



Załącznik nr 1

do Zarządzenia Rektora PG nr 30/2022 z kwietnia 2022 r.

The author of the doctoral dissertation: mgr. Ahmer Bin Hafeez

Scientific discipline: Chemical Sciences

DOCTORAL DISSERTATION

Title of doctoral dissertation: Pharmacological Potential of Bee Products Microbiota

**Title of doctoral dissertation (in Polish): Potencjał Farmakologiczny Mikrobioty
Produktów Pszczelich**

Supervisor

signature

Dr hab. inż. Piotr Szweda, prof. uczelni

Gdańsk, year 2026



STATEMENT

The author of the doctoral dissertation: mgr. Ahmer Bin Hafeez

I, the undersigned, declare that I am aware that in accordance with the provisions of Art. 27 (1) and (2) of the Act of 4th February 1994 on Copyright and Related Rights (Journal of Laws of 2021, item 1062), the university may use my doctoral dissertation entitled: *Investigation of the antimicrobial potential and microbiota of bee pollen and bee bread derived from Polish apiaries*. for scientific or didactic purposes.¹

Gdańsk, ...

.....
signature of the PhD student

Aware of criminal liability for violations of the Act of 4th February 1994 on Copyright and Related Rights and disciplinary actions set out in the Law on Higher Education and Science (Journal of Laws 2021, item 478),² as well as civil liability, I declare that the submitted doctoral dissertation is my own work.

I declare that the submitted doctoral dissertation is my own work performed under and in cooperation with the supervision of Dr. hab. inż. Piotr Szweda.

This submitted doctoral dissertation has never before been the basis of an official procedure associated with the awarding of a PhD degree.

All the information contained in the above thesis, which is derived from written and electronic sources are documented in a list of relevant literature in accordance with Art. 34 of the Copyright and Related Rights Act.

I confirm that this doctoral dissertation is identical to the attached electronic version.

Gdańsk, ...

.....
signature of the PhD student

I, the undersigned, agree to include an electronic version of the above doctoral dissertation in the open, institutional, digital repository of Gdańsk University of Technology.

Gdańsk, ...

.....
signature of the PhD student

¹ Art 27. 1. Educational institutions and entities referred to in art. 7 sec. 1 points 1, 2, and 4–8 of the Act of 20 July 2018 – Law on Higher Education and Science, may use the disseminated works in the original and in translation for the purposes of illustrating the content provided for didactic purposes or in order to conduct research activities, and to reproduce for this purpose disseminated minor works or fragments of larger works.

² If the works are made available to the public in such a way that everyone can have access to them at the place and time selected by them, as referred to in para. 1, is allowed only for a limited group of people learning, teaching, or conducting research, identified by the entities listed in paragraph 1.



DESCRIPTION OF DOCTORAL DISSERTATION

The Author of the doctoral dissertation: Ahmer Bin Hafeez

Title of doctoral dissertation: Pharmacological Potential of Bee Products Microbiota

Title of doctoral dissertation (in Polish): Potencjał Farmakologiczny Mikrobioty
Produktów Pszczelich

Language of doctoral dissertation: English

Supervisor: Dr. hab. inż. Piotr Szweda

Date of Doctoral Defense:

Summary of Doctoral Dissertation

Antimicrobial resistance (AMR) is one of the most significant challenges in modern medicine globally. The increasing prevalence of resistant pathogens has outpaced the introduction of novel antibiotics into clinical practice, necessitating the identification and development of effective alternative therapeutics.

Natural products have historically been the primary source of clinically approved antibiotics and remain a structurally diverse and biologically validated reservoir for the discovery of novel antimicrobial agents. Over the past two decades, renewed scientific interest has emerged in the antimicrobial potential of natural products, especially those derived from bees.

The primary objective of this doctoral research was to systematically investigate the antimicrobial potential of the microbiota associated with three bee products: honey, bee pollen, and bee bread, using an integrated approach combining phenotypic screening, whole-genome sequencing, genome mining, functional annotation, and computational structural modeling. Bacterial isolates from all three sources, predominantly *Bacillus* spp., showed significant antimicrobial activity



against clinically and veterinary relevant pathogens, including *Staphylococcus aureus* and *Listeria monocytogenes*, and harbored dense arrays of biosynthetic gene clusters encoding lipopeptides, polyketides, siderophores, and a broad repertoire of ribosomally synthesized and post-translationally modified peptides (RiPPs). Paralichenidin, a novel two-component lantibiotic produced by *Bacillus paralicheniformis* isolated from honey, was identified through BGC mining and characterized using molecular dynamics simulations, which confirmed the distinct and complementary roles of its two peptide components in lipid II recognition and membrane targeting. Moreover, strains from bee pollen and bee bread exhibited considerable probiotic potential, meeting criteria for gastric acid and bile tolerance, surface adhesion, and cholesterol assimilation, while a multi-tool annotation pipeline assigned putative functions to 56 previously hypothetical proteins, identifying roles in sporulation, biofilm formation, motility, secretion, and metabolic regulation.

These findings collectively establish the bee-associated microbiota as a reproducible, biosynthetically rich source of novel antimicrobial and probiotic candidates, providing a rigorous scientific foundation for future experimental validation and downstream biotechnological development.

The outcomes of this research have been disseminated through five peer-reviewed scientific publications.

Keywords of Doctoral Dissertation: Bee Products, Microbiota, Resistance, Honey, Bee pollen, Bee bread, Bacteriocins, Lantibiotics, *Staphylococcus* spp.,

Streszczenie

Oporność na środki przeciwdrobnoustrojowe (AMR, antimicrobial resistance) stanowi jedno z najpoważniejszych wyzwań współczesnej medycyny. Tempo wprowadzania do praktyki klinicznej nowych leków nie nadąża za tempem selekcji mikroorganizmów (głównie bakterii i grzybów) niewrażliwych na stosowane aktualnie chemioterapeutyki przeciwdrobnoustrojowe. Stwarza to pilną potrzebę identyfikacji i opracowania nowych, alternatywnych i przede wszystkim skutecznych metod terapii chorób infekcyjnych. Ciekawym i bardzo zróżnicowanym, aczkolwiek ciągle niedocenianym rezerwuarem substancji o aktywności przeciwdrobnoustrojowej są produkty naturalne. Zawierają one wiele składników o unikalnej budowie chemicznej (zdecydowanie różniące się od stosowanych aktualnie leków) charakteryzujących się wysoką aktywnością

przeciwbakteryjną i/lub przeciwgrzybową oraz pożądanymi właściwościami użytkowymi (np. dobrą rozpuszczalnością w wodzie, niską cytotoksycznością). W ciągu ostatnich dwóch dekad nastąpił wyraźny ponowny wzrost zainteresowania potencjałem przeciwdrobnoustrojowym produktów naturalnych, w tym także produktów pszczelich. Badania z ostatnich lat wskazują, że na szczególną uwagę zasługuje także mikrobiota tych produktów – populacja zasiedlających je szczepów mikroorganizmów. Głównym celem niniejszej rozprawy doktorskiej była systematyczna ocena potencjału przeciwdrobnoustrojowego mikrobioty związanej z trzema produktami pszczelimi: miodem, pyłkiem oraz pierzgą. Aktywność przeciwdrobnoustrojową metabolitów szczepów bakteryjnych wyizolowanych z ww. produktów oceniano za pomocą metody rozcieńczeniowej - ustalenie wartości minimalnego stężenia hamującego – MIC oraz za pomocą metody dyfuzyjno-krażkowej – pomiar stref zahamowania wzrostu. Potencjał probiotyczny izolatów oceniano na podstawie wyników testów tolerancji warunków panujących w układzie pokarmowym człowieka (np. niskie pH i obecność soli kwasów żółciowych) i ewentualnych zagrożeń dla pacjentów (np. lekooporność, aktywność hemolityczna). Analizy genomów wybranych izolatów (głównie bakterii z rodzaju *Bacillus* spp.) potwierdziły ich potencjał do syntezy substancji o aktywności przeciwdrobnoustrojowej. W genomie każdego z badanych szczepów zidentyfikowano operony „odpowiedzialne” za syntezę szerokiego spektrum metabolitów: lipopeptydów, poliketydów, syderoforów, peptydów syntetyzowanych rybosomalnie i modyfikowanych potranslacyjnie o aktywności przeciwbakteryjnej i/lub przeciwgrzybowej. Dzięki zdolności do wytwarzania powyższych związków badane izolaty wykazały znaczną aktywność hamującą wzrost ważnych patogenów ludzi i zwierząt, w tym *Staphylococcus aureus* i *Listeria monocytogenes*. W przypadku wyodrębnionych z miodu izolatów *Bacillus paralicheniformis* SZA16 i SZA14 scharakteryzowano nowy dwuskładnikowy lantibiotyk - paralichenidynę. Wyniki uzyskane w trakcie realizacji pracy jednoznacznie wskazują, że mikrobiota produktów pszczelich stanowi cenne i wciąż niedostatecznie poznane źródło metabolitów o znacznym potencjale przeciwdrobnoustrojowym, które zasługują na dalsze badania w kontekście opracowywania nowych strategii terapeutycznych przeciwko patogennym bakteriom i grzybom w tym szczepom lekoopornym. Rezultaty przedstawionych badań zostały opublikowane w pięciu recenzowanych artykułach naukowych.

Słowa kluczowe: Bee Products, Microbiota, Resistance, Honey, Bee pollen, Bee bread, Bacteriocins, Lantibiotics, *Staphylococcus* spp.,

I want to express my deepest gratitude to my supervisor, Dr. hab. inż. Piotr Szweda, for the invaluable guidance and support you provided throughout my PhD studies.

I would like to extend my sincere thanks to my Parents and family, without whom this would not have been possible – thank you for the endless encouragement, support, and attention.

Furthermore, I could not have undertaken this journey without my dearest wife, who has been a source of strength, patience, and motivation throughout this entire experience. I am truly blessed to have you as my partner in this dance called life.

I am also grateful to my friends for supporting me through this journey.

I would be remiss in not mentioning my lab colleagues, especially Dr. Karolina Pelka – thank you for a great time spent together in the lab and during social meetings.

Table of Contents

1. Introduction	11
1.1. The Emerging Challenge of Bacterial Resistance.....	11
1.2. Antibiotic Discovery	12
1.3. Development of Resistance to Antibiotics	16
1.3.1 Natural Resistance	16
1.3.2 Acquired Resistance.....	17
1.3.3 Adaptive Drug Resistance.....	18
1.3.4 Mechanisms of Antibacterial Drug Resistance	19
1.4 ESKAPE Pathogens: General Information and Clinical Significance.....	20
1.4.1 ESKAPE Bacteria	22
1.5 Honey Bees (<i>Apis mellifera</i>)	24
1.5.1 Honey.....	25
1.5.2 Bee Pollen (BP).....	27
1.5.3 Bee Bread.....	28
1.5.4 Propolis	29
1.5.5 Royal Jelly	29
1.6 Microflora of Honey and Other Bee Products.....	31
1.6.1 Taxonomic Characterization of the Bee Product Microbiota	33
Bacteria	33
1.7 Functional Classification of Antimicrobial Peptides and Specialized Metabolites in <i>Bacillus</i> and <i>Lactobacillus</i>.....	36
1.7.1 Ribosomally Synthesized and Post-translationally Modified Peptides (RiPPs).....	37
1.7.2 Non-Ribosomally Synthesized Peptides and Specialized Metabolites (NRPs).....	37
1.8 Profiles of Ribosomal Specialized Metabolites (RiPPs)	38
1.8.1 One-Component Lantibiotics	38
1.8.2 Two-Component Lantibiotics	39
1.8.3 Lasso Peptides.....	39
1.8.4 Sactipeptides	39
1.9 Profiles of Non-Ribosomal Specialized Metabolites (NRPs).....	40
1.9.1 Fengycin.....	40
1.9.2 Bacitracin	40
1.9.3 Lichenysin.....	41
1.9.4 Butirosin A/Butirosin B	41
1.9.5 Plipastatin.....	41
1.9.6 Bacilysin	41
1.9.7 Bacillibactin	42
1.9.8 Surfactin.....	42

2. Aim and Objectives of the Research	45
2.1 Aim	45
2.2 Objectives	45
Included Publications	47
Whole-genome Sequencing and Antimicrobial Potential of Bacteria Isolated from Polish Honey	50
Probiotic Potential of <i>Bacillus</i> Isolates from Polish Bee Pollen and Bee Bread	54
In Silico Safety Assessment of <i>Bacillus</i> Isolated from Polish Bee Pollen and Bee Bread as Novel Probiotic Candidates	58
<i>In Silico</i> Functional Annotation and Structural Characterization of Hypothetical Proteins in <i>Bacillus paralicheniformis</i> and <i>Bacillus subtilis</i> Isolated from Honey	63
Genomic and Functional Landscape of Antimicrobial Peptides and Lipopeptides from Ginger Honey-derived <i>Bacillus subtilis</i>	68
3. Unpublished Data and Results	72
3.1 Discovery of a Novel Two-Component Lantibiotic, Paralichenidin (Pld): A Relative of Amyloliquecin GF610	72
3.1.1 Bioinformatic Identification of the Biosynthetic Gene Cluster	72
3.1.2. Organization and Architecture of the Paralichenidin Operon	72
3.1.3. Transport and Immunity Determinants	73
3.1.4. Regulatory and Processing Elements	74
3.2 Deduced Peptide Sequences, Post-Translational Modifications, and Phylogenetic Analysis of Paralichenidin Prepropeptides and Core Peptides	75
3.2.1 Sequence Conservation and Variation in the Pld α Core Peptides	77
3.2.2. Sequence Conservation and Variation in the Pld β Core Peptides	78
3.2.3. Phylogenetic Analysis of Prepropeptide and Core Peptide Sequences	79
3.3. Predicted Structure and Bridge Formations	82
3.4. Structural modeling and Molecular dynamics simulations of Lipid II and α-/β-peptide–Lipid II complexes	83
4. Overview of Key Research Results from the Constituent Publications	90
4.1. Conclusions	93
Acknowledgments	96
Scientific Achievements	97
5. References	99
List of Figures	119
List of Tables	123

List of Important Symbols and Abbreviations

AIDS – Acquired Immunodeficiency Syndrome
AMR – Antimicrobial Resistance
AOI – Areas of Interest
BP – Bee Pollen
BB – Bee Bread
BHI – Brain Heart Infusion Broth
BLAST – Basic Local Alignment Search Tool
MRSA - Methicillin-resistant *Staphylococcus aureus*
CLSI – Clinical and Laboratory Standards Institute
CTAB – Cetyl Trimethylammonium Bromide
DNA – Deoxyribonucleic Acid
EEA – European Economic Area,
EARS-Net – European Antimicrobial Resistance Surveillance Network
GBDP – Genome Blast Distance Phylogeny
GIT – Gastrointestinal Tract
GRAS – Generally Recognized as Safe
GRAS – Global Antibiotic Resistance Surveillance Report
HIV – Human Immunodeficiency Virus
iTOL – Interactive Tree of Life
LA – Luria Bertani Agar
LAB – Lactic Acid Bacteria
LB – Luria Bertani Broth
MD – Molecular Dynamics
MDR – Multi-drug resistant
MHB – Mueller Hinton Broth
MIC – Minimal Inhibitory Concentration
MRJP1 – Major Royal Jelly Protein 1
MUSCLE – Multiple Sequence Comparison by Log- Expectation
MW – Molecular Weight
NCBI – National Center for Biotechnology Information

OD₆₀₀ – Optical Density at 600nm

ORF – Open Reading Frame

OrthoANI – Orthologous Average Nucleotide Identity

Paralichenidin – Pld

PBPs – Penicillin-Binding Proteins

PBS – Phosphate Buffer Saline

PGAP – Prokaryotic Genome Annotation Pipeline

ROS – Reactive Oxygen Species

RNA – Ribonucleic Acid

SD – Standard Deviation

SDS-PAGE – Sodium Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis

SPAdes – St. Petersburg Genome Assembler

TYGS – Type Genome Server

WHO – World Health Organization

VRSA - Vancomycin-Resistant *Staphylococcus aureus*

YPD – Yeast Extract-Peptone-Dextrose Broth

1. Introduction

1.1. The Emerging Challenge of Bacterial Resistance

Antibiotic resistance is a significant global health problem. Pathogenic microorganisms, especially bacteria, have exhibited increased resistance to current antibiotics over the past two to three decades [1]. A predicted statistical model published in *The Lancet* (2021) revealed antibiotic resistance as a leading cause of mortality globally, particularly prevalent in low-resource environments [2]. In 2019, an estimated 4.95 million deaths were associated with bacterial resistance [2]. The analysis included data from 204 countries, covering 471 million individual records or isolates and 7585 study locations [2]. It evaluated 23 major pathogens, 18 antibiotic classes or combinations, and 88 pathogen drug combinations [2].

Recent surveillance data further confirm the escalation of resistance. The Global Antibiotic Resistance Surveillance Report (GRAS) 2025 indicated that between 2018 and 2023, resistance levels increased in nearly 44% of monitored pathogen-antibiotic combinations [3]. This report focused on eight common bacterial pathogens, including *Acinetobacter* spp., *Escherichia coli*, *Klebsiella pneumoniae*, *Neisseria gonorrhoeae*, non-typhoidal *Salmonella* spp., *Shigella* spp., *Staphylococcus aureus*, and *Streptococcus pneumoniae*. The analysis evaluated resistance to 22 antibiotics or 11 combinations of antibiotic classes and examined 93 pathogen-antibiotic combinations across various illness types in both global and regional assessments [3].

Following the so-called golden age of antibiotics, the development of new antibiotics has declined sharply [4]. Between 2000 and 2020, only 27 antibiotics were introduced for commercial use [5]. In contrast, the FDA approved approximately 270 antibiotics for clinical application between 1928 and the 1970s [6]. This decline, combined with increasing resistance, has intensified the global health challenge. A significant concern is associated with the ESKAPE group of pathogens, which includes *Enterococcus faecium*, *S. aureus*, *K. pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species. These organisms are classified as priority pathogens due to their ability to evade the immune system and cause severe healthcare-associated infections. Global estimates indicate that ESKAPE pathogens accounted for more than 70% of antimicrobial resistance-related deaths in 2019 [2,7].

At the regional level, the burden of antimicrobial resistance remains substantial. Data from the European Antimicrobial Resistance Surveillance Network (EARS-Net) reported more than 800,000 infections caused by antibiotic-resistant bacteria, resulting in over 35,000 deaths

attributable to these infections. This corresponds to approximately 181 infections per 100,000 population in the EU and EEA, with more than 70% occurring in healthcare settings. The attributable mortality rate was estimated at 7.9 per 100,000 population. The leading pathogens contributing to this burden included third-generation cephalosporin-resistant *E. coli*, methicillin-resistant *S. aureus* (MRSA), and carbapenem-resistant *K. pneumoniae*. The overall disease burden is comparable to that of influenza, tuberculosis, and HIV/AIDS combined. Although some pathogens, such as MRSA, have shown declining trends in certain regions, infections caused by carbapenem-resistant *K. pneumoniae* increased by nearly 50% between 2019 and 2022. Overall, the burden of antimicrobial resistance in the EU and EEA increased between 2016 and 2020 [8,9]. Thus, there is an urgent need for new, inexpensive, effective, and safe antibiotics or other alternative antimicrobial agents (e.g., bacteriocins, phages, or natural compounds) [10].

1.2. Antibiotic Discovery

Before the discovery of antibiotics in the early 20th century, infectious diseases were mainly treated through a diverse array of traditional, herbal, and alchemical remedies, many of which had been used for thousands of years. These treatments aimed to boost the body's natural defenses, reducing visible signs of infection, or act as antiseptics, often guided by the doctrine of signatures or empirical knowledge passed down through generations. During the medieval era, species of the genus *Scabiosa* (such as *Scabiosa columbaria* and *Scabiosa succisa*) were considered near-universal remedies. Their infusions were commonly used to treat skin infections, plague, and various internal inflammatory conditions [11].

Honey bee products, particularly honey and propolis, have been used for thousands of years in the treatment of wounds and infections. Modern research has substantiated these traditional applications, demonstrating that honey, bee pollen, and bee bread exhibit antimicrobial activity against pathogens such as *S. aureus* [12]. Clay from Lemnos, known historically as *Terra Sigillata* ("sealed earth"), was valued for over 2,500 years as a medicinal remedy. Referred to as Lemnian Earth, it was traditionally used to treat poisoning, dysentery, plague, and wound infections. Recent scientific investigations have suggested that this clay, particularly when associated with specific fungi or microbial communities, can exhibit antibacterial activity against pathogens such as *S. aureus* and *P. aeruginosa*, supporting aspects of its longstanding therapeutic reputation [13].

In 1910, Paul Ehrlich synthesized arsphenamine, also known as Salvarsan, the arsenic compound referred to as "compound 606". It showed strong activity against *Treponema pallidum* and became

the first effective treatment for syphilis. This work marked one of the earliest examples of rational drug design [14]. In 1932, chemists at Bayer, including Josef Klarer, Fritz Mietzsch, and Gerhard Domagk, developed the sulfonamide dye derivative sulfonamidochrysoidine, later marketed as Prontosil. This compound demonstrated antibacterial activity *in vivo* and initiated the sulfonamide era. The discovery of penicillin represents a pivotal moment in antimicrobial therapy. In 1928, Alexander Fleming observed that a mold of the genus *Penicillium* inhibited the growth of *S. aureus* on an agar plate. Later, Howard Florey and Ernst Boris Chain developed methods to purify and produce penicillin in a stable, water-soluble form suitable for clinical use [14]. The success of penicillin established the concept of exploiting microorganisms to combat infectious pathogens. Building on this principle, Selman Waksman investigated soil *Actinomycetes* and identified several antimicrobial compounds, including streptomycin. His work significantly contributed to the period known as the Golden Era of antibiotics, spanning roughly from the 1940s to the 1960s [1]. An overview of the timeline regarding the discovery and approval of various antibiotics is presented in **Figure 1**; further details regarding the antibiotics, including their respective mechanisms of action, origins, and target pathogens, are summarized in **Table 1**.

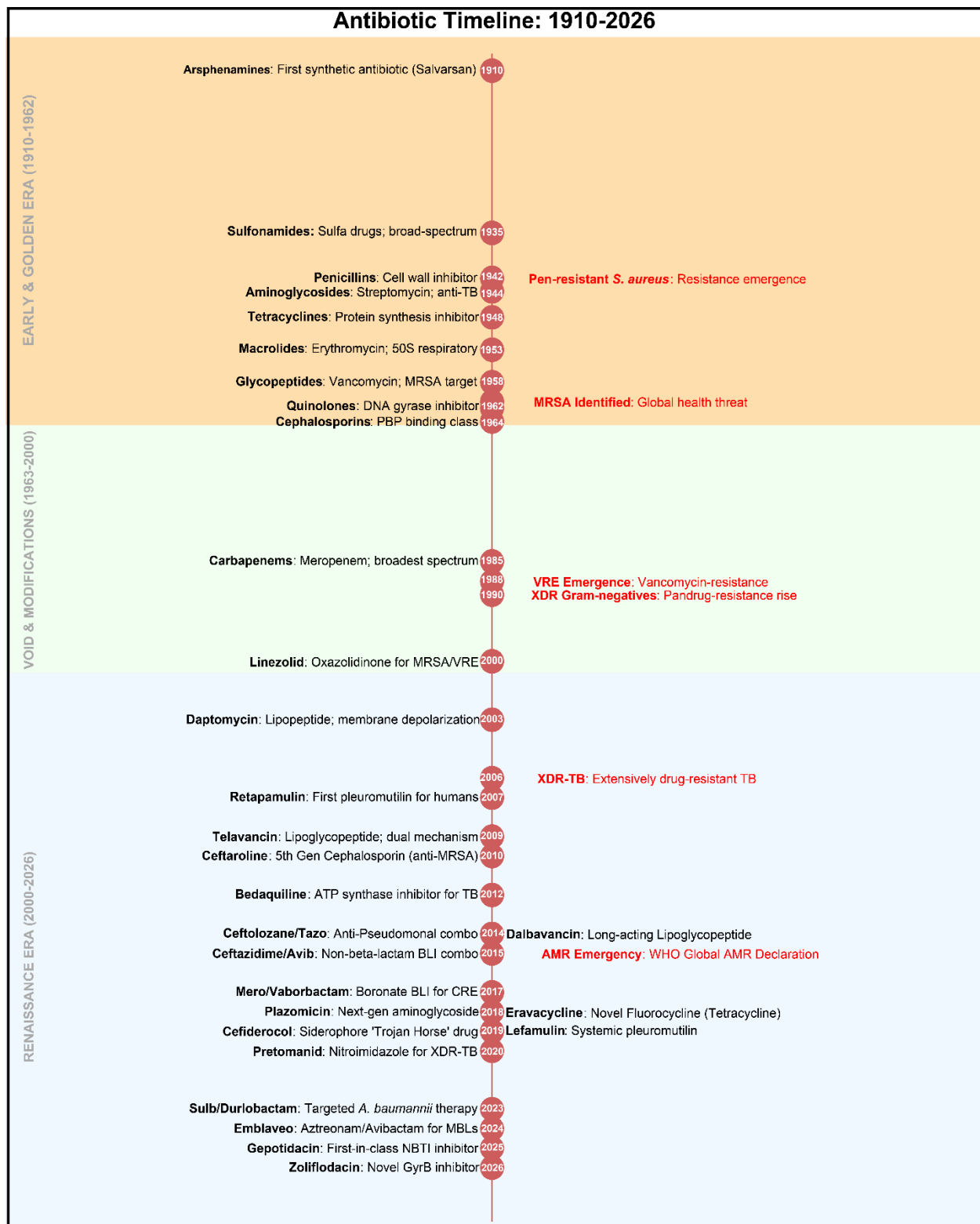


Figure 1. The Evolution of Antimicrobial Innovation and Resistance Milestones. A vertical chronology from 1910 to 2026, highlighting the "Golden Era" of discovery, the subsequent "Discovery Void," and the modern "Renaissance" era. Figure Created in Adobe Illustrator.

Table 1. Classification of selected antibiotics: mechanisms of action, sources, and antimicrobial spectra.

Drug Name	Mode of Action	Source	Target Pathogen	References
Penicillin G	Inhibits cell wall synthesis (PBPs)	Natural (<i>Penicillium chrysogenum</i>)	Gram-positive/negative cocci	[15]
Vancomycin	Inhibits cell wall synthesis (Lipid II binding)	Natural (<i>Amycolatopsis orientalis</i>)	Gram-positive (MRSA, VRE)	[16]
Fosfomycin	Inhibits MurA enzyme (cell wall initiation)	Natural (<i>Streptomyces</i> spp.)	Gram-negative/Gram-positive	[17]
Pivmecillinam	Specifically inhibits PBP2 (cell wall)	Semi-synthetic	Gram-negative uUTI (<i>E. coli</i>)	[18]
Cefiderocol	Siderophore-mediated PBP inhibition	Synthetic/Semi-synthetic	MDR Gram-negative (CR-E, CR-PA)	[19]
Gentamicin	30S Ribosome (induces mRNA misreading)	Natural (<i>Micromonospora purpurea</i>)	Gram-negative pathogens	[20]
Doxycycline	30S Ribosome (blocks aminoacyl-tRNA)	Semi-synthetic (from <i>Streptomyces</i>)	Broad-spectrum/Intracellulars	[21]
Erythromycin	50S Ribosome (inhibits translocation)	Natural (<i>Saccharopolyspora erythraea</i>)	Gram-positive/Atypical bacteria	[22]
Linezolid	50S Ribosome (inhibits initiation complex)	Synthetic	MDR Gram-positives (VRE, MRSA)	[23]
Ciprofloxacin	Inhibits DNA Gyrase and Topoisomerase IV	Synthetic	Broad-spectrum/Gram-negative	[24]
Zoliflodacin	Inhibits Gyrase B subunit (novel site)	Synthetic (Spiropyrimidinetrione)	<i>Neisseria gonorrhoeae</i>	[25]
Gepotidacin	Balanced Gyrase/Topo IV inhibition	Synthetic (Triazaacenaphthylene)	UTI pathogens (<i>E. coli</i>)	[25]
Rifampin	Inhibits RNA Polymerase (Transcription)	Natural (<i>Amycolatopsis rifamycinica</i>)	<i>M. tuberculosis</i> , Gram-positive	[26]

Drug Name	Mode of Action	Source	Target Pathogen	References
Pretomanid	Mycolic acid inhibition/Nitrogen release	Synthetic	MDR/XDR <i>Mycobacterium tuberculosis</i>	[27]
Sulfonamides	Inhibits Dihydropteroate synthase	Synthetic	Broad-spectrum	[28]
Trimethoprim	Inhibits Dihydrofolate reductase	Synthetic	Broad-spectrum	[29]
Daptomycin	Cell membrane depolarization	Natural (<i>Streptomyces roseosporus</i>)	Gram-positive (MRSA, VRE)	[30]
Colistin	LPS binding and membrane disruption	Natural (<i>Paenibacillus polymyxa</i>)	MDR Gram-negative	[31]
Sulopenem	Inhibits cell wall synthesis (Oral Penem)	Synthetic	ESBL <i>Enterobacteriaceae</i> (uUTI)	[32]
Emblaveo	PBP3 inhibition + BLI protection	Synthetic	MBL-producing <i>Enterobacterales</i>	[33]

1.3. Development of Resistance to Antibiotics

The overuse and misuse of antibiotics in human medicine, inappropriate prescribing practices, and their extensive application in agriculture have significantly accelerated the emergence and dissemination of antibiotic resistance [34]. Bacterial resistance can be broadly classified into three main categories based on the mechanism of development: intrinsic resistance, acquired resistance, and adaptive resistance [35–37].

1.3.1 Natural Resistance

Natural resistance includes intrinsic and inducible (naturally induced) resistance. In both cases, resistance genes are naturally present within the bacterial genome. Intrinsic resistance is a species-specific, inherent characteristic that exists independently of prior antibiotic exposure and is not associated with horizontal gene transfer (HGT) [38–40]. The most common mechanisms of intrinsic resistance include reduced outer membrane permeability, particularly due to lipopolysaccharides (LPS) in Gram-negative bacteria, restricted drug entry through porins, and the

natural expression of efflux pumps. For example, cell wall–deficient bacteria, such as *Mycoplasma* and *Ureaplasma*, are intrinsically resistant to β -lactam antibiotics [41]. Gram-negative bacteria are intrinsically resistant to vancomycin due to the impermeability of the outer membrane to large glycopeptides [42].

Anaerobic bacteria exhibit intrinsic resistance to aminoglycosides because drug uptake requires oxygen-dependent transport mechanisms [43], whereas obligate aerobic bacteria are resistant to metronidazole due to their inability to reduce the drug to its active form [44]. Intrinsic resistance is clinically important because it defines the inherent susceptibility profile of bacterial species, thereby guiding empirical therapy selection.

1.3.2 Acquired Resistance

Acquired resistance develops when a previously susceptible bacterium becomes resistant through genetic changes, such as mutations or HGT [37]. It arises from changes, such as mutations, within the chromosomal sequence or through the acquisition of mobile genetic elements (MGEs), such as plasmids and transposons [45]. Chromosomal resistance may alter drug targets, reduce membrane permeability, or modify metabolic pathways [37]. Extrachromosomal resistance involves MGEs, such as plasmids and transposons, which are often associated with integrons. Plasmids frequently carry resistance genes encoding diverse mechanisms, including enzymatic inactivation of antibiotics, efflux pumps, and target modification. Transposons are mobile sequences capable of inserting resistance genes into chromosomes or plasmids, which facilitates their dissemination. Integrons facilitate the accumulation and expression of multiple resistance gene cassettes at specific integration sites. Because these elements transfer between bacteria, extrachromosomal resistance spreads rapidly and often leads to multidrug resistance [37]. The accumulation of resistance through acquired and, to a lesser extent, intrinsic and adaptive mechanisms can ultimately manifest as cross-resistance, multidrug resistance, and pan-drug resistance.

Cross Resistance

Cross-resistance occurs when resistance to one antibiotic confers resistance to other agents with similar mechanisms of action. This is common among structurally related drugs, such as penicillins and cephalosporins. However, cross-resistance may also occur between unrelated drug classes, such as erythromycin and lincomycin [37].

Multi-drug Resistance and Pan-resistance

Multidrug-resistant bacteria resist at least three classes of antimicrobials. This phenotype develops through the accumulation of multiple resistance genes or the overexpression of broad-spectrum mechanisms such as efflux pumps and enzymatic inactivation. Extensively drug-resistant (XDR) strains are resistant to all but one or two antimicrobial classes, whereas pan-drug-resistant (PDR) strains are resistant to all available antibiotics [41,46].

1.3.3 Adaptive Drug Resistance

Adaptive resistance is a reversible, transient response to environmental stimuli. Bacteria temporarily adjust gene expression in response to stress, nutrient limitation, pH shifts, ionic stress, growth phase, or sub-inhibitory antibiotic exposure. Once the stimulus is removed, susceptibility typically returns. Unlike intrinsic or acquired resistance, adaptive resistance does not involve stable genetic changes [47,48]. **Figure 2** illustrates the classification of antibacterial resistance, categorized by resistance type and specific mechanisms.

