insects, on both an industrial and artisanal scale (Melgar-Lalanne et al. 2019).

11 Added fresh to food products and recipes

Fresh mealworms can be used to make processed foods like burgers by adding them whole to beef (Elhassan et al. 2019). Insects are also valuable for food formulations like meatballs or burger patties because they are 'unseen as insects' and hence more acceptable to European customers (Caparros Megido et al. 2018). Insect-based ingredients are also sold for the manufacture of cookies, chocolates, tortilla-style chips, and other snacks (Melgar-Lalanne et al. 2019), as well as being added to a variety of goods, including energy drinks (Hartmann & Siegrist 2017, 2018) (Figure 2).

12 Some recipes from the world

Mexico is the Latin American country with the longest tradition of eating insects. Grasshoppers and crickets (also known as "chapulines") are frequently toasted or roasted in a skillet without oil until crispy; they are harvested from late spring until early winter, when the rainy season begins (Cohen et al. 2009). Lemon, salt, and chili are frequently added after toasting, when the insects are still hot. The water used to boil the insects can be acidified with lemon juice or vinegar, and flavoring elements like garlic and onion added. During boiling, the insects turn a reddish color and aromatic compounds form. Chapulines are used in a variety of traditional foods, including tacos (corn tortillas filled with insects and hot sauce), hot chili sauces (chili sauce with chapulines (Hernández-Álvarez et al. 2014)), and "chiles relleno" (chiles stuffed with chapulines). Chapulines are frequently served with alcoholic beverages as a snack (Cohen et al. 2009). In Thailand, about 200 species of insects are consumed, and they are prepared in a variety of ways other than the traditional (roasting, frying, and steaming), including curried, dipping (combined with chili paste), and salted (Halloran et al. 2015; Raheem et al. 2019). There is no tradition of cooking insects in Western countries except for the use of insects as food colorants, particularly cochineal (*Dactylopius coccus* C. (Hemiptera: Dactylopiidae)). Cochineal dye, made from the dried bodies of female cochineal insects, has been used for centuries as red colourants for food industry (Deveoglu 2020).

In Italy, Casu marzu, a Traditional Agri-food Product (TAP) of Sardinia, consists of a sheep's milk cheese whose sensory characteristics originate (during the ripening period) from the development of the of the small larvae of *Piophila casei* L. (Diptera: Tephritidae) (Regione autonoma della Sardegna 2014). Once the cheese has become creamy, it is consumed by lifting the lid and removing the product with a spoon. The TAPs are products whose processing methods, preservation, and maturation have been consolidated over time according to traditional rules and are included in a list prepared by the Ministry of Agricultural Food and Forestry Policies at the indication of the regions. Regulated initially by the decree of 18 July 2000 and the sixteenth revision of the national list of traditional agri-food products published in the Official Gazette of the Italian Republic No 143 of 21 June 2016 this cheese already has its own promotion committee (based in Ossi, Sardinia) of companies, public bodies, producers and the University of Sassari, that supports obtaining of the Protected Designation of Origin (PDO), requiring a production specification based on compliance with specific health and hygiene requirements, such as that of breeding *P. casei* in a controlled environment. Many Japanese individuals raise wasps in their homes. The wasps are given meat, fish, and sugar water, and their nests are placed in a safe location. The larvae and pupae are gathered for food in the fall. The majority are cooked in soy sauce with sugar and sake (rice wine) and served with cooked rice. Canned wasp larvae and pupae are available in Japanese supermarkets and are considered a high-priced delicacy (van Huis & Dicke 2014). Termites are regarded as a unique and nutritious diet in the tropics, as they are rich in protein, amino acids and fatty acids, iron, calcium, and other micronutrients. Termites are frequently fried in their own fat, and the remaining oil can be utilized to cook other meat dishes. Otherwise, termites can be steamed after being wrapped in banana leaves or dried for storage after cooking. In Kenya, powdered, sundried termites are used to replace up to 5% of the wheat flour in cakes (Gordon 2013). A popular strong liquor in Latin America is made with a 2-inch (5 cm) "worm", a caterpillar, at the bottom of the bottle. Mezcal is a distilled beverage made entirely of Mexican agave plants with a minimum alcohol concentration of 45%. Mezcal is mostly manufactured in the state of Oaxaca, where thirty different species of agave are utilized to create the different flavors. Tequila, on the other hand, is only 51% agave and does not come with a caterpillar. The mezcal caterpillars are essentially an agave pest (*Comadia redtenbacheri* L. (Lepidoptera: Cossidae)). They eat the plant stems and leaves and are not regulated with pesticides; instead, they are harvested and used in traditional Oaxacan cuisine. They are not just found at the bottom of mezcal bottles, but they are also regarded as a delicacy: fried and served with guacamole on a tortilla, or ground and mixed with tomatoes and chilies in a spicy sauce (van Huis & Dicke 2014). A curious dish is Mexican-style Escamoles, known as Mexican caviar or insect caviar: fried edible larvae and pupae of the ant *Liometopum apiculatum* L. (Hymenoptera: Formicidae) and seasoned with spices and vegetables and often served with tacos (Ramos-Elorduy 1998).

13 A look into the near future: cultured meat from insect cells

While the utilization of insects in the food industry represents an innovative approach to addressing nutritional requirements while reducing environmental impact, another frontier lies in the potential production of cultured meat derived from insect cells. This emerging technology offers promising possibilities for sustainable protein production, leveraging the biological efficiency of insects to meet the growing global demand for meat while mitigating the environmental challenges associated with traditional livestock farming. In recent years, increasing attention has been paid to the identification of alternative sources for the production of edible proteins. One possible solution is the use of insect cell lines to produce cultivated meat (CM, also known as cell-based or cultured meat) via tissue engineering methods (Giglio et al. 2024) (Figure 3).

CM is meat produced from animal stem cells by replicating the process of cells growing and dividing in the body to obtain a product with the same nutritional and organoleptic attributes as traditional meat. In 2002, the first experimental evidence of CM was published, demonstrating that cultivated fish cells might help the growth of a goldfish muscle explant. In 2013, Dr. Mark Post's team, at Maastricht University, made a widely publicised cheeseburger containing the first documented taste of CM (Post 2012). Cells, cell culture media, scaffolds, and bioreactors are required for the development of CM. In practice, cells are expanded in bioreactors

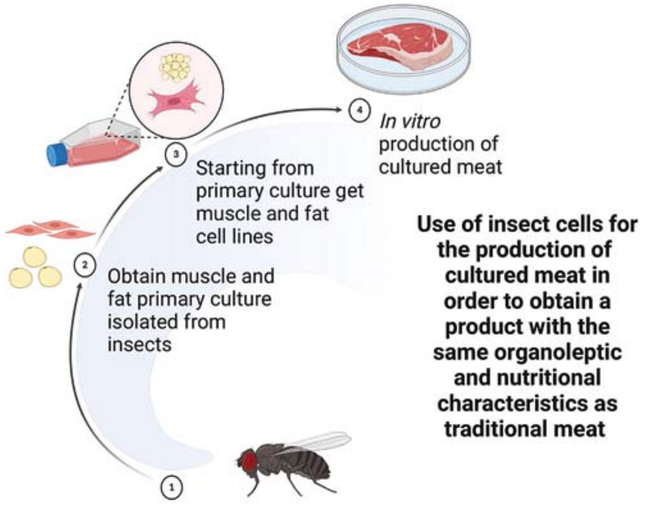

Fig. 3. Schematic representation of different steps for the *in vitro* cultured meat production, starting from muscle and fat cells of insect embryo, to achieve organoleptic and nutritional characteristics similar to traditional meat. The steps include: (1) isolating muscle and fat primary cultures from insects, (2) obtaining muscle and fat primary cultures, (3) culturing these primary cultures to develop muscle and fat cell lines, and (4) producing cultured meat in vitro.