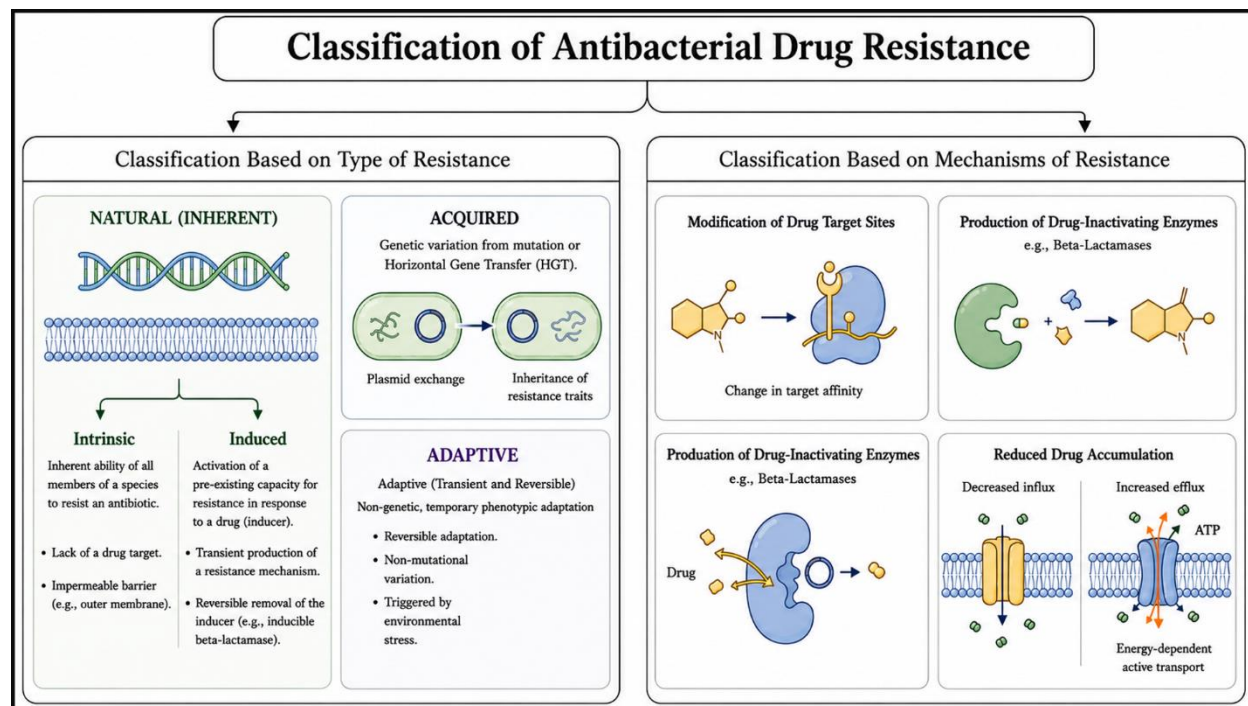


FIGURE 2. Systematic classification of antibacterial drug resistance. The schematic illustrates the major categories of antibacterial drug resistance based on two complementary criteria: **(Left Panel)** the type of resistance, comprising natural (intrinsic and induced), acquired, and adaptive resistance; and **(Right Panel)** the underlying mechanisms of resistance, including modification of drug target sites, enzymatic inactivation of antimicrobial agents, and reduced

intracellular drug accumulation through decreased membrane permeability (reduced influx) or enhanced efflux pump activity. Together, these mechanisms enable bacterial survival and persistence under antimicrobial stress. Figure Created in Adobe Illustrator.

1.3.4 Mechanisms of Antibacterial Drug Resistance

With the global rise in antimicrobial resistance (AMR), understanding the molecular and physiological mechanisms by which bacteria resist antibacterials has become essential for mitigating this crisis [49]. For an antibacterial to be effective, it must reach its target at sufficient concentration, bind appropriately, and disrupt a vital cellular process. Bacteria can survive antibacterial exposure by interfering with one or more of these critical steps [50]. **Figure 3** provides a schematic representation of various antimicrobial target sites alongside the diverse biochemical strategies employed by bacteria to achieve resistance.

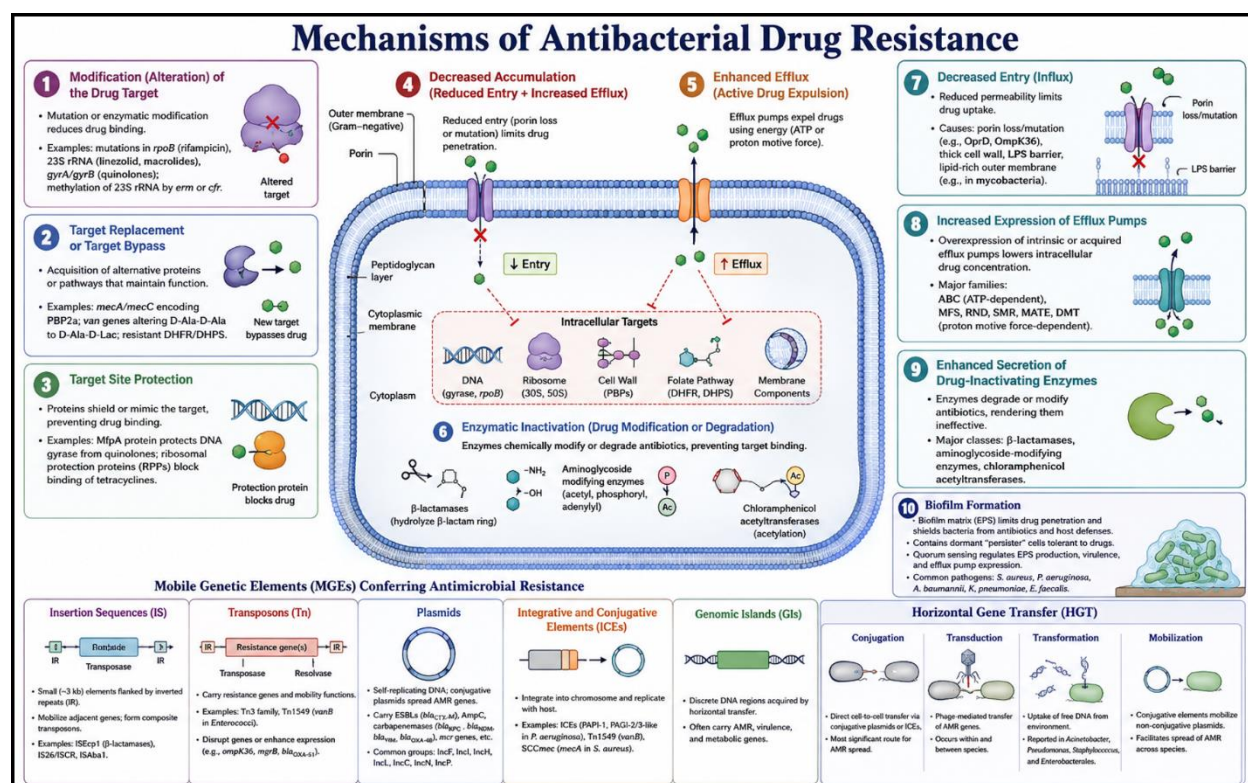


Figure 3. Mechanisms of antibacterial drug resistance and dissemination of antimicrobial resistance determinants. Schematic representation of the major molecular, physiological, and genetic mechanisms by which bacteria resist antibacterial agents. **(1)** Modification (alteration) of the drug target reduces antibacterial binding through mutations or enzymatic modification of cellular targets, including ribosomal RNA, DNA gyrase, RNA polymerase, and penicillin-binding proteins. **(2)** Target replacement or target bypass involves the acquisition of alternative proteins or metabolic pathways that maintain essential cellular functions despite antibacterial exposure, such as PBP2a-mediated methicillin

resistance and resistant variants of DHFR and DHPS. **(3)** Target site protection occurs when bacterial proteins shield or mimic the antibacterial target, preventing effective drug binding, as observed with ribosomal protection proteins and the DNA gyrase-protecting protein MfpA. **(4)** Decreased accumulation results from a combination of reduced drug uptake and enhanced drug efflux, leading to subinhibitory intracellular antibacterial concentrations. **(5)** Enhanced efflux is mediated by energy-dependent transport systems that actively export antibacterials from the bacterial cell, including members of the ABC, MFS, RND, SMR, MATE, and DMT transporter families. **(6)** Enzymatic inactivation involves degradation or chemical modification of antibacterial molecules through mechanisms such as β -lactam hydrolysis, aminoglycoside acetylation, phosphorylation, adenylation, and chloramphenicol acetylation. **(7)** Decreased entry (influx) occurs through reduced membrane permeability caused by loss or alteration of porins, changes in outer membrane composition, or other permeability barriers that limit antibacterial penetration. **(8)** Increased expression of efflux pumps further reduces intracellular drug concentrations and contributes substantially to multidrug resistance, particularly in Gram-negative pathogens. **(9)** Enhanced secretion or production of antibacterial-inactivating enzymes enables extracellular or periplasmic degradation of antibacterials before they reach their targets, with β -lactamases representing the most clinically significant example. **(10)** Biofilm formation protects bacterial populations through the production of an extracellular polymeric substance (EPS) matrix, reduced drug penetration, altered metabolic activity, quorum-sensing-regulated responses, and the persistence of dormant cells. The lower section illustrates the principal mobile genetic elements (MGEs) responsible for the acquisition and dissemination of antimicrobial resistance genes, including insertion sequences (ISs), transposons (Tn), plasmids, integrative and conjugative elements (ICEs), and genomic islands (GIs). These elements facilitate the mobilization and spread of resistance determinants, including genes encoding extended-spectrum β -lactamases (ESBLs), carbapenemases, aminoglycoside-modifying enzymes, and colistin resistance factors. Dissemination of antimicrobial resistance is further driven by horizontal gene transfer (HGT) through conjugation, transduction, transformation, and plasmid mobilization. Collectively, these interconnected mechanisms contribute to the emergence, persistence, and global spread of multidrug-resistant bacteria. Figure Created in Adobe Illustrator.

1.4 ESKAPE Pathogens: General Information and Clinical Significance

Multidrug-resistant (MDR) ESKAPE pathogens, including *E. faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *Enterobacter* species, represent a major global health threat [51]. These organisms are responsible for a large proportion of healthcare-associated infections, with carbapenem-resistant *A. baumannii* and *P. aeruginosa* contributing substantially to morbidity and mortality in intensive care units. Their persistence is frequently linked to suboptimal infection control practices, which enable the maintenance and dissemination of resistance plasmids and facilitate recurrent or polymicrobial infections that are increasingly difficult to treat [52]. The World Health Organization (WHO) has designated ESKAPE pathogens as priority organisms for research and drug development due to their extensive resistance and limited treatment options [53].

The clinical success of ESKAPE pathogens is multifactorial. A key feature is their ability to form biofilms, which enhance surface adherence and protect against antimicrobial agents and host immune responses [51]. These pathogens also produce toxins and degradative enzymes that promote tissue damage and immune evasion. In addition, extensive genomic plasticity and horizontal gene transfer through plasmids and other mobile genetic elements facilitate rapid acquisition and dissemination of resistance determinants [54].

Despite extensive antimicrobial research, few fundamentally new antibiotic classes have reached clinical use. Most recently approved therapies involve reformulations or combinations of existing drugs rather than novel scaffolds. This limitation is concerning because MDR ESKAPE pathogens are increasingly associated with community-acquired infections in addition to healthcare settings [55]. Resistance in these organisms is linked to treatment failure, prolonged hospitalization, increased mortality, and major economic burden [10,56]. New therapeutic strategies include pathogen-directed approaches that target virulence mechanisms and host-directed therapies that modulate immune responses [57]. Antimicrobial peptides (AMPs) have also attracted interest because of their broad-spectrum activity, rapid bactericidal effects, and lower tendency to induce resistance [58–60]. AMPs primarily target negatively charged bacterial membranes, although intracellular effects have also been reported. However, their clinical application remains limited by proteolytic instability, low bioavailability, and potential cytotoxicity. Structural optimization and chemical modification are being explored to overcome these limitations [10]. **Figure 4** illustrates the ESKAPE pathogen consortium and associated infections.

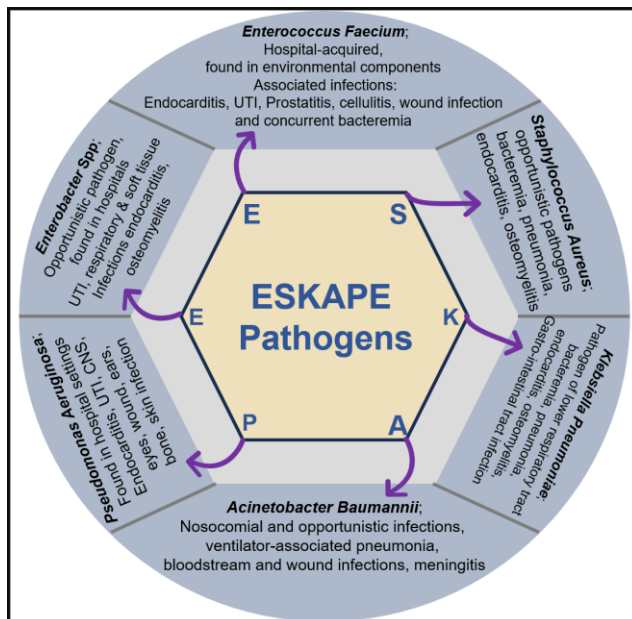


Figure 4. The ESKAPE pathogen consortium and its role in biofilm-associated infections. Figure Created in Adobe Illustrator.

1.4.1 ESKAPE Bacteria

i. E. faecium

E. faecium has emerged since the 1980s as a dominant nosocomial pathogen, largely due to its adaptive capacity within hospital environments. This adaptation is mediated by pathogenicity islands, mobile genetic elements, and the accumulation of resistance determinants such as *vanA*, alongside virulence attributes including biofilm formation [61]. Although typically a commensal organism of the gastrointestinal microbiota, *E. faecium* can cause severe infections in immunocompromised individuals, including urinary tract infections, bacteremia, and endocarditis. Clinical isolates frequently exhibit resistance to penicillins, fluoroquinolones, macrolides, and tetracyclines [62,63]. The emergence of vancomycin-resistant *E. faecium* (VREfm) significantly narrows therapeutic options and enhances environmental persistence in hospital settings, contributing to recurrent outbreaks. Consequently, VREfm has been designated a high-priority pathogen by the WHO for antimicrobial research and development [62,63].

ii. *S. aureus*

S. aureus colonizes the skin and mucosal surfaces and is capable of causing invasive diseases such as bacteremia, pneumonia, and endocarditis [64]. Its pathogenicity is supported by an extensive repertoire of immune evasion mechanisms, including inhibition of B- and T-cell proliferation, disruption of phagocyte function, suppression of oxidative killing, and complement interference [65]. These mechanisms facilitate recurrent and persistent infections. The organism demonstrates resistance to multiple antimicrobial classes, including β -lactams, glycopeptides, and oxazolidinones. Vancomycin-resistant *S. aureus* (VRSA) strains commonly harbor the *vanA* operon within the *Tn1546* transposon, underscoring the role of horizontal gene transfer in resistance dissemination [66].

iii. *K. pneumoniae*

K. pneumoniae is a Gram-negative opportunistic pathogen responsible for pneumonia, bloodstream infections, urinary tract infections, and liver abscesses in both hospital and community settings. Strains are broadly categorized as opportunistic, hypervirulent, or MDR based on accessory genome composition [67]. MDR strains, frequently carrying plasmid-encoded β -lactamases and carbapenemases, represent a substantial clinical concern. The compromised efficacy of cephalosporins and carbapenems due to enzymatic hydrolysis has contributed to increased mortality and therapeutic failure [7].

iv. *A. baumannii*

A. baumannii is particularly problematic in intensive care units, where it causes respiratory tract, bloodstream, urinary tract, and wound infections [68]. Its resistance profile includes β -lactamases, such as OXA-23, OXA-24/40, and OXA-58, as well as resistance to aminoglycosides, quinolones, and carbapenems [69]. The organism's environmental resilience and biofilm-forming capacity facilitate prolonged survival on hospital surfaces and promote nosocomial transmission. Therapeutic management often necessitates last-resort antimicrobials, increasing both treatment complexity and healthcare costs [70].

v. *P. aeruginosa*

P. aeruginosa exhibits intrinsic and acquired resistance mechanisms that collectively limit therapeutic efficacy [71]. Multidrug efflux systems (*MexAB-OprM*, *MexXY*, *MexCD-OprJ*, *MexEF-OprN*) reduce intracellular antibiotic concentrations [72,73]. β -lactamase production, including AmpC and extended-spectrum β -lactamases, inactivates β -lactam antibiotics [74]. Loss or modification of the OprD porin restricts carbapenem uptake [74], while mutations in *gyrA*, *gyrB*, *parC*, and *parE* confer quinolone resistance [75]. Aminoglycoside-modifying enzymes such as AAC(6')-Ib and APH(3')-IIa further diminish ribosomal binding of aminoglycosides [76]. Robust biofilm formation enhances tolerance to both antimicrobial agents and host immune defenses [77].

vi. *Enterobacter* Species

Enterobacter spp. are increasingly implicated in healthcare-associated infections, including urinary tract infections, pneumonia, bloodstream infections, and wound infections [78]. These organisms possess intrinsic resistance to several β -lactams and fluoroquinolones and have acquired additional mechanisms, such as ESBL and carbapenemase production [79,80]. Their capacity for biofilm formation contributes to persistence on medical devices and complicates eradication strategies.

1.5 Honey Bees (*Apis mellifera*)

Insects play a fundamental role in ecosystem stability, particularly through pollination. Honey bees (*Apis mellifera*) represent one of the most ecologically and economically significant pollinator species. It is estimated that 87.5% of flowering plants rely on animal-mediated pollination [44], and approximately one-third of the global human dietary supply depends directly or indirectly on bee-pollinated crops [81]. Consequently, the ecological services provided by *A. mellifera* extend beyond biodiversity maintenance to global food security. Beyond pollination, honey bees produce a diverse array of biologically active products, including honey, propolis, beeswax, venom, royal jelly, bee pollen, and bee bread. For centuries, people have utilized these products for their nutritional properties and documented biological activities, such as antibacterial, anti-inflammatory, and antioxidant effects [82]. Their functional properties arise from complex

biochemical compositions derived either from plant sources, bee metabolism, or a combination of both.

Honey bees and their products also function as bioindicators of environmental contamination, as they can accumulate pollutants present in their foraging environment, thereby reflecting ecosystem health [83]. From a biological standpoint, beeswax, venom, and royal jelly are synthesized directly by the bees through specialized glands, whereas honey, propolis, pollen, and bee bread originate from plant materials that are subsequently collected, enzymatically modified, and biochemically transformed by the bees [82]. **Figure 5** illustrates the locations of glands in honey bees.

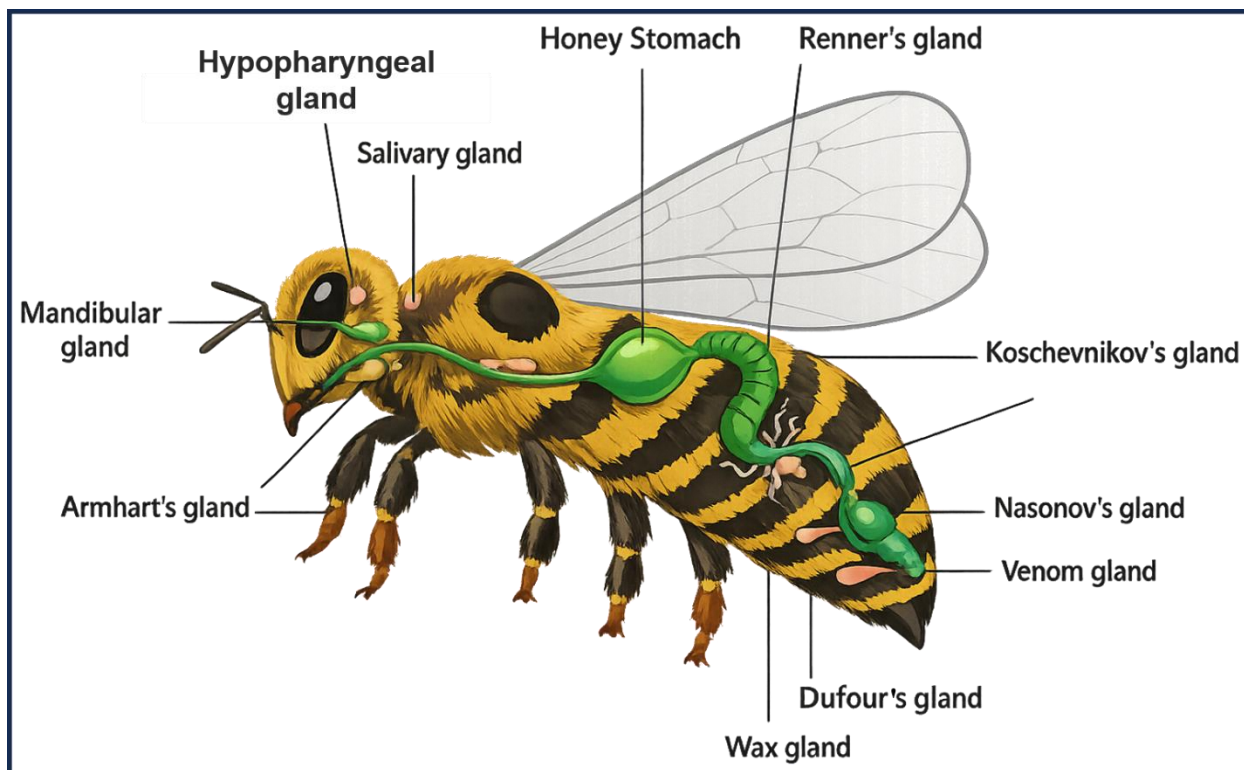


Figure 5. Schematic representation of the anatomical distribution of *Apis mellifera* (Honey bee) exocrine glands associated with colonial ontogeny and the synthesis of bee-derived products. Figure Created in Biorender.

1.5.1 Honey

For centuries, Egyptians, Greeks, Romans, and Chinese civilizations have utilized honey for its various therapeutic properties in wound healing, gastrointestinal ailments, coughs, sore throats, and earaches [26]. As a functional food, it provides energy and nourishment, supporting vital organs within the body [27]. The quality of honey is influenced by its active components, such as glucose, fructose, flavonoids, polyphenols, organic acids, and some enzymes [28]. Various

constituents, including phenolic acids, flavonoids, ascorbic acid, proteins, carotenoids, and specific enzymes like glucose oxidase and catalase, contribute to its health-promoting properties [29].

Composition

Natural honey is a sweet, supersaturated solution consisting of 82.4% carbohydrates, 38.5% fructose, 31% glucose, 12.9% other sugars, and 17.1% water. It also contains 0.5% protein, organic acids, essential minerals, amino acids, vitamins, phenols, and traces of various other compounds [84]. Honey also contains enzymes like glucose oxidase, amylase, catalase, peroxidase, invertase, and lysozyme. Important ingredients of this product are organic acids, e.g., gluconic acid, citric acid, malic acid, and lactic acid. The group of phenolic acids includes derivatives of hydroxycinnamic acid and hydroxybenzoic acid [85]. In honey, naringenin, hesperetin, pinocembrin, chrysin, galangin, quercetin, and kaempferol represent the flavonoid class [86].

Health effects of honey

Honey exhibits several biological activities. Honey has a long-standing tradition in wound care due to its therapeutic properties. Scientific studies have confirmed its efficacy in treating wounds, insect bites, burns, skin disorders, sores, and boils [87]. Honey has shown promising results in healing infected wounds unresponsive to antiseptics or antibiotics, including those infected with antibiotic-resistant bacteria such as methicillin-resistant *S. aureus* (MRSA) [88]. It also plays a beneficial role in wound malodor, often associated with anaerobic bacteria like *Bacteroides* spp. and *Peptostreptococcus* spp., by providing a substantial amount of glucose as a preferred substrate for metabolism over amino acids and replacing malodorous compounds such as ammonia, amines, and sulfur with lactic acid [89]. It has also been used for the management of diabetic foot ulcers [90,91]. Moreover, it has also been used for treating gastrointestinal disorders caused by various pathogenic bacteria and enteropathogens, including *Salmonella* spp., *E. coli*, and *Shigella* spp. [92]. Honey exhibits antimicrobial activity against *Porphyromonas gingivalis*, a gram-negative bacterium responsible for periodontitis [93]. Manuka honey has shown efficacy in treating sore throats. By coating the inner lining of the throat, honey effectively eliminates microbes while providing soothing relief [94,95]. Moreover, honey has also demonstrated its positive effects on metabolic and cardiovascular health [96–99] and exhibits anticancer activity [100–104].

1.5.2 Bee Pollen (BP)

Bee pollen (BP) is flower pollen grains collected by bees and agglutinated using salivary secretions, nectar, and honey to form granules of 1.4 to 4 mm. Pollen is essential for the sexual reproduction of flowering plants and those that produce cones. The primary function of pollen is to provide a nutrient source (particularly amino acids and proteins) for the colony, supporting its growth and maintenance [105,106].

Composition

BP is rich in proteins, essential amino acids, vitamins, fatty acids, and other compounds with functional properties. Plant species, geographical region, and the season of pollen collection significantly influence the specific composition of bee pollen[105–107].

BP is primarily composed of carbohydrates, which constitute two-thirds of its dry weight [108,109]. Monosaccharides, fructose, and glucose are the predominant sugars, accounting for approximately 94% of total sugars in BP [105]. Unlike plant pollen, BP contains a high amount of reducing sugars [110]. Other disaccharides such as sucrose, maltose, turanose, erlose, and trehalose are also present [105]. Additionally, cellulose constitutes around 3–4% of BP and is mainly found in the layers of pollen grains, affecting their digestibility [105]. Proteins are the second most abundant component of BP, comprising 10–40% of its dry weight [106,109,111].

Lipids are another essential nutritional component of BP. Recent studies have reported lipid contents ranging from 1% to 24.4% of dry weight in BP samples [112–116].

BP contains a wide array of polyphenolic compounds, particularly flavonoids and phenolic acids [107]. Ferulic acid and 2-hydroxycinnamic acid are notable for their content levels [105,106,117].

BP also displays a diverse profile of flavonoids, with a predominance of flavonols, flavones, flavanones, flavanonols, and flavan-3-ols [105,106].

Therapeutic properties

BP exhibits several biological activities. It is rich in antioxidants and has been widely utilized to combat oxidative stress [118–121]. BP possesses potential antidiabetic and anti-hyperglycemic effects [122–124]. It has also demonstrated antibacterial, antiviral, and antifungal [125–127] activities, as well as anti-inflammatory activity [120,128].

1.5.3 Bee Bread

Bee bread (BB) is formed when bees collect pollen, mix it with saliva and honey, and pack this mixture into honeycomb cells. Over a period of two weeks, under specific conditions of temperature (35-36°C), moisture, enzyme activity from glandular secretions, and the presence of microorganisms (including bacteria and yeasts), bee bread is formed. BP is processed into BB. During the winter and early spring, BB, along with honey, serves as the sole food source for the bee colony. BB is a lesser-known and less-studied bee product [105,109].

Composition

BB exhibits significant variations in its nutritional composition (including macro- and micronutrients) and non-nutritional bioactive compounds, depending on the geographical and botanical origin of the pollen and the presence of microbiota within [105,109,129]. BB can contain up to 30% water in its fresh weight. Studies have reported carbohydrate proportions ranging from 13% to 72% of its dry weight [105,109]. Proteins in BB constitute approximately 17% to 30% of its dry weight [130]. The lipid content of BB can range from 1.1% to 11.55% of its dry weight [131,132].

Therapeutic properties of BB

BB contains moderate levels of organic acids, including gluconic, formic, acetic, propionic, and butyric acids [133], and may even harbor probiotics [134]. Several studies have identified numerous phenolic molecules in BB [109,129,131,134]. Urcan et al. [135] found that the phenolic profile of BB is similar to that of BP, although some transformations occur during the production of BB. Rutin is the predominant component observed, with significant concentrations of quercetin as well [136]. The antioxidant capacity of BB has been extensively studied due to its phenolic compounds and other constituents. BB has been documented to exhibit comparable or even superior antioxidant capacity relative to honey or propolis [131]. The antibacterial properties of BB have been examined, demonstrating susceptibility to many bacterial strains and fungi in hydromethanolic BB extracts. BB extracts demonstrate superior efficiency against gram-positive bacteria compared to gram-negative bacteria [137]. *In vitro* studies have examined the anticancer efficacy of BB, while research is being conducted on its anti-inflammatory and immunomodulatory properties, antihypertensive effects, hypolipidemic actions, and hepatoprotective capabilities [129].

1.5.4 Propolis

Propolis, commonly known as "bee glue," is a resinous substance collected by bees from diverse plant sources. The term "propolis" originates from the Greek words "pro," meaning "defense," and "polis," meaning "city" or "community," indicating its role in protecting the beehive [138]. Bees utilize propolis to seal openings, repair cracks, and strengthen the structural integrity of the hive. It also serves to smooth the inner surface, maintain the hive's internal temperature at around 35°C, and shield it from external elements and potential predators [138].

Composition

Propolis is a mixture of various chemical constituents. Its composition typically includes lipids (50%), beeswax (30%), essential oils (10%), pollen (5%), and organic components (5%) [139,140]. The organic content of propolis consists of carboxylic acids (20%), terpenoids (15%), steroids (12%), hydrocarbons (10%), sugars (6%), alkaloids (6%), flavonoids (4%), phenols (3%), vitamins (2%), amino acids (2%), ketones (2%), proteins (1%), and other compounds (14%) [141,142]. Among these constituents, flavonoids, polyphenols, carboxylic acids, quercetins, fatty acids, cinnamic acid, esters, and terpenoids such as pinocembrin, galangin, carboxylic acids, caffeic acid, caffeic acid phenethyl ester (CAPE), saponin, phorbol, naringenin, gallic acid, naringin, benzoic acids, amino acids, apigenin, coumaric acid, steroids, vitamins, reducing sugar, and essential oils play significant roles in the biological activities of propolis [143].

Health effects of propolis

Several biological activities have been reported for propolis. It has been used in the treatment of gastrointestinal (GI) disorders [144], bacterial vaginosis (BV) and vulvovaginal candidiasis (VVC) [145,146], gingivitis and periodontitis [147,148], and diabetes mellitus (Type 2) [149,150]. It has also displayed strong anticancer [142,151], anti-inflammatory [142,152,153], and antiviral activity [154,155].

1.5.5 Royal Jelly

Royal jelly (RJ) is a gelatinous and creamy secretion produced by the hypopharyngeal and mandibular glands of young worker bees, known as nurses [156]. It serves as the primary food source for bee larvae until their third day of life. However, the queen bee relies exclusively on RJ for her entire lifespan [157–159]. It has a moderately acidic pH ranging from 3.1 to 3.9, a density

of 1.1g/mL, varies in color from white to yellow, and has a slightly pungent odor and taste [140,160].

Composition

RJ primarily consists of water (60-70%), carbohydrates, proteins, free amino acids, lipids, minerals, vitamins, and polyphenols [161]. The carbohydrate content ranges from 7% to 18%, with glucose and fructose being the predominant sugars. The protein content varies between 9% and 18%, with the major royal jelly proteins (MRJPs) comprising approximately 80% of the total proteins. MRJPs consist of nine members (MRJP1–MRJP9) and play essential roles in the development of the queen bee [162,163]. RJ contains other proteins in small amounts, such as aspimin, jelleines, royalisin, and small peptides like dipeptides, which possess antioxidative and antibacterial activities [164,165]. The lipid fraction of RJ comprises 3-8% of its composition and includes fatty acids, waxes, phenols, steroids, and phospholipids [166].

Health Benefits of Royal Jelly

RJ possesses various biological activities, including antibacterial, immunomodulatory, vasodilative, hypotensive, antihypercholesterolemic, and antitumor properties [142,152]. It has shown positive effects on reproductive health, and has demonstrated its potential in neurodegenerative and aging diseases [142,152]. **Figure 6** presents a Venn diagram showing the shared and distinct bioactive and therapeutic properties of honey, bee pollen, and bee bread.