(devices used to grow cells) with nutrients and growth signals and incorporated into a scaffold that mimics the texture of traditional meat products. This workflow begins with the source of cells, which can be obtained in one of two ways: the first, and most common, is to perform a tissue biopsy on the animal of interest or to use post-mortem tissues; the second option, is to use a source of pluripotent stem cells (Reiss et al. 2021). For the generation of CM, cells from a range of species have been considered, primarily bovine, porcine, and avian. The necessity for suitable growth conditions for a variety of cell types and the high cost of media are two technological obstacles that prevent the development and expansion of cell culture (Rubio et al. 2019b). Cells originating from less common species may be able to overcome these obstacles. Numerous properties of insect cells suggest their potential for large-scale production, including easier adaptation to suspension and serum-free culture, lower volume and simpler culture media requirements, and favourable growth conditions (including growth in suspension, at room temperature without CO_2, more adaptable to changing environments, avoiding the need for serum or expensive growth factors) when compared to mammalian cells (Geden & Hogsette 1994; Global Market Insights 2021). Animal-derived meat is primarily composed of muscle and fat tissue; thus, muscle and fat cells must be cultivated to create CM products. Finding a robust source of insect muscle and fat cells is a substantial obstacle to the synthesis of insect tissues for food. Primary myoblasts immortalised from *Drosophila melanogaster* L. (Diptera: Drosophilidae) and contractile myotubes from *Manduca sexta* L. (Lepidoptera: Sphingidae) have been described (Baryshyan et al. 2012; Dequéant et al. 2015). Insect muscle cells were isolated and cultivated using *D. melanogaster* and *M. sexta* embryos as well as *M. sexta* pupae of second stage (Simcox et al. 2008; Dequéant et al. 2015). In contrast to primary embryonic myoblasts and pupal myoblasts, which are capable of both proliferation (extended growth to generate many cells) and differentiation (to form specific cell types like muscle or fat for foods), the genetically immortalised adult muscle precursor cells of *D. melanogaster* have a high proliferative ability but a restricted differentiation potential (Dequéant et al. 2015; Rubio et al. 2019a). Since there is evidence that insect myoblasts need direct contact with neurons for complete development, the lack of support cell types in the early cultures may be the cause of the limited differentiation of immortalised insect muscle cells. The ability to regulate the proliferation and differentiation of cultured muscle cells is crucial for large-scale manufacturing. Significant progress has been achieved despite the fact that the hormonal pathways involved in the development of insect muscles are not as well understood as those in humans and other animals. For instance, it has been shown that ecdysteroids, which are steroid hormones, govern muscle growth (Tischler et al. 1989). Low quantities of 20-hydroxyecdysone enhance myoblast proliferation in *M. sexta*, but amounts beyond the critical threshold limit myoblast development. The analogue of juvenile hormone, methoprene, inhibits the ability of large doses of ecdysteroids to induce proliferative arrest and differentiation. The hormonal regulators of the differentiation process may be used to control cell proliferation during production (Rubio et al. 2019b).

While muscle provides the majority of meat biomass, fat is essential for flavour and nutrition in cellular agriculture. Numerous insect species have significant levels of omega-3 and omega-6 essential fatty acids. Insect fat body tissue contains proteins and carbohydrates in addition to lipids. It is necessary to cultivate both the muscle and fat tissue of insects in order to provide nutritious and tasty food. Other insect cells may be generated in vitro utilizing cells from the insect's fat body (Chapman 2012). The accumulation and release of nutrients by fat cells may prolong the in vitro survival and contraction of muscle cells for months without modifying the media (Baryshyan et al. 2012). In a similar manner, procedures conditioned by the fat cells may improve in vitro embryonic cell growth. One of the first types of insect tissue to be cultured in vitro for the study of protein synthesis was the fat body (Nowock et al. 1975; Raikhel et al. 1997). Fat body cells produce essential proteins such as vitellogenin, the precursor protein of the egg yolk, and growth hormones that bind to proteins (Nowock et al. 1975; Wyatt 1988). Numerous papers detail the process of isolating insect fat cells. The majority of treatments use advanced-stage larvae and ethanol or sodium hypochlorite to sterilise the insects. Adipose tissue is taken from the abdomen and homogenised into a culture medium or buffer solution. In most treatments, explants were generated without tissue digestion. A number of media formulations, including basal media with or without fetal bovine serum and antibiotics, have been used to develop fat body cells. Most of the techniques included reseeding the medium every one to two weeks and culturing the cells between 25 and 27 °C. In contrast to the enzymatic procedures often applied to vertebrate cells, passage may usually be accomplished with a slight mechanical perturbation. One study established a cell line from *Spilarctia seriatopunctata* L. (Lepidoptera: Erebidae) that grew in suspension, as opposed to the majority of cell lines that are grown as adherent culture (Mitsuhashi 1984). There is evidence that fat-tissue-specific cells develop slowly at the beginning but may form continuous lines, and a fat body cell line generated from *Mamestra brassicae* L. (Lepidoptera: Noctuidae) was passaged 100 times over the course of nine months, 26 days after isolation. The replication rates of fat cell lines varied from 48 to 72 hours. Fat cells may be preserved temporarily at 5 °C (Mitsuhashi 1981). The innovative concept of CM enables the manufacturing of meat without the need for animals by employing tissue engineering techniques. In terms of economics, health, animal welfare, and the environment, CM production may be better than conventional meat production. CM has the ability to significantly minimise animal suffering while also satisfying customers' nutritional requirements. The in vitro meat production technology is predicted to be both time and energy efficient compared with animal-derived meat, requiring only a few weeks as compared to many months or years. Producing CM will also reduce land for raising animals. The use of insect cell cultures in cell farming shows potential as a means of overcoming technological limitations and generating nutrient-rich food in a more cost-effective approach. The durability of insect cell culture techniques, simplicity of immortalization, high density proliferation, serum-free nature, and flexibility of suspension culture with respect to mammalian cells make them important candidates for inclusion into meat cultures and other innovative products.

Acknowledgements: This study was carried out within the Ecosystem of Innovation "Tech4You – Technologies for climate change adaptation and quality of life improvement" and received funding from the European Union Next-GenerationEU (PIANO NAZIONALE DI RIPRESA E RESILIENZA (PNRR) – MISSIONE 4 COMPONENTE 2, INVESTIMENTO 1.4 – DD 1049/23.06.22 – ECS_00000009). This manuscript reflects only the authors' views and opinions; neither the European Union nor the European Commission can be considered responsible for them. This work was supported by Basilicata Region within the framework of FSC "Fondo per lo Sviluppo e la Coesione" ("Gestione del ciclo di scarti e sottoprodotti della filiera agroalimentare attraverso la loro bio-conversione in prodotti di valore" – D.G.R. n. 652 30/09/2022).

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Manuscript received: April 10, 2024
Revisions requested: May 28, 2024
Revised version received: June 10, 2024
Manuscript accepted: June 27, 2024

Article

In Vitro Evaluation of the Antibacterial Activity of the Peptide Fractions Extracted from the Hemolymph of *Hermetia illucens* (Diptera: Stratiomyidae)

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Simple Summary: Antibiotic resistance is a worldwide social and health crisis. The search for therapeutic alternatives, including the use of antimicrobial peptides (AMPs), is critical. AMPs are small molecules synthesized by a wide range of living organisms. Microbiological and mass spectrometric techniques were used to examine peptides in the hemolymph of larvae of the scavenger insect *Hermetia illucens* (Diptera, Stratiomyidae) after infection with *Escherichia coli* or *Micrococcus flavus*, as well as uninfected larvae, used as control. Microbiological assays allowed us to confirm antimicrobial activity of *H. illucens* AMPs, while via mass spectrometry we identified a set of 33 AMPs, expressed in different conditions: 20 AMPs were expressed in all the analyzed conditions, while 13 were differentially expressed after Gram negative or Gram positive bacterial challenge. Differentially expressed AMPs may be responsible for a more specialized action.

Abstract: Antimicrobial peptides (AMPs) are a chemically and structurally heterogeneous family of molecules produced by a large variety of living organisms, whose expression is predominant in the sites most exposed to microbial invasion. One of the richest natural sources of AMPs is insects which, over the course of their very long evolutionary history, have adapted to numerous and different habitats by developing a powerful innate immune system that has allowed them to survive but also to assert themselves in the new environment. Recently, due to the increase in antibiotic-resistant bacterial strains, interest in AMPs has risen. In this work, we detected AMPs in the hemolymph of *Hermetia illucens* (Diptera, Stratiomyidae) larvae, following infection with *Escherichia coli* (Gram negative) or *Micrococcus flavus* (Gram positive) and from uninfected larvae. Peptide component, isolated via organic solvent precipitation, was analyzed by microbiological techniques. Subsequent mass spectrometry analysis allowed us to specifically identify peptides expressed in basal condition and peptides differentially expressed after bacterial challenge. We identified 33 AMPs in all the analyzed samples, of which 13 are specifically stimulated by Gram negative and/or Gram positive bacterial challenge. AMPs mostly expressed after bacterial challenge could be responsible for a more specific activity.

Keywords: antibiotic resistance; black soldier fly; AMPs; *Escherichia coli*; *Micrococcus flavus*

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Citation: Scieuzo, C.; Giglio, F.; Rinaldi, R.; Lekka, M.E.; Cozzolino, F.; Monaco, V.; Monti, M.; Salvia, R.; Falabella, P. *In Vitro* Evaluation of the Antibacterial Activity of the Peptide Fractions Extracted from the Hemolymph of *Hermetia illucens* (Diptera: Stratiomyidae). *Insects* **2023**, *14*, 464. https://doi.org/10.3390/insects14050464

Academic Editor: Diana Martín

Received: 11 March 2023
Revised: 9 May 2023
Accepted: 10 May 2023
Published: 15 May 2023

1. Introduction

Infectious diseases have always been one of the major threats to human and animal health and a major cause of morbidity and mortality [1,2]. The discovery of antibiotics was a powerful tool to support medicine for the treatment of bacterial infections and the associated complications. The progressive misuse of antibiotics has unfortunately favored the selection and spread of resistant populations of bacterial agents [3,4]. The drug-resistance phenomenon has a heavy impact on the world community [5,6]. Following the development of antibiotic-resistant bacterial strains and the reduced availability of effective antibiotics, a need to identify new molecules to be used for the development of alternative therapies is growing [7,8]. Antimicrobial peptides (AMPs) are small cationic molecules, containing from 10 to 50 amino acids, able to selectively bind the membranes of bacteria, disrupting them and inducing cell death [9,10]. They constitute one of the first lines of defense of organisms against a great variety of external agents [11,12].

Several characteristics of AMPs make them particularly interesting as potential therapeutic tools, as they manifest synergies with the acquired immune system [13] and demonstrate specificity towards prokaryotic cells [14]: due to their positive charge, the AMPs establish an electrostatic interaction with the surface of the pathogens that exposes a net negative charge [15,16]; they demonstrate broad-spectrum activity against viruses, bacteria and fungi [17], kill rapidly (99.9% of bacteria treated in 20 min) [18], show synergies with conventional antibiotics [19,20], are effective against antibiotic-resistant bacteria and they do not cause the selection of new resistant mutants, as they act on bacterial cell membranes with mechanisms different from those of common drugs [21].

AMPs are essential components of the first line of defense systems of bacteria, plants and animals, including mammals [22,23]. Their production within various organisms is specific and can occur in a constitutive manner or can be induced in response to an external insult by pathogens [23,24]. Among invertebrates, insects, with more than one million species described, represent a source of great interest. AMPs are part of the humoral immune response of insects [25–27]. In holometabolous species AMPs are biosynthesized mainly in the fat body and transferred into the hemolymph [28] from which they can spread and act throughout the organism; in heterometabolous species, they are produced by haemocytes and secreted into the hemolymph following infection [28–31]. One of the most appealing insects for the AMP production is the Diptera *Hermetia illucens* (Linnaeus, 1758), commonly known as black soldier fly. *H. illucens* larvae, attracted by specific volatile organic compounds [32], feed on decaying organic substrates of vegetable and animal origin [33–35], converting them into a high-value biomass made up of proteins and lipids that can be used in a variety of applications, including feed, energy and cosmetics industry [36,37], as well as to extract high-value compounds for application in biomedical and pharmaceutical fields [38–40]. Because of their nutritional substrates, they are exposed to a high and constant concentration of pathogenic microorganisms such as bacteria and fungi present in these substrates [41]. In order to survive, larvae have developed a powerful immune system, with high production of AMPs [42]. These molecules can be constitutively expressed, or their expression can be strongly influenced both by the microorganisms they come into contact with and by the composition of the diet itself [43]. The analysis of one of the *H. illucens* transcriptomes allowed the identification of 57 putatively active AMPs, also characterized by bioinformatic tools, belonging to different classes (defensins, cecropins, attacins, diptericins, knottin-like, stomoxyn-like, alo-1 like and lysozyme) [44,45]. Recent studies have also highlighted the potential antimicrobial activity of some *H. illucens* AMPs against *Staphylococcus aureus*, methicillin-resistant *S. aureus* and *Pseudomonas aeruginosa* [46,47]. *H. illucens* can potentially be an excellent source of new compounds to use alone or in synergy with common antibiotics, especially against resistant strains [48,49]. The aim of this work was to identify the AMPs in *H. illucens* hemolymph, both from uninfected larvae and from larvae infected with *Escherichia coli* (Gram negative) or *Micrococcus flavus* (Gram positive). The peptide component isolated from the hemolymph was analyzed via preliminary microbiological tests and via mass spectrometry to specifically identify constitutive and induced peptides, differentially expressed after bacterial challenge. These peptides could have potential application in biomedical and pharmacological fields, to make an innovative contribution to counteract the antibiotic-resistance issue.

2. Materials and Methods

2.1. *Hermetia illucens* Rearing

Hermetia illucens larvae were provided by Xflies s.r.l (Potenza, Italy). After egg hatching, larvae were fed on a standard Gainesville diet (30% alfalfa, 50% wheat bran, 20% corn meal) [50] at 70% moisture under controlled conditions of temperature (27 ± 1.0 °C), relative humidity (70% ± 5%) and photoperiod (12L:12D (h)) [35].

2.2. *H. illucens* Larval Infection and Hemolymph Collection

Escherichia coli (Gram negative, LMG:2092 strain) and *Micrococcus flavus* (Gram positive, DSM 19079) were incubated in 10 mL of Luria Bertani (LB) broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl), at 37 °C for 24 h, under shaking. A total of 1 mL of each bacterial culture was inoculated into a fresh LB broth, incubated at 37 °C and used for the experiment once the optical density (OD) at 600 nm reached 1. Last instar larvae of *H. illucens* were firstly washed with sterile water and then infected via a capillary dipped into the cell suspension of *E. coli* or *M. flavus* [51,52] in order to stimulate the production of different antimicrobial peptides (AMPs). Following the bacterial challenge, larvae were left in a controlled chamber at 27 °C for 24 h. A group of uninfected larvae was used as control. For each treatment, 100 larvae were used. To facilitate the spill of hemolymph, larval abdomens were punctured by a sterile capillary and the hemolymph from infected and uninfected larvae was collected, using a pipette (Gilson, Middleton, WI, USA), in ice-cold tubes, containing a fixed-minimum quantity of L-ascorbic acid (0.015 g) (Merck Millipore, Burlington, MA, USA), to prevent hemolymph melanization. To recover only the plasma and remove the cellular components, the extracted hemolymph was subjected to centrifugation at 10,000 rcf for 5 min at 4 °C. The recovered supernatant (cell-free hemolymph) was stored at −80 °C until use.

2.3. Peptide Fraction Precipitation by Organic Solvents

In order to separate the putative AMPs in the hemolymph from the higher molecular weight proteins, the plasma recovered from both uninfected and infected larvae was subjected to a precipitation protocol with methanol (Merck Millipore, Burlington, MA, USA), acetic acid (Merck Millipore, Burlington, MA, USA) and water in a 90:1:9 *v/v* ratio. Sample and solvent were mixed in a 1:9 *v/v* ratio. The sample was centrifuged for 45 min at 16,000 rcf at 4 °C. The obtained supernatant, containing compounds with a molecular weight lower than 30 kDa, was then vacuum dried to remove the organic solvents and resuspended in a volume of sterile water equal to the original plasma volume. To remove possible traces of lipids that could be co-extracted due to the use of methanol, a further treatment with hexane was performed. Specifically, an equal volume of hexane (Merck Millipore, Burlington, MA, USA) was added to each extract. The samples were vortexed and centrifuged at 16,000 rcf for 20 min at 4 °C [53]. The upper fraction, possibly containing lipids, was removed and stored for the subsequent evaluation via antibiogram assay (Section 2.5). All samples were subsequently stored at 4 °C until next use.

2.4. Protein Quantification via Bradford Assay

The concentrations of all samples were quantified with Bio-Rad Protein Assay, Dye Reagent Concentrate (Bio-Rad, Hercules, CA, USA), according to the Bradford method [54]. To calculate the concentration of the proteins of interest, a standard calibration using known concentrations of the Bovine Serum Albumin (BSA) protein (Merck Millipore, Burlington, MA, USA) was set up. The absorbance of the samples was measured at a wavelength of 595 nm using a spectrophotometer (Thermo Scientific, Waltham, MA, USA).