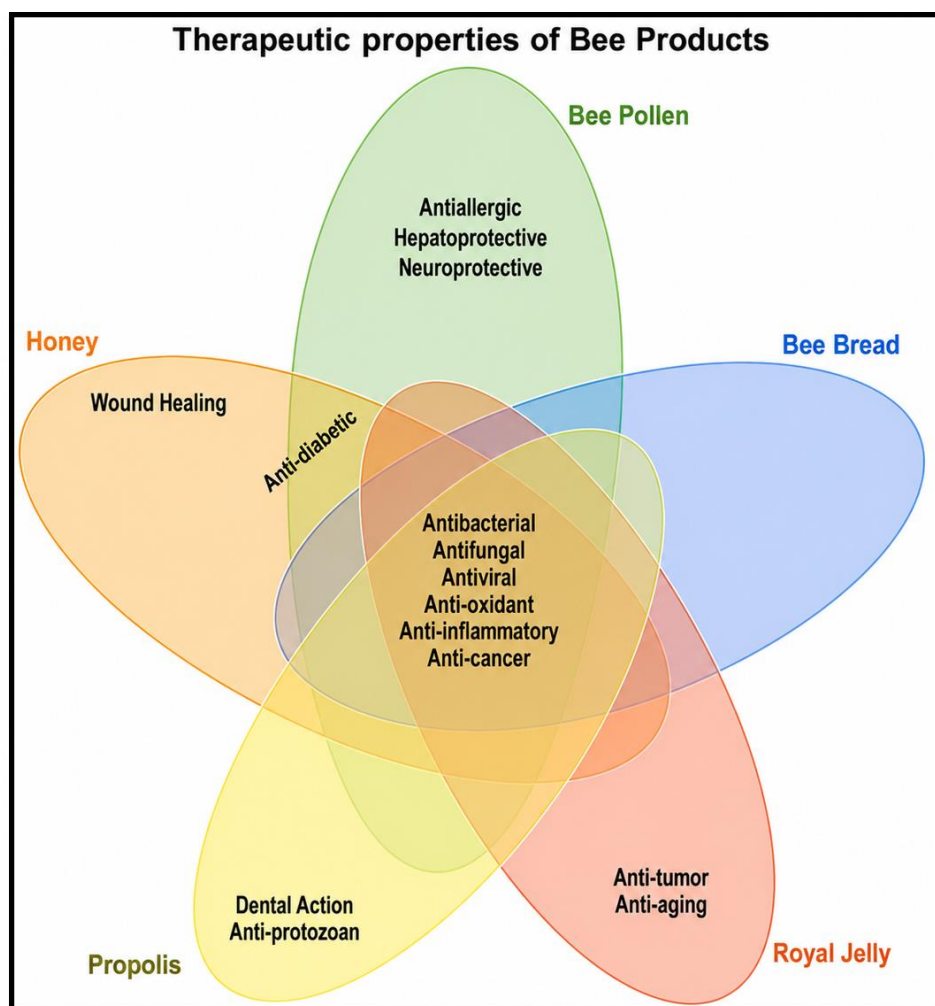


Figure 6. Schematic representation of the bioactive properties and therapeutic potential of honey, bee pollen, and bee bread. Figure Created in Adobe Illustrator.

1.6 Microflora of Honey and Other Bee Products

The microbial ecology of beehive products represents a highly specialized and dynamic interface in which botanical resources, insect physiology, and environmental microbiology converge. Honey bees (*A. mellifera*) do not simply collect nectar and pollen; rather, they function as biological mediators, introducing and selecting microorganisms that contribute to preservation, nutrient transformation, and colony defense. Through enzymatic activity, regurgitation, and hive-associated processing, bees inoculate raw substrates with microbial communities that influence the biochemical maturation of honey, bee pollen, and bee bread.

The isolation and characterization of microorganisms from honey and related bee products give insight into this complex socio-ecological system. The resulting microbiome reflects a balance

between environmental acquisition and host-driven selection pressures, including osmotic stress, low pH, antimicrobial compounds, and hive-specific conditions. This dynamic microbial equilibrium plays a role not only in product stability but also in shaping the overall health of the colony. Recently, increasing attention has been directed toward the microflora of pollinating insects, particularly in the context of declining populations of honey bees (*Apis mellifera* L.) and bumblebees (*Bombus* spp.) [167]. Understanding the composition and function of bee-associated microbial communities is therefore essential for elucidating factors contributing to colony health and resilience. A diverse array of microorganisms—including bacteria, molds, and yeasts—has been identified in association with honey bees and their products [168]. These microbial populations may originate from floral sources, environmental exposure, or the bee gut, and their persistence is influenced by both ecological conditions and bee-mediated selective mechanisms. **Figure 7** presents Core Microbial Consortia shared across honey, bee pollen, and bee bread.

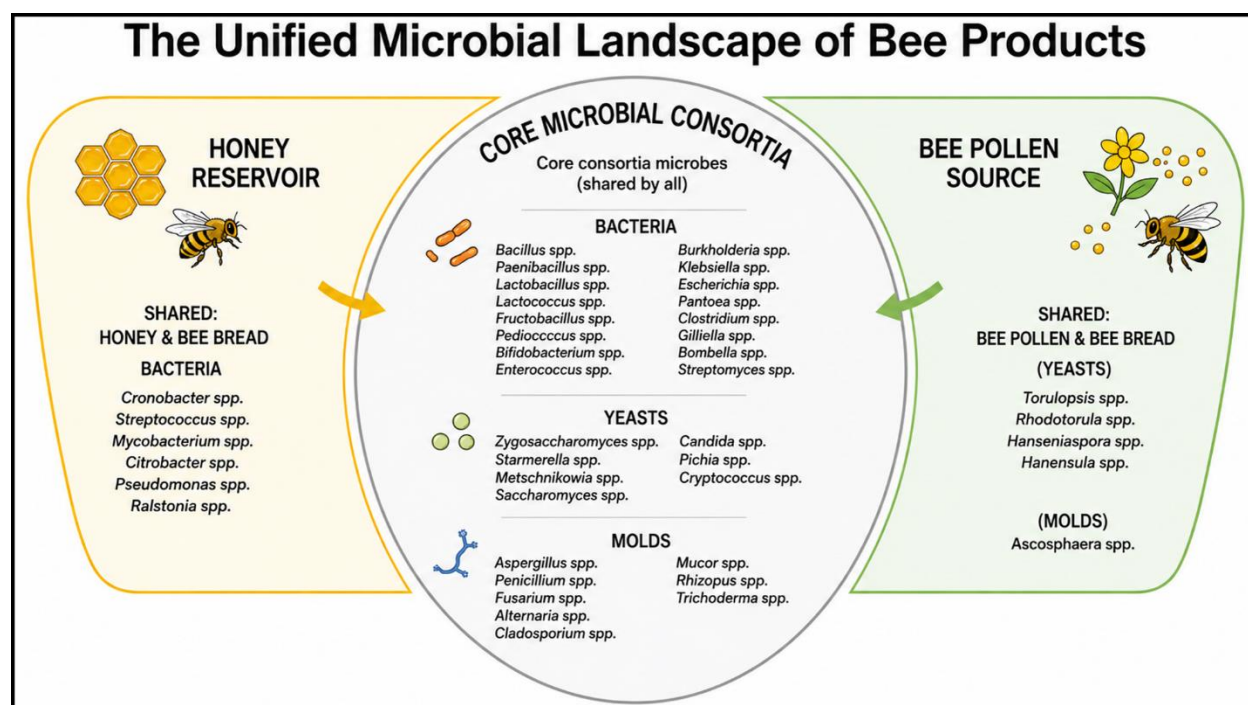


Figure 7. Diagrammatic overview of the shared and unique microbiota of honey, bee pollen, and bee bread. The central node delineates the Core Microbial Consortia shared across all three matrices, systematically categorized into distinct bacterial, yeast, and mold genera (e.g., *Lactobacillus* spp., *Zygosaccharomyces* spp., and *Aspergillus* spp.). **Left:** The Honey Reservoir highlights taxa unique to honey or selectively shared with bee bread (e.g., *Cronobacter* spp., *Pseudomonas* spp.). **Right:** The Bee Pollen source traces the introduction of environmental fungi and yeasts (e.g., *Torulopsis* spp., *Ascosphaera* spp.). Figure Created in Adobe Illustrator.

1.6.1 Taxonomic Characterization of the Bee Product Microbiota

The bacterial community in honey reflects the honey bee's interactions with its environment and its own internal flora. Despite its inhibitory properties, honey is not sterile. Microbiological surveys have identified a diverse range of bacteria, primarily in dormant or spore forms, that enter the product during nectar collection, processing, and storage.

Bacteria

i. Bacillaceae

The *Bacillaceae* family comprises rod-shaped, spore-forming bacteria, including aerobic or facultatively anaerobic *Bacillus* spp. and anaerobic *Clostridium* spp. [169]. Endospore formation enables survival under harsh conditions such as high osmolarity, low water activity, and acidity [170], and antimicrobial compounds present in honey [171,172], bee pollen, and bee bread [173]. Members of *Bacillaceae* were detected across all three products, although abundance varied by product type and geographic origin. In honey, *Bacillus* species isolated from wild samples included strains closely related to *Bacillus safensis* [174], *Bacillus velezensis* [175], *Bacillus paralicheniformis* [171], and *Bacillus subtilis* [172]. These isolates showed antibacterial and antifungal activity [175], demonstrating functional activity rather than simple presence.

In bee pollen, *Bacillus cereus* [176], *Bacillus thuringiensis* [177], and other *Bacillus* spp. were identified, likely originating from plant surfaces and soil [178,179]. In bee bread, *Bacillus* spp. were consistently reported but usually at lower relative abundance compared to dominant fermentative bacteria such as *Lactobacillus* [178,180]. *Paenibacillus* species were also detected. *Paenibacillus alvei* MP1 exhibited antimicrobial activity against *S. aureus* and *Listeria monocytogenes*, whereas *Paenibacillus larvae* was identified in some bee bread datasets as a bee pathogen [181]. Reported *Bacillus* species in bee products include *Bacillus megaterium*, *B. subtilis*, *Bacillus licheniformis*, *Bacillus pumilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *B. cereus*, *Bacillus altitudinis*, and *Bacillus circulans* [172,182–189]. These species produce diverse secondary metabolites such as lipopeptides, including surfactins, iturins, and fengycins, peptide antibiotics, bacteriocins, polyketides, and extracellular enzymes [171,172,175,178,190]. Such metabolites contribute to antibacterial, antifungal, antibiofilm, and enzymatic activities and may support pollen degradation and bee bread biotransformation [173]. Several *Bacillus* strains

from bee products demonstrate probiotic potential and are classified as Generally Recognized as Safe (GRAS) [178,180], except pathogenic species such as *B. cereus* and *Bacillus anthracis* [184]. Probiotic candidates tolerate gastric acid and bile salts, adhere to epithelial cells, and modulate host immunity [178]. However, probiotic designation requires the exclusion of toxin genes and antibiotic resistance determinants through extensive validations [180].

ii. Lactic Acid Bacteria

Lactic acid bacteria (LAB) are core members of the gut microbiota of *A. mellifera* and are widely distributed in honey, bee pollen, bee bread, wax, and comb [134,191]. They perform homofermentative or heterofermentative metabolism, converting carbohydrates into lactic acid and other organic acids that contribute to preservation and pathogen inhibition.

LAB were dominant across honey, pollen, and bee bread, although distribution differed by matrix. In honey, *Lactobacillus kunkeei* was frequently the most abundant species [192]. Other species included *Lactobacillus apis*, *Lactobacillus apinorum*, *Lactobacillus delbrueckii*, *Lactobacillus plantarum*, *Lactobacillus johnsonii*, *Lactobacillus brevis*, *Lactobacillus rhamnosus*, *Lactobacillus casei*, and *Lactobacillus panisapium* [193,194]. In some stingless bee honeys, *Lactobacillus* accounted for up to 64% of total bacterial diversity. *Lactococcus lactis* dominated certain raw honey samples [195,196].

In bee pollen, *Lactobacillus* spp. were detected, but were not always dominant. Pollen samples also contained *E. faecalis*, *Enterococcus casseliflavus*, and other LAB members, reflecting early-stage fermentation and plant-associated microbiota [197,198]. In bee bread, LAB clearly dominated, often representing more than 50% of relative abundance, depending on the floral source [199]. Genera such as *Fructobacillus*, *Pediococcus*, *Bifidobacterium*, and *Lactococcus* were also detected. Bee bread fermentation is strongly driven by LAB metabolism, leading to acidification and stabilization [200].

LAB produces a broad range of secondary metabolites, including bacteriocins, organic acids, hydrogen peroxide, siderophores, proteases, and volatile fatty acids. These compounds inhibit pathogens such as *P. larvae*, *Melissococcus plutonius*, *Spiroplasma melliferum*, and agents of chalkbrood, American and European foulbrood, varroosis, and nosemosis [201]. LAB isolated

from bee products also exhibits probiotic potential. They survive gastrointestinal conditions, adhere to epithelial cells, produce antioxidant metabolites, reduce reactive oxygen species, and modulate cytokine responses and immune cell activation. They can inhibit pathogens such as *Helicobacter pylori*, *Clostridium difficile*, *S. enterica*, *P.s aeruginosa*, *E. faecium*, *E. coli*, and *S. aureus* [201]. However, not all LAB are inherently beneficial. Some *Enterococcus* and *Lactobacillus* strains may harbor resistance genes, and functional and genomic validation is required before probiotic application, as these resistance genes can potentially lead to adverse effects in patients if the strains are not properly evaluated.

iii. Other Bacterial families

Beyond *Bacillaceae* and LAB, honey, pollen, and bee bread harbor diverse Gram-negative and environmental bacteria. In honey, Proteobacteria often dominated. Some Vietnamese honey samples showed more than 95% Proteobacteria, with *Burkholderia*, *Ralstonia*, *Atlantibacter*, *Klebsiella*, and *Enterobacter* as dominant genera. Identified species included *Burkholderia contaminans*, *Ralstonia pickettii*, *Atlantibacter hermannii*, and *K. pneumoniae*. Honey also contained core bee gut symbionts such as *Gilliamella*, *Frischella*, *Bartonella*, *Bombella*, *Apibacter*, and *Arsenophonus*. In bee pollen, *Enterobacteriaceae* were common, including *Pantoea agglomerans*, *Pantoea ananatis*, *Cronobacter malonicus*, *Escherichia vulneris*, and *S. enterica*. *Clostridium perfringens* was reported as highly abundant in some pollen datasets. These taxa likely originate from plant surfaces and environmental exposure. In bee bread, microbial diversity was high and included *Enterobacteriaceae*, *Streptomycetaceae*, *Bacillaceae*, and *Streptococcaceae*. Genera such as *Streptomyces*, *Streptococcus*, *Bacillus*, *Synechococcus*, and *Rosenbergiella* varied depending on the floral source and region. Certain detected taxa are opportunistic or pathogenic, including *Melissococcus plutonius*, *P. larvae*, *Spiroplasma melliferum*, *K. pneumoniae*, and *E. coli*. Their presence may indicate environmental contamination or compromised hive health. However, metagenomic detection alone does not confirm viability or pathogenic activity. Validation based on culture or transcriptomics is essential for accurate functional interpretation [185,202–205].

Yeasts, molds, and fungi

Fungal communities differed between honey, pollen, and bee bread but played important roles in fermentation and ecology. In honey, yeasts frequently dominated fungal profiles. *Zygosaccharomyces mellis*, *Zygosaccharomyces pseudorouxii*, and *Zygosaccharomyces rouxii* were often predominant, with some stingless bee honeys showing more than 70% relative abundance of *Zygosaccharomyces*. Other detected genera included *Saccharomyces*, *Kluyveromyces*, *Zygorhizopus*, *Candida*, *Yarrowia lipolytica*, *Starmerella*, and *Cryptococcus neoformans* in certain datasets [185].

In bee pollen, filamentous fungi such as *Aspergillus*, *Fusarium*, *Penicillium*, *Alternaria*, *Cladosporium*, and *Mucor* were frequently detected, largely reflecting plant-associated and environmental sources [206–208]. In bee bread, yeast diversity was influenced by the fermentation stage. *Metschnikowia* species were common in pollen loads. In fresh bee bread, *Starmerella* and *Zygosaccharomyces* dominated. During maturation, *Starmerella* and *Zygosaccharomyces* remained prevalent. Yeasts contribute to fermentation through the production of ethanol, organic acids, enzymes, and volatile metabolites that influence preservation and flavor. Molds associated with bee products include *Penicillium*, *Aspergillus*, *Fusarium*, *Cladosporium*, *Rhizopus*, and *Trichoderma*. Some species, such as *Ascosphaera apis* and *Aspergillus* spp., are bee pathogens and cause chalkbrood or stonebrood. Therefore, the presence of fungi reflects a balance between beneficial fermentative activity and potential spoilage or pathogenic risk. DNA-based detection must be complemented with functional assays to distinguish resident fermentative fungi from transient contaminants [209–211].

1.7 Functional Classification of Antimicrobial Peptides and Specialized Metabolites in *Bacillus* and *Lactobacillus*

The genera *Bacillus* and *Lactobacillus* represent two of the most significant taxonomic groups in industrial microbiology and clinical probiotics due to their expansive secondary metabolomes. These microorganisms have evolved sophisticated chemical defense systems categorized fundamentally by their biosynthetic origins: the ribosomally synthesized and post-translationally modified peptides (RiPPs) and the non-ribosomally synthesized peptides (NRPs) and specialized metabolites. This division reflects divergent evolutionary strategies for niche competition.

Ribosomal synthesis allows for rapid structural diversification through genetic mutation, facilitating the emergence of precision-targeted bacteriocins and lantibiotics that often exhibit activity against closely related competitors [10]. Conversely, non-ribosomal synthesis, governed by massive multi-modular enzyme complexes, enables the incorporation of non-proteinogenic amino acids and fatty acid moieties, producing exceptionally stable and structurally diverse molecules like cyclic lipopeptides and siderophores that serve as broad-spectrum surfactants or membrane disruptors [212].

1.7.1 Ribosomally Synthesized and Post-translationally Modified Peptides (RiPPs)

Ribosomally synthesized and post-translationally modified peptides, or RiPPs, are defined by a shared biosynthetic logic where a precursor peptide is translated by the ribosome and subsequently transformed into a bioactive molecule through dedicated enzymatic modifications. In the context of *Bacillus* and *Lactobacillus*, these modifications include the formation of thioether bridges (lanthionines), sulfur-to-alpha-carbon cross-links (sactipeptides), and the creation of mechanically interlocked topologies (lasso peptides). These structural features confer remarkable stability against proteolytic degradation, extreme pH, and thermal stress, essential for survival in competitive environments such as the soil or the gastrointestinal tract [213]. The RiPP biosynthetic gene clusters typically include a structural gene encoding the precursor peptide, enzymes for modification, a leader peptidase for maturation, and dedicated immunity and transport proteins [214].

1.7.2 Non-Ribosomally Synthesized Peptides and Specialized Metabolites (NRPs)

Non-ribosomally synthesized peptides (NRPs) and related metabolites are produced via the thiotemplate mechanism of Non-Ribosomal Peptide Synthetases (NRPS), which operate independently of the ribosomal machinery [215]. These assembly lines are composed of repetitive modules, each responsible for the selection, activation, and condensation of a specific substrate, allowing for the inclusion of D-amino acids and β -hydroxy fatty acids [215]. This flexibility results in the production of highly stable cyclic lipopeptides, such as surfactin and fengycin, which act as powerful biosurfactants and membrane-active agents [216,217]. Beyond peptides, this category also includes aminoglycosides like butirosin [218] and catecholate siderophores like bacillibactin

[219]. In *Bacillus* and *Lactobacillus*, NRPs are critical for biofilm architecture, swarming motility, and the inhibition of a broad range of competitors, including bacteria and filamentous fungi. **Figure 8** classifies the secondary metabolites based on biosynthetic origin and primary mechanism of action.

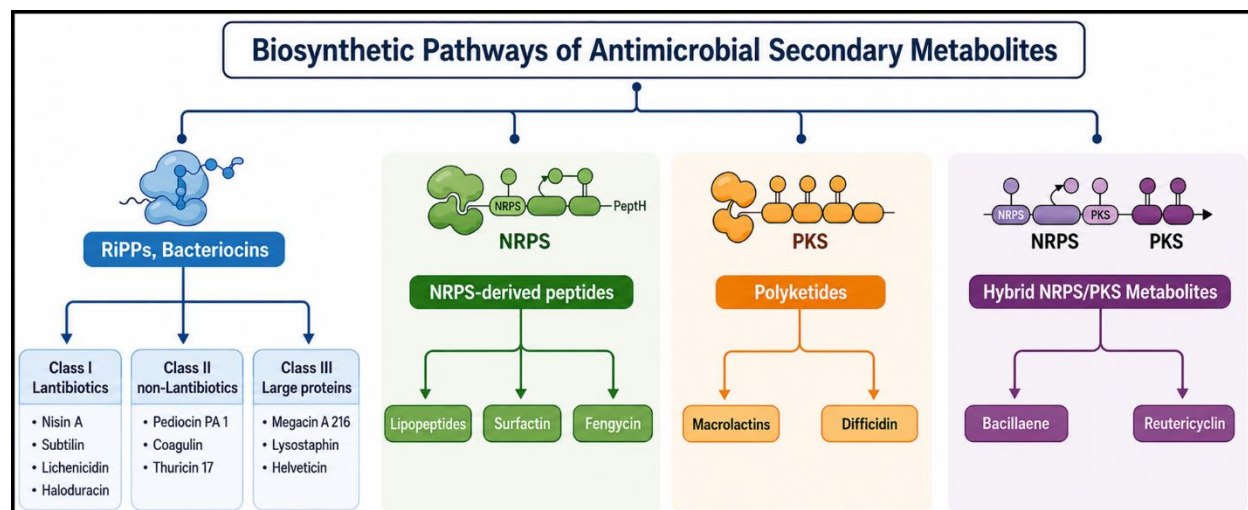


Figure 8. Classification and biosynthetic pathways of antimicrobial secondary metabolites in *Bacillus* and *Lactobacillus*. The pathways are color-coded into four primary categories: Ribosomally synthesized and post-translationally modified peptides (RiPPs) and bacteriocins (**blue**); Non-ribosomal peptide synthetase (NRPS)-derived peptides (**green**); Polyketides (**orange**); and Hybrid NRPS/PKS metabolites (**purple**). Subclasses and representative antimicrobial compounds, such as lantibiotics, lipopeptides, macrolactins, and hybrid structures, are delineated hierarchically to illustrate structural and biosynthetic diversity. Figure Created in Adobe Illustrator.

1.8 Profiles of Ribosomal Specialized Metabolites (RiPPs)

1.8.1 One-Component Lantibiotics

One-component lantibiotics are small, heat-stable peptides characterized by intramolecular thioether rings [220], with Nisin (produced by *L. lactis* and other LAB) and Subtilin (from *B. subtilis*) being the primary examples. Nisin has a molecular weight of 3331.05 Da [221], while Subtilin is approximately 3321.7 Da [222]. Other examples from these genera include Ericin A and Ericin S from *B. subtilis* [223] and Plantaricin C from *L. plantarum* [224]. These molecules exert antibacterial activity by binding to the pyrophosphate unit of lipid II, inhibiting cell wall synthesis, often followed by pore formation in the cytoplasmic membrane. They exhibit potent activity against Gram-positive bacteria such as *L. monocytogenes* and *S. aureus* [225]. They are

unique in their ability to inhibit the outgrowth of spores from *Bacillus* and *Clostridium* species [226,227]. Furthermore, they demonstrate significant antibiofilm activity by preventing initial surface attachment [228].

1.8.2 Two-Component Lantibiotics

Two-component lantibiotics require the synergistic action of two distinct peptides for optimal activity, acting in a 1:1 ratio [229]. This class is exemplified by Lichenicidin (Lch α 3249.51 Da, Lch β 3019.36 Da) from *B. licheniformis* [229], Haloduracin (Hal α 3043.2802 Da and Hal β 2330.0456 Da) from *B. halodurans* [230], and Amyloliquecidin (GF610 α 3026 Da and GF610 β 2451 Da) from *B. amyloliquefaciens* [190]. Additionally, Lacticin 3147 (LtnA1 3306 Da and LtnA2 2847 Da) is a paradigm of this class produced by the lactic acid bacterium *L. lactis* [231], while *L. plantarum* produces Plantaricin W [232]. Their mode of action involves a "division of labor" where one peptide binds to lipid II to inhibit cell wall synthesis, while the second peptide is recruited to form a stable, high-conductance pore in the membrane [233]. These systems confer exceptional antibacterial potency against Gram-positive pathogens like MRSA and VRE and act as highly effective antibiofilm agents by depleting intracellular ATP [234,235].

1.8.3 Lasso Peptides

Lasso peptides are unique RiPPs with a mechanically interlocked structure where the C-terminal tail is threaded through a macrocyclic ring [236]. A prominent example from the genus *Bacillus* is LP-2514, produced by *B. licheniformis* MCC 2514, which has a molecular weight of approximately 2000 Da. Their mode of action is highly target-specific; LP-2514 inhibits bacterial growth by acting as an inhibitor of RNA biosynthesis [237]. Another lasso peptide, lariocidin (LAR), binds to the ribosome, inhibits translation elongation, and induces miscoding. Moreover, LAR exhibits broad antimicrobial activity against pathogenic Gram-positive and Gram-negative bacteria [238].

1.8.4 Sactipeptides

Sactipeptides are characterized by sulfur-to-alpha-carbon thioether cross-links formed by radical S-adenosylmethionine (SAM) enzymes [239]. The most studied member is Subtilosin A, a circular

sactipeptide produced ribosomally by various *Bacillus* species, notably *B. subtilis* 168 and *B. amyloliquefaciens*, with a molecular weight of 3015.5 Da [240]. Its mode of action involves binding to the cytoplasmic membrane of sensitive cells via electrostatic interactions, creating transient pores that cause the immediate depletion of the transmembrane pH gradient and leakage of intracellular ions and ATP [241]. Subtilisin A exhibits potent broad-spectrum antibacterial activity against human and food-borne pathogens, including both Gram-positive and Gram-negative pathogens, such as *Gardnerella vaginalis* [242] and *L. monocytogenes* [243]. They also show high antibiofilm activity by interfering with quorum sensing [244].

1.9 Profiles of Non-Ribosomal Specialized Metabolites (NRPs)

1.9.1 Fengycin

Fengycins are cyclic lipodecapeptides produced non-ribosomally by *Bacillus* species such as *B. subtilis*, *B. amyloliquefaciens*, and *B. velezensis*, with molecular weights typically between 1463 Da to 1515 Da [216]. Their mode of action involves interacting with sterol components in the target membrane, which induces a burst of reactive oxygen species (ROS) and loss of mitochondrial membrane potential in fungi [245]. Fengycin is a potent antifungal agent against filamentous fungi like *Magnaporthe grisea* and *Botrytis cinerea* [245]. While its direct antibacterial activity is moderate, it plays a critical role in antibiofilm strategies by preventing fungal colonization and destabilizing mixed-species biofilms through the inhibition of quorum sensing (Agr system) [245].

1.9.2 Bacitracin

Bacitracin is a non-ribosomally synthesized antibiotic produced primarily by *B. licheniformis* and *B. subtilis*, with a molecular weight of approximately 1422 Da. It exerts its antibacterial activity by binding to undecaprenyl pyrophosphate (the C55-lipid carrier), thereby inhibiting the transport of peptidoglycan precursors across the membrane and blocking cell wall synthesis [246]. It is highly effective against Gram-positive bacteria, including pathogens like *Streptococcus mutans* and *Pantoea ananatis* [246,247].

1.9.3 Lichenysin

Lichenysin is a cyclic lipopeptide biosurfactant produced by *Bacillus licheniformis* with a molecular weight ranging from 1000 Da to 1500 Da [248]. Its mode of action involves reducing surface tension and permeabilizing biological membranes by interacting with phospholipid bilayers [248]. Lichenysin exhibits significant antibacterial activity, particularly against Gram-positive organisms, and is a potent antibiofilm agent capable of preventing the adhesion of pathogens like *E. coli* and *S. aureus* [249]. Additionally, it also exhibits antifungal activity [250].

1.9.4 Butirosin A/Butirosin B

Butirosins are naturally occurring aminoglycoside antibiotics produced by *Niallia* (formerly *Bacillus*) *circulans* and *Bacillus vitellinus*, with Butirosin A having a molecular weight of approximately 555 Da [251]. They are unique for their (S)-4-amino-2-hydroxybutyrate (AHBA) side chain, which allows the molecule to bind to the 30S ribosomal subunit and interfere with protein synthesis while resisting common aminoglycoside-modifying enzymes [252]. Butirosins exhibit broad-spectrum antibacterial activity against both Gram-positive and Gram-negative pathogens, including *Pseudomonas aeruginosa*. They have been shown to impede the proliferation of bacterial biofilms by suppressing the synthesis of essential matrix proteins [253].

1.9.5 Plipastatin

Plipastatin is a cyclic lipodecapeptide structurally similar to fengycin, produced non-ribosomally by *Bacillus subtilis* and *B. amyloliquefaciens*, with a molecular weight of 1463 Da to 1500 Da [254]. Its mode of action involves the disruption of the fungal cell membrane and interference with lipid signaling pathways through the inhibition of phospholipase A2 [254]. Plipastatin acts as a potent antifungal agent against pathogens such as *Fusarium* species, *Podosphaera fusca*, and *Aspergillus carbonarius* [254].

1.9.6 Bacilysin

Bacilysin, also known as tetaine and bacillin, is a simple non-ribosomal dipeptide (L-alanine and L-anticapsin) produced by *Bacillus* spp. including *B. subtilis* and *B. pumilus*, with a low molecular

weight of 271.1 Da [255]. After intracellular transport, it is hydrolyzed to release anticapsin, which irreversibly inhibits glucosamine-6-phosphate synthase, thereby blocking the synthesis of peptidoglycan in bacteria and chitin in fungi [255]. This dual mechanism confers broad-spectrum antibacterial and antifungal activity against pathogens like *Staphylococcus aureus* [256,257] and *Xanthomonas oryzae* [258].

1.9.7 Bacillibactin

Bacillibactin is a catecholate siderophore produced via an NRPS pathway by *Bacillus* species such as *B. subtilis*, *B. amyloliquefaciens*, and *B. velezensis*, with a molecular weight of 881.2 Da [259]. Its primary mode of action is the high-affinity chelation of ferric iron (Fe^{3+}), which starves competing microorganisms of this essential nutrient [259], or by direct antibiosis [260]. This nutrient deprivation results in both antibacterial and antifungal activity against diverse pathogens, including MRSA and *P. aeruginosa* [261].

1.9.8 Surfactin

Surfactin is a powerful cyclic lipopeptide produced by many *Bacillus* strains, such as *B. subtilis* and *B. velezensis*, with a molecular weight of approximately 1036.3 Da [262]. It operates through a surfactant-based mode of action, incorporating into the phospholipid bilayer to cause dehydration of head groups and bilayer instability, leading to pore formation. While its direct antibacterial activity is modest, it is essential for biofilm architecture and exceptional antibiofilm activity through surface modification and interference with microbial adhesion [263]. Surfactin also possesses potent antiviral properties against lipid-enveloped viruses [264] and acts synergistically with fengycin to enhance overall antifungal efficacy [265]. **Table 2** provides an overview of bioactive secondary metabolites produced by *Bacillus* species and lactic acid bacteria (LAB), including their molecular weights, producer strains, and primary mechanisms of action.

Table 2. Profiles of bioactive secondary metabolites produced by *Bacillus* and Lactic Acid Bacteria (LAB): Comparative analysis of molecular weight, producing species, and primary mechanisms of action.

Metabolite	Class	M.W. (Da)	Source Genus	Mode of Action
Subtilin	RiPP	3321.7	<i>Bacillus</i>	Lipid II binding
Lichenicidin	RiPP	~3000–3300	<i>Bacillus</i>	Synergistic pore formation
Haloduracin	RiPP	~3000	<i>Bacillus</i>	Synergistic pore formation
Amyloliuecidin	RiPP	2451–3026	<i>Bacillus</i>	Synergistic pore formation
Lacticin 3147	RiPP	2847–3306	<i>Lactococcus/LAB</i>	Synergistic pore formation
Plantaricin W	RiPP	~3000	<i>Lactobacillus</i>	Synergistic pore formation
LP-2514	RiPP	~2000	<i>Bacillus</i>	RNA synthesis inhibition
Subtilosin A	RiPP	3015.5	<i>Bacillus</i>	PMF disruption/ATP efflux
Fengycin	NRP	1463–1515	<i>Bacillus</i>	Sterol interaction/ROS burst
Bacitracin	NRP	1422	<i>Bacillus</i>	C55-lipid carrier binding
Lichenysin	NRP	1000–1500	<i>Bacillus</i>	Membrane permeabilization
Butirosin A	Specialized	~555	<i>Bacillus</i>	30S Ribosomal binding
Plipastatin	NRP	1463–1500	<i>Bacillus</i>	Phospholipase A2 inhibition
Bacilysin	NRP	271.1	<i>Bacillus</i>	Glucosamine-6-P synthase inhibition
Surfactin	NRP	1058.6	<i>Bacillus</i>	Bilayer destabilization

Pelgipeptin	NRP	1072–1100	<i>Paenibacillus</i>	Membrane disruption
Difficidin	PKS	544	<i>Bacillus</i>	Ribosomal protein inhibition
Macrolactin	PKS	402	<i>Bacillus</i>	EF-Tu inhibition
Bacillaene	Hybrid	580	<i>Bacillus</i>	Protein synthesis inhibition
Reutericyclin	Hybrid	349	<i>Lactobacillus</i>	Proton ionophore

2. Aim and Objectives of the Research

2.1 Aim

The primary aim of this research was to evaluate the pharmacological potential of the microbiota associated with bee products, with particular emphasis on probiotic properties and antimicrobial metabolite production. This aim was addressed through the following research directions:

1. Screening of isolates for probiotic potential and antimicrobial metabolite production.
2. Optimization of culture conditions to enhance production and purification of metabolites exhibiting the highest antimicrobial activity (five to ten producing strains).
3. Genome sequencing and bioinformatic analysis of selected high-producing strains to identify genetic determinants responsible for antimicrobial metabolite biosynthesis.
4. Structural elucidation of metabolites demonstrating the strongest antimicrobial activity.

2.2 Objectives

To achieve the stated aim, the following specific objectives have been established:

A. Screening of Isolates with Probiotic Potential and Antimicrobial Metabolite Production

- Preparation and establishment of a collection of honey samples obtained from Polish apiaries.
- Isolation of bacterial strains and evaluation of their antibacterial activity, including testing crude extracts against reference strains of clinically relevant pathogens
- Determination of antistaphylococcal activity of crude extracts against clinical isolates of *S. aureus*, including MSSA and MRSA strains.

B. Optimization of Metabolite Production and Purification

- Cultivation of selected isolates in various growth media to evaluate the influence of nutrient composition on metabolite production.
- Determination of optimal temperature conditions for maximal production of antimicrobial compounds and associated enzymes.
- Selection of five to ten strains demonstrating the highest antimicrobial potential for further downstream purification studies.

C. Genome sequencing and bioinformatic characterization

- Taxonomic identification and classification of the most promising isolates using 16S rRNA gene sequencing and Whole Genome Sequencing (WGS).
- Bioinformatic mining of genomic data to identify biosynthetic gene clusters responsible for secondary metabolite and enzyme production.
- Assessment of the probiotic potential of *Bacillus* strains isolated from bee pollen and bee bread samples.

D. Structural characterization of antimicrobial metabolites

- Preparation of crude extracts from selected *Bacillus* strains.
- Determination of antimicrobial activity of crude extracts prior to purification.
- Chemical analysis and structural elucidation of active compounds using HPLC, MALDI, and high-resolution mass spectrometry (HRMS).
- Determination of antimicrobial activity of purified fraction.

Included Publications

Articles Presented in the Dissertation with the Author's Contribution

1. ARTICLE 1

Whole-genome sequencing and antimicrobial potential of bacteria isolated from Polish honey by **Ahmer Bin Hafeez**, Karolina Pełka, Kamila Buzun, Randy W. Worobo, and Piotr Szweda

Applied Microbiology and Biotechnology 2023; doi.org/10.1007/s00253-023-12732-9

IF2026=4.3, Q1, Ministry of Science and Higher Education Points (MHES): 100

Accepted: 16/08/2023

Published: 04/09/2023

Ahmer Bin Hafeez's contribution to this article was the initial screening of bacterial isolates from Polish honey samples, evaluation of their antimicrobial activity, and selection of the three most promising strains for detailed characterization. He performed whole-genome sequencing, de novo genome assembly, and comprehensive genomic analyses, including ANI analysis, phylogenomic reconstruction, COG functional annotation, and comparative genomics. He also conducted secondary metabolite biosynthetic gene cluster identification using antiSMASH and BAGEL4 pipelines, structural homology modeling of predicted antimicrobial peptides, and CAZyme profiling. He was primarily responsible for data interpretation and preparation of the manuscript.

2. ARTICLE 2

Probiotic Potential of *Bacillus* Isolates from Polish Bee Pollen and Bee Bread.

Karolina Pełka, **Ahmer Bin Hafeez**, Randy W. Worobo, Piotr Szweda

Probiotics and Antimicrobial Proteins 2023; doi:10.1007/s12602-023-10157-4

IF 2026=4.4, Q1, MHES Points: 70

Accepted: 12/09/2023

Published: 19/09/2023

Ahmer Bin Hafeez's contribution to this article was the genomic and phylogenomic analyses of the three selected probiotic candidate strains (PY2.3, BP20.15, and BB10.1). This included whole-genome sequencing analysis, taxonomic classification, and bioinformatic investigation of genes encoding antimicrobial compounds such as bacteriocins and secondary metabolites. The genomic data generated by Ahmer Bin Hafeez provided the molecular foundation that confirmed and

supported the experimental laboratory findings obtained by the co-authors. He was also responsible for the preparation of the manuscript.

3. ARTICLE 3

In Silico Safety Assessment of *Bacillus* Isolated from Polish Bee Pollen and Bee Bread as Novel Probiotic Candidates

by **Ahmer Bin Hafeez**, Karolina Pelka, Randy W. Worobo, Piotr Szweda

International Journal of Molecular Sciences 2024; doi.org/10.3390/ijms25010666

IF 2026=4.9, Q1, MHES Points: 140

Accepted: 25/12/2023

Published: 04/01/2024

Ahmer Bin Hafeez's contribution to this article was the design and execution of the entire *in silico* probiogenomics analysis. He performed genome quality assessment using BUSCO, pangenome analysis of *Bacillus subtilis* and *Bacillus velezensis* genomes, functional genome annotation, and CAZyme profiling. He conducted the safety evaluation of the isolates, which included antimicrobial resistance gene detection using CARD and KEGG databases, virulence factor analysis using VirulenceFinder and VFDB, pathogenicity probability assessment using PathogenFinder, plasmid detection using PlasmidFinder, and prophage identification. He was primarily responsible for data interpretation and preparation of the manuscript.

4. Article 4

In Silico Functional Annotation and Structural Characterization of Hypothetical Proteins in *Bacillus paralicheniformis* and *Bacillus subtilis* Isolated from Honey

by **Ahmer Bin Hafeez**, Subrahmanyam Sappati, Radoslaw Krzemieniecki, Randy Worobo, and Piotr Szweda

ACS Omega 2025; doi.org/10.1021/acsomega.4c07105

IF 2026=4.3, Q2, MHES Points: 70

Accepted: 25/12/2024

Published: 27/02/2025

Ahmer Bin Hafeez's contribution to this article was the conception and execution of the computational analysis of hypothetical proteins identified in honey-derived *Bacillus* genomes. He performed functional annotation using multiple databases, including InterPro, CDD BLAST, SUPERFAMILY, SMART, CATH, and SCANPROSITE, as well as physicochemical

characterization using ProtParam and subcellular localization prediction. He conducted protein structure prediction using SWISS-MODEL and AlphaFold2, molecular dynamics simulations including RMSD and RMSF analyses, protein-protein interaction network analysis using the STRING database, and ROC analysis to validate the prediction pipeline. He was primarily responsible for data interpretation and preparation of the manuscript.

5. Article 5

Genomic and functional landscape of antimicrobial peptides and lipopeptides from ginger honey-derived *Bacillus subtilis*

by **Ahmer Bin Hafeez**, and Piotr Szweda

Molecular Genetics and Genomics 2026; doi.org/10.1007/s00438-026-02411-5

IF 2026=2.1, Q2, MHES Points: 100

Accepted: 15/03/2026

Published: 01/04/2026

Ahmer Bin Hafeez's contribution to this article was the design and execution of the entire study. He was responsible for the isolation of *Bacillus subtilis* strains Miodo8 and Miodo10 from ginger honey, phenotypic characterization, fermentation optimization across multiple growth media, and evaluation of antimicrobial activity against a panel of bacterial and fungal pathogens. He performed whole-genome sequencing, functional annotation, CAZyme profiling, resistome analysis, biosynthetic gene cluster mining, pangenome analysis, comparative synteny analysis, prophage and mobile genetic element identification, and in silico antimicrobial peptide screening. He was solely responsible for data interpretation and preparation of the manuscript.

Copyright Disclaimer: The following publication is reproduced with permission from Springer Nature. The article "Genomic and functional landscape of antimicrobial peptides and lipopeptides from ginger honey-derived *Bacillus subtilis*" by Bin Hafeez et al., published in **Molecular Genetics and Genomics** (April 2026), is included in its entirety as part of this thesis under **License Number 6277671348832**, granted by Copyright Clearance Center on May 28, 2026. Reproduced with permission from Springer Nature.

SUMMARY IMPACT FACTOR (IF)

The summary IF of the articles presented in the dissertation is 20

Whole-genome Sequencing and Antimicrobial Potential of Bacteria Isolated from Polish Honey

Ahmer Bin Hafeez¹, Karolina Pelka¹, Kamila Buzun², Randy Worobo³, Piotr Szweda¹

¹ Department of Pharmaceutical Technology and Biochemistry, Faculty of Chemistry, Gdańsk University of Technology, Ul. G. Narutowicza 11/12, 80-233 Gdańsk, Poland

² Department of Biotechnology, Faculty of Pharmacy, Medical University of Białystok, 15-089 Białystok, Poland

³ Department of Food Science, Cornell University, Ithaca, NY 14853, USA

Raw honey is a reservoir for several microbial species, such as mold, yeast, and spore-forming bacteria. The composition of the microbiota in bee products might differ with respect to botanical and geographical origins.

This study focused on investigating the microbial diversity of Polish honey with a particular focus on the isolation, characterization, and determination of the antimicrobial potential of isolates recovered from Polish honey. Given the increasing need for novel antimicrobials and the recognition of bee products as reservoirs of bioactive microorganisms, the aim was to isolate bacterial strains from honey, evaluate their antimicrobial activity, and characterize their biosynthetic potential using whole-genome sequencing and genome mining approaches.

Three multifloral honey samples collected from northern Poland were screened, yielding a total of 132 bacterial isolates. Initial selection of antimicrobial producers was based on inhibition of *Staphylococcus aureus* reference strains. Further verification was performed using cell-free after-culture medium (CFAM) to confirm secretion of active metabolites. Among the isolates, three strains—SZA14, SZA16, and SZB3—demonstrated the strongest anti-staphylococcal activity and were selected for detailed genomic analysis.

Whole-genome sequencing using Illumina MiSeq technology enabled de novo assembly and comprehensive genomic characterization. Average Nucleotide Identity (ANI) analysis and phylogenomic reconstruction classified isolates SZA14 and SZA16 as *Bacillus paralicheniformis*, while isolate SZB3 was identified as *Bacillus subtilis*. The two *B. paralicheniformis* isolates exhibited 98.96% nucleotide identity, indicating close relatedness but not clonality. Genome sizes ranged from 4.24 to 4.51 Mb with GC contents typical for *Bacillus* species.

Functional genome annotation revealed that approximately 91–94% of predicted genes could be assigned to Clusters of Orthologous Genes (COG) categories, with a substantial proportion

classified as “function unknown,” highlighting the potential for undiscovered metabolic capabilities. Comparative genomic analyses demonstrated high overall synteny with type strains but also revealed genomic regions suggestive of horizontal gene transfer, including prophage elements, which may contribute to antimicrobial gene acquisition and diversification.

Secondary metabolite biosynthetic gene clusters (BGCs) were identified using antiSMASH and BAGEL4 pipelines. Each genome contained 14 predicted BGCs, including non-ribosomal peptide synthetases (NRPS), polyketide synthases (PKS), ribosomally synthesized and post-translationally modified peptides (RiPPs), and hybrid clusters. In the *B. paralicheniformis* isolates SZA14 and SZA16, gene clusters encoding lichenysin, fengycin, bacitracin, bacillibactin, lanthipeptides, and thiopeptides were identified. BAGEL4 analysis revealed putative RiPP clusters coding for compounds such as enterocin Nkr-5-3B, haloduracin- α , sonorensin, bottromycin, lasso peptides, and sactipeptides.

In the *B. subtilis* isolate SZB3, NRPS clusters encoding surfactin and plipastatin were detected, alongside RiPP clusters responsible for subtilosin A, sporulation-killing factor (skfA), competence factors, and sactipeptides. Structural homology modeling further supported the presence of bacteriocin-like peptides, showing high similarity to known antimicrobial peptides such as enterocin NKR-5-3B, lichenicidin, and subtilosin A. Minor structural deviations observed in modeled peptides suggest possible sequence variation that could influence bioactivity and specificity.

Beyond classical antimicrobial compounds, additional short peptides predicted *in silico* displayed sequence similarity to known antimicrobial peptides, including paenibacterin, polymyxin, gramicidin S, and brevivacillin, further expanding the antimicrobial repertoire of these strains.

Carbohydrate-active enzyme (CAZyme) analysis revealed numerous glycoside hydrolases, transferases, esterases, and polysaccharide lyases, several containing signal peptides indicative of secretion. These findings suggest additional biotechnological potential, including applications in biomass degradation and industrial biocatalysis.

Collectively, this study demonstrates that honey-derived *Bacillus* species constitute a rich source of biosynthetic diversity, encoding a broad spectrum of antimicrobial compounds spanning lipopeptides, siderophores, RiPPs, and small antimicrobial peptides. The combination of phenotypic screening and whole-genome sequencing enabled precise taxonomic classification and comprehensive mapping of biosynthetic gene clusters. Importantly, the presence of multiple

bacteriocin-related clusters and structural variants indicates the potential for novel antimicrobial molecules.

These findings reinforce the concept of honey as a valuable ecological niche for spore-forming bacteria with significant antimicrobial capabilities and support further biochemical isolation and structural characterization of the predicted compounds. The genomic insights provided here establish a foundation for future work aimed at purification, structural elucidation, and activity assessment of novel antimicrobial agents derived from honey microbiota.

Article 1.

**Whole-genome sequencing and antimicrobial potential of bacteria isolated from Polish
honey (2023)**

Applied Microbiology and Biotechnology. 2023 Oct;107(20):6389-406

<https://doi.org/10.1007/s00253-023-12732-9>

Accepted: 16 August 2023 / Published online: 4 September 2023

IF2026 = 4.3, Q1, MHES Points: 100

Probiotic Potential of *Bacillus* Isolates from Polish Bee Pollen and Bee Bread

Karolina Pelka¹, Ahmer Bin Hafeez¹, Randy W. Worobo² and Piotr Szweda¹

¹ Department of Pharmaceutical Technology and Biochemistry, Faculty of Chemistry, Gdansk University of Technology, ul. G. Narutowicza 11/12, 80-233 Gdansk, Poland;

² Department of Food Science, Cornell University, Ithaca, NY 14853, USA

This study investigated the probiotic potential of *Bacillus* strains isolated from bee pollen and bee bread collected in Poland. Bee products are widely recognized for their nutritional value and biological activity. Bee pollen and bee bread contain proteins, amino acids, lipids, vitamins, minerals, and many bioactive compounds. These products also host diverse microbial communities. Some of these microorganisms may provide beneficial effects for both bees and humans. *Bacillus* species are particularly interesting because they form spores and can survive harsh environmental conditions. Many *Bacillus* strains are used in food biotechnology, and some are applied as probiotic microorganisms. The aim of this study was to isolate *Bacillus* strains from bee pollen and bee bread and evaluate their probiotic potential using physiological, biochemical, and genomic analyses.

Ten *Bacillus* isolates were obtained from the analyzed samples. Seven strains were isolated from bee pollen and three from bee bread. Identification using 16S rRNA gene sequencing confirmed that all isolates belonged to the *Bacillus* genus. The first stage of the study focused on the antagonistic activity of these isolates against selected pathogenic microorganisms. The tested pathogens included *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella enterica*, *Pseudomonas aeruginosa*, *Candida albicans*, *Candida krusei*, and *Candida glabrata*. The *Bacillus* isolates showed clear inhibitory activity against several bacterial pathogens. The strongest inhibition was observed against *staphylococci* and *P. aeruginosa*. *L. monocytogenes* and *E. coli* were less sensitive to the *Bacillus* strains. Antifungal activity was weaker, and the isolates showed limited inhibition of *Candida* species, especially *C. albicans*.

A key requirement for probiotic bacteria is the ability to survive conditions present in the human gastrointestinal tract. The isolates were therefore tested for tolerance to acidic conditions and bile salts. Acid tolerance was examined at pH 2 to simulate the stomach environment. The survivability

of the isolates under these conditions ranged from 35.5% to 68.23%. Bile salt tolerance was tested using a 0.3% bile salt solution that simulates conditions in the small intestine. All isolates showed strong tolerance to bile salts, and survival rates ranged from 84.63% to 110.15%. These results indicate that the tested *Bacillus* strains are capable of surviving conditions similar to those encountered during passage through the gastrointestinal tract.

Adhesion-related characteristics were also evaluated because adhesion to intestinal surfaces is an important feature of probiotic microorganisms. Cell surface hydrophobicity was measured using affinity to xylene. The results varied among isolates and ranged from 5.69% to 61.08%. Higher hydrophobicity values indicate stronger potential adhesion to epithelial cells. Auto-aggregation ability was measured after 4 hours and 24 hours of incubation. After 4 hours, the auto-aggregation values ranged from 21.55% to 46.75%. After 24 hours, the aggregation ability increased significantly and ranged from 47.08% to 88.52%. The isolates were also tested for co-aggregation with selected pathogens, including *S. aureus*, *E. coli*, *L. monocytogenes*, and *S. enterica*. The strongest co-aggregation was observed with *L. monocytogenes*, followed by *S. aureus* and *S. enterica*. These interactions suggest that the *Bacillus* isolates may interfere with pathogen colonization in the intestinal environment.

Safety evaluation is essential when considering bacteria for probiotic use. Antibiotic susceptibility was examined using the disk diffusion method with ten antibiotics. Most isolates were sensitive to the tested antibiotics. However, six strains showed resistance to penicillin, and two strains were resistant to clindamycin. None of the isolates produced DNase enzyme. Five strains exhibited no hemolytic activity on blood agar. Lack of DNase activity and absence of hemolysis indicate that these isolates do not possess common virulence-related traits.

Another functional property examined in this study was cholesterol assimilation. This characteristic is often associated with probiotic bacteria because it may contribute to lowering cholesterol levels in the host. All *Bacillus* isolates were able to assimilate cholesterol from the culture medium. The level of cholesterol assimilation ranged from 10.74% to 36.45% depending on the strain. Carbohydrate metabolism was analyzed using API 50 CH biochemical tests. The results showed that the isolates were metabolically versatile. The *Bacillus* strains were able to utilize 30 out of 49 tested carbohydrates. These included different monosaccharides, disaccharides,

polysaccharides, glycosides, and triols. The ability to metabolize a broad range of carbohydrates indicates that these bacteria can adapt to different nutrient conditions within the gastrointestinal environment.

Based on the combined results of antimicrobial activity, survival under gastrointestinal conditions, adhesion-related properties, safety evaluation, cholesterol assimilation, and metabolic capacity, three isolates were selected as the most promising probiotic candidates. These strains were PY2.3, BP20.15, and BB10.1. Whole genome sequencing and phylogenomic analysis were performed to determine their precise taxonomic classification and to investigate their genetic potential. Genome analysis identified strain PY2.3 as *Bacillus velezensis*, while strains BP20.15 and BB10.1 were classified as *Bacillus subtilis*. Genomic investigation revealed the presence of genes responsible for the production of antimicrobial compounds such as bacteriocins and other secondary metabolites. These compounds may contribute to the observed inhibition of pathogenic microorganisms. The genomic data, therefore, supported the experimental results obtained during laboratory testing.

The findings of this study demonstrate that *Bacillus* strains isolated from bee pollen and bee bread possess several characteristics associated with probiotic microorganisms. These include tolerance to gastrointestinal conditions, antimicrobial activity against pathogens, cholesterol assimilation, adhesion-related properties, and the ability to metabolize a wide range of carbohydrates. The results also indicate that bee-derived products can serve as a valuable source of beneficial *Bacillus* strains. However, further laboratory and *in vivo* studies are required to confirm the safety and probiotic effects of these strains in the human host.

Article 2.

Probiotic Potential of *Bacillus* Isolates from Polish Bee Pollen and Bee Bread

Probiotics and Antimicrobial Proteins (2023);

<https://doi.org/10.1007/s12602-023-10157-4>

Accepted: 12/09/2023 / Published: 19/09/2023

IF2026 = 4.4, Q1, MHES Points: 70

Author Contribution Statement: In this publication, Ahmer Bin Hafeez was responsible for the genomic and phylogenomic analyses of the three selected probiotic candidate strains (PY2.3, BP20.15, and BB10.1). This included whole-genome sequencing analysis, taxonomic classification, and bioinformatic investigation of genes encoding antimicrobial compounds such as bacteriocins and secondary metabolites. The genomic data I generated provided the molecular foundation that confirmed and supported the experimental laboratory findings obtained by the co-authors.

In Silico Safety Assessment of *Bacillus* Isolated from Polish Bee Pollen and Bee Bread as Novel Probiotic Candidates

Ahmer Bin Hafeez¹, Karolina Pelka¹, Randy Worobo² and Piotr Szweda^{1*}

¹ Department of Pharmaceutical Technology and Biochemistry, Faculty of Chemistry, Gdansk University of Technology, Ul. G. Narutowicza 11/12, 80-233 Gdansk, Poland

² Department of Food Science, Cornell University, Ithaca, NY 14853, USA

This study investigated the genomic safety and probiotic potential of *Bacillus* strains isolated from bee pollen and bee bread using a comprehensive in silico probiogenomics approach. Bee products such as bee pollen and bee bread are increasingly recognized for their nutritional value and therapeutic properties. These products contain diverse microbial communities that may contribute to beneficial health effects. *Bacillus* species are commonly found in bee-related products, and several strains are widely used as probiotics due to their stability, ability to form spores, and tolerance to environmental stress. However, before probiotic application, it is essential to evaluate their safety and genomic characteristics. The objective of this study was therefore to perform an in-depth genomic analysis of selected *Bacillus* isolates in order to assess their probiotic potential, safety attributes, antibiotic resistance profile, and the presence of genes involved in beneficial metabolic functions.

The genomic analysis focused on three *Bacillus* isolates that were previously identified as promising probiotic candidates based on laboratory experiments. These strains included BB10.1 and BP20.15 isolated from bee bread and PY2.3 isolated from bee pollen. Whole-genome sequencing data of these isolates were analyzed using multiple bioinformatics tools to investigate genome completeness, gene annotation, virulence factors, antimicrobial resistance genes, metabolic pathways, and secondary metabolite biosynthetic clusters.

Genome quality and completeness were first evaluated using the BUSCO analysis tool. The results demonstrated a high level of genome completeness for all three isolates. Genome completeness values were 99.6% for BB10.1, 99.5% for BP20.15, and 100% for PY2.3. Additionally, most genes were present as single copy orthologs, with 99.6%, 99.3%, and 99.7% single copy BUSCO genes for BB10.1, BP20.15, and PY2.3 respectively. Fragmented genes were minimal, ranging from 0

to 0.4%, and no missing BUSCO genes were detected. These results confirmed that the genome assemblies were highly complete and suitable for further functional and comparative genomic analyses.

Pangenome analysis was conducted to examine the genomic diversity of these isolates in comparison with other *Bacillus* strains. The analysis included 180 *Bacillus subtilis* genomes and 204 *Bacillus velezensis* genomes. The pan genome analysis of *Bacillus subtilis* evaluated 752,925 genes and identified 10,810 orthologous groups, while the *Bacillus velezensis* pangenome analysis included 790,409 genes and 11,759 orthologous groups. The results indicated that the *Bacillus* pangenome is open, meaning that additional genes continue to appear as more genomes are analyzed. This open pangenome suggests that *Bacillus* species possess high genetic diversity and adaptability to different environmental conditions. However, no novel genes associated with pathogenicity or safety risks were identified in the analyzed isolates.

Functional genome annotation and Carbohydrate-Active enZymes (CAZyme) profiling revealed the presence of genes involved in various probiotic-related functions. These included genes responsible for stress tolerance, adhesion, nutrient metabolism, antioxidant activity, and vitamin biosynthesis. Several genes encoding universal stress proteins and other stress response mechanisms were detected, suggesting that these *Bacillus* strains are capable of surviving harsh conditions such as acidic pH, temperature fluctuations, and other environmental stresses encountered in the gastrointestinal tract. In addition, adhesion-related proteins were identified, which may facilitate the attachment of bacteria to intestinal epithelial surfaces and support colonization of the gut environment.

Carbohydrate active enzyme profiling showed that glycoside hydrolases were the most abundant enzyme group among the detected CAZymes. These enzymes play an important role in the degradation of complex carbohydrates such as starch, cellulose, and hemicellulose. The presence of these enzymes indicates that the studied *Bacillus* strains have the capacity to metabolize a wide range of dietary carbohydrates, which may contribute to improved digestion and nutrient availability in the host.

A critical part of the study involved evaluating the safety of these *Bacillus* isolates through the detection of antimicrobial resistance genes and potential virulence factors. Genome analysis identified several antimicrobial resistance-related genes in the isolates. Genes associated with resistance to vancomycin, beta-lactam antibiotics, and cationic antimicrobial peptides were detected through KEGG analysis. Further investigation using the CARD database revealed 18 resistance gene hits in the BB10.1 genome, 15 hits in BP20.15, and 9 hits in PY2.3. These genes were mainly related to antibiotic target alteration, antibiotic efflux systems, antibiotic target protection, reduced permeability, and antibiotic inactivation mechanisms. However, many of these genes are commonly present in non-pathogenic *Bacillus* species and may represent intrinsic resistance rather than transferable resistance traits.

Virulence gene analysis was performed using VirulenceFinder and the Virulence Factor Database. VirulenceFinder did not detect any known virulence genes associated with pathogenic bacteria in the genomes of the studied isolates. Nevertheless, VFDB analysis predicted 69 virulence-associated genes in BB10.1, 86 genes in BP20.15, and 70 genes in PY2.3. These genes were primarily associated with basic cellular functions such as adhesion, enzyme activity, immune evasion, iron acquisition, secretion systems, and stress adaptation. These genes are commonly found in many non-pathogenic bacteria and may contribute to survival and adaptation in host environments rather than pathogenicity.

Further genomic safety evaluation included the detection of undesirable genes related to toxin production, hemolytic activity, and biogenic amine synthesis. Genome annotation identified genes encoding hemolysin proteins; however, previous laboratory tests showed that the isolates exhibited γ hemolysis, which indicates the absence of hemolytic activity and is considered safe. Only genes encoding L-lactate dehydrogenase were detected, while genes responsible for D-lactate production were absent. The absence of D-lactate production is considered beneficial because excessive D-lactate accumulation may cause metabolic complications in humans.

Additional analysis of genome plasticity revealed the presence of bacteriophage-related sequences in some isolates. For example, the genome of BB10.1 contained the *Brevibacillus* phage Osiris, while the PY2.3 genome contained *Brevibacillus* phage Jimmer 1. These prophage elements

contribute to genetic diversity and bacterial evolution, but did not appear to introduce harmful genes related to pathogenicity.

The probability of the isolates being human pathogens was also evaluated using the PathogenFinder tool. The predicted probability values were very low, indicating minimal pathogenic potential. The probability scores were 0.24 for BB10.1, 0.129 for BP20.15, and 0.118 for PY2.3. Furthermore, no plasmids were detected in the genomes using the PlasmidFinder tool, which reduces the risk of horizontal transfer of antibiotic resistance genes.

Genome mining also revealed many biosynthetic gene clusters responsible for the production of antimicrobial compounds. The BB10.1 genome contained 18 biosynthetic gene clusters, including 13 clusters encoding antimicrobial peptides. The BP20.15 genome contained 15 clusters, while the PY2.3 genome contained 13 clusters. These clusters were associated with the production of antimicrobial metabolites such as surfactin, bacillaene, bacilysin, plipastatin, fengycin, bacillibactin, and subtilosin A. These compounds are well known for their antimicrobial and antifungal properties and may contribute to the ability of *Bacillus* strains to inhibit pathogenic microorganisms.

Overall, the results of this study demonstrate that the *Bacillus* isolates obtained from bee pollen and bee bread possess several genomic features associated with probiotic microorganisms. The genomes contain genes responsible for stress tolerance, adhesion, carbohydrate metabolism, vitamin synthesis, and antimicrobial compound production. At the same time, no major virulence genes or pathogenic determinants were detected, and the probability of these strains being human pathogens was extremely low. These findings support the potential use of these *Bacillus* strains as safe probiotic candidates. However, additional in vitro and in vivo studies are necessary to confirm their safety and functional probiotic properties before their application in food or pharmaceutical products.

Article 3

In Silico Safety Assessment of *Bacillus* Isolated from Polish Bee Pollen and Bee Bread as Novel Probiotic Candidates (2024);

<https://doi.org/10.3390/ijms25010666>

Accepted: 25/12/2023 / Published: 04/01/2024

IF2026 = 4.9, Q1, MHES Points: 140

***In Silico* Functional Annotation and Structural Characterization of Hypothetical Proteins in *Bacillus paralicheniformis* and *Bacillus subtilis* Isolated from Honey**

Ahmer Bin Hafeez^{1*}, Subrahmanyam Sappati^{1*}, Radoslaw Krzemieniecki¹, Randy Worobo², and Piotr Szweda¹

¹Department of Pharmaceutical Technology and Biochemistry, Faculty of Chemistry, Gdańsk University of Technology, Poland

²Department of Food Science, Cornell University, Ithaca, New York 14853, United States

This study focused on the functional annotation and structural characterization of hypothetical proteins present in *Bacillus* strains isolated from honey. *Bacillus* species are widely distributed in nature and frequently occur in bee-related products, where they can contribute to antimicrobial activity and other beneficial biological functions. Genome sequencing of *Bacillus* isolates often reveals a large number of proteins annotated as hypothetical proteins. These proteins are predicted from genomic sequences, but their biological functions remain unknown. Functional characterization of these proteins is important because they may participate in essential cellular processes, stress response, metabolism, or antimicrobial activity. The aim of this study was therefore to analyze hypothetical proteins identified in the genomes of *Bacillus paralicheniformis* and *Bacillus subtilis* isolates obtained from honey and to predict their potential functions using a comprehensive in silico approach.

Three *Bacillus* strains previously isolated from honey with antimicrobial potential were used in this investigation. Two isolates were identified as *B. paralicheniformis* (SZA14 and SZA16), while one isolate was classified as *B. subtilis* (SZB3). Genome sequencing revealed a large number of predicted proteins in these strains. *B. paralicheniformis* SZA14 encoded 4462 proteins, of which 529 proteins (11.9%) were annotated as hypothetical proteins. *B. paralicheniformis* SZA16 encoded 4270 proteins with 385 hypothetical proteins (9.0%). *B. subtilis* SZB3 contained 4348 proteins, among which 478 proteins (10.9%) were classified as hypothetical proteins. Because SZA14 and SZA16 shared 98.96% genomic similarity, the hypothetical proteins from SZA14 together with those from SZB3 were selected for detailed computational analysis.

To determine the potential biological roles of these proteins, multiple bioinformatics tools and databases were applied for functional annotation and structural prediction. Conserved domains and

motifs were identified using databases such as InterPro, CDD BLAST, SUPERFAMILY, SMART, CATH, and SCANPROSITE. Only proteins that showed functional domains in at least three independent tools were selected for further investigation. This filtering process resulted in the selection of 56 hypothetical proteins for detailed annotation. The functions of these proteins were further analyzed using PSI BLAST and the Database of Essential Genes to identify proteins involved in essential biological pathways. In addition, physicochemical parameters such as molecular weight, isoelectric point, instability index, aliphatic index, and hydrophobicity were predicted using the ProtParam tool.

Subcellular localization analysis was conducted to determine the cellular position of the identified proteins because protein localization often provides clues about biological function. Several prediction tools, including BUSCA, PSORTb, and Gpos mPLoc, were used. The results indicated that 25 proteins were predicted to be cytoplasmic, seven proteins were associated with the cytoplasmic membrane, and several proteins remained of unknown localization. Additional analyses revealed the presence of signal peptides and membrane-associated regions in a subset of proteins. Eight proteins were predicted to contain signal peptides involved in secretion processes, while seven proteins possessed transmembrane alpha helices that are typically associated with membrane transport, receptor activity, and molecular signaling pathways.

Physicochemical analysis of the 56 annotated hypothetical proteins provided further insights into their structural characteristics. The results demonstrated that the majority of the proteins were hydrophilic. Specifically, 49 proteins (87.5%) showed negative GRAVY values, indicating hydrophilic and globular characteristics, while seven proteins (12.5%) were predicted to be hydrophobic and likely associated with cellular membranes. These structural characteristics are consistent with the functional roles of many bacterial proteins, involved in enzymatic activity, metabolic processes, and cellular regulation.

Functional classification of the annotated proteins revealed that these hypothetical proteins were involved in diverse biological processes. The predicted proteins included enzymes, binding proteins, membrane proteins, transport-related proteins, lipoproteins, and regulatory proteins. Many of these proteins were associated with essential cellular mechanisms such as DNA replication, transcription, metabolic regulation, ion transport, and cellular signaling. Several

proteins were also linked to bacterial survival mechanisms, including sporulation, biofilm formation, and motility. These processes are particularly important for *Bacillus* species because they allow the bacteria to adapt to environmental stress conditions and maintain long-term survival in competitive ecological niches.