2.5. Evaluation of the Antibacterial Activity of Hemolymph via Antibiogram Assay

The *in vitro* evaluation of the antimicrobial activity of hemolymph extracts was carried out via antibiogram (agar diffusion test), using a solution of LB-Agar. A colony of *E. coli* and a colony of *M. flavus* were transferred each to 10 mL of LB and incubated overnight at 37 °C, under shaking. The bacterial culture was uniformly distributed on the agar-containing plates, using a cotton swab. Following its adsorption, 5 µL of each sample, the peptide fractions of the hemolymph extracted from infected and uninfected larvae, was dispensed onto the plate. As a negative control, 5 µL of sterile water was used. All tests were performed in triplicate, incubating the plate overnight at 37 °C.

2.6. Evaluation of the Hemolymph Antibacterial Activity via Bioautography (SDS Gel Overlay Method) Experiment

The antibacterial activity of the peptide fraction recovered from the plasma of infected and uninfected larvae was also evaluated via a bioautography experiment [55]. Briefly, two polyacrylamide gels were prepared (4% stacking, 12% running); one of the two gels was stained with a solution of Blue Coomassie (Merck Millipore, Burlington, MA, USA) in order to visualize the bands corresponding to the peptide samples, while the second gel was washed with Triton X-100 (Bio Rad, Hercules, CA, USA) at 2.5% for 1 h to remove the SDS and with Tris-HCl 50 mM pH 7.5 for 2 h to allow the renaturation of the peptides; finally, the gel was incubated in LB culture medium for 1 h. At the end of the incubation in LB, solid nutrient LB-agar culture medium (0.7%) containing *E. coli* or *M. flavus* cells was transferred onto the gel and incubated for 24 h at 37 °C. For each experimental condition, 20 µL of sample was loaded.

2.7. Evaluation of the Hemolymph Antibacterial Activity via Microdilution Assay

For the microdilution assay, performed against both *E. coli* and *M. flavus* cultures, the major quantity used in the antibiogram assay was used as a starting quantity (4.5 µg) that was subsequently subjected to serial dilution for a total of 6 serial dilutions (2.24 µg, 1.13 µg, 0.56 µg, 0.28 µg, 0.14 µg). Experimentally, cultures of both *E. coli* and *M. flavus* were seeded in 96-well plates (1×10^6 cells per well) and treated with the serial dilutions, reaching a final volume of 200 µL. Wells containing water and culture alone were used as controls. Plates were incubated at 37 °C to allow bacterial growth for 24 h of incubation, the absorbance of the samples under examination was measured using a spectrophotometer (Thermo Scientific, Waltham, MA, USA) at a wavelength of 600 nm. The experiments were carried out in three technical replicates for each of the three biological replicates. Results were reported as percentage of bacterial culture treated in different conditions compared to culture alone (control), whose value was considered as 100%.

2.8. SDS-PAGE and In Situ Hydrolysis

The peptide fraction extracted from *H. illucens* larvae infected with *E. coli*, *M. flavus* and from uninfected larvae (control) was fractionated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In detail, at 15 µL for each protein extract, the loading buffer 1X, composed of 2% SDS (Bio-Rad, Hercules, CA, USA), 50 mM TRIS-HCl pH 6.8 (Merck Millipore, Burlington, MA, USA), 10% Glycerol (Merck Millipore, Burlington, MA, USA) and bromophenol blue (Bio-Rad, Hercules, CA, USA), was added, and they were separated on a 20% SDS-PAGE gel. After the run, the gel was stained with GelCode™ Blue Safe Protein Stain (Thermo Fisher Scientific, Waltham, MA, USA) and destained with Milli-Q water. A total of 3 bands for each condition (*E. coli*, *M. flavus*, control) were cut and *in situ* hydrolyzed with trypsin as previously described [56]. Peptide mixtures were extracted in 0.2% formic acid (HCOOH) (Merck Millipore, Burlington, MA, USA) and acetonitrile (ACN) (Merck Millipore, Burlington, MA, USA) and vacuum dried via a SpeedVac System (Thermo Fisher Scientific, Waltham, MA, USA).

2.9. LC-MS/MS Analysis and Protein Identification

Each peptide mixture was dissolved in 10 µL of 0.2% HCOOH (Merck Millipore, Burlington, MA, USA) and analyzed via nano LC-MS/MS on an LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) coupled to a nanoLC system nano Easy II. Each peptide mixture was concentrated and desalted onto a trapping column (C18 Easy Column L = 2 cm, ID = 100 mm, Nano Separations, Nieuwkoop, the Netherlands), and then fractionated on a C18 reverse-phase capillary column (C18 Easy Column L = 20 cm, ID = 7.5 µm, 3 µm, (Nano Separations, Nieuwkoop, The Netherlands) with a flow rate of 250 nL/min. The gradient used for peptide elution ranged from 10% to 60% of eluent B in 69 min [57]. Eluents A and B have the following composition: 2% ACN LC-MS grade and 0.2% HCOOH, and 95% ACN LC-MS grade and 0.2% HCOOH, respectively. The MS/MS method was set up in a data-dependent acquisition mode (DDA), with a full scan ranging from 300 to 1800 *m/z* range, followed by fragmentation in CID modality of the top 5 ions (MS/MS scan) selected by intensity and charge state (+2, +3, +4 charges), and applying a dynamic exclusion time of 40 s [58]. The peak list generated was uploaded in Mascot software (version 2.4.0) and research was performed by using the in-house database named the "*Hermetia illucens* database". The parameters for protein identification were as follows: "trypsin" as enzyme with at least one missed cleavage, "carbamidomethyl" as a fixed modification, "oxidation of Met" and "pyro-Glu at N-term if Gln" as variable modifications, 0.6 Da as MS/MS tolerance and 10 ppm as peptide tolerance. Scores threshold of matches for MS/MS data was fixed at 10 for all peptides.

2.10. Statistical Analysis

All experiments were performed in triplicates (three independent biological replicates) and results were expressed as means ± standard error. Data were analyzed via GraphPad Prism 6.0 software (GraphPad Software, Inc., La Jolla, CA, USA) using one-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* test.

3. Results

3.1. Evaluation of Sample Concentration

The concentration of the samples obtained following precipitation with organic solvents was evaluated via the Bradford assay. The values obtained are shown in the following table (Table 1):

Table 1. Concentrations of the samples obtained via precipitation with organic solvents from plasma extracted from uninfected larvae or larvae infected with *E. coli* or *M. flavus*. Data are expressed as mean ± standard errors of three independent biological replicates.

	Uninfected Larvae	**Larvae Infected with *E. coli***	**Larvae Infected with *M. flavus***
Precipitation with organic solvents	0.583 ± 0.02 µg/µL	0.739 ± 0.07 µg/µL	0.930 ± 0.03 µg/µL

3.2. Evaluation of the Antibacterial Activity of Peptide Fraction of Hemolymph via Antibiogram Assay

The peptide fractions recovered following precipitation with methanol/acetic acid/water (90:1:9 *v/v* ratio) of the plasma extracted from uninfected larvae and from larvae infected with *E. coli* or *M. flavus*, were first analyzed via agar diffusion test to evaluate their antibacterial effect against *E. coli* and *M. flavus*. The test performed both against *E. coli* and *M. flavus* revealed the presence of an inhibition zone, in correspondence with all the analyzed samples (Figure 1). Differences were detected against the two analyzed strains: halos were wider (Table 2) and well defined in the plate with *M. flavus*, compared to the *E. coli* plate, in which the bacterial growth was not completely inhibited, as demonstrated by a patina of bacterial cells on the halo surface.