Protein structure prediction was performed to gain deeper insight into the structural organization and stability of the selected proteins. Homology-based structural models were generated using the SWISS MODEL server, while de novo structural predictions were obtained using AlphaFold2. The predicted 3-dimensional structures were visualized and analyzed using UCSF Chimera. To further refine the structural models, molecular dynamics simulations were carried out to examine the stability of the proteins under physiological conditions. During these simulations, root mean square deviation and root mean square fluctuation analyses were conducted to evaluate structural stability and flexibility. These simulations improved the reliability of the predicted models and allowed identification of regions critical for protein activity.

Protein-protein interaction analysis was also performed using the STRING database to explore the potential interaction networks of the annotated proteins. Interaction networks provide important information regarding functional associations between proteins within the cell. The predicted interaction partners suggested that many of the analyzed proteins participate in metabolic pathways, DNA replication, transcriptional regulation, and cellular stress responses. Some proteins were linked to essential enzymatic activities such as ribonucleoside diphosphate reductase, DNA primase, and ribonuclease enzymes, which play crucial roles in DNA synthesis and gene expression.

To evaluate the reliability of the functional prediction pipeline, receiver operating characteristic analysis was performed using a curated dataset of experimentally validated proteins. The analysis demonstrated a high level of predictive accuracy for the computational tools used in this study. The overall average accuracy of the employed bioinformatics tools was 95.3%, with an average sensitivity of 94.05% and specificity of 99.25%. The average area under the ROC curve was 0.96, indicating strong reliability and robustness of the computational predictions used for functional annotation of hypothetical proteins.

Overall, the results of this study demonstrate that a large proportion of previously uncharacterized hypothetical proteins in *Bacillus* genomes can be functionally annotated using integrated bioinformatics approaches. The analysis successfully predicted functions for 56 hypothetical proteins and revealed their involvement in essential cellular processes, such as sporulation, biofilm formation, metabolic regulation, motility, and DNA replication. These findings provide valuable insights into the biological roles of previously unknown proteins and contribute to a better understanding of *Bacillus* cell biology. Furthermore, the identified proteins may represent potential targets for future experimental validation and biotechnological applications. However, experimental studies including biochemical assays, structural analysis, and molecular characterization will be necessary to confirm the predicted functions and fully elucidate the biological significance of these proteins.

Article 4

***In Silico* Functional Annotation and Structural Characterization of Hypothetical Proteins
in *Bacillus paralicheniformis* and *Bacillus subtilis* Isolated from Honey (2025)**

<https://doi.org/10.1021/acsomega.4c07105>

Accepted: 25/12/2024 / Published: 27/02/2025

IF2026 = 4.3, Q1, MHES Points: 70

Genomic and Functional Landscape of Antimicrobial Peptides and Lipopeptides from Ginger Honey-derived *Bacillus subtilis*

Ahmer Bin Hafeez¹, and Piotr Szweda^{1*}

¹ Department of Pharmaceutical Technology and Biochemistry, Faculty of Chemistry, Gdańsk University of Technology, ul. G. Narutowicza 11/12, Gdańsk 80-233, Poland

This study examined two *Bacillus subtilis* strains, Miodo8 and Miodo10, isolated from multifloral ginger honey, to determine their antimicrobial potential. It combined phenotypic testing, fermentation optimization, genome sequencing, pangenome analysis, and in silico screening of antimicrobial peptides to build a complete picture of the strains' bioactive potential.

A small number of colonies from the honey sample exhibited strong antagonistic activity, and Miodo8 and Miodo10 were selected for further study due to their strongest anti-staphylococcal effects. Fermentation in several growth media, including LB, BHI, MHB, TSB, YPD, and MDB, showed that yeast peptone dextrose (YPD) supported the highest antimicrobial output in both strains, producing the largest inhibition zones against *Staphylococcus aureus*. This indicates that medium composition strongly affects metabolite production.

Functionally, both strains were active mainly against Gram-positive bacteria. Their crude extracts inhibited methicillin-resistant *S. aureus*, methicillin-sensitive *S. aureus*, *Staphylococcus epidermidis*, and *Listeria monocytogenes* most effectively, while activity against Gram-negative bacteria and yeasts was absent or weak. This pattern is consistent with the known behavior of many *Bacillus*-derived lipopeptides and bacteriocins, which often act more strongly against Gram-positive cells because of differences in cell envelope structure. The extracts also inhibited *Bacillus subtilis* ATCC 6633, which reflects the competitive antimicrobial capacity of the isolates.

The safety and physiological tests added nuance to the study. Both strains exhibited β -hemolysis on blood agar, but quantitative assays indicated that hemolytic activity was limited, and Miodo10 showed no detectable hemolysis within the tested range. It indicated that species identity alone does not guarantee safety, because *Bacillus* isolates can carry hemolysin-related genes or other cytotoxic determinants. On the other hand, both strains were broadly susceptible to several antibiotics, though they were resistant to bacitracin and showed strain-specific differences for

rifampicin and penicillin. They also displayed broad carbohydrate utilization, moderate tolerance to salt, sensitivity to high glucose, and best growth at neutral to slightly alkaline pH. These findings suggest adaptation to the honey environment, while also showing that further safety validation is still required before any translational application.

Genome sequencing provided strong evidence for biosynthetic potential. Miodo8 and Miodo10 each had genomes of about 4.1 Mb, with similar GC content, similar gene counts, and no plasmids detected. Functional annotation identified around 112 carbohydrate-active enzymes in each genome, indicating substantial metabolic flexibility. More importantly, genome mining revealed numerous biosynthetic gene clusters linked to surfactin, fengycin, plipastatin, bacillibactin, bacillaene, subtilosin A, bacilysin, and pelgipeptin. Comparative synteny showed that several of these clusters, especially subtilosin A, surfactin, bacillibactin, bacilysin, and fengycin, were highly conserved relative to *Bacillus subtilis* 168. The genomes also contained mobile genetic elements and intact prophages, which point to genomic plasticity and possible horizontal gene transfer. Resistome analysis detected acquired and intrinsic resistance determinants, including genes associated with resistance to streptomycin, macrolides, and other antibiotic classes.

The comparative genomic analysis placed both isolates within an open *B. subtilis* pan genome, meaning that the species continues to gain new genes through evolution and niche adaptation. Miodo8 and Miodo10 were extremely close to each other at the nucleotide level, but each retained a few strain-specific genes, which suggests minor specialization within a shared genetic background. This matters because the strains were not just routine *Bacillus* isolates. They belong to a dynamic species known for metabolic versatility and secondary metabolite production. The *in silico* AMP screening strengthened that conclusion by identifying several high-confidence peptide candidates with the expected cationic and hydrophobic features typical of antimicrobial peptides.

Overall, the study shows that ginger honey can serve as a source of bioactive *B. subtilis* strains with real antimicrobial promise. The antimicrobial activity was supported by biosynthetic gene clusters, secretion potential, and predicted antimicrobial peptides. Future research efforts should focus on validating the predicted metabolites and antimicrobial peptides through LC-MS/MS analysis, peptide isolation, and structural characterization. Additional studies are also needed to evaluate toxicity and safety in greater detail, particularly because hemolytic activity and resistance

markers were detected. These approaches will help determine the therapeutic and biotechnological potential of the identified *B. subtilis* strains.

5. Article 5

Genomic and functional landscape of antimicrobial peptides and lipopeptides from ginger honey-derived *Bacillus subtilis* (2026)

<https://doi.org/10.1007/s00438-026-02411-5>

IF 2026=2.1, Q2, MHES Points: 100

Accepted: 15/03/2026

Published: 01/04/2026

Copyright Disclaimer: The following publication is reproduced with permission from Springer Nature. The article "Genomic and functional landscape of antimicrobial peptides and lipopeptides from ginger honey-derived *Bacillus subtilis*" by Bin Hafeez et al., published in ***Molecular Genetics and Genomics*** (April 2026), is included in its entirety as part of this thesis under **License Number 6277671348832**, granted by Copyright Clearance Center on May 28, 2026. Reproduced with permission from Springer Nature.

3. Unpublished Data and Results

3.1 Discovery of a Novel Two-Component Lantibiotic, Paralichenidin (Pld): A Relative of Amyloliuecidin GF610

3.1.1 Bioinformatic Identification of the Biosynthetic Gene Cluster

To identify bacteriocin-encoding operons within the genomes of *Bacillus paralicheniformis* SZA14 and *B. paralicheniformis* SZA16 [171], biosynthetic gene cluster (BGC) mining was performed using two complementary tools: BAGEL4 and antiSMASH. BAGEL4 scans for small open reading frames (ORFs) that may encode bacteriocins and subsequently examines the surrounding genomic context for associated components, including transporters and immunity proteins, enabling the full bacteriocin operon to be delineated [266]. antiSMASH was additionally employed to identify genes encoding antibiotics and secondary metabolites [267], which are frequently associated with members of the *Bacillus* genus. antiSMASH analysis revealed a BGC with significant similarity to the previously characterized two-component lantibiotic amyloliuecidin GF610, produced by *Bacillus velezensis* [190]. This cluster was subsequently studied in detail to gain mechanistic insight into the biosynthetic machinery of what appeared to be a novel two-component lantibiotic, herein designated Paralichenidin (Pld).

3.1.2. Organization and Architecture of the Paralichenidin Operon

The Pld operon spans approximately 29.25 kbp and is located on contig one of the draft genome of *B. Paralicheniformis* SZA16 [171]. It encodes two putative lantibiotic structural peptides: ORF1 (*PldA1*), which encodes a 68-amino-acid prepropeptide (*Pld* α), and ORF3 (*PldA2*), which encodes a 71-amino-acid prepropeptide (*Pld* β). *Pld* α shares 77.9% amino acid identity with the GF610 α prepropeptide, while *Pld* β shares 70.4% identity with the GF610 β prepropeptide (**Table 3**), supporting their classification as related two-component lantibiotics [190,230] (**Figure 9**). As expected for a two-peptide lantibiotic system, two dedicated modification enzymes are encoded within the operon [230]. ORF2 (*PldM1*) encodes a 1,105-residue modification enzyme sharing 69.33% identity with the amyloliuecidin GF610M1[190], 40.04% with the haloduracin HalM1 enzyme [230], and 38.19% with the lichenicidin modification enzyme LchM1 [268]. ORF4 (*PldM2*) encodes a 1,030-residue modification enzyme sharing 66.5% identity with amyloliuecidin GF610M2 [190] and 34.42% with haloduracin HalM2 [230]. Together, these

enzymes are predicted to carry out the dehydration and cyclization reactions required for lanthionine bridge formation in both peptide components [230].

Table 3. BLAST results of the α and β components of Paralichenidin, including both prepropeptide and core peptide sequences, alongside their closest characterized homologues, producer strains, percentage identity, alignment length, and E-value.

Description	Species	Percentage Identity	Accession Length	E-value
α-PrePropeptide				
Amyloliuecidin GF610 α	<i>B. velezensis</i>	77.941	68	1.96E-40
Plantaricin α	<i>L. plantarum</i>	52.941	59	2.42E-12
Haloduracin α	<i>B. Halodurans</i>	48.485	69	2.93E-21
Lichenicidin α	<i>B. licheniformis</i>	42.647	74	2.10E-15
Lacticin α	<i>L. lactis</i>	41.304	59	4.52E-08
Mersacidin	<i>Bacillus</i> sp. strain	37.705	68	1.89E-10
β-PrePropeptide				
Amyloliuecidin GF610 β	<i>B. velezensis</i>	70.423	71	4.20E-38
Lichenicidin β	<i>B. licheniformis</i>	49.254	72	2.21E-14
Haloduracin β	<i>B. Halodurans</i>	46.552	65	2.15E-12
Lacticin β	<i>L. lactis</i>	24.615	65	1.98E-04
Plantaricin β	<i>L. plantarum</i>	27.87	67	6.07E-01
α-Core peptide				
Amyloliuecidin GF610 α	<i>B. velezensis</i>	77.78	28	3.00E-22
Plantaricin α	<i>L. plantarum</i>	66.67	28	4.00E-14
Haloduracin α	<i>B. Halodurans</i>	74.07	28	2.00E-18
Lichenicidin α	<i>B. licheniformis</i>	66.67	26	5.00E-07
Lacticin α	<i>L. lactis</i>	66.67	30	2.00E-08
Mersacidin	<i>Bacillus</i> sp. strain	85.71	20	2.00E-04
β-Core peptide				
Amyloliuecidin GF610 β	<i>B. velezensis</i>	69.23%	26	2.00E-14
Lichenicidin β	<i>B. licheniformis</i>	46.67	32	2.00E-03
Haloduracin β	<i>B. Halodurans</i>	47.62	24	7.00E-05
Lacticin β	<i>L. lactis</i>	50	29	3.10E-02

3.1.3. Transport and Immunity Determinants

Downstream of the modification enzymes, ORF5 (*PldT1*) encodes a predicted ABC transporter ATP-binding protein (**Figure 9**) with 81.12% identity to the amylioluecidin transporter GF610T

and 53.62% to the haloduracin transporter HalT, suggesting a conserved role in propeptide export [230]. Following the pattern established in lichenicidin, it is likely that this LchT-like transporter also cleaves the N-terminal glycine leader sequence from both propeptides upon secretion [269], while the PldP-like protease (ORF14, see below 4.1.4) subsequently removes the six newly exposed N-terminal residues from the β peptide to yield the mature bacteriocin [269,270].

Several ORFs in the cluster appear to encode components of a bacteriocin immunity system [269]. ORF7 (*PldT2*, 306 amino acid residues) and ORF11 (*PldT3*, 301 amino acid residues) share 61.1% and 53.42% identity, respectively, with the bacitracin ABC transporter ATP-binding protein of *Halalkalibacterium halodurans* (formerly known as *Bacillus halodurans*) [230]. ORF8 (*PldF1*, 235 amino acid residues) and ORF9 (*PldG1*, 246 amino acid residues) encode ABC transporter permease-related proteins [271] sharing 54.04% and 63.16% identity with *H. halodurans* homologues, respectively. Similarly, ORF12 (*PldF2*, 232 amino acid residues) and ORF13 (*PldG2*, 252 amino acid residues) share 42.92% and 52.23% identity with ABC transporter permeases from the same organism.

3.1.4. Regulatory and Processing Elements

Embedded between the two transporter clusters, ORF10 (*PldE2*) encodes a 77-amino-acid helix-turn-helix (HTH) transcriptional regulator [269] sharing 84.72% identity with the amyloliquecidin ORF11 (*PldT3*) homologue and 61.97% with the corresponding protein from the haloduracin operon. HTH regulators of this family play a pivotal role in binding DNA and modulating gene expression, suggesting ORF10 functions as a local transcriptional regulator of the Pld operon. ORF14 (*PldP*) encodes a 467-residue lanthionine-specific protease belonging to the S8 family of serine peptidases, sharing 67.18% identity with the amyloliquecidin LanP and 27.13% with the haloduracin protease. This enzyme is predicted to cleave the leader sequence from the prepropeptides, a critical step in activating the mature lantibiotic [272]. ORF6 (*PldE1*) encodes a 100-amino-acid hypothetical protein of unknown function (**Figure 9**).

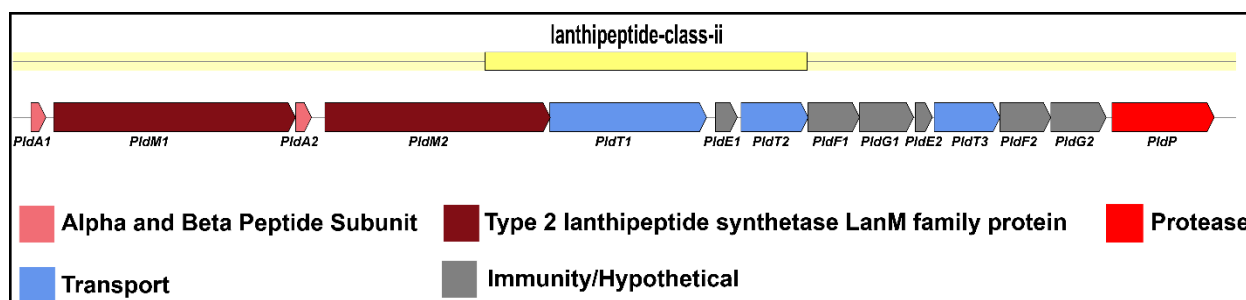


Figure 9. Proposed biosynthetic gene cluster of Paralichenidin. Bacteriocin homologues are identified using the accepted nomenclature for describing lantibiotics. For Paralichenidin, *PldA1* and *PldA2* encode the putative bacteriocins, *PldM1* and *PldM2* encode the accompanying modification enzymes and *PldT* and *PldP* are involved in bacteriocin transport and leader cleavage. Genes are color-coded as per antiSMASH, indicating the putative role of each protein. Figure Created in Adobe Illustrator.

3.2 Deduced Peptide Sequences, Post-Translational Modifications, and Phylogenetic Analysis of Paralichenidin Prepropeptides and Core Peptides

The structural gene products of the *Pld* operon were deduced from the predicted ORF sequences and analyzed in terms of their prepropeptide organization, post-translational modification patterns, and evolutionary relationships with known two-component lantibiotics.

The *PldA* prepropeptide comprises 68 amino acids in total, consisting of a 40-residue N-terminal leader sequence followed by a 28-residue core peptide. The leader sequence is predicted to direct the prepropeptide for post-translational modification and subsequent export, consistent with the biosynthetic logic established for related two-component lantibiotics. The unmodified core peptide sequence (CEWYNISCQLGNKGQWCTLTKECQRSCK) is predicted to undergo a series of post-translational modifications characteristic of class II lantibiotics. Specifically, serine and threonine residues within the core are predicted to be dehydrated to 2,3-didehydroalanine (Dha) and (Z)-2,3-didehydrobutyrine (Dhb), respectively, by the cognate *PldM1* modification enzyme, prior to intramolecular cyclization to form lanthionine and methyllanthionine thioether bridges. The resulting modified core peptide is predicted to have the sequence CEWYNIDhaCQLGNKGQWCDhbLDhbKECQRSCK, with the dehydrated residues at positions 7 (Dha), 18 (Dhb), and 20 (Dhb) flanking the conserved cysteine residues involved in ring formation.

The Pld β prepropeptide comprises 71 amino acids, consisting of a 45-residue leader sequence and a 26-residue core peptide. As observed for the elongated β peptides of related two-component lantibiotics such as Hal β and Lch β , the Pld β leader sequence is considerably longer than that of Pld α , a feature thought to reflect the additional processing steps required for the β component. The unmodified Pld β core peptide sequence (TTPSSLPCGTLVTAVWCPSNACTSDC) is similarly predicted to undergo extensive post-translational modification by the PldM2 enzyme. The modified core peptide is predicted as DhbDhbPDhaDhaLPCGDhbLVDhbAVWCPDhaNACDhbSDC, with dehydration of Thr-1, Thr-2, Ser-4, Ser-5, Thr-10, Thr-13, Ser-19, and Thr-23 to their respective Dhb and Dha derivatives, and subsequent cyclization to generate four methyllanthionine bridges. Together, the predicted modification patterns of Pld α and Pld β are fully consistent with those observed in the closely related peptides, further supporting the assignment of Paralichenidin as a member of this lantibiotic subfamily.

To investigate the evolutionary relationships of Paralichenidin within the broader family of two-component lantibiotics, phylogenetic analyses were performed on both the prepropeptide and core peptide sequences of the α and β components independently. The reference sequences included in the analysis were selected to represent the major characterized members of this lantibiotic subfamily: Amyloliquecidin GF610 α/β [190], Plantaricin (Pls) α/β [273], Haloduracin (Hal) α/β [230], Lichenicidin (Lch) α/β [229], Lacticin (Ltn) 3147 α/β [231], and Mersacidin [274], alongside the corresponding Pld α and Pld β sequences. Mersacidin, a single-peptide lantibiotic, was included in the α peptide analysis specifically on the basis of its sequence homology with the α components of two-component lantibiotics, as independently confirmed by BLAST analysis.

Sequence alignment was performed using MAFFT (v7) [275] with automatic method selection, a gap opening penalty of 1.53, and a gap extension penalty of 0.123, using the BLOSUM substitution matrix and viewed in the Bioedit software. All alignments were carried out via the phylogeny.fr (<https://www.phylogeny.fr/>) advanced analysis pipeline [276]. Phylogenetic trees were inferred using IQ-TREE under the ultrafast bootstrap approximation (UFBoot) with 1,000 bootstrap replicates, a perturbation strength of 0.5, and a stopping rule of 100 iterations. The substitution model was selected automatically from protein substitution models using the built-in ModelFinder algorithm. Trees were generated separately for α and β sequences, and in each case for both the

full prepropeptide (including leader sequences) and the mature core peptides, yielding four trees in total. The resulting Newick-format tree files were imported into the Interactive Tree of Life (iTOL) platform for visualization [277]. Trees were rooted at the midpoint, and final figures were refined in Adobe Illustrator for clarity and publication quality (**Figure 10**).

Multiple sequence alignment of Pld α and Pld β with representative two-component lantibiotics, including Amyloliquecidin GF610, Haloduracin, Lichenicidin, Plantaricin, and Lacticin (Ltn), revealed a high degree of conservation within the mature core regions, particularly at cysteine residues predicted to participate in lanthionine and methyllanthionine ring formation (**Figure 10**). Despite this overall conservation, several amino acid substitutions were identified within the Pld peptides at positions flanking these conserved cysteines.

3.2.1 Sequence Conservation and Variation in the Pld α Core Peptides

To place Paralichenidin within the broader context of characterized two-component lantibiotics, multiple sequence alignments were performed on both the prepropeptide (**Figure 10A**) and mature core peptide sequences (**Figure 10B**) of Pld α and Pld β alongside GF610 α/β , Hal α/β , Lichenicidin α/β , Pls α/β , Ltn 3147 α/β , and Mersacidin. The alignments revealed a high degree of conservation within the mature core regions, particularly at the cysteine residues involved in lanthionine and methyllanthionine bridge formation, consistent with the essential structural role of these residues in defining the characteristic lanthipeptide fold. Despite this overall conservation, several amino acid substitutions were identified at specific positions flanking the conserved cysteine framework, providing insight into the sequence features that distinguish Paralichenidin from its closest relatives.

In the Pld α core peptide, cysteine residues at positions 1, 8, 17, 23, and 27, together with their structurally equivalent partners across the homologues, were near-universally conserved across all sequences examined, confirming that the overall bridging topology is conserved across the family. The observed variation was instead concentrated at a discrete set of inter-cysteine positions (**Figure 10B**). At position 2, Pld α carries a glutamic acid, whereas GF610 α , Hal α , and Lch α each carry an alanine. At position 9, Pld α carries a glutamine, while GF610 α carries a lysine, Hal α carries an arginine, and Lch α carries a proline. At position 15, Pld α carries a glutamine, whereas

GF610 α and Hal α share an alanine, and Lch α carries a tyrosine. At position 21, Pld α and Lch α both carry a lysine, while GF610 α and Hal α carry a valine. At position 25, Pld α carries an arginine, compared to a serine in GF610 α and a proline in both Hal α and Lch α . Finally, at position 28, Pld α carries a lysine, while GF610 α , Hal α , and Lch α each carry an asparagine. Since none of these variable positions coincide with a bridge-forming cysteine, they are unlikely to disrupt the core lanthipeptide fold and instead likely represent surface-exposed residues that contribute to the fine-scale sequence differences distinguishing Paralichenidin from its closest homologues.

3.2.2. Sequence Conservation and Variation in the Pld β Core Peptides

In the Pld β core peptide, the conserved cysteine residues and several neighboring positions were likewise retained across all compared β peptides, confirming preservation of the methyllanthionine bridge architecture (**Figure 10 E, F**). Variable positions were distributed across two functionally distinct regions of the peptide: the N-terminal hydrophobic stretch and the C-terminal region. Within the N-terminal region, Pld β carries a threonine at position 12, whereas GF610 β , Hal β , and Lch β all retain a valine. At position 13, Pld β carries a leucine, compared to a phenylalanine in GF610 β , a serine in Hal β , and a threonine in Lch β . At position 17, Pld β carries a valine while both GF610 β and Hal β carry an alanine, and at position 18, Pld β carries a tryptophan while GF610 β carries a phenylalanine. In the C-terminal region of Pld β , at position 22, Pld β carries an asparagine, whereas GF610 β , Hal β , and Lch β all carry a threonine. At position 23, Pld β carries an alanine, compared to a lysine conserved across GF610 β , Hal β , and Lch β , a substitution that introduces a notable difference in charge at this position, since lysine carries a positive charge while alanine is neutral. Most strikingly, at position 27, preceding the terminal cysteine of the β peptide, Pld β carries an aspartic acid, while GF610 β carries a serine, Hal β carries a glutamine, and Lch β carries an arginine (**Figure 10F**). This position thus shows not only variation in residue identity but also considerable variation in charge character across the family, with Pld β being the only member to carry a negatively charged residue at this site. Taken together, the charge differences at positions 23 and 27 within the C-terminal region suggest that the interaction between Pld β and the Pld α :lipid II complex may be electrostatically distinct from equivalent interactions in other two-component lantibiotic systems, potentially contributing to the specificity of the Paralichenidin two-peptide complex and its unique antimicrobial profile.

3.2.3. Phylogenetic Analysis of Prepropeptide and Core Peptide Sequences

To further establish the evolutionary position of Paralichenidin within the two-component lantibiotic family, phylogenetic analyses were conducted on both the α (**Figure 10C, D**) and β peptide sequences in their prepropeptide and core peptide forms (**Figure 10 G, H**). The reference sequences included representatives of all major characterized members of this subfamily: Amyloliuecidin GF610, Plantaricin, Haloduracin, Lichenicidin, and Lacticin 3147. Mersacidin, a single-peptide lantibiotic, was additionally included in the α peptide analysis based on its sequence homology with the α components of two-component lantibiotics, as independently confirmed by BLAST analysis [278].

In the α prepropeptide tree (**Figure 10C**), Pld α grouped most closely with GF610 α , while Pls α clustered with Hal α . Together, these four sequences formed a broader, well-supported clade. Lch α and Ltn α formed a separate clade alongside Mersacidin, reflecting their greater divergence from the Amyloliuecidin/Haloduracin lineage. The topology of this tree suggests that the leader sequences of Pld α and GF610 α are most closely related, pointing to a shared biosynthetic ancestry at the prepropeptide level.

In contrast, the phylogenetic analysis based on the mature core peptide sequence (**Figure 10D**) revealed a different topology. Once the leader sequences were removed, Pld α , GF610 α , Hal α , and Pls α converged into a single tightly supported clade with notably short branch lengths and high bootstrap support (>80), reflecting a high degree of sequence conservation among these mature core peptides relative to the other lantibiotics included in the analysis. This tight clustering suggests that despite their distinct sequences, these core peptides have been maintained within a narrow sequence space, most likely driven by the structural constraints imposed by their shared lipid II-binding mechanism. In other words, while the leader sequences have diverged sufficiently to reflect lineage-specific differences, the core peptides have been maintained within a narrow sequence space that preserves their functional architecture. The Lichenicidin/Lacticin/Mersacidin grouping remained distinct and separate in the core peptide tree as well, consistent with their placement in the prepropeptide analysis.

For the β component, the phylogenetic signal was strikingly more consistent across both trees. In both the prepropeptide tree (**Figure 10G**) and the core peptide tree (**Figure 10H**), Pld β grouped clearly and consistently with GF610 β , supported by bootstrap values greater than 80. Lch β and Hal β formed a closely adjacent sister clade in both trees, while Ltn β and Pls β resolved as a more distantly related group in each case. The stability of this topology across both prepropeptide and core peptide analyses indicates that the evolutionary relationship between Pld β and GF610 β is robust and is preserved in both the leader and mature peptide sequences, reflecting a closer and more unambiguous phylogenetic affiliation than that observed for the α component.

Taken together, the phylogenetic analyses reveal a nuanced picture of Paralichenidin's evolutionary history. The β peptide places Paralichenidin unambiguously within the Amyloliqecidin lineage, while the α peptide analysis highlights the strong conservation of mature core peptide sequences across the broader Amyloliqecidin/Haloduracin/Plantaricin group — a conservation that likely reflects shared functional constraints rather than strict vertical descent. These findings, combined with the sequence identity data and operon organization described above, firmly support the classification of Paralichenidin as a novel member of the amyloliqecidin/haloduracin subfamily of two-component lantibiotics, biosynthesized by *Bacillus paralicheniformis* SZA14 and SZA16.

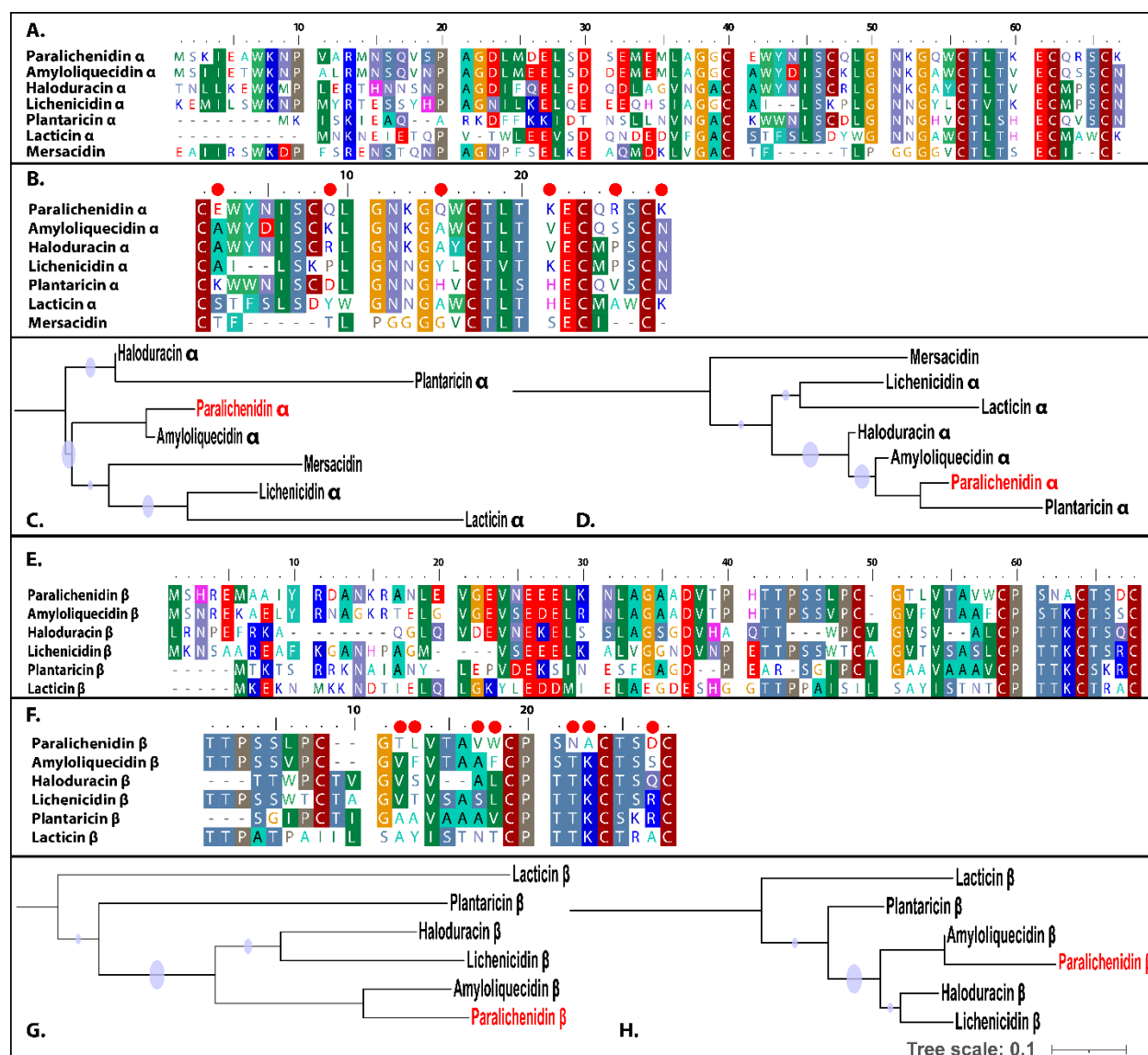


Figure 10. Multiple sequence alignment and phylogenetic analysis of Pld α and β peptides with representative two-component lantibiotics. **(A)** Multiple sequence alignment of the Pld α prepropeptide with Amyloliquecidin α , Haloduracin α , Lichenicidin α , Plantaricin α , Lacticin α , and Mersacidin. **(B)** Multiple sequence alignment of the Pld α mature core peptide with the same set of α peptides; red dots indicate positions of amino acid variation among Pld α and its closest homologues, flanked by conserved cysteine residues involved in lanthionine and methylanthionine bridge formation. **(C)** Maximum-likelihood phylogenetic tree of α prepropeptide sequences, rooted at the midpoint, showing Pld α clustering with Amyloliquecidin α . **(D)** Maximum-likelihood phylogenetic tree of α core peptide sequences, rooted at the midpoint, showing Pld α within a tightly supported clade alongside Haloduracin α , Amyloliquecidin α , and Plantaricin α . **(E)** Multiple sequence alignment of the Pld β prepropeptide with Amyloliquecidin β , Haloduracin β , Lichenicidin β , Plantaricin β , and Lacticin β . **(F)** Multiple sequence alignment of the Pld β mature core peptide with the same set of β peptides; red dots indicate positions of amino acid variation among Pld β and its closest homologues. **(G)** Maximum-likelihood phylogenetic tree of β prepropeptide sequences, rooted at the midpoint, showing Pld β clustering with Amyloliquecidin β . **(H)** Maximum-likelihood phylogenetic tree of β core peptide sequences, rooted at the midpoint, showing Pld β clustering closely and consistently with Amyloliquecidin β . Figure Created in Adobe Illustrator.

3.3. Predicted Structure and Bridge Formations

Sequence comparisons with known two-peptide lantibiotics revealed that Paralichenidin conforms to the conserved structural patterns observed across this bacteriocin family. Amyloliquecidin GF610 exhibited the greatest sequence similarity, consistent with a shared mode of action centered on lipid II binding [229,230]. Conserved residues in the lanthionine and methyllanthionine bridge-forming regions imply a shared structural scaffold among the α peptides consistent with their established role in lipid II recognition and binding. In two-component lantibiotics, the α peptide binds lipid II with high affinity, anchoring the complex at the membrane surface, whereupon the β peptide docks onto the α :lipid II complex to assemble the lethal pore-forming complex [233,279].

Using the GF610 α and Hal α peptides as structural templates, the following bridge topology was predicted for Pld α : a disulfide bridge between Cys-1 and Cys-8, a lanthionine bridge between Ser-7 and Cys-17, and methyllanthionine bridges between Thr-18 and Cys-23, and between Thr-20 and Cys-27, with Ser-26 remaining unmodified (**Figure 11A**). For Pld β , modelled on the GF610 β and Lch β peptides, methyllanthionine bridges are predicted between Thr-1 and Cys-8, Thr-13 and Cys-17, Ser-19 and Cys-22, and Thr-23 and Cys-26. Residues Thr-2, Ser-4, Ser-5, and Thr-10 are predicted to undergo dehydration to di-dehydrobutyrine (Dhb) or di-dehydroalanine (Dha), respectively, while Ser-24 remains unmodified (**Figure 11B**). Taken together, the sequence conservation, MSA variation analysis, phylogenetic positioning, predicted post-translational modifications, and structural modelling collectively support the classification of Paralichenidin as a novel two-component lantibiotic belonging to the amyloliquecidin/haloduracin subfamily, biosynthesised by *B. paralicheniformis* SZA14 and SZA16. The unique combination of substitutions identified in the C-terminal region of Pld β , in particular, warrants further experimental investigation to determine their functional consequences for the antimicrobial activity and target specificity of this novel lantibiotic.

TTPSSLPCGTLVTAVWCPSNACTSDC for the Pld β peptide. Subsequently, steered molecular dynamics was used to bring selected side-chain sulfur atoms into proximity and generate conformations consistent with the expected thioether-constrained topology. For the Pld α peptide, restraints corresponding to the following cross-links were applied: Cys1–Cys8, Ser7–Cys17, Thr18–Cys23, and Thr20–Cys27. For the Pld β peptide, restraints corresponding to Thr1–Cys8, Thr13–Cys17, Ser19–Cys22, and Thr23–Cys26 were used. After generation of the modified topologies, CHARMM-compatible parameters for the dehydrated amino acids Dha, Dhb, and Abu were introduced according to Turpin et al. [281]. The resulting peptide structures were energy-minimized and equilibrated for 50 ns. Representative structures obtained from this procedure are shown in **Figure 12**.

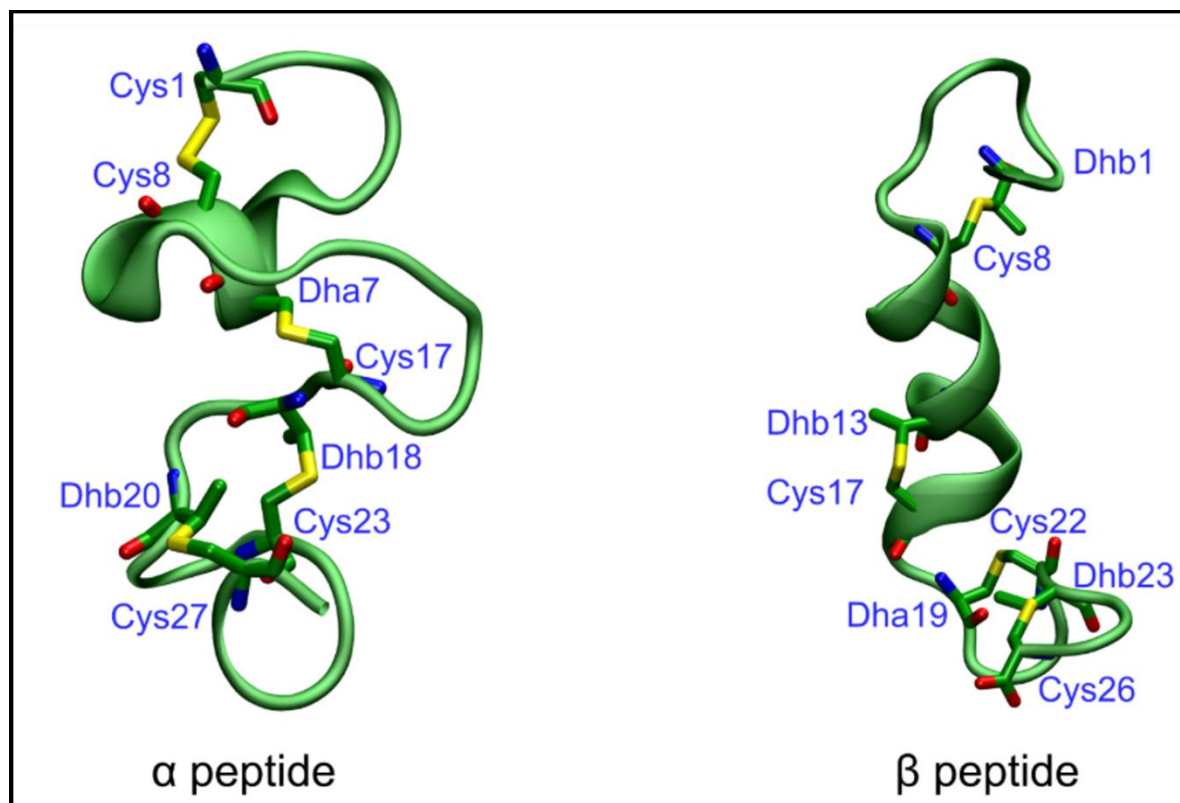


Figure 12. The structures of Pld α and Pld β peptides, obtained from equilibrium simulations based on their post translational modification sequences. Figure Created in VMD.

Molecular dynamics simulations of a single Lipid II molecule embedded in a lipid bilayer were first performed to generate representative Lipid II conformations for subsequent simulations of α - and β -peptide–Lipid II complexes. All simulations were carried out using the CHARMM36

additive force field [282]. The membrane systems were prepared with CHARMM-GUI Membrane Builder [283], using a 3:7 mixture of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) to mimic a Gram-negative bacterial inner membrane. Each leaflet contained 60 POPG and 140 POPE molecules, giving an initial membrane area of $109 \times 109 \text{ \AA}^2$ in the XY plane. The system was solvated with a 50 $\text{\AA}$ water layer on each side of the bilayer and neutralized with 219 sodium and 99 chloride ions, corresponding to an ionic strength of approximately 0.15 M. Initial Lipid II coordinates were taken from Sheridan et al. [284]. The Lipid II molecule was oriented with its polyprenyl tail approximately parallel to the bilayer normal and inserted into the membrane such that the headgroup was positioned near the membrane–water interface. Water molecules and POPG or POPE molecules overlapping with the Lipid II headgroup were removed.

Energy minimization, equilibration, and production simulations were performed with GROMACS [285] following the standard CHARMM-GUI protocol [286]. A 2 fs integration time step and the leap-frog Verlet algorithm were used. Periodic boundary conditions were applied in all three dimensions, with initial box dimensions of $109 \text{ \AA} \times 109 \text{ \AA} \times 140 \text{ \AA}$. Covalent bonds involving hydrogen atoms were constrained with the P-LINCS algorithm [287], whereas water molecules were constrained using SETTLE [288]. A 12 $\text{\AA}$ cutoff was used for short-range nonbonded interactions, and the neighbor list was updated every time step. Long-range electrostatic interactions were treated with the particle-mesh Ewald method [289]. Production simulations were performed at 303.15 K in the NPT ensemble. Temperature was maintained with the Nosé–Hoover thermostat [290] using a coupling time of 1 ps, and pressure was controlled semi-isotropically at 1 bar with the Parrinello–Rahman barostat [291] using a coupling time of 5 ps.

The Pld α - and Pld β -peptide structures were generated from the modified sequences proposed above, namely CysEWYNIDhaCysQLGNKGQWCysDhbLDhbKECysQRSCysK for the α peptide and DhbDhbPDhaDhaLPCysGDhbLVDhbAVWCysPDhaNACysDhbSDCys for the β peptide.

The α - and β -peptide–Lipid II complexes were simulated in hydrated lipid bilayers using the same simulation parameters as described above, unless stated otherwise. Initial coordinates of the complexes were generated by superposing the prepared α - or β -peptide and Lipid II structures onto complex models obtained from Boltz-2 docking predictions [292]. Because the Pld α and Pld β peptides contain noncanonical post-translational modifications that are not natively represented

in Boltz-2, docking calculations were performed using the unmodified peptide sequences while retaining restraints corresponding to the expected sulfur-containing cross-links. Lipid II was included as a ligand represented by its pyrophosphate-containing headgroup (SMILES: C/C(C)=C/COP(=O)([O-])OP(=O)([O-])OC1OC(CO)CCC1NC(C)O, see **Figure 13**).

To characterize α - and β -peptide binding to Lipid II, we calculated the free energy as a function of the center-of-mass distance, r , between the pyrophosphate moiety of Lipid II and the C-terminal thioether-constrained Lipid II-binding region of the peptide. For the Pld α peptide, this region was defined as residues 17–24 (with TLTKCQ motif), encompassing the two predicted thioether-constrained rings. For the Pld β peptide, the corresponding region was defined as residues 1–8. Replica-exchange umbrella sampling simulations were performed with the PLUMED 2.8 plugin [293] coupled to GROMACS. The reaction coordinate was sampled from 0.4 nm, representing the tightly bound state, to 4.0 nm, corresponding to the fully dissociated state, using 20 uniformly distributed umbrella windows separated by 0.2 nm. Initial configurations for the umbrella windows were extracted from an additional 500 ns unbiased MD simulations in which the Pld α - or Pld β -peptide–Lipid II complex progressively sampled dissociated configurations. Each umbrella window was simulated for 0.5 μ s with a harmonic biasing potential of 480 kcal mol⁻¹ nm⁻² applied along r . Exchanges between neighboring windows were attempted every 2 ps, resulting in an average acceptance probability of approximately 9%. Free-energy profiles were reconstructed from the last 400 ns of each trajectory using the weighted histogram analysis method, WHAM 2.0.9 [294].

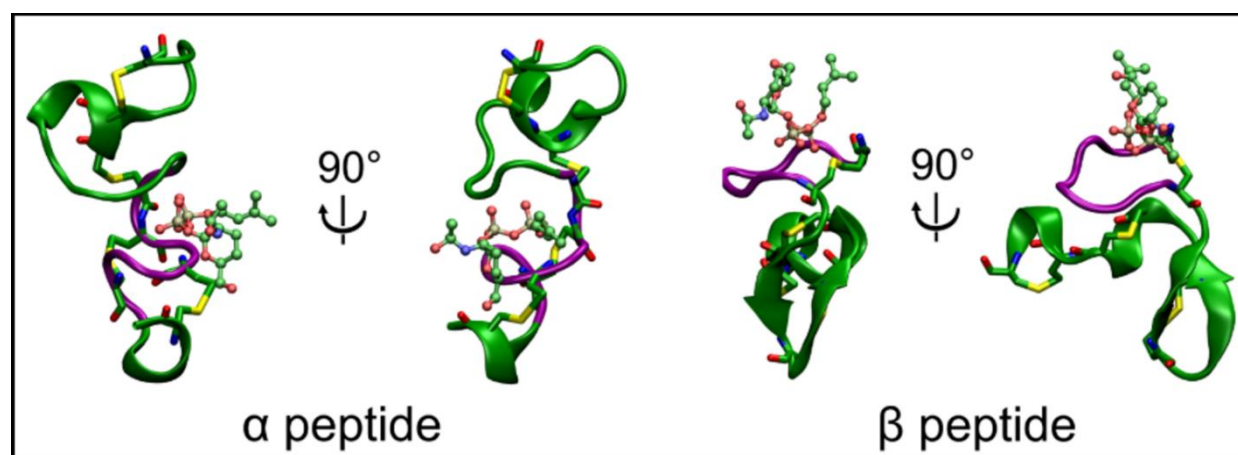


Figure 13. Pld α - and Pld β -peptide interaction models generated using the Boltz-2 predictor, subsequently used as a framework for free energy simulations. The peptide fragments that interact with pyrophosphate are highlighted in purple. Lipid II is represented by pyrophosphate, GlcNAc, and an isoprenoid fragment. Figure Created in VMD.

Binding of α and β peptides to Lipid II

Boltz-2 predictions suggested that both the Pld α and Pld β peptides bind Lipid II with comparable micromolar apparent affinities. However, the predicted probability of being a binder was markedly higher for the Pld α peptide, approximately 60%, than for the Pld β peptide (**Figure 14B**), for which the probability was only about 20%. This difference suggested that, although both peptides may form transient contacts with Lipid II, the α peptide is more likely to establish a stable and specific complex.

To quantitatively compare the affinity of the Pld α and Pld β peptides for Lipid II, we performed free-energy molecular dynamics simulations, as described in the Methods section. The resulting free-energy profiles, shown in **Figure 14**, demonstrate that only the Pld α peptide exhibits substantial affinity for Lipid II. The Pld α peptide profile shows a well-defined free-energy minimum at a center-of-mass distance of approximately 0.7 nm between the peptide binding region and the pyrophosphate moiety of Lipid II (**Figure 14A**). The depth of this minimum is approximately 10 kcal mol⁻¹, indicating formation of a stable bound state.

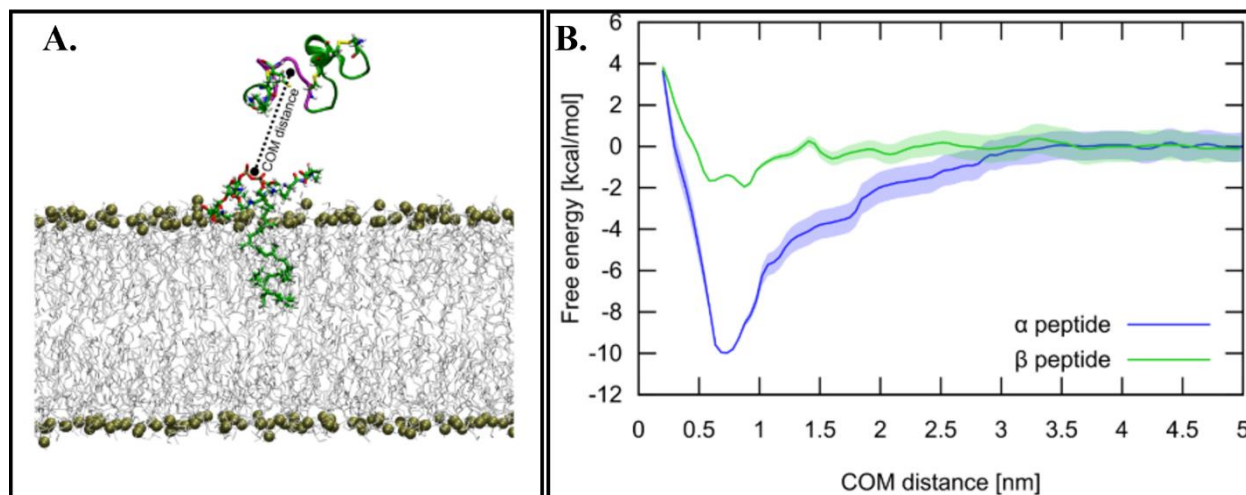


Figure 14. (A) Definition of the reaction coordinate used in free energy simulations. The reaction coordinate was defined as the center-of-mass (DOM) distance between the Lipid II pyrophosphate moiety and the peptide Lipid II-binding region. (B) Free energy profiles of Pld α - and Pld β -peptide binding to Lipid II. The profiles were calculated along the center-of-mass (COM) distance between the peptide binding region and the Lipid II pyrophosphate moiety. Figure Created in VMD

The magnitude of this interaction is consistent with experimental data reported for other Lipid II-binding lantibiotics. For example, nisin binds Lipid II with high affinity and uses Lipid II as a docking molecule for pore formation and inhibition of cell-wall biosynthesis; reported affinities

are in the nanomolar to submicromolar range depending on the membrane system and experimental approach. A dissociation constant in the range of 10–300 nM corresponds approximately to a binding free energy of -9 to -11 kcal mol⁻¹ at room temperature, which is close to the value obtained here for the α peptide [295].

In contrast, the Pld β peptide displayed only marginal affinity for Lipid II. Its free-energy profile showed a shallow minimum of approximately <2 kcal mol⁻¹, which is consistent with weak, nonspecific, or short-lived interactions rather than formation of a stable complex. This weak binding provides a plausible molecular explanation for the lack of experimentally observed membrane-disrupting effects for the Pld β peptide.

The different Lipid II affinities of the two peptides can be rationalized, at least in part, by their net charges and the electrostatic character of the interaction. The Pld α peptide carries a net positive charge of approximately $+2e$, mainly due to Lys13, Lys21, and Arg25, partially compensated by Glu21. In contrast, the Pld β peptide has an overall charge of approximately $-1e$, mainly due to Asp26. Because the Lipid II headgroup contains a highly anionic pyrophosphate moiety, favorable electrostatic interactions are expected to stabilize the Pld α -peptide–Lipid II complex, whereas the Pld β peptide lacks the same electrostatic complementarity. This interpretation is also consistent with the established role of the pyrophosphate group as a key recognition element in Lipid II-binding lantibiotics such as nisin.

Finally, we examined whether the Pld α and Pld β peptides could interact with each other and form a heteromeric complex. To this end, additional Boltz-2 predictions were performed using the previously identified Lipid II-interacting motif as a structural reference. The predicted models, shown in **Figure 15**, suggest that the Pld α and Pld β peptides are capable of forming a complex. Importantly, in the predicted α/β -peptide complex, residues 17–24 of the Pld α peptide remain accessible to the Lipid II-binding motif, indicating that heteromeric complex formation does not necessarily occlude the putative Lipid II-interacting region.

These results suggest a possible model in which the Pld α and Pld β peptides may associate with each other while retaining the ability to interact with Lipid II. However, the binding affinity between the Pld α and Pld β peptides, as well as the affinity of the resulting α/β -peptide complex for Lipid II, remains to be quantified. Further computational studies will therefore be required to determine whether such a complex forms under physiological conditions and whether it contributes to Lipid II recognition or membrane-associated activity.

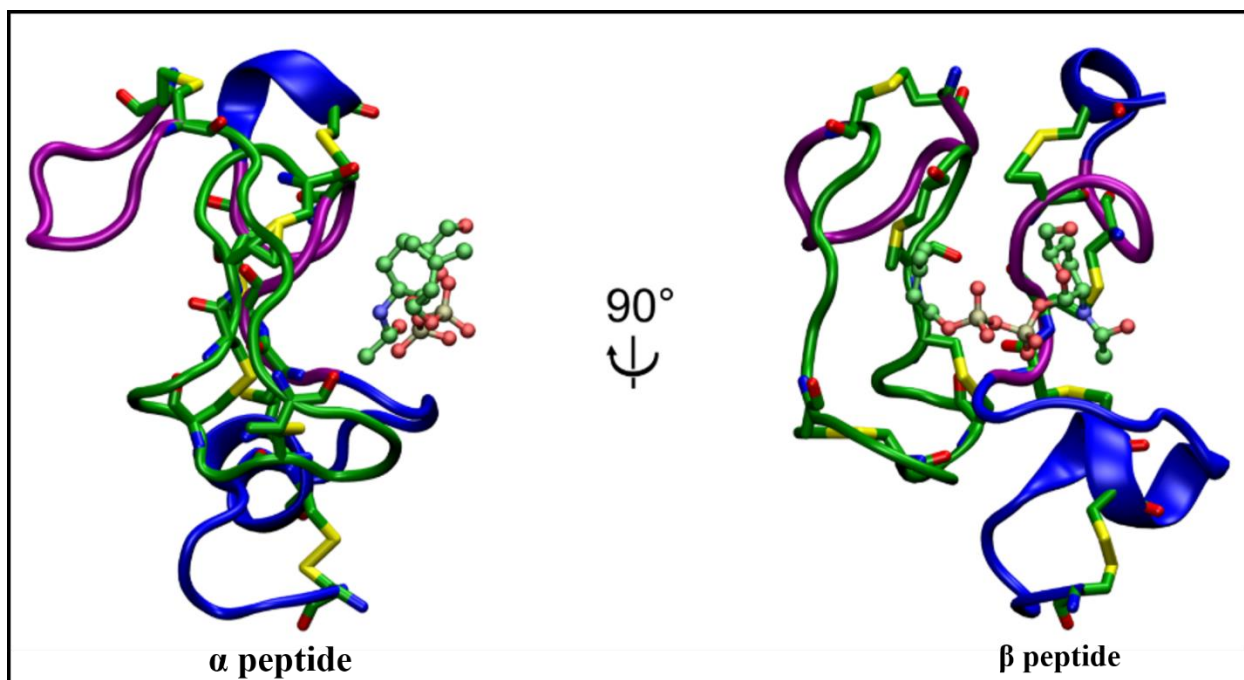


Figure 15. Structural models predicted by Boltz-2 for Pld α - and Pld β -peptide binding to Lipid II. The primary Lipid II-interacting motifs are highlighted in purple, while the Pld α peptide is blue and the Pld β peptide is shown in green. Figure Created in VMD

4. Overview of Key Research Results from the Constituent Publications

The core idea across all five publications is the discovery and characterization of antimicrobial-producing and probiotic *Bacillus* species associated with Polish bee products. Together, the studies move from isolation and phenotypic screening through genomic characterization, safety validation, and functional annotation, building a cumulative body of evidence for these microorganisms as candidates for novel antimicrobial and probiotic applications.

The first and most consistent finding is that bee products — honey, bee pollen, and bee bread — selectively enrich *Bacillus* strains harboring unusually dense arrays of secondary metabolite biosynthetic gene clusters (BGCs). Whether the source was multifloral honey or ginger honey, the recovered strains (*B. paralicheniformis* and *B. subtilis*) consistently harboured 13–18 BGCs per genome. This is not merely a genomic observation: phenotypic screening confirmed that cell-free culture filtrates inhibited clinically relevant Gram-positive pathogens, including MRSA and *L. monocytogenes*, providing direct functional validation of the genomic predictions. The convergence of antimicrobial phenotype and biosynthetic genotype, observed independently across honey-derived and pollen/bee bread-derived strains, supports bee products as a reproducible ecological source of biosynthetically competent antimicrobial-producing strains.

The second major finding concerns the identity and breadth of the antimicrobial compounds encoded. Genome mining consistently detected BGCs for lipopeptides (surfactin, fengycin/plipastatin, lichenysin), siderophores (bacillibactin), polyketides (bacillaene), and an unexpectedly broad repertoire of RiPPs, including subtilosin A, lasso peptides, sactipeptides, lanthipeptides, thiopeptides, and putative bacteriocins structurally related to enterocin NKR-5-3B, haloduracin, sonorensin, and bottromycin — several of which have not previously been reported from honey-associated *Bacillus* strains. Structural homology modelling of predicted bacteriocin-like peptides revealed minor sequence deviations from characterized reference compounds, suggesting that these isolates may produce structural variants with potentially distinct bioactivity or target specificity, warranting further experimental characterization. The additional *in silico* identification of short peptides with similarity to paenibacterin, gramicidin S, and brevivacillin further extends the predicted antimicrobial chemical diversity attributable to these strains.

The third key result is that a subset of strains — PY2.3 (*B. velezensis*), BP20.15, and BB10.1 (*B. subtilis*) — simultaneously fulfilled multiple criteria for probiotic candidacy. These isolates

survived simulated gastric conditions (pH 2), tolerated 0.3% bile salts, demonstrated surface hydrophobicity and auto-aggregation values consistent with intestinal adhesion, and co-aggregated with relevant pathogens, including *L. monocytogenes* and *S. aureus*. Cholesterol assimilation and broad carbohydrate utilization (30 of 49 substrates) add functional dimensions relevant to host benefit. Critically, these probiotic properties are not incidental to the antimicrobial capacity; they are encoded by the same genomes that carry the BGC arsenal, making these strains dual-function candidates with both competitive and host-beneficial predicted roles.

In silico safety evaluation provided the most direct evidence that the probiotic candidates are unlikely to pose a hazard. PathogenFinder scores were near 0 for all three strains; no transferable virulence genes and plasmids were detected, and hemolytic phenotyping confirmed γ -hemolysis exclusively. Resistome analysis detected multiple resistance-associated genes, but these were attributable to intrinsic chromosomal mechanisms rather than acquired mobile elements, a distinction relevant to biosafety assessment. Collectively, the safety data support these strains as candidates for further experimental evaluation.

Functional annotation of hypothetical proteins revealed that approximately 10–12% of the coding capacity of the genomes characterized in this study remained uncharacterized prior to this work, reflecting the current limitations of annotation databases rather than an absence of biological function. The integrated bioinformatic pipeline, validated at 95.3% accuracy, successfully assigned putative functions to 56 of these hypothetical proteins, many linked to sporulation, biofilm formation, motility, and metabolic regulation — processes directly relevant to probiotic fitness and environmental persistence. A subset carried secretion signals or transmembrane helices, raising the possibility that some contribute to the secreted antimicrobial activity observed phenotypically, and indicating that the biosynthetic inventories reported across the constituent publications likely underestimate the full coding capacity of these strains.

Fermentation optimization experiments revealed that YPD medium supported the highest antimicrobial metabolite output among tested media for ginger honey-derived *B. subtilis* strains, yet even under these conditions, activity remained restricted to Gram-positive targets, with no inhibition of Gram-negative bacteria or fungi observed despite the genomes harboring BGCs with the theoretical capacity to produce broad-spectrum compounds. This observation has an important implication: genomic potential and phenotypic output are not synonymous, and medium

composition is only one of several variables — alongside regulatory networks, growth phase, and environmental signals — that govern which BGCs are actually expressed. It also suggests that isolates screened in standard bacteriological media across the earlier studies may have expressed only a fraction of their true biosynthetic capacity.

Beyond the five constituent publications, the results presented in Chapter 4 extend these findings through the discovery of Paralichenidin (Pld), a novel two-component lantibiotic identified through BGC mining of *B. paralicheniformis* SZA14 and SZA16. The 29.25 kbp Pld operon encodes two structural peptides — Pld α (77.9% identity to GF610 α) and Pld β (70.4% identity to GF610 β) — alongside the full biosynthetic, transport, and immunity machinery characteristic of the amyloliquocidin/haloduracin subfamily. MSA and phylogenetic analysis confirmed near-universal conservation of bridge-forming cysteines across both peptides while identifying six discrete inter-cysteine substitutions in each. Free-energy molecular dynamics simulations established that Pld α binds lipid II with approximately $-10 \text{ kcal mol}^{-1}$ affinity while Pld β displayed only marginal affinity ($<2 \text{ kcal mol}^{-1}$), consistent with the established two-component model in which the α :lipid II complex serves as the docking platform for the β peptide. Pld α carries a net positive charge of $+2e$, providing electrostatic complementarity with the anionic pyrophosphate moiety of lipid II and offering a plausible basis for its strong binding affinity. In contrast, Pld β carries a net negative charge of $-1e$ driven by the unique Asp26 in its C-terminal region, whereas all characterized homologues examined in this study are neutral to positive, suggesting that the interaction of Pld β with the α :lipid II complex may be electrostatically distinct from those of its closest homologues and warrants experimental investigation.

Taken together, the findings of this dissertation support bee products as a reproducible ecological source of biosynthetically competent *Bacillus* populations whose antimicrobial and probiotic potential were validated through an integrated pipeline of phenotypic, genomic, and computational approaches. The identification of Paralichenidin, combined with molecular dynamics simulations revealing the differential lipid II-binding affinities of its two peptide components, extends these findings to the mechanistic level and demonstrates that bee product-associated strains are capable of producing structurally novel lantibiotics not previously described from this ecological source.

4.1. Conclusions

1. Polish honey, bee pollen, and bee bread constitute a reproducible and taxonomically consistent reservoir of *Bacillus* species (*B. subtilis*, *B. paralicheniformis*, and *B. velezensis*), suggesting a functional relationship between the bee product environment and the selective enrichment of biosynthetically competent spore-formers with antimicrobial and probiotic properties.
2. Whole-genome sequencing combined with genome mining — encompassing antiSMASH, BAGEL4, resistome profiling, pangenome reconstruction, and in silico AMP screening — consistently revealed biosynthetic potential that phenotypic screening alone cannot capture, and represents a necessary and complementary strategy for the comprehensive characterization of bee product microbiota.
3. Bee product-associated *Bacillus* strains harbor dense arrays of biosynthetic gene clusters (13–18 per genome) encoding lipopeptides, siderophores, polyketides, and a broad repertoire of RiPPs, including lanthipeptides, thiopeptides, sactipeptides, lasso peptides, and bacteriocin-like compounds; several of these represent structural variants not previously reported from this ecological source, exhibiting sequence deviations from characterized reference molecules that suggest potentially distinct bioactivity or target specificity.
4. BGC mining of *B. paralicheniformis* SZA14 and SZA16 identified Paralichenidin (Pld), a novel two-component lantibiotic belonging to the amyloliquecidin/haloduracin subfamily, whose operon encodes the complete biosynthetic, transport, and immunity machinery required for lantibiotic production and self-resistance. Phylogenetic and MSA analyses confirmed six discrete inter-cysteine substitutions in each peptide component relative to characterized homologues, with Pld β uniquely carrying a negatively charged aspartic acid at position 26 in its C-terminal region, contrasting with the neutral to positive charges reported for Lichenicidin β and Haloduracin β , respectively. Free-energy molecular dynamics simulations established that Pld α binds lipid II with approximately -10 kcal mol $^{-1}$ affinity while Pld β displayed only marginal affinity (<2 kcal mol $^{-1}$), consistent with the established two-component model in which the α :lipid II complex serves as the docking platform for the β peptide, and warrants experimental investigation of Pld β 's electrostatically distinct C-terminal docking interface.

5. Probiotic and antimicrobial functionalities co-occur within the same strains and are jointly encoded in their genomes: selected isolates simultaneously tolerated simulated gastrointestinal conditions, inhibited clinically relevant pathogens including MRSA and *L. monocytogenes*, adhered to surfaces, assimilated cholesterol, and utilized a broad range of carbohydrates, establishing them as dual-function candidates with both competitive and host-beneficial modes of action.
6. Comprehensive *in silico* safety profiling — encompassing virulence gene screening, resistome analysis, hemolytic phenotyping, plasmid detection, and pathogenicity prediction — demonstrated low pathogenic potential for the leading probiotic candidates, with no transferable virulence determinants, no plasmids, and PathogenFinder scores below 0.25, collectively supporting their suitability for further safety and efficacy evaluation.
7. Approximately 10–12% of the coding capacity of the *Bacillus* genomes characterized in this study remained functionally uncharacterized, reflecting the current limitations of annotation databases rather than an absence of biological function; the integrated multi-tool annotation pipeline, validated at 95.3% accuracy, successfully assigned putative functions to 56 of these hypothetical proteins, identifying roles in sporulation, biofilm formation, motility, secretion, and metabolic regulation, and demonstrating that this uncharacterized coding capacity represents a biologically significant layer of genomic information.
8. The open pangenome architecture, horizontal gene transfer, and prophage-mediated genomic plasticity observed across bee-associated *Bacillus* genomes indicate that these strains are genomically dynamic, continuously acquiring novel genetic material; consequently, the biosynthetic inventories reported here likely represent an underestimate of the true bioactive potential of these populations, which will become more fully resolved as reference databases expand and additional genomes are characterized.
9. The dissertation presents an integrated methodological framework — spanning ecological sampling, phenotypic screening, whole-genome sequencing, genome mining, functional annotation, and *in silico* safety evaluation — that provides a reproducible and transferable approach for the systematic investigation of antimicrobial and probiotic potential in bee product-associated microbiota and analogous natural environments; where applicable,

structural modelling and molecular dynamics simulations further extended this framework to the mechanistic characterization of novel compounds, as demonstrated through the computational analysis of Paralichenidin. The findings collectively lay a scientific foundation for future work focused on the purification, structural characterization, and functional validation of predicted antimicrobial compounds, and on the further experimental evaluation of bee-associated *Bacillus* strains as probiotic and bioactive candidates for potential biotechnological and therapeutic applications.