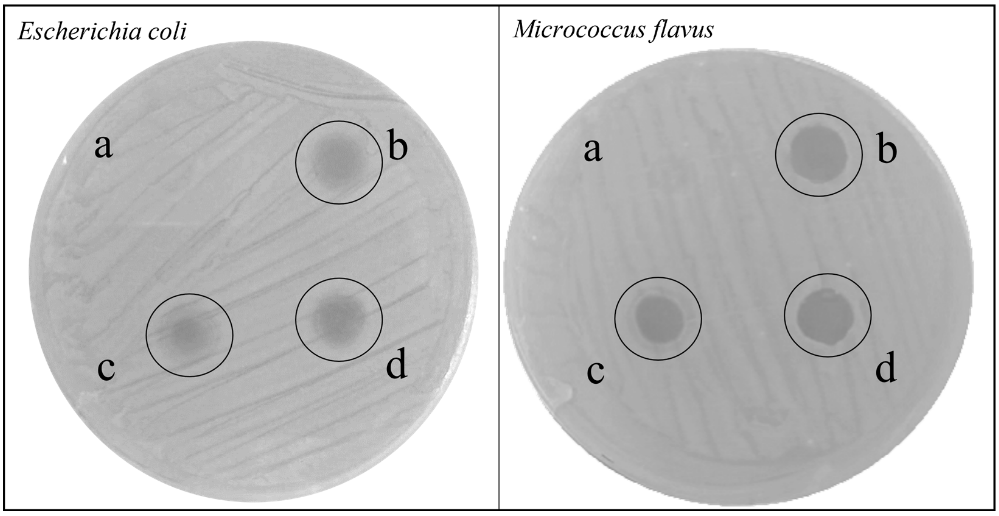

Figure 1. Agar diffusion test of peptide fractions obtained via precipitation with organic solvents, performed against *E. coli* (on the left) or *M. flavus* (on the right). (**a**) H_2O, negative control; (**b**) peptide fraction from larvae infected with *E. coli*; (**c**) peptide fraction from uninfected larvae; (**d**) peptide fraction from larvae infected with *M. flavus*. The experiments were carried out in triplicate (three independent biological replicates).

Table 2. Diameters (mm) of inhibition zones formed by peptide fraction, obtained via precipitation with organic solvents, from uninfected larvae, larvae infected with *E. coli* or larvae infected with *M. flavus*. Data are expressed as mean ± standard errors of diameters measured via antibiogram of three independent biological replicates. Different letters indicate significant differences between the same sample against the different strains (capital letters) and among different samples against the same strains (lowercase letters). Data are analyzed with one-way ANOVA and Bonferroni post *hoc test* (p value *E. coli* = 0.2690, *M. flavus* = 0.0046) and unpaired t-test with Welch's correction (p value uninfected larvae = 0.8113, larvae infected with *E. coli* = 0.3868 and larvae infected with *M. flavus* = 0.0824).

	Uninfected Larvae	**Larvae Infected with *E. coli***	**Larvae Infected with *M. flavus***
E. coli	6.67 ± 1.2 ^{aA}	8.67 ± 0.3 ^{aA}	8.00 ± 0.5 ^{aA}
M. flavus	6.33 ± 0.3 ^{bA}	8.00 ± 0.6 ^{aA}	9.67 ± 0.3 ^{aA}

3.3. Evaluation of the Antibacterial Activity of Peptide Fraction of the Hemolymph via Bioautography (SDS Gel Overlay Method) Assay

An electrophoretic analysis of the infected and uninfected samples, treated with methanol, acetic acid and water in a 90:1:9 ratio *v/v* was performed. Three identical gels (12% acrylamide) were prepared and at the end of the electrophoretic run, one of the gels was stained with Coomassie Blue, while on the other gels a bioautography test against *E. coli* and *M. flavus* was performed. Results in Figures 2a and 3a show the presence of low molecular weight bands, around 10 kDa. Figures 2b and 3b show an inhibition zone in correspondence with low molecular weight bands relative to the peptide fraction obtained following precipitation of the plasma extracted from all samples, and tested against *E. coli* and *M. flavus*, respectively. Figures 2c and 3c show the overlay between the gel and the inhibition zone observed on bioautography, to confirm that the obtained inhibition comes from peptides around 10 kDa.

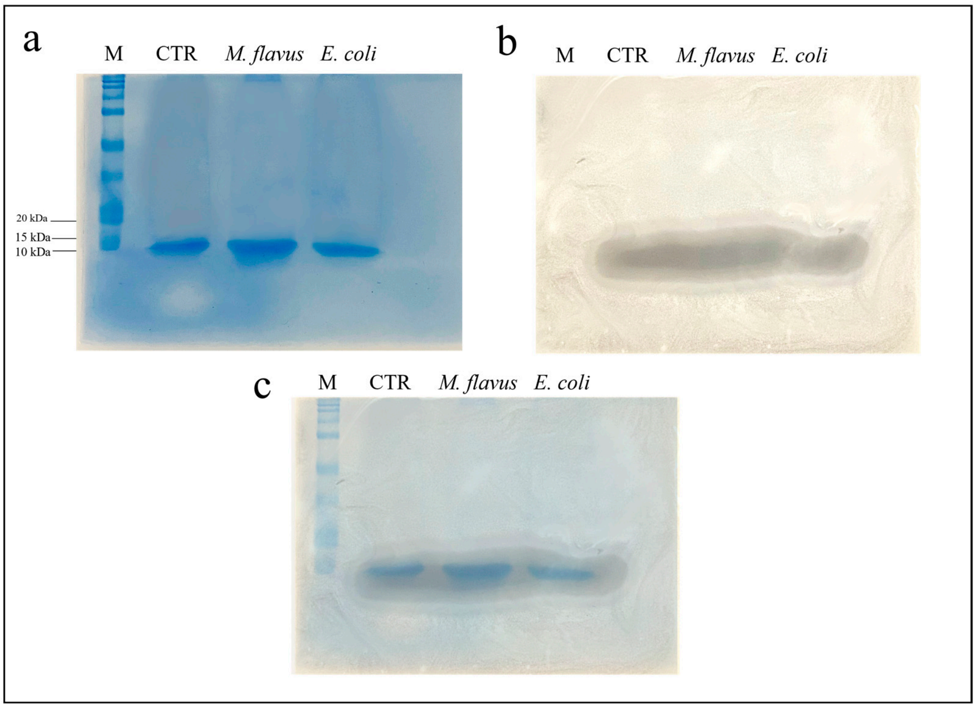

Figure 2. SDS-PAGE (**a**) and bioautography (**b**) performed against *E. coli* of the samples obtained following precipitation with organic solvents. In (**c**), an overlay of the previous images is presented. M = marker, "All Blue Standards Biorad" (Biorad, Hercules, CA, USA). CTR = peptide fraction from uninfected larvae; *M. flavus* = peptide fraction from larvae infected with *M. flavus*; *E. coli* = plasma from larvae infected with *E. coli*. The experiments were carried out in triplicate (three independent biological replicates).

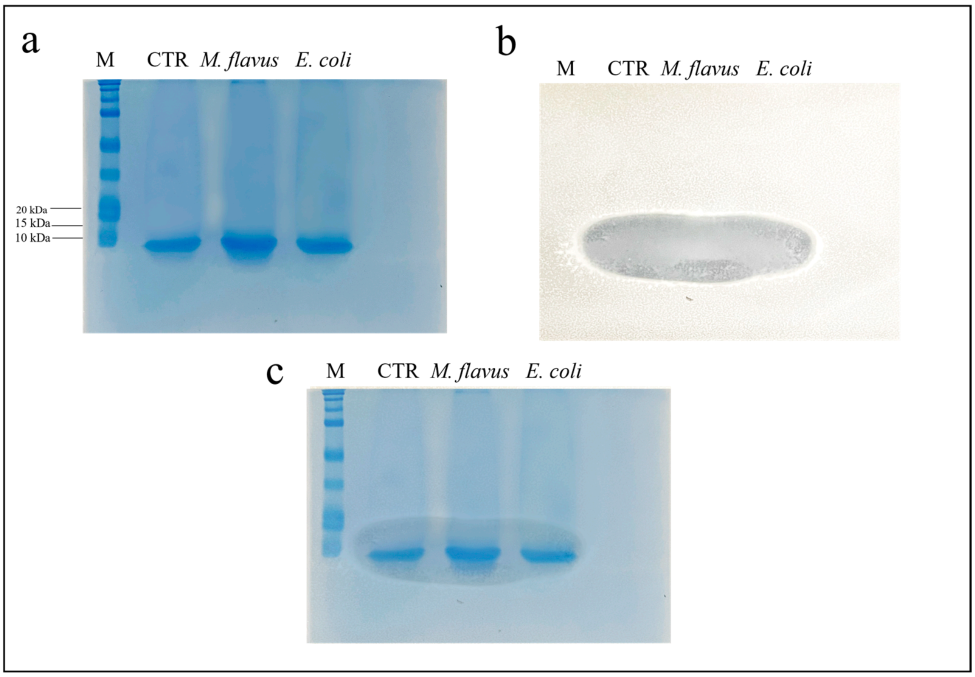

Figure 3. SDS-PAGE (**a**) and bioautography (**b**) performed against *M. flavus* of the samples obtained following precipitation with organic solvents. In (**c**), an overlay of the previous images is presented. M = marker, "All Blue Standards Biorad" (Biorad, Hercules, CA, USA). CTR = plasma from uninfected larvae; *M. flavus* = plasma from larvae infected with *M. flavus*; *E. coli* = plasma from larvae infected with *E. coli*. The experiments were carried out in triplicate (three independent biological replicates).