Acknowledgments

This research was funded by the National Science Centre, Poland, under grant UMO-2021/41/B/NZ9/03929, "Biotechnological and Pharmacological Potential of the Microbiota of Bee Products," led by Dr. Hab. inż. Piotr Szweda.

I would also like to express my sincere gratitude to Dr. Mateusz Kogut and Prof. Jacek Czub for their invaluable assistance with the predicted structure and bridge formation analyses, as well as the structural modeling and molecular dynamics simulations of Lipid II and α -/ β -peptide–Lipid II complexes presented in Section 4 of this thesis.

Scientific Achievements

1. Articles Published in Scientific Journals

1.  Springer

Hafeez, A.B., Pelka K., Buzun K., Worobo R.W., Szweda P. Whole genome sequencing and antimicrobial potential of bacteria isolated from Polish honey. *Applied Microbiology and Biotechnology* (2023) doi:10.1007/s00253-023-12732-9

2.  Springer

Pelka K., **Hafeez, A.B.**, Worobo R.W., Szweda P. Probiotic Potential of *Bacillus* Isolates from Polish Bee Pollen and Bee Bread. *Probiotics and Antimicrobial Proteins* (2023) doi:10.1007/s12602-023-10157-4

3. 

Hafeez, A.B., Karolina Pelka, Randy Worobo, and Piotr Szweda. "In silico safety assessment of *Bacillus* isolated from polish bee pollen and bee bread as novel probiotic candidates." *International Journal of Molecular Sciences* 25, no. 1 (2024): 666. doi.org/10.3390/ijms25010666

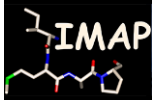
4. 

Hafeez, A.B., Subrahmanyam Sappati, Radoslaw Krzemieniecki, Randy Worobo, and Piotr Szweda. "In Silico Functional Annotation and Structural Characterization of Hypothetical Proteins in *Bacillus paralicheniformis* and *Bacillus subtilis* Isolated from Honey." *ACS omega* 10, no. 9 (2025): 8993-9006. doi/10.1021/acsomega.4c07105.

5.  Springer

Hafeez, A.B., and Piotr Szweda. "Genomic and functional landscape of antimicrobial peptides and lipopeptides from ginger honey-derived *Bacillus subtilis*." *Molecular Genetics and Genomics* 301, no. 1 (2026): 86. doi.org/10.1007/s00438-026-02411-5

2. Scientific Conferences

1. 

Rapid Oral and Poster Presentation – 11th International Meeting on Antimicrobial Peptides (IMAP 2024), King's College London, UK
Authors: **Hafeez, A.B.**, Connor, P., Szweda, P.

2. 

Poster Presentation – 3rd Polish-Czech Probiotic Conference, Hirsfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences (2024)
Authors: **Hafeez, A.B.**, Szweda, P.

3. 

Oral Presentation – Trends in Natural Products Research: A PSE Young Scientists' Meeting, Paris, France. Title: "Bee products microbiota as a source of novel antimicrobials". (2023)
Authors: **Hafeez, A.B.**, Szweda, P.

3. Scientific Internships

1. 

15/02/2025 – 18/05/2025 - Internship at the Department of Chemistry, Faculty of Natural, Mathematical & Engineering Sciences, King's College London, UK
Supervisor: Prof. Sarah M. Barry

2. 

12/09/2024 – 12/11/2024 - Internship at the DTU Bioengineering, Department of Biotechnology and Biomedicine, Technical University of Denmark
Supervisor: Prof. Ling Ding

3. 

Internship at the Department of Biochemistry and Biotechnology, University of Thessaly, Greece
Supervisor: Prof. Grigorios Amoutzias

4. Scholarships & Awards

- European Molecular Biology Organization (EMBO) Scientific Exchange Grant, 2025
- NAWA Stipendium Scholarship for the Best Doctoral Students Carrying Out Doctoral Projects in International Cooperation, 2024
- Francium International Doctoral Fellowship, 2022–2026
- Polonium International Doctoral Fellowship, 2021–2022
- Mini-Grant Award for Doctoral Students, 2023–2024
- Mini-Grant for Young Scientific Staff, 2023–2024
- Travel Bursary, Trends in Natural Products Research Meeting, Paris, France

5. References

1. Hutchings, M.; Truman, A.; Wilkinson, B. Antibiotics: Past, Present and Future. *Curr. Opin. Microbiol.* **2019**, *51*, 72–80, doi:10.1016/J.MIB.2019.10.008.
2. Murray, C.J.; Ikuta, K.S.; Sharara, F.; Swetschinski, L.; Robles Aguilar, G.; Gray, A.; Han, C.; Bisignano, C.; Rao, P.; Wool, E.; et al. Global Burden of Bacterial Antimicrobial Resistance in 2019: A Systematic Analysis. *Lancet* **2022**, *399*, 629–655, doi:10.1016/S0140-6736(21)02724-0.
3. Organization, W.H. Global Antibiotic Resistance Surveillance Report 2025 WHO Global Antimicrobial Resistance and Use Surveillance System (GLASS). **2025**.
4. da Cunha, B.R.; Fonseca, L.P.; Calado, C.R.C. Antibiotic Discovery: Where Have We Come from, Where Do We Go? *Antibiotics* **2019**, *8*, doi:10.3390/ANTIBIOTICS8020045.
5. Wetzel, C.; Lonneman, M.; Wu, C. Polypharmacological Drug Actions of Recently FDA Approved Antibiotics. *Eur. J. Med. Chem.* **2021**, *209*, 112931, doi:10.1016/J.EJMECH.2020.112931.
6. Power, E. Impact of Antibiotic Restrictions: The Pharmaceutical Perspective. *Clin. Microbiol. Infect.* **2006**, *12*, 25–34, doi:10.1111/J.1469-0691.2006.01528.X.
7. De Oliveira, D.M.P.; Forde, B.M.; Kidd, T.J.; Harris, P.N.A.; Schembri, M.A.; Beatson, S.A.; Paterson, D.L.; Walker, M.J. Antimicrobial Resistance in ESKAPE Pathogens. *Clin. Microbiol. Rev.* **2020**, *33*, doi:10.1128/CMR.00181-19.
8. Brüssow, H. The Antibiotic Resistance Crisis and the Development of New Antibiotics. *Microb. Biotechnol.* **2024**, *17*, doi:10.1111/1751-7915.14510.
9. *Surveillance of Antimicrobial Resistance in Europe, 2024 Data; 2025*;
10. Bin Hafeez, A.; Jiang, X.; Bergen, P.J.; Zhu, Y. Antimicrobial Peptides: An Update on Classifications and Databases. *Int. J. Mol. Sci.* **2021**, *22*, doi:10.3390/ijms22211691.
11. Wen, R.; Feng, W.; Wang, N.; Zhang, C.; Ruhan, A.; Myadagbadam, U.; Chimedtseren, C.; Yi, L.; Li, M. Research Advances in the Genus *Scabiosa*: A Comprehensive Review of Botany, Traditional Uses, Phytochemistry and Pharmacology. *Chem. Biodivers.* **2025**, *2025*, e202500229, doi:10.1002/cbdv.202500229.
12. Jenkins, R. A Review of Selected Bee Products as Potential Anti-Bacterial, Anti-Fungal, and Anti-Viral Agents. *Med. Res. Arch.* **2016**, *4*, doi:10.18103/mra.v4i8.887.
13. Milling, S.; Ijaz, U.Z.; Venieri, D.; Christidis, G.E.; Rattray, N.J.W.; Gounaki, I.; Andrusaite, A.; Hareendran, A.; Knapp, C.W.; Jones, A.X.; et al. Beneficial Modulation of the Gut Microbiome by Leachates of *Penicillium Purpurogenum* in the Presence of Clays: A Model for the Preparation and Efficacy of Historical Lemnian Earth. *PLoS One* **2024**, *19*, e0313090, doi:10.1371/journal.pone.0313090.
14. Aminov, R.I. A Brief History of the Antibiotic Era: Lessons Learned and Challenges for the Future. *Front. Microbiol.* **2010**, *1*, 134, doi:10.3389/fmicb.2010.00134.
15. Lima, L.M.; Silva, B.N.M. da; Barbosa, G.; Barreiro, E.J. β -Lactam Antibiotics: An Overview from a Medicinal Chemistry Perspective. *Eur. J. Med. Chem.* **2020**, *208*, 112829, doi:10.1016/j.ejmech.2020.112829.
16. Rubinstein, E.; Keynan, Y. Vancomycin Revisited - 60 Years Later. *Front. Public Heal.* **2014**, *2*, 77697, doi:10.3389/fpubh.2014.00217.
17. Michalopoulos, A.S.; Livaditis, I.G.; Gougoutas, V. The Revival of Fosfomycin. *Int. J. Infect. Dis.* **2011**, *15*, e732–e739, doi:10.1016/j.ijid.2011.07.007.
18. Hawkins, B.K.; Ding, H.; Harriott, M.M.; Wang, J.L. New Perspectives on Antimicrobial Agents:

- Pivmecillinam. *Antimicrob. Agents Chemother.* **2025**, 69, doi:10.1128/aac.01824-24.
19. McCreary, E.K.; Heil, E.L.; Tamma, P.D. New Perspectives on Antimicrobial Agents: Cefiderocol. *Antimicrob. Agents Chemother.* **2021**, 65, doi:10.1128/AAC.02171-20.
 20. Chen, C.; Chen, Y.; Wu, P.; Chen, B. Update on New Medicinal Applications of Gentamicin: Evidence-Based Review. *J. Formos. Med. Assoc.* **2014**, 113, 72–82, doi:10.1016/j.jfma.2013.10.002.
 21. Singh, S.; Khanna, D.; Kalra, S. Minocycline and Doxycycline: More Than Antibiotics. *Curr. Mol. Pharmacol.* **2021**, 14, 1046–1065, doi:10.2174/1874467214666210210122628.
 22. Platon, V.M.; Dragoi, B.; Marin, L. Erythromycin Formulations—A Journey to Advanced Drug Delivery. *Pharm. 2022, Vol. 14*, **2022**, 14, doi:10.3390/pharmaceutics14102180.
 23. Hashemian, S.M.R.; Farhadi, T.; Ganjparvar, M. Linezolid: A Review of Its Properties, Function, and Use in Critical Care. *Drug Des. Devel. Ther.* **2018**, 12, 1759–1767, doi:10.2147/DDDT.S164515.
 24. Sharma, P.C.; Jain, A.; Jain, S.; Pahwa, R.; Yar, M.S. Ciprofloxacin: Review on Developments in Synthetic, Analytical, and Medicinal Aspects. *J. Enzyme Inhib. Med. Chem.* **2010**, 25, 577–589, doi:10.3109/14756360903373350.
 25. Osheroff, N. Gyrase and Topoisomerase IV as Antibacterial Targets for Gepotidacin and Zoliflodacin: Teaching Old Enzymes New Tricks. *Int. J. Mol. Sci.* **2026, Vol. 27**, **2026**, 27, doi:10.3390/ijms27010496.
 26. Floss, H.G.; Yu, T.W. Rifamycin Mode of Action, Resistance, and Biosynthesis. *Chem. Rev.* **2005**, 105, 621–632, doi:10.1021/cr030112j.
 27. Nguyen, T.V.A.; Nguyen, Q.H.; Nguyen, T.N.T.; Anthony, R.M.; Vu, D.H.; Alffenaar, J.W.C. Pretomanid Resistance: An Update on Emergence, Mechanisms and Relevance for Clinical Practice. *Int. J. Antimicrob. Agents* **2023**, 62, 106953, doi:10.1016/j.ijantimicag.2023.106953.
 28. Zafar, W.; Sumrra, S.H.; Hassan, A.U.; Chohan, Z.H. A Review on ‘Sulfonamides’: Their Chemistry and Pharmacological Potentials for Designing Therapeutic Drugs in Medical Science. *J. Coord. Chem.* **2023**, 76, 546–580, doi:10.1080/00958972.2023.2208260.
 29. Carter, C.; Wain, J.; Langridge, G.C. Trimethoprim: Bactericidal or Bacteriostatic Activity Is Dependent on Bacterial Growth Conditions. *J. Med. Microbiol.* **2026**, 75, 002129, doi:10.1099/jmm.0.002129.
 30. Heidary, M.; Khosravi, A.D.; Khoshnood, S.; Nasiri, M.J.; Soleimani, S.; Goudarzi, M. Daptomycin. *J. Antimicrob. Chemother.* **2018**, 73, 1–11, doi:10.1093/jac/dkx349.
 31. Andrade, F.F.; Silva, D.; Rodrigues, A.; Pina-Vaz, C. Colistin Update on Its Mechanism of Action and Resistance, Present and Future Challenges. *Microorg. 2020, Vol. 8*, **2020**, 8, 1–12, doi:10.3390/microorganisms8111716.
 32. Rusu, A.; Oancea, O.L.; Donici, E.; Uncu, L. Recent Developments in Penem Antibiotics: Structural and Therapeutic Perspectives. *Mol.* **2025, Vol. 30**, **2025**, 30, doi:10.3390/molecules30102126.
 33. Shirley, M.; Macgowan, A.P. Aztreonam-Avibactam: A Review in the Treatment of Serious Bacterial Infections Caused by Aerobic Gram-Negative Organisms. *Drugs* **2025**, 86, 79–91, doi:10.1007/s40265-025-02258-x.
 34. Ventola, C.L. The Antibiotic Resistance Crisis: Part 1: Causes and Threats. *Pharm. Ther.* **2015**, 40, 277, doi:Article.
 35. Lee, J.H. Perspectives towards Antibiotic Resistance: From Molecules to Population. *J. Microbiol.* **2019**, 57, 181–184, doi:10.1007/s12275-019-0718-8.

36. Bo, L.; Sun, H.; Li, Y.D.; Zhu, J.; Wurpel, J.N.D.; Lin, H.; Chen, Z.S. Combating Antimicrobial Resistance: The Silent War. *Front. Pharmacol.* **2024**, *15*, 1347750, doi:10.3389/fphar.2024.1347750.
37. Belay, W.Y.; Getachew, M.; Tegegne, B.A.; Teffera, Z.H.; Dagne, A.; Zeleke, T.K.; Abebe, R.B.; Gedif, A.A.; Fenta, A.; Yirdaw, G.; et al. Mechanism of Antibacterial Resistance, Strategies and next-Generation Antimicrobials to Contain Antimicrobial Resistance: A Review. *Front. Pharmacol.* **2024**, *15*, 1444781, doi:10.3389/fphar.2024.1444781.
38. Cox, G.; Wright, G.D. Intrinsic Antibiotic Resistance: Mechanisms, Origins, Challenges and Solutions. *Int. J. Med. Microbiol.* **2013**, *303*, 287–292, doi:10.1016/j.ijmm.2013.02.009.
39. Martinez, J.L. General Principles of Antibiotic Resistance in Bacteria. *Drug Discov. Today Technol.* **2014**, *11*, 33–39, doi:10.1016/j.ddtec.2014.02.001.
40. Uddin, T.M.; Chakraborty, A.J.; Khusro, A.; Zidan, B.R.M.; Mitra, S.; Emran, T. Bin; Dhama, K.; Ripon, M.K.H.; Gajdács, M.; Sahibzada, M.U.K.; et al. Antibiotic Resistance in Microbes: History, Mechanisms, Therapeutic Strategies and Future Prospects. *J. Infect. Public Health* **2021**, *14*, 1750–1766, doi:10.1016/j.jiph.2021.10.020.
41. Nikaido, H. Multidrug Resistance in Bacteria. *Annu. Rev. Biochem.* **2009**, *78*, 119–146, doi:10.1146/annurev.biochem.78.082907.145923.
42. Miller, W.R.; Munita, J.M.; Arias, C.A. Mechanisms of Antibiotic Resistance in Enterococci. *Expert Rev. Anti. Infect. Ther.* **2014**, *12*, 1221–1236, doi:10.1586/14787210.2014.956092.
43. Ramirez, M.S.; Tolmasky, M.E. Aminoglycoside Modifying Enzymes. *Drug Resist. Updat.* **2010**, *13*, 151–171, doi:10.1016/j.drug.2010.08.003.
44. Hecht, D.W. Prevalence of Antibiotic Resistance in Anaerobic Bacteria: Worrisome Developments. *Clin. Infect. Dis.* **2004**, *39*, 92–97, doi:10.1086/421558.
45. Cesur, S.; Demiröz, A.P. Antibiotics and the Mechanisms of Resistance to Antibiotics. *Med. J. Islam. World Acad. Sci.* **2013**, *109*, 1–5.
46. Manchanda, V.; Sanchaita, S.; Singh, N. Multidrug Resistant Acinetobacter. *J. Glob. Infect. Dis.* **2010**, *2*, 291, doi:10.4103/0974-777x.68538.
47. Sandoval-Motta, S.; Aldana, M. Adaptive Resistance to Antibiotics in Bacteria: A Systems Biology Perspective. *Wiley Interdiscip. Rev. Syst. Biol. Med.* **2016**, *8*, 253–267, doi:10.1002/wsbm.1335.
48. D'Aquila, P.; Rango, F. De; Paparazzo, E.; Passarino, G.; Bellizzi, D. Epigenetic-Based Regulation of Transcriptome in Escherichia Coli Adaptive Antibiotic Resistance. *Microbiol. Spectr.* **2023**, *11*, doi:10.1128/spectrum.04583-22.
49. Zhu, Y.G.; Johnson, T.A.; Su, J.Q.; Qiao, M.; Guo, G.X.; Stedtfeld, R.D.; Hashsham, S.A.; Tiedje, J.M. Diverse and Abundant Antibiotic Resistance Genes in Chinese Swine Farms. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110*, 3435–3440, doi:10.1073/pnas.1222743110.
50. Peterson, E.; Kaur, P. Antibiotic Resistance Mechanisms in Bacteria: Relationships between Resistance Determinants of Antibiotic Producers, Environmental Bacteria, and Clinical Pathogens. *Front. Microbiol.* **2018**, *9*, 426686, doi:10.3389/fmicb.2018.02928.
51. de Pontes, J.T.C.; Borges, A.B.T.; Roque-Borda, C.A.; Pavan, F.R. Antimicrobial Peptides as an Alternative for the Eradication of Bacterial Biofilms of Multi-Drug Resistant Bacteria. *Pharm.* **2022**, *Vol. 14*, **2022**, *14*, doi:10.3390/PHARMACEUTICS14030642.
52. da Costa de Souza, G.; Roque-Borda, C.A.; Pavan, F.R. Beta-Lactam Resistance and the Effectiveness of Antimicrobial Peptides against KPC-Producing Bacteria. *Drug Dev. Res.* **2022**, *83*, 1534–1554, doi:10.1002/DDR.21990;CTYPE:STRING:JOURNAL.

53. WHO World Health Organization Bacterial Priority Pathogens List, 2024: Bacterial Pathogens of Public Health Importance to Guide Research, Development and Strategies to Prevent and Control Antimicrobial Resistance; World Health Organization Geneva, 2024;
54. Dale, J.L.; Cagnazzo, J.; Phan, C.Q.; Barnes, A.M.T.; Dunny, G.M. Multiple Roles for Enterococcus Faecalis Glycosyltransferases in Biofilm-Associated Antibiotic Resistance, Cell Envelope Integrity, and Conjugative Transfer. *Antimicrob. Agents Chemother.* **2015**, *59*, 4094–4105, doi:10.1128/AAC.00344-15;ISSUE:ISSUE:DOI.
55. Bopp, T.C.; Marchesi, M.; Zihlmann, R.; Sax, H.; Wolfensberger, A. Identifying Patients at High Risk for Multidrug-Resistant Organisms after Hospitalization Abroad. *Infect. Control Hosp. Epidemiol.* **2023**, *44*, 1281–1288, doi:10.1017/ICE.2022.256.
56. Roque-borda, C.A.; da Silva, P.B.; Rodrigues, M.C.; Azevedo, R.B.; Di Filippo, L.; Duarte, J.L.; Chorilli, M.; Vicente, E.F.; Pavan, F.R. Challenge in the Discovery of New Drugs: Antimicrobial Peptides against WHO-List of Critical and High-Priority Bacteria. *Pharm. 2021, Vol. 13*, **2021**, *13*, doi:10.3390/PHARMACEUTICS13060773.
57. Parmanik, A.; Das, S.; Kar, B.; Bose, A.; Dwivedi, G.R.; Pandey, M.M. Current Treatment Strategies Against Multidrug-Resistant Bacteria: A Review. *Curr. Microbiol.* **2022**, *79*, 388–, doi:10.1007/S00284-022-03061-7.
58. Martinez, M.; Gonçalves, S.; Felício, M.R.; Maturana, P.; Santos, N.C.; Semorile, L.; Hollmann, A.; Maffía, P.C. Synergistic and Antibiofilm Activity of the Antimicrobial Peptide P5 against Carbapenem-Resistant Pseudomonas Aeruginosa. *Biochim. Biophys. Acta - Biomembr.* **2019**, *1861*, 1329–1337, doi:10.1016/J.BBAMEM.2019.05.008.
59. Mohan, N.M.; Zorgani, A.; Jalowicki, G.; Kerr, A.; Khaldi, N.; Martins, M. Unlocking NuriPep 1653 From Common Pea Protein: A Potent Antimicrobial Peptide to Tackle a Pan-Drug Resistant Acinetobacter Baumannii. *Front. Microbiol.* **2019**, *10*, 479732, doi:10.3389/FMICB.2019.02086/TEXT.
60. Kang, D.D.; Park, J.; Park, Y. Therapeutic Potential of Antimicrobial Peptide PN5 against Multidrug-Resistant E. Coli and Anti-Inflammatory Activity in a Septic Mouse Model. *Microbiol. Spectr.* **2022**, *10*, doi:10.1128/SPECTRUM.01494-22;WGROU:STRING:PUBLICATION.
61. Zhou, X.; Willems, R.J.L.; Friedrich, A.W.; Rossen, J.W.A.; Bathoorn, E. Enterococcus Faecium: From Microbiological Insights to Practical Recommendations for Infection Control and Diagnostics. *Antimicrob. Resist. Infect. Control* **2020**, *9*, 130–, doi:10.1186/S13756-020-00770-1.
62. Ayobami, O.; Willrich, N.; Reuss, A.; Eckmanns, T.; Markwart, R. The Ongoing Challenge of Vancomycin-Resistant Enterococcus Faecium and Enterococcus Faecalis in Europe: An Epidemiological Analysis of Bloodstream Infections. *Emerg. Microbes Infect.* **2020**, *9*, 1180–1193, doi:10.1080/22221751.2020.1769500.
63. O'Toole, R.F.; Leong, K.W.C.; Cumming, V.; Van Hal, S.J. Vancomycin-Resistant Enterococcus Faecium and the Emergence of New Sequence Types Associated with Hospital Infection. *Res. Microbiol.* **2023**, *174*, 104046, doi:10.1016/J.RESMIC.2023.104046.
64. Howden, B.P.; Giulieri, S.G.; Wong Fok Lung, T.; Baines, S.L.; Sharkey, L.K.; Lee, J.Y.H.; Hachani, A.; Monk, I.R.; Stinear, T.P. Staphylococcus Aureus Host Interactions and Adaptation. *Nat. Rev. Microbiol.* **2023**, *21*, 380–395, doi:10.1038/s41579-023-00852-y.
65. Thammavongsa, V.; Kim, H.K.; Missiakas, D.; Schneewind, O. Staphylococcal Manipulation of Host Immune Responses. *Nat. Rev. Microbiol.* **2015**, *13*, 529–543, doi:10.1038/NRMICRO3521;SUBJMETA.
66. Mlynarczyk-Bonikowska, B.; Kowalewski, C.; Krolak-Ulinska, A.; Marusza, W. Molecular Mechanisms of Drug Resistance in Staphylococcus Aureus. *Int. J. Mol. Sci.* **2022**, *23*, **2022**,

- 23, doi:10.3390/IJMS23158088.
67. Wang, G.; Zhao, G.; Chao, X.; Xie, L.; Wang, H. The Characteristic of Virulence, Biofilm and Antibiotic Resistance of *Klebsiella Pneumoniae*. *Int. J. Environ. Res. Public Heal.* **2020**, *Vol. 17*, **2020**, *17*, 1–17, doi:10.3390/IJERPH17176278.
 68. Lucidi, M.; Visaggio, D.; Migliaccio, A.; Capecchi, G.; Visca, P.; Imperi, F.; Zarrilli, R. Pathogenicity and Virulence of *Acinetobacter Baumannii*: Factors Contributing to the Fitness in Healthcare Settings and the Infected Host. *Virulence* **2024**, *15*, doi:10.1080/21505594.2023.2289769.
 69. Shi, J.; Cheng, J.; Liu, S.; Zhu, Y.; Zhu, M. *Acinetobacter Baumannii*: An Evolving and Cunning Opponent. *Front. Microbiol.* **2024**, *15*, 1332108, doi:10.3389/FMICB.2024.1332108/FULL.
 70. Karampatakis, T.; Tsergouli, K.; Behzadi, P. Pan-Genome Plasticity and Virulence Factors: A Natural Treasure Trove for *Acinetobacter Baumannii*. *Antibiot.* **2024**, *Vol. 13*, **2024**, *13*, doi:10.3390/ANTIBIOTICS13030257.
 71. Olivares, E.; Badel-Berchoux, S.; Provot, C.; Prévost, G.; Bernardi, T.; Jehl, F. Clinical Impact of Antibiotics for the Treatment of *Pseudomonas Aeruginosa* Biofilm Infections. *Front. Microbiol.* **2020**, *10*, 487974, doi:10.3389/FMICB.2019.02894/TEXT.
 72. Reynolds, D.; Kollef, M. The Epidemiology and Pathogenesis and Treatment of *Pseudomonas Aeruginosa* Infections: An Update. *Drugs* **2021**, *81*, 2117–2131, doi:10.1007/S40265-021-01635-6.
 73. Lorusso, A.B.; Carrara, J.A.; Barroso, C.D.N.; Tuon, F.F.; Faoro, H. Role of Efflux Pumps on Antimicrobial Resistance in *Pseudomonas Aeruginosa*. *Int. J. Mol. Sci.* **2022**, *Vol. 23*, **2022**, *23*, doi:10.3390/IJMS232415779.
 74. Nasser, M.; Ogaili, M.; Palwe, S.; Kharat, A.S. Molecular Detection of Extended Spectrum β -Lactamases, Metallo β -Lactamases, and Amp-C β -Lactamase Genes Expressed by Multiple Drug Resistant *Pseudomonas Aeruginosa* Isolates Collected from Patients with Burn/Wound Infections. *Burn. Open* **2020**, *4*, 160–166, doi:10.1016/J.BURNSO.2020.07.003.
 75. Khademi, F.; Maarofi, K.; Arzanlou, M.; Peeri-Dogaheh, H.; Sahebkar, A. Which Missense Mutations Associated with DNA Gyrase and Topoisomerase IV Are Involved in *Pseudomonas Aeruginosa* Clinical Isolates Resistance to Ciprofloxacin in Ardabil? *Gene Reports* **2021**, *24*, 101211, doi:10.1016/J.GENREP.2021.101211.
 76. Ahmadian, L.; Norouzi Bazgir, Z.; Ahanjan, M.; Valadan, R.; Goli, H.R. Role of Aminoglycoside-Modifying Enzymes (AMEs) in Resistance to Aminoglycosides among Clinical Isolates of *Pseudomonas Aeruginosa* in the North of Iran. *Biomed Res. Int.* **2021**, *2021*, 7077344, doi:10.1155/2021/7077344.
 77. Tuon, F.F.; Dantas, L.R.; Suss, P.H.; Tasca Ribeiro, V.S. Pathogenesis of the *Pseudomonas Aeruginosa* Biofilm: A Review. *Pathog.* **2022**, *Vol. 11*, **2022**, *11*, doi:10.3390/PATHOGENS11030300.
 78. Ramirez, D.; Giron, M. Enterobacter Infections. *StatPearls* **2025**.
 79. Zong, Z.; Feng, Y.; McNally, A. Carbapenem and Colistin Resistance in Enterobacter: Determinants and Clones. *Trends Microbiol.* **2021**, *29*, 473–476, doi:10.1016/j.tim.2020.12.009.
 80. Khademi, F.; Vaez, H.; Neyestani, Z.; Sahebkar, A. Prevalence of ESBL-Producing Enterobacter Species Resistant to Carbapenems in Iran: A Systematic Review and Meta-Analysis. *Int. J. Microbiol.* **2022**, *2022*, 8367365, doi:10.1155/2022/8367365.
 81. Khalifa, S.A.M.; Elshafiey, E.H.; Shetaia, A.A.; El-Wahed, A.A.A.; Algethami, A.F.; Musharraf, S.G.; Alajmi, M.F.; Zhao, C.; Masry, S.H.D.; Abdel-Daim, M.M.; et al. Overview of Bee Pollination

- and Its Economic Value for Crop Production. *Insects* **2021**, Vol. 12, **2021**, 12, doi:10.3390/INSECTS12080688.
82. Dumitru, C.D.; Neacsu, I.A.; Grumezescu, A.M.; Andronesu, E. Bee-Derived Products: Chemical Composition and Applications in Skin Tissue Engineering. *Pharm.* **2022**, Vol. 14, **2022**, 14, doi:10.3390/PHARMACEUTICS14040750.
 83. Bargańska, Z.; Ĺebioda, M.; Namieśnik, J. Honey Bees and Their Products: Bioindicators of Environmental Contamination. *Crit. Rev. Environ. Sci. Technol.* **2016**, 46, 235–248, doi:10.1080/10643389.2015.1078220.
 84. Ferreres, F.; García-Viguera, C.; Tomás-Lorente, F.; Tomás-Barberán, F.A. Hesperetin: A Marker of the Floral Origin of Citrus Honey. *J. Sci. Food Agric.* **1993**, 61, 121–123, doi:10.1002/JSFA.2740610119.
 85. Da Silva, P.M.; Gauche, C.; Gonzaga, L.V.; Costa, A.C.O.; Fett, R. Honey: Chemical Composition, Stability and Authenticity. *Food Chem.* **2016**, 196, 309–323, doi:10.1016/J.FOODCHEM.2015.09.051.
 86. Truchado, P.; Ferreres, F.; Bortolotti, L.; Sabatini, A.G.; Tomás-Barberán, F.A. Nectar Flavonol Rhamnosides Are Floral Markers of Acacia (Robinia Pseudacacia) Honey. *J. Agric. Food Chem.* **2008**, 56, 8815–8824, doi:10.1021/JF801625T.
 87. Molan, P.C. The Evidence Supporting the Use of Honey as a Wound Dressing. <http://dx.doi.org/10.1177/1534734605286014> **2016**, 5, 40–54, doi:10.1177/1534734605286014.
 88. Natarajan, S.; Williamson, D.; Grey, J.; Harding, K.G.; Cooper, R.A. Healing of an MRSA-Colonized, Hydroxyurea-Induced Leg Ulcer with Honey. *J. Dermatolog. Treat.* **2001**, 12, 33–36, doi:10.1080/095466301750163563.
 89. Scepankova, H.; Combarros-Fuertes, P.; Fresno, J.M.; Tornadijo, M.E.; Dias, M.S.; Pinto, C.A.; Saraiva, J.A.; Estevinho, L.M. Role of Honey in Advanced Wound Care. *Molecules* **2021**, 26, 4784, doi:10.3390/MOLECULES26164784.
 90. Falanga, V. Wound Healing and Its Impairment in the Diabetic Foot. *Lancet (London, England)* **2005**, 366, 1736–1743, doi:10.1016/S0140-6736(05)67700-8.
 91. Mohamed, H.; Salma, M.A.; Al Lenjawi, B.; Mohammad, A.; Jouda, Z.; Abdi, S.; Abraham, S.; Abou Ayash, H. Enhancing Primary Healing Post Ray Amputation in a Diabetic Patient: Efficacy of Natural Honey. *J. Diabet. Foot Complicat.* **2014**, 6, 13–18.
 92. Adebolu, T.T. Effect of Natural Honey on Local Isolates of Diarrhea-Causing Bacteria in Southwestern Nigeria. *African J. Biotechnol.* **2005**, 4, 1172.
 93. Eick, S.; Schäfer, G.; Kwieciński, J.; Atrott, J.; Henle, T.; Pfister, W. Honey - a Potential Agent against Porphyromonas Gingivalis: An in Vitro Study. *BMC Oral Health* **2014**, 14, doi:10.1186/1472-6831-14-24.
 94. Gupta, R.K.; Stangaciu, S. Apitherapy: Holistic Healing through the Honeybee and Bee Products in Countries with Poor Healthcare System. In *Beekeeping for poverty alleviation and livelihood security*; Springer, Dordrecht, 2014; pp. 413–446.
 95. Patel, S.; Cichello, S. Manuka Honey: An Emerging Natural Food with Medicinal Use. *Nat. Products Bioprospect.* **2013**, 3, 121–128.
 96. Rakha, M.K.; Nabil, Z.I.; Hussein, A.A. Cardioactive and Vasoactive Effects of Natural Wild Honey against Cardiac Malperformance Induced by Hyperadrenergic Activity. *J. Med. Food* **2008**, 11, 91–98, doi:10.1089/JMF.2006.172.
 97. Afroz, R.; Tanvir, E.M.; Karim, N.; Hossain, M.S.; Alam, N.; Gan, S.H.; Khalil, M.I. Sundarban