3.4. Evaluation of the Biological Activity of the Peptide Fractions via Liquid Microdilution Assays

Starting from the qualitative results obtained by agar diffusion and bioautography tests, microdilution assays against *E. coli* (Figure 4) and *M. flavus* (Figure 5) were performed.

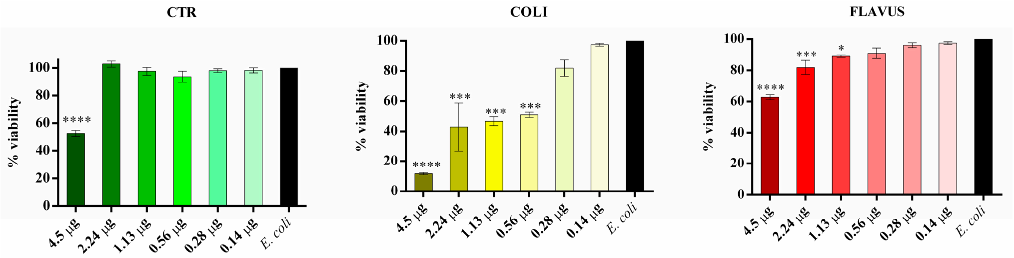

Figure 4. Microdilution assay against *E. coli* performed with the peptide fractions obtained via precipitation with organic solvents. CTR = peptide fractions from uninfected larvae; COLI = peptide fractions from larvae infected with *E. coli*; FLAVUS = peptide fractions from larvae infected with *M. flavus*. The black bars represent the untreated *E. coli* cell culture. Results are presented as percentage of viability of bacterial culture treated in different conditions compared to culture alone (control), whose value was considered as 100%. Data are expressed as means ± standard error of three independent biological replicates and statistical significance was evaluated with one-way ANOVA followed by Bonferroni *post hoc* test (* $p < 0.1$, *** $p < 0.001$, **** $p < 0.0001$).

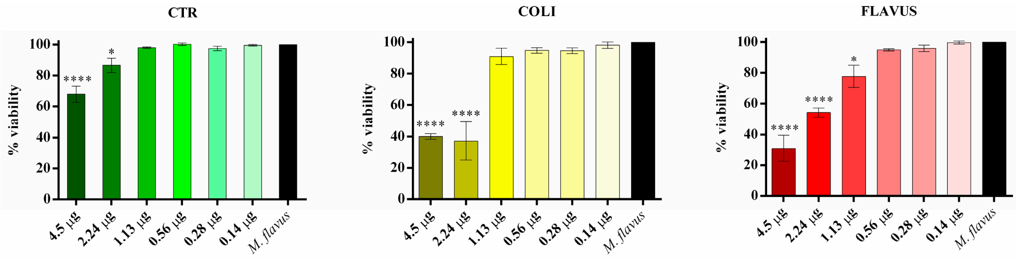

Figure 5. Microdilution assay against *M. flavus* performed with the peptide fractions obtained via precipitation with organic solvents. CTR = peptide fractions from uninfected larvae; COLI = peptide fractions from larvae infected with *E. coli*; FLAVUS = peptide fractions from larvae infected with *M. flavus*. The black bars represent the untreated *M. flavus* cell culture. Results are presented as percentage of viability of bacterial culture treated in different conditions compared to culture alone (control), whose value was considered as 100%. Data are expressed as means ± standard error of three independent biological replicates and statistical significance was evaluated with one-way ANOVA followed by Bonferroni post hoc test (* $p < 0.1$, **** $p < 0.0001$).

All the analyzed samples are able to inhibit *E. coli* cell growth, although with different minimum inhibitory concentrations (MICs) and percentage of reduction. Indeed, sample control can inhibit cell growth by 42%, exclusively at the highest quantity tested (4.5 µg). The MIC of peptide fractions obtained from larvae infected with *E. coli* is 0.56 µg, with a reduction in cell growth of 50%, while as concerns peptide fractions obtained from larvae infected with *M. flavus* MIC is 1.13 µg, with a reduction in cell growth of 11%. The highest quantity of peptides obtained from larvae infected with *E. coli* is able to reduce the growth by 89%, while the highest quantity of peptides obtained from larvae infected with *M. flavus* is able to reduce the growth by 32%.

All the examined samples may prevent *M. flavus* cell development, albeit at varying MICs and reduction rates. Indeed, the highest tested quantity for the sample control (4.5 µg) can inhibit cell growth by 33%, whereas the MIC value (2.24 µg) inhibits the 14% growth. Differently to the MIC against *E. coli*, in the peptide fractions obtained from larvae infected with *E. coli* this value is 2.24 µg, with a reduction in cell growth of 63%, the same percentage obtained by the highest quantity used. As concerns peptide fractions obtained from larvae infected with *M. flavus*, the MIC value is 1.13 µg, with a reduction in cell growth of 33%. The highest quantity of this peptide fraction is able to reduce the growth by 69%.

3.5. Mass Spectrometry Analysis

After SDS analysis, bands were *in situ* hydrolyzed via trypsin, and the peptide mixtures were analyzed via LC-MS/MS. The raw data from mass spectrometry analysis were converted to mgf files and then inserted into the MASCOT software for protein identification. The protein database used consists of contigs containing putative protein sequences derived from *H. illucens* transcriptomes. Six putative protein sequences, each with a single reading frame, are presented for each contig. In Table S1a–d, the identified peptides are presented including the following information: experimental *m/z* value of the peptide, experimental mr value, the mascot score, the sequence of identified peptides, the contig code, the amino acid sequence frame (in red the peptides found by LC-MS/MS) and the frame number of the transcriptomic sequence obtained with SEQtools that match with the LC-MS/MS.

We identified 33 AMPs (Figure 6): 20 expressed in all the analyzed conditions, 6 absent in control and expressed only after infection with *E. coli* or *M. flavus*, 1 differentially expressed after infection of *E. coli* and 6 differentially expressed after infection with *M. flavus.* The 6 AMPs differentially expressed after the infection of both bacteria were 4 defensins, 1 attacin and 1 uncharacterized protein; the AMPs expressed after *M. flavus* infection were 4 cecropins and 2 defensins, while the differentially AMP expressed after *E. coli* infection was a defensin.

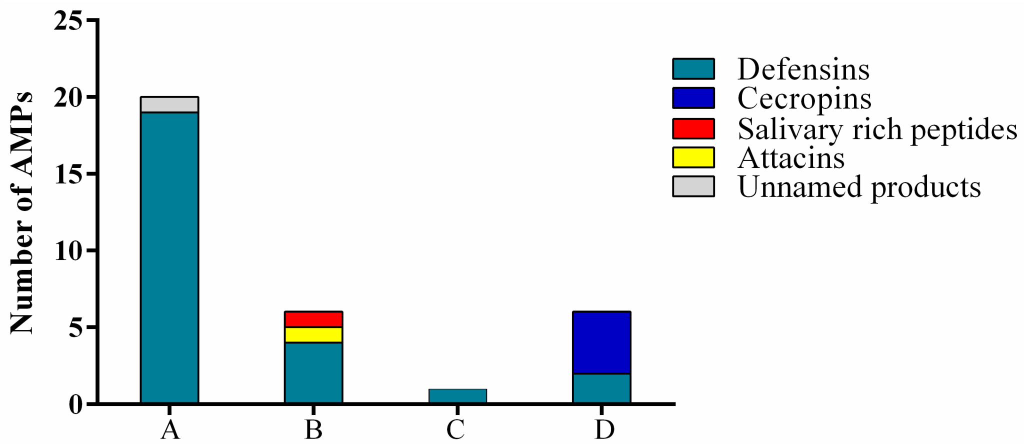

Figure 6. Number and classes of AMPs identified via the LC-MS/MS in different experimental conditions: A = peptides identified both in infected and uninfected larvae; B = peptides identified in larvae infected with *E. coli* or *M. flavus*; C = peptide identified exclusively in larvae infected with *E. coli*; D = peptides identified exclusively in larvae infected with *M. flavus.*

4. Discussion

In recent decades, the excessive and inappropriate use of antibiotics in human and veterinary medicine has contributed to an increase in the natural selection of resistant bacteria and a decrease in drug efficacy [59,60]. Few classes of antibiotics are now effective against some multi-resistant pathogenic bacteria and the worldwide spread of resistance genes is considered a scenario of extreme emergency [61]. For this reason, the search for new molecules with antibacterial activity represents one of the major current challenges for the scientific community. Antimicrobial peptides (AMPs) represent an excellent alternative to modern antibiotics [62,63]. AMPs are small molecules positively charged that selectively interact with the negatively charged bacterial surface [64]. One of the richest sources of AMPs is represented by the class of insects which is characterized by the large quantity and the diversity of its molecules and processes. Insects are organisms extremely well adapted to diverse habitats, primarily due to their innate immune system, which provides them with a range of cellular and humoral responses against microorganisms [65]. Moreover, insects can also feed on substances with different levels of contaminations, so they synthesize AMPs to fight such infections and survive in dangerous conditions [66]. AMPs extracted from insects have the potential to fight the microorganisms that act as hazards to human health [66,67]. One of the most interesting insect species is Diptera *Hermetia illucens*, which is able to produce a number of AMPs, far superior to that of other insects [44]. The wide spectrum of produced AMPs is directly related to the remarkable variety of substrates on which the larva feeds on. The present work is part of a broader project of identification and structural and functional characterizations of the AMPs produced by *H. illucens* larvae, in order to use them as highly innovative antimicrobial molecules. In this work, we focused on the *in vitro* evaluation of the antimicrobial activity of the peptide fraction of the hemolymph of *H. illucens*, following infection with the Gram negative bacterium *Escherichia coli* or the Gram positive *Micrococcus flavus*, via microbiological tests performed against *E. coli* and *M. flavus* themselves. Following the precipitation of the peptide fraction, a further extraction step in hexane was performed to ensure the absence of any traces of lipids, which have antimicrobial activity due to the presence of lauric acid [37]. As shown in Figure S1, no activity was detected in the upper fraction of the hexane extract, the fraction that should contain lipid traces, demonstrating that the antimicrobial activity detected is exclusively attributable to the AMPs. From our data, it is possible to highlight that AMPs produced by *H. illucens* are effective both against Gram positive and Gram negative bacteria, and that the expression of some AMPs can be induced following the stimulation by specific bacteria. Although the microbiological analyses (antibiogram, bioautography and microdilution assay) were the starting point of our experiments, for the identification of constitutive and inducible AMPs and the differential expression after the bacterial challenge with Gram negative and Gram positive bacteria, a mass spectrometry analysis was also performed. With a combined transcriptomic and proteomic approach, we identified 20 AMPs constitutively expressed, whose expression could increase after bacterial infection, and 13 inducible. The bacterial infection, indeed, stimulates the expression of specific peptides. Both *E. coli* and *M. flavus* induced the expression of 6 AMPs (defensins, attacins and cysteine-rich peptide), while a defensin was induced specifically by *E. coli*, cecropins and defensins by *M. flavus*. As expected, the most detected AMPs were defensins [45].

Usually, insect defensins are more active against Gram positive bacteria such as *S. aureus* [47] or *Bacillus subtilis* [68]; however, some of them also exhibit antimicrobial activity against Gram negative bacteria, in particular *E. coli* [47,69]. Defensin expression can be induced by Gram negative [70–72] or positive bacteria [70,73,74], as also recorded via experiments carried out in this work.

Cecropins, α-helical AMPs, are indiscriminately active against Gram negative bacteria, such as *E. coli*, *Klebsiella pneumoniae*, *Salmonella typhimurium* and *Pseudomonas aeruginosa* [75–77], or Gram positive bacteria, such as *Staphylococcus* and *Bacillus* species [75–77]. Their expression can be induced by both Gram positive and negative bacteria [78]: for example, in Lepidoptera, different microbial infections result in different patterns of cecropin gene expression, indicating that various signaling pathways can contribute to the same immune gene expression.

The results obtained from our experiments suggested that, depending on the bacteria used for the infection, different AMPs could be induced, as previously reported for *Drosophila melanogaster* [79,80], *Diatraea saccharalis* [65], *Galleria mellonella* [81], *Rhynchophorus ferrugineus* [82].

In Rocha *et al.*, *D. saccharalis* larvae were challenged with *E. coli* and *B. subtilis*. The infection with the Gram positive bacteria induced more pronounced antibacterial activity (evaluated via antibiogram against *B. subtilis*) corresponding to an increase in the expression of 2 AMPs, a defensin and an attacin. Infection with Gram negative bacterium, on the other hand, induced an exclusive increase in the levels of the attacin [65].

In Mak et al. [81], *Galleria mellonella* larvae were challenged with *E. coli* and *M. luteus* and tested against *E. coli*, finding stronger activity by larvae challenged with the Gram negative bacterium. Then differentially expressed peptides were analyzed via HPLC analysis: firstly, it was observed that with the *E. coli* challenge a higher concentration of peptides was obtained, as also observed in our experiments, then it was detected that the most stimulated peptides were a proline-rich peptide, a cecropin-d-like peptide and an anionic peptide-3, this last stimulated also by Gram positive bacterium injection.

Similarly to our work, in Meghashree et al. [83], the infection with *E. coli* and *S. aureus* of *D. melanogaster* and *Drosophila ananassae* larvae showed an increase in protein concentration in hemolymph, and stronger antimicrobial activity, compared to uninfected larvae, in which no inhibition zones in the agar diffusion test were detected. HPLC analysis and the SDS PAGE for high molecular weight proteins showed a differential expression of induced peptides: 3 and 2 peptides were more expressed after *E. coli*/*S. aureus* infection in *D. ananassae* and *D. melanogaster,* respectively, while the SDS PAGE for low molecular weight proteins showed a single protein differentially expressed in both species exclusively after the *E. coli* infection. The LC-MS/MS analysis demonstrated that this protein was a cecropin. As reported by Meghashree et al. [83], the effect of non-induced AMPs is not always easily identifiable: for example, in contrast to our experiments, in experiments on the American Cockroach, *Periplaneta americana*, non-induced hemolymph also did not show any activity against both Gram positive and Gram negative bacteria, whereas induced hemolymph exhibited high activity against *Micrococcus luteus* but less against *E. coli* [84]. However, it is important to notice that, in our experiments, the control sample has a lower antimicrobial effect than the peptide fraction deriving from hemolymph of infected larvae.

The activity of hemolymph both against *E. coli* and *M. flavus* was consistent with what has been reported in the literature, even though not many studies analyze the activity of *H. illucens* hemolymph.

In many cases, an extract from larvae *in toto* is analyzed: for example, in Choi et al. and Auza et al., the antibacterial activity of methanolic extract of *H. illucens* larvae was detected against Gram negative bacteria (*Klebsiella pneumoniae*, *Neisseria gonorrhoeae*, *Shigella sonnei*, *Salmonella typhimurium*, *E. coli* and *P. aeruginosa*) [41,85].

To the best of our knowledge, only a few papers have focused on *H. illucens* hemolymph extract, testing it against few bacterial strains, *E. coli* (strain D31), *M. luteus* and *S. aureus* [55,86]. In Lee *et al*., *H. illucens* larvae were also immunized by *Lactobacillus* species, showing an increase in antimicrobial activity after the infection, as also reported in our paper in which larvae stimulated with Gram positive showed a major reduction in bacteria cell viability against this bacterial group [86]. In Zdybicka-Barabas *et al*., larvae infected with a Gram positive (*M. luteus*) or Gram negative bacterium (*E. coli* D31) and not infected larvae showed good activity exclusively against the Gram positive bacterium strain [55]. Slight activity against *E. coli* was detected exclusively in *E. coli*-challenged larvae, suggesting a higher sensitivity of the *H. illucens* AMPs towards Gram positive bacteria and that also the specific strain that is used for stimulation, and towards which the putative activity is to be detected, is fundamental.

In general, in our experiments the strongest antibacterial activity related to Gram negative or positive bacteria is related to the species used for the infection: indeed, the strongest activity against Gram positive bacteria was recorded in a peptide fraction derived from hemolymph of larvae infected with Gram positive bacteria, with also a lower MIC (1.13 μg) compared to control samples and samples derived from *E. coli* infection, whose MIC is 2.24 μg in both cases. Peptide fraction derived from hemolymph of larvae infected with *E. coli* showed the strongest activity against Gram negative bacteria, with a lower MIC (0.56 μg), compared to control samples and samples derived from larvae infected with *M. flavus*, whose MIC is 4.45 μg and 1.13 μg, respectively. Literature data together with results obtained in our studies encourage testing hemolymph extracts towards different strains of the same bacteria and other bacteria, pathogenic and not.

Further studies are needed, using different bacteria both for the infection that can stimulate the production of different AMPs, and the bacteria against which these peptides could be tested, since the same pool of molecules can have different inhibitory effects. The antimicrobial activity of some peptides (or peptide fractions), indeed, can be displayed in a different way, even on the same bacterial species, but deriving from a different strain. This specific expression could result from the activation of many signaling pathways that control the production of specific defense peptide genes.

The identification of the AMPs of *H. illucens* in the hemolymph and the subsequent production is the first step to find new molecules to use as therapeutic alternatives or in synergy with current antibiotics for applications in the pharmacological and biotechnological fields. Further investigation will include microbiological experiments on the specific peptides differentially expressed and an *in silico* molecular docking against bacterial proteins [44].

5. Conclusions

The peptide fraction of hemolymph of *Hermetia illucens* larvae showed antibacterial activity against both Gram negative *Escherichia coli* and Gram positive *Micrococcus flavus* bacteria, depending on used doses and larval infection: although uninfected larvae exhibit antibacterial activity, it can be improved with bacterial infection, inducing a major expression of specific AMPs. After microbiological assays, via mass spectrometry technique we identified 20 AMPs constitutively expressed and 13 inducible by *M. flavus* and *E. coli* infection. The identification of the AMPs of *H. illucens* in the hemolymph could be the starting point to discover alternative molecules to current antibiotics to overcome the problem of antimicrobial resistance.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/insects14050464/s1, Figure S1. Agar diffusion test of peptide fractions obtained by precipitation with organic solvents, performed against *E. coli* (on the left) or *M. flavus* (on the right). In the figure are reported samples pre- (A) and post- (B) treatment by hexane, as well as the upper fraction possibly containing lipids (C). (a) negative control (H_2O for A and B, hexane for C); (b) peptide fraction from larvae infected with *E. coli*; (c) peptide fraction from uninfected larvae; (d) peptide fraction from larvae infected with *M. flavus*. The experiments were carried out in triplicate (three independent biological replicates). Table S1a, peptides identified both in infected and uninfected larvae; Table S1b, peptides identified in larvae infected with *E. coli* or *M. flavus*; Table S1c, peptide identified exclusively in larvae infected with *E. coli*; Table S1d, peptides identified exclusively in larvae infected with *M. flavus*. The table reports experimental m/z value of the peptide, experimental mr value, mascot score, the sequence of identified peptides, the contig code, the amino acid sequence frame (in red the peptides found by LC-MS/MS) and the frame number of the transcriptomic sequence obtain with SEQtools that match with the LC-MS/MS.

Author Contributions: Conceptualization, P.F.; funding acquisition, P.F.; data curation, P.F., C.S., R.S.; methodology, P.F., C.S., R.S., F.G., R.R., F.C., V.M., M.M.; supervision, P.F., R.S.; validation, P.F., C.S., R.S.; writing—original draft, P.F., C.S.; writing—review and editing, P.F., C.S., F.G., R.R., M.E.L., F.C., V.M., M.M., R.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the Italian Ministry University and Research (MUR) (PRIN2017, protocol Prot. 2017AHTCK7) and Basilicata Region (PO FESR, BASILICATA 2014–2020 “AAA: SAFE SOS” D.D. 12AF.2020/D.01255-9/11/2020).

Data Availability Statement: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

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Acknowledgements

Questa tesi non rappresenta un punto di arrivo, ma il risultato di un lungo viaggio, umano e scientifico, arricchito da incontri e momenti che hanno lasciato un segno indelebile. Durante questi anni ho avuto la fortuna di poter contare su persone che, con il loro tempo, la loro disponibilità e il loro sapere, mi hanno aiutata a chiarire dubbi, rafforzare idee e affrontare sfide.

Un ringraziamento particolare va innanzitutto alla Professoressa Patrizia Falabella, che ha svolto un ruolo centrale nel mio percorso di dottorato. La sua guida, dedizione e competenza sono state per me un punto di riferimento costante durante tutto il mio lavoro di ricerca. Mi ha trasmesso l'entusiasmo e la curiosità scientifica necessari per affrontare con determinazione anche le sfide più complesse. Il suo esempio di rigore, passione per la ricerca e attenzione ai dettagli ha arricchito non solo il mio progetto di studio, ma anche la mia formazione personale e professionale.

Esprimo la mia sincera gratitudine al Professore Željko Popović per avermi accolto presso l'Università di Novi Sad e per il prezioso supporto offerto durante il mio periodo di ricerca all'estero. La sua disponibilità, i suoi consigli scientifici e la possibilità di confrontarmi con lui hanno contribuito in modo significativo al completamento del mio lavoro.

Desidero inoltre ringraziare la Professoressa Donnarumma, dell'Università della Campania "Luigi Vanvitelli", e Alessandra per avermi accolta con calore e per aver creato un ambiente in cui mi sono sentita come a casa. La loro capacità di trasmettere con passione le loro conoscenze e di condividere il loro sapere in modo aperto e generoso è stata per me una fonte di grande arricchimento. Inoltre, la fiducia dimostratami nel concedermi piena autonomia ha rappresentato un importante stimolo per la mia crescita.

Un grazie speciale va anche a Rosanna e Carmen, la cui competenza e disponibilità hanno rappresentato un supporto indispensabile durante il mio percorso. La loro presenza è stata per me un riferimento prezioso, sia dal punto di vista professionale che umano.

Un enorme grazie va a tutte le persone che hanno reso possibile questa tesi. Il loro supporto e il loro contributo non si sono limitati a far quadrare tutto (e a farmi sopravvivere alle scadenze!), ma mi hanno insegnato qualcosa di fondamentale: la ricerca scientifica non è solo questione di impegno individuale, ma è soprattutto un gioco di squadra fatto di dialoghi, collaborazioni autentiche e momenti di confronto.

Ai miei genitori, che hanno creduto in me, anche prima che potessi farlo io, con il loro ineguagliabile "sostegno alla ricerca". Grazie per aver riposto in me la stessa fiducia che si riserva a una terapia innovativa: con pazienza, determinazione e un inesauribile supporto. Siete stati il mio "fondo di ricerca" più affidabile e il mio principale comitato etico. Spero che i risultati siano all'altezza delle aspettative… o almeno ci proverò!

Ringrazio te, Antonietta, amica e sorella, e Paolo, perché mi ricordate ogni giorno quanto io valga.

Agli inquilini della stanza "afidi", o della stanza a 18-28 gradi a seconda della stagione, nota anche come la stanza del brainstorming, un grazie speciale.

Un ringraziamento particolare a Valentina, incontrata per la prima volta all'esame di statistica e poi ritrovata nel gruppo "i gel si preparano con l'acqua e usando la spruzzetta". Quello che era iniziato come un rapporto di lavoro è diventato una vera amicizia: compagna di avventure, segreti e disavventure. E quando sentirai che sta per arrivare il momento di svenire, ricorda che per me sei una fonte di ispirazione… e prova a resistere almeno fino alla fine!

Un grazie speciale anche a Doris, abitante fondatrice della stanza, che mi ha dato conforto nei momenti più difficili, quando tutto si concludeva con un semplice: "Eh, va beh, è andata così". La tua presenza è stata un sostegno fondamentale.

Alla mia Barbie preferita, Sofia, Mariarita e a Miriam, grazie per la vostra capacità di rendere tutto più ironico e per le battute (sì, anche quelle su certi legami di parentela non confermati). Vi ringrazio per aver curato la grafica, per le risate e per le immancabili cofecchie.

Ringrazio il gruppo AMP Roberta, Emine e Federica: questo consorzio ha reso i cetrioli, anche i più amari, con tutto un altro sapore.

Ringrazio tutti: Micaela, Francus, Andrea, Giovanni, Maddalena, Anna, Francesco, Antonio, Ilaria, grazie per avermi ricordato che il vero esperimento è capire come si possa sopravvivere a questo laboratorio insieme.

Grazie ad Agata e Ilenia, perché, sebbene tutti possano prendere percorsi differenti nella vita, non importa dove andremo: porteremo una piccola parte degli altri un po' ovunque (ed io voi).

Ed infine, ma non per questo meno importante, anche stavolta dico grazie alla mia “capa tosta”.