- Honey Confers Protection against Isoproterenol-Induced Myocardial Infarction in Wistar Rats. *Biomed Res. Int.* **2016**, 2016, doi:10.1155/2016/6437641.
98. Khalil, M.I.; Sulaiman, S.A.; Boukraa, L. Antioxidant Properties of Honey and Its Role in Preventing Health Disorder. *Open Nutraceuticals J.* **2014**, 3, 6–16, doi:10.2174/18763960010030100006.
 99. Ajibola, A.; Olusakin, J.; Oyewale, A.A. Growth and Metabolic Response of Suckling Rats Fed with Natural Honey Supplements. *Ommega Int.* **2016**, 3, 1–8.
 100. Erejuwa, O.O.; Sulaiman, S.A.; Ab Wahab, M.S. Effects of Honey and Its Mechanisms of Action on the Development and Progression of Cancer. *Molecules* **2014**, 19, 2497–2522, doi:10.3390/MOLECULES19022497.
 101. Tsiapara, A. V.; Jaakkola, M.; Chinou, I.; Graikou, K.; Tolonen, T.; Virtanen, V.; Moutsatsou, P. Bioactivity of Greek Honey Extracts on Breast Cancer (MCF-7), Prostate Cancer (PC-3) and Endometrial Cancer (Ishikawa) Cells: Profile Analysis of Extracts. *Food Chem.* **2009**, 116, 702–708, doi:10.1016/J.FOODCHEM.2009.03.024.
 102. Fauzi, A.N.; Norazmi, M.N.; Yaacob, N.S. Tualang Honey Induces Apoptosis and Disrupts the Mitochondrial Membrane Potential of Human Breast and Cervical Cancer Cell Lines. *Food Chem. Toxicol.* **2011**, 49, 871–878, doi:10.1016/J.FCT.2010.12.010.
 103. Abdel Aziz, A.; Rady, H.M.; Amer, M.A.; Kiwan, H.S. Effect of Some Honey Bee Extracts on the Proliferation, Proteolytic and Gelatinolytic Activities of the Hepatocellular Carcinoma Hepg2 Cell Line. *Aust J Basic Appl Sci* **2009**, 3, 2754–2769.
 104. Wen, C.T.P.; Hussein, S.Z.; Abdullah, S.; Karim, N.A.; Makpol, S.; Yusof, Y.A.M. Gelam and Nenas honeys inhibit proliferation of HT 29 colon cancer cells by inducing DNA damage and apoptosis while suppressing inflammation. *Asian Pac. J. Cancer Prev.* **2012**, 13, 1605–1610, doi:10.7314/APJCP.2012.13.4.1605.
 105. Kieliszek, M.; Piwowarek, K.; Kot, A.M.; Błażej, S.; Chlebowska-Śmigiel, A.; Wolska, I. Pollen and Bee Bread as New Health-Oriented Products: A Review. *Trends Food Sci. Technol.* **2018**, 71, 170–180, doi:10.1016/J.TIFS.2017.10.021.
 106. Thakur, M.; Nanda, V. Composition and Functionality of Bee Pollen: A Review. *Trends Food Sci. Technol.* **2020**, 98, 82–106, doi:10.1016/J.TIFS.2020.02.001.
 107. De-Melo, A.A.M.; de Almeida-Muradian, L.B. Chemical Composition of Bee Pollen. *Bee Prod. - Chem. Biol. Prop.* **2017**, 221–259, doi:10.1007/978-3-319-59689-1_11/TABLES/2.
 108. Li, Q.Q.; Wang, K.; Marcucci, M.C.; Sawaya, A.C.H.F.; Hu, L.; Xue, X.F.; Wu, L.M.; Hu, F.L. Nutrient-Rich Bee Pollen: A Treasure Trove of Active Natural Metabolites. *J. Funct. Foods* **2018**, 49, 472–484, doi:10.1016/J.JFF.2018.09.008.
 109. Aylanc, V.; Falcão, S.I.; Ertosun, S.; Vilas-Boas, M. From the Hive to the Table: Nutrition Value, Digestibility and Bioavailability of the Dietary Phytochemicals Present in the Bee Pollen and Bee Bread. *Trends Food Sci. Technol.* **2021**, 109, 464–481, doi:10.1016/J.TIFS.2021.01.042.
 110. Almeida-Muradian, L.B.; Pamplona, L.C.; Coimbra, S.; Barth, O.M. Chemical Composition and Botanical Evaluation of Dried Bee Pollen Pellets. *J. Food Compos. Anal.* **2005**, 18, 105–111, doi:10.1016/J.JFCA.2003.10.008.
 111. Liolios, V.; Tananaki, C.; Dimou, M.; Kanelis, D.; Goras, G.; Karazafiris, E.; Thrasyvoulou, A. Ranking Pollen from Bee Plants According to Their Protein Contribution to Honey Bees. <http://dx.doi.org/10.1080/00218839.2016.1173353> **2016**, 54, 582–592, doi:10.1080/00218839.2016.1173353.
 112. De-Melo, A.A.M.; Estevinho, L.M.; Moreira, M.M.; Delerue-Matos, C.; Freitas, A. da S. de; Barth,

- O.M.; Almeida-Muradian, L.B. de A Multivariate Approach Based on Physicochemical Parameters and Biological Potential for the Botanical and Geographical Discrimination of Brazilian Bee Pollen. *Food Biosci.* **2018**, *25*, 91–110, doi:10.1016/J.FBIO.2018.08.001.
113. Martins, M.C.T. Physicochemical Composition of Bee Pollen from Eleven Brazilian States. **2011**.
 114. Odoux, J.F.; Feuillet, D.; Aupinel, P.; Loublier, Y.; Tasei, J.N.; Mateescu, C. Territorial Biodiversity and Consequences on Physico-Chemical Characteristics of Pollen Collected by Honey Bee Colonies. *Apidologie* **2012**, *43*, 561–575, doi:10.1007/S13592-012-0125-1/FIGURES/5.
 115. Liolios, V.; Tananaki, C.; Kanelis, D.; Rodopoulou, M.A.; Dimou, M.; Argana, N. The Determination of Fat Content and Fatty Acid Composition of Bee Pollen Based on Its Botanical Origin. In Proceedings of the Proceedings of the 5th International Symposium on Bee Products; 2019; pp. 7–10.
 116. Mărgăoan, R.; Mărghițaș, L. Al; Dezmirean, D.S.; Dulf, F. V.; Bunea, A.; Socaci, S.A.; Bobiș, O. Predominant and Secondary Pollen Botanical Origins Influence the Carotenoid and Fatty Acid Profile in Fresh Honeybee-Collected Pollen. *J. Agric. Food Chem.* **2014**, *62*, 6306–6316, doi:10.1021/JF5020318/SUPPL_FILE/JF5020318_SI_001.PDF.
 117. Negri, G.; Teixeira, E.W.; Alves, M.L.T.M.F.; De Camargo Carmello Moreti, A.C.; Otsuk, I.P.; Borguini, R.G.; Salatino, A. Hydroxycinnamic Acid Amide Derivatives, Phenolic Compounds and Antioxidant Activities of Extracts of Pollen Samples from Southeast Brazil. *J. Agric. Food Chem.* **2011**, *59*, 5516–5522, doi:10.1021/JF200602K.
 118. Aabed, K.; Bhat, R.S.; Moubayed, N.; Al-Mutiri, M.; Al-Marshoud, M.; Al-Qahtani, A.; El-Ansary, A. Ameliorative Effect of Probiotics (*Lactobacillus Paracaseii* and Protexin®) and Prebiotics (Propolis and Bee Pollen) on Clindamycin and Propionic Acid-Induced Oxidative Stress and Altered Gut Microbiota in a Rodent Model of Autism. *Cell. Mol. Biol.* **2019**, *65*, 1–7, doi:10.14715/CMB/2019.65.1.1.
 119. Carpes, S.T.; Begnini, R.; Alencar, S.M. de; Masson, M.L. Study of Preparations of Bee Pollen Extracts, Antioxidant and Antibacterial Activity. *Ciência e Agrotecnologia* **2007**, *31*, 1818–1825, doi:10.1590/S1413-70542007000600032.
 120. Pascoal, A.; Rodrigues, S.; Teixeira, A.; Feás, X.; Estevinho, L.M. Biological Activities of Commercial Bee Pollens: Antimicrobial, Antimutagenic, Antioxidant and Anti-Inflammatory. *Food Chem. Toxicol.* **2014**, *63*, 233–239, doi:10.1016/J.FCT.2013.11.010.
 121. Pari, L.; KarthiKesan, K. Protective Role of Caffeic Acid against Alcohol-Induced Biochemical Changes in Rats. *Fundam. Clin. Pharmacol.* **2007**, *21*, 355–361, doi:10.1111/J.1472-8206.2007.00505.X.
 122. Mohamed, N.A.; Ahmed, O.M.; Hozayen, W.G.; Ahmed, M.A. Ameliorative Effects of Bee Pollen and Date Palm Pollen on the Glycemic State and Male Sexual Dysfunctions in Streptozotocin-Induced Diabetic Wistar Rats. *Biomed. Pharmacother.* **2018**, *97*, 9–18, doi:10.1016/J.BIOPHA.2017.10.117.
 123. Senyuk, B.; Boreyko, L.D.; Yurnyuk, S. CORRECTION OF CLINICAL AND BIOCHEMICAL PARAMETERS USING BEE POLLEN IN PATIENTS WITH DIABETES. **2016**.
 124. Alkhalidy, H.; Moore, W.; Wang, Y.; Luo, J.; McMillan, R.P.; Zhen, W.; Zhou, K.; Liu, D. The Flavonoid Kaempferol Ameliorates Streptozotocin-Induced Diabetes by Suppressing Hepatic Glucose Production. *Molecules* **2018**, *23*, doi:10.3390/MOLECULES23092338.
 125. Didaras, N.A.; Karatasou, K.; Dimitriou, T.G.; Amoutzias, G.D.; Mossialos, D. Antimicrobial Activity of Bee-Collected Pollen and Beebread: State of the Art and Future Perspectives. *Antibiot. 2020, Vol. 9, Page 811* **2020**, *9*, 811, doi:10.3390/ANTIBIOTICS9110811.

126. Kacániová, M.; Vuković, N.; Chlebo, R.; Haščík, P.; Rovná, K.; Cubon, J.; Dzugan, M.; Pasternakiewicz, A. The Antimicrobial Activity of Honey, Bee Pollen Loads and Beeswax from Slovakia. *Arch. Biol. Sci.* **2012**, *64*, 927–934, doi:10.2298/ABS1203927K.
127. Kyselka, J.; Bleha, R.; Dragoun, M.; Bialasová, K.; Horáčková, Š.; Schätz, M.; Sluková, M.; Filip, V.; Synytsya, A. Antifungal Polyamides of Hydroxycinnamic Acids from Sunflower Bee Pollen. *J. Agric. Food Chem.* **2018**, *66*, 11018–11026, doi:10.1021/ACS.JAFC.8B03976.
128. Komosinska-Vassev, K.; Olczyk, P.; Kaźmierczak, J.; Mencner, L.; Olczyk, K. Bee Pollen: Chemical Composition and Therapeutic Application. *Evid. Based. Complement. Alternat. Med.* **2015**, *2015*, doi:10.1155/2015/297425.
129. Khalifa, S.A.M.; Elashal, M.; Kieliszek, M.; Ghazala, N.E.; Farag, M.A.; Saeed, A.; Xiao, J.; Zou, X.; Khatib, A.; Göransson, U.; et al. Recent Insights into Chemical and Pharmacological Studies of Bee Bread. *Trends Food Sci. Technol.* **2020**, *97*, 300–316, doi:10.1016/J.TIFS.2019.08.021.
130. Mohammad, S.M.; Mahmud-Ab-Rashid, N.K.; Zawawi, N. Botanical Origin and Nutritional Values of Bee Bread of Stingless Bee (*Heterotrigona itama*) from Malaysia. *J. Food Qual.* **2020**, *2020*, doi:10.1155/2020/2845757.
131. Bakour, M.; Fernandes, Â.; Barros, L.; Sokovic, M.; Ferreira, I.C.F.R.; Badiia lyoussi Bee Bread as a Functional Product: Chemical Composition and Bioactive Properties. *LWT* **2019**, *109*, 276–282, doi:10.1016/J.LWT.2019.02.008.
132. Kaplan, M.; Karaoglu, Ö.; Eroglu, N.; Silici, S. Fatty Acid and Proximate Composition of Bee Bread. *Food Technol. Biotechnol.* **2016**, *54*, doi:10.17113/FTB.54.04.16.4635.
133. Dranca, F.; Ursachi, F.; Oroian, M. Bee Bread: Physicochemical Characterization and Phenolic Content Extraction Optimization. *Foods* **2020**, *Vol. 9*, Page 1358 **2020**, *9*, 1358, doi:10.3390/FOODS9101358.
134. Vásquez, A.; Olofsson, T.C. The Lactic Acid Bacteria Involved in the Production of Bee Pollen and Bee Bread. <http://dx.doi.org/10.3896/IBRA.1.48.3.07> **2015**, *48*, 189–195, doi:10.3896/IBRA.1.48.3.07.
135. Urcan, A.; Al MĂRGHITAŞ, L.; Dezmirean, D.S.; Bobiş, O.; Bonta, V.; Mureşan, C.I.; Mărgăoan, R. Chemical Composition and Biological Activities of Beebread-Review. *Bull. UASVM Anim. Sci. Biotechnol.* **2017**, *74*, 1843–536, doi:10.15835/buasvmcn-asb.
136. Bayram, N.E.; Gercek, Y.C.; Çelik, S.; Mayda, N.; Kostić, A.; Dramićanin, A.M.; Özkök, A. Phenolic and Free Amino Acid Profiles of Bee Bread and Bee Pollen with the Same Botanical Origin – Similarities and Differences. *Arab. J. Chem.* **2021**, *14*, 103004, doi:10.1016/J.ARABJC.2021.103004.
137. Pełka, K.; Otlowska, O.; Worobo, R.W.; Szweda, P. Bee Bread Exhibits Higher Antimicrobial Potential Compared to Bee Pollen. *Antibiot. (Basel, Switzerland)* **2021**, *10*, 1–14, doi:10.3390/ANTIBIOTICS10020125.
138. Castaldo, S.; Capasso, F. Propolis, an Old Remedy Used in Modern Medicine. *Fitoterapia* **2002**, *73*, doi:10.1016/S0367-326X(02)00185-5.
139. Lotfy, M. Biological Activity of Bee Propolis in Health and Disease. *Asian Pac J Cancer Prev* **2006**, *7*, 22–31.
140. Ramadan, M.F.; Al-Ghamdi, A. Bioactive Compounds and Health-Promoting Properties of Royal Jelly: A Review. *J. Funct. Foods* **2012**, *4*, 39–52, doi:10.1016/J.JFF.2011.12.007.
141. Sumi, C.D.; Yang, B.W.; Yeo, I.C.; Hahm, Y.T. Antimicrobial Peptides of the Genus *Bacillus*: A New Era for Antibiotics. *Can. J. Microbiol.* **2015**, *61*, 93–103, doi:10.1139/CJM-2014-0613/ASSET/IMAGES/CJM-2014-0613TAB2.GIF.

142. Pasupuleti, V.R.; Sammugam, L.; Ramesh, N.; Gan, S.H. Honey, Propolis, and Royal Jelly: A Comprehensive Review of Their Biological Actions and Health Benefits. *Oxid. Med. Cell. Longev.* **2017**, *2017*, doi:10.1155/2017/1259510.
143. Salatino, A.; Teixeira, É.W.; Negri, G.; Message, D. Origin and Chemical Variation of Brazilian Propolis. *Evid. Based. Complement. Alternat. Med.* **2005**, *2*, 33–38, doi:10.1093/ECAM/NEH060.
144. Freitas, S.F.; Shinohara, L.; Sforcin, J.M.; Guimarães, S. In Vitro Effects of Propolis on Giardia Duodenalis Trophozoites. *Phytomedicine* **2006**, *13*, 170–175, doi:10.1016/J.PHYMED.2004.07.008.
145. Imhof, M.; Lipovac, M.; Kurz, C.H.; Barta, J.; Verhoeven, H.C.; Huber, J.C. Propolis Solution for the Treatment of Chronic Vaginitis. *Int. J. Gynecol. Obstet.* **2005**, *89*, 127–132.
146. Capoci, I.R.G.; Bonfim-Mendonça, P.D.S.; Arita, G.S.; Pereira, R.R.D.A.; Consolaro, M.E.L.; Bruschi, M.L.; Negri, M.; Svidzinski, T.I.E. Propolis Is an Efficient Fungicide and Inhibitor of Biofilm Production by Vaginal Candida Albicans. *Evid. Based. Complement. Alternat. Med.* **2015**, *2015*, doi:10.1155/2015/287693.
147. Santos, V.R.; Pereira, E.M.R.; Da Silva, J.L.D.C.; Silva, F.F.; De Luca, M.P.; Ferreira, E.F.E.; Lorentz, T.C.M. Clinical Evidence of the Efficacy of a Mouthwash Containing Propolis for the Control of Plaque and Gingivitis: A Phase II Study. *Evid. Based. Complement. Alternat. Med.* **2011**, *2011*, doi:10.1155/2011/750249.
148. Bertolini, P.F.R.; Biondi Filho, O.; Pomilio, A.; Pinheiro, S.L.; de Carvalho, M.S. Antimicrobial Capacity of Aloe Vera and Propolis Dentifrice against Streptococcus Mutans Strains in Toothbrushes: An in Vitro Study. *J. Appl. Oral Sci.* **2012**, *20*, 32–37, doi:10.1590/S1678-77572012000100007.
149. Zakerkish, M.; Jenabi, M.; Zaeemzadeh, N.; Hemmati, A.A.; Neisi, N. The Effect of Iranian Propolis on Glucose Metabolism, Lipid Profile, Insulin Resistance, Renal Function and Inflammatory Biomarkers in Patients with Type 2 Diabetes Mellitus: A Randomized Double-Blind Clinical Trial. *Sci. Rep.* **2019**, *9*, doi:10.1038/S41598-019-43838-8.
150. Daleprane, J.B.; Abdalla, D.S. Emerging Roles of Propolis: Antioxidant, Cardioprotective, and Antiangiogenic Actions. *Evid. Based. Complement. Alternat. Med.* **2013**, *2013*, doi:10.1155/2013/175135.
151. Altabbal, S.; Athamnah, K.; Rahma, A.; Wali, A.F.; Eid, A.H.; Iratni, R.; Al Dhaheri, Y. Propolis: A Detailed Insight of Its Anticancer Molecular Mechanisms. *Pharmaceuticals (Basel)*. **2023**, *16*, doi:10.3390/PH16030450.
152. Giampieri, F.; Quiles, J.L.; Cianciosi, D.; Forbes-Hernández, T.Y.; Orantes-Bermejo, F.J.; Alvarez-Suarez, J.M.; Battino, M. Bee Products: An Emblematic Example of Underutilized Sources of Bioactive Compounds. *J. Agric. Food Chem.* **2022**, *70*, 6833–6848, doi:10.1021/ACS.JAFC.1C05822.
153. Ibáñez, B.; Melero, A.; Montoro, A.; San Onofre, N.; Soriano, J.M. Radioprotective Effects from Propolis: A Review. *Molecules* **2023**, *28*, doi:10.3390/MOLECULES28155842.
154. Berretta, A.A.; Silveira, M.A.D.; Córdor Capcha, J.M.; De Jong, D. Propolis and Its Potential against SARS-CoV-2 Infection Mechanisms and COVID-19 Disease: Running Title: Propolis against SARS-CoV-2 Infection and COVID-19. *Biomed. Pharmacother.* **2020**, *131*, doi:10.1016/J.BIOPHA.2020.110622.
155. Zulhendri, F.; Chandrasekaran, K.; Kowacz, M.; Ravalia, M.; Kripal, K.; Fearnley, J.; Perera, C.O. Antiviral, Antibacterial, Antifungal, and Antiparasitic Properties of Propolis: A Review. *Foods (Basel, Switzerland)* **2021**, *10*, doi:10.3390/FOODS10061360.

156. Alvarez-Suarez, J.M. *Bee Products-Chemical and Biological Properties*; Springer, 2017; ISBN 3319596896.
157. Boselli, E.; Caboni, M.F.; Sabatini, A.G.; Marcazzan, G.L.; Lercker, G. Determination and Changes of Free Amino Acids in Royal Jelly during Storage. *Apidologie* **2003**, *34*, 129–137, doi:10.1051/APIDO:2003011.
158. Ferioli, F.; Armaforte, E.; Caboni, M.F. Comparison of the Lipid Content, Fatty Acid Profile and Sterol Composition in Local Italian and Commercial Royal Jelly Samples. *JAOCS, J. Am. Oil Chem. Soc.* **2014**, *91*, 875–884, doi:10.1007/S11746-014-2446-X/TABLES/3.
159. Wang, Y.; Ma, L.; Zhang, W.; Cui, X.; Wang, H.; Xu, B. Comparison of the Nutrient Composition of Royal Jelly and Worker Jelly of Honey Bees (*Apis Mellifera*). *Apidologie* **2016**, *47*, 48–56, doi:10.1007/S13592-015-0374-X/TABLES/2.
160. Nabas, Z.; Haddadin, M.S.Y.; Haddadin, J.; Nazer, I.K. Chemical Composition of Royal Jelly and Effects of Synbiotic with Two Different Locally Isolated Probiotic Strains on Antioxidant Activities. *Polish J. Food Nutr. Sci.* **2014**, *64*.
161. Balkanska, R.; Karadjova, I.; Ignatova, M. Comparative Analyses of Chemical Composition of Royal Jelly and Drone Brood. *Bulg. Chem. Commun* **2014**, *46*, 412–416.
162. Drapeau, M.D.; Albert, S.; Kucharski, R.; Prusko, C.; Maleszka, R. Evolution of the Yellow/Major Royal Jelly Protein Family and the Emergence of Social Behavior in Honey Bees. *Genome Res.* **2006**, *16*, 1385–1394, doi:10.1101/GR.5012006.
163. Bărnăuțiu, L.I.; Mărghițaș, L. Al; Dezmirean, D.S.; Mihai, C.M.; Bobiș, O. Chemical Composition and Antimicrobial Activity of Royal Jelly-REVIEW. *Sci. Pap. Anim. Sci. Biotechnol.* **2011**, *44*, 67–72.
164. Kunugi, H.; Ali, A.M. Royal Jelly and Its Components Promote Healthy Aging and Longevity: From Animal Models to Humans. *Int. J. Mol. Sci.* **2019**, *20*, doi:10.3390/IJMS20194662.
165. Bilikova, K.; Huang, S.C.; Lin, I.P.; Šimuth, J.; Peng, C.C. Structure and Antimicrobial Activity Relationship of Royalisin, an Antimicrobial Peptide from Royal Jelly of *Apis Mellifera*. *Peptides* **2015**, *68*, 190–196, doi:10.1016/J.PEPTIDES.2015.03.001.
166. Xue, X.; Wu, L.; Wang, K. Chemical Composition of Royal Jelly. *Bee Prod. - Chem. Biol. Prop.* **2017**, 181–190, doi:10.1007/978-3-319-59689-1_8/TABLES/1.
167. Lozo, J.; Berić, T.; Terzić-Vidojević, A.; Stanković, S.; Fira, D.; Stanisavljević, L. Microbiota Associated with Pollen, Bee Bread, Larvae and Adults of Solitary Bee *Osmia Cornuta* (Hymenoptera: Megachilidae). *Bull. Entomol. Res.* **2015**, *105*, 470–476, doi:10.1017/S0007485315000292.
168. Gilliam, M. Identification and Roles of Non-Pathogenic Microflora Associated with Honey Bees1. *FEMS Microbiol. Lett.* **1997**, *155*, 1–10, doi:10.1111/J.1574-6968.1997.TB12678.X.
169. McBride, B.W.; Turnbull, P.C.B. *Bacillus*, Infection and Immunity. *Encycl. Immunol.* **1998**, 311–315, doi:10.1006/RWEI.1999.0080.
170. Cho, W. Il; Chung, M.S. *Bacillus* Spores: A Review of Their Properties and Inactivation Processing Technologies. *Food Sci. Biotechnol.* **2020**, *29*, 1447–1461, doi:10.1007/S10068-020-00809-4/TABLES/4.
171. Hafeez, A. Bin; Pelka, K.; Buzun, K.; Worobo, R.; Szweda, P. Whole-Genome Sequencing and Antimicrobial Potential of Bacteria Isolated from Polish Honey. *Appl. Microbiol. Biotechnol.* **2023**, *107*, 6389–6406, doi:10.1007/S00253-023-12732-9.
172. Hafeez, A. Bin; Szweda, P. Genomic and Functional Landscape of Antimicrobial Peptides and

- Lipopeptides from Ginger Honey-Derived *Bacillus Subtilis*. *Mol. Genet. Genomics* 2026 3011 **2026**, 301, 86-, doi:10.1007/S00438-026-02411-5.
173. Pełka, K.; Worobo, R.W.; Walkusz, J.; Szweda, P. Bee Pollen and Bee Bread as a Source of Bacteria Producing Antimicrobials. *Antibiot. (Basel, Switzerland)* **2021**, 10, doi:10.3390/ANTIBIOTICS10060713.
 174. Shin, C.J.; O'Connor, T.J. Complete Genome Sequence of an African Raw Honey Bacterial Isolate, *Bacillus Safensis* Strain AHB11. *Microbiol. Resour. Announc.* **2022**, 11, e00516-22.
 175. Xiong, Z.R.; Cobo, M.; Whittall, R.M.; Snyder, A.B.; Worobo, R.W. Purification and Characterization of Antifungal Lipopeptide Produced by *Bacillus Velezensis* Isolated from Raw Honey. *PLoS One* **2022**, 17, e0266470, doi:10.1371/journal.pone.0266470.
 176. Flores, J.L.H.; Landaverde, D.S.; Huerta, Y.P.; Castillo, V.L.G.; Sánchez, M. de los Á.B.; Hernández, I.A.; López, M.Á.R.; Hidalgo, E.Á.; Jones, G.H.; Guillén, J.C. Phylogenetic Analysis of *Bacillus Cereus* Ssensu Lato Isolates from Commercial Bee Pollen Using TRNACys-PCR. *Microorg.* 2020, Vol. 8, **2020**, 8, doi:10.3390/MICROORGANISMS8040524.
 177. Saccà, M.L.; Manici, L.M. Honey Bee-Associated Bacteria as Producers of Bioactive Compounds for Protecting Hives. A Biosynthetic Gene-Based Approach. *Microbiol. Res.* **2021**, 252, 126860, doi:10.1016/J.MICRES.2021.126860.
 178. Pełka, K.; Hafeez, A. Bin; Worobo, R.W.; Szweda, P. Probiotic Potential of *Bacillus* Isolates from Polish Bee Pollen and Bee Bread. *Probiotics Antimicrob. Proteins* **2023**, 1, 1–14, doi:10.1007/S12602-023-10157-4/FIGURES/4.
 179. Pełka, K.; Worobo, R.W.; Walkusz, J.; Szweda, P. Bee Pollen and Bee Bread as a Source of Bacteria Producing Antimicrobials. *Antibiot. (Basel, Switzerland)* **2021**, 10, doi:10.3390/ANTIBIOTICS10060713.
 180. Bin Hafeez, A.; Pełka, K.; Worobo, R.; Szweda, P. In Silico Safety Assessment of *Bacillus* Isolated from Polish Bee Pollen and Bee Bread as Novel Probiotic Candidates. *Int. J. Mol. Sci.* **2024**, 25, 666, doi:10.3390/IJMS25010666/S1.
 181. Pajor, M.; Xiong, Z.R.; Worobo, R.W.; Szweda, P. *Paenibacillus Alvei* Mp1 as a Producer of the Proteinaceous Compound with Activity against Important Human Pathogens, Including *Staphylococcus Aureus* and *Listeria Monocytogenes*. *Pathogens* **2020**, 9, doi:10.3390/pathogens9050319.
 182. Martín-González, D.; Bordel, S.; Solis, S.; Gutierrez-Merino, J.; Santos-Beneit, F. Characterization of *Bacillus* Strains from Natural Honeybee Products with High Keratinolytic Activity and Antimicrobial Potential. *Microorganisms* **2023**, 11, 456, doi:10.3390/MICROORGANISMS11020456/S1.
 183. López, A.C.; Alippi, A.M. Diversity of *Bacillus Megaterium* Isolates Cultured from Honeys. *LWT - Food Sci. Technol.* **2009**, 42, 212–219, doi:10.1016/J.LWT.2008.05.001.
 184. Zulkhairi Amin, F.A.; Sabri, S.; Ismail, M.; Chan, K.W.; Ismail, N.; Mohd Esa, N.; Mohd Lila, M.A.; Zawawi, N. Probiotic Properties of *Bacillus* Strains Isolated from Stingless Bee (*Heterotrigona Itama*) Honey Collected across Malaysia. *Int. J. Environ. Res. Public Health* **2019**, 17, doi:10.3390/IJERPH17010278.
 185. Xiong, Z.R.; Sogin, J.H.; Worobo, R.W. Microbiome Analysis of Raw Honey Reveals Important Factors Influencing the Bacterial and Fungal Communities. *Front. Microbiol.* **2023**, 13, 1099522, doi:10.3389/FMICB.2022.1099522/FULL.
 186. Baharudin, M.M.A.A.; Ngalimat, M.S.; Shariff, F.M.; Yusof, Z.N.B.; Karim, M.; Baharum, S.N.; Sabri, S. Antimicrobial Activities of *Bacillus Velezensis* Strains Isolated from Stingless Bee

- Products against Methicillin-Resistant *Staphylococcus Aureus*. *PLoS One* **2021**, *16*, e0251514, doi:10.1371/JOURNAL.PONE.0251514.
187. Prakit, B.; Chaeyod, R.; Khongkool, K.; Chanasit, W.; Lertworapreecha, M. Multifunctional Probiotic and Safety Attributes *Heyndrickxia Coagulans* Isolated from Stingless Bee Honey. *Ann. Microbiol.* **2025**, *75*, 3-, doi:10.1186/S13213-025-01791-0.
 188. López, A.C.; Fernández, L.A.; Alippi, A.M. Traceability of Potential Enterotoxigenic *Bacillus Cereus* in Bee-Pollen Samples from Argentina throughout the Production Process. *Int. J. Food Microbiol.* **2020**, *334*, doi:10.1016/j.ijfoodmicro.2020.108816.
 189. Ngalmat, M.S.; Rahman, R.N.Z.R.A.; Yusof, M.T.; Syahir, A.; Sabri, S. Characterisation of Bacteria Isolated from the Stingless Bee, *Heterotrigona Itama*, Honey, Bee Bread and Propolis. *PeerJ* **2019**, *7*, doi:10.7717/PEERJ.7478.
 190. Gerst, M.M.; Somogyi, Á.; Yang, X.; Yousef, A.E. Detection and Characterization of a Rare Two-Component Lantibiotic, Amyloliqucedin GF610 Produced by *Bacillus Velezensis*, Using a Combination of Culture, Molecular and Bioinformatic Analyses. *J. Appl. Microbiol.* **2022**, *132*, 994–1007, doi:10.1111/JAM.15290.
 191. Iorizzo, M.; Letizia, F.; Ganassi, S.; Testa, B.; Petrarca, S.; Albanese, G.; Di Criscio, D.; De Cristofaro, A. Functional Properties and Antimicrobial Activity from Lactic Acid Bacteria as Resources to Improve the Health and Welfare of Honey Bees. *Insects* **2022**, *13*, doi:10.3390/INSECTS13030308.
 192. Tamarit, D.; Ellegaard, K.M.; Wikander, J.; Olofsson, T.; Vásquez, A.; Andersson, S.G.E. Functionally Structured Genomes in *Lactobacillus Kunkeei* Colonizing the Honey Crop and Food Products of Honeybees and Stingless Bees. *Genome Biol. Evol.* **2015**, *7*, 1455, doi:10.1093/GBE/EVV079.
 193. Brar, G.; Ngor, L.; McFrederick, Q.S.; Torson, A.S.; Rajamohan, A.; Rinehart, J.; Singh, P.; Bowsher, J.H. High Abundance of *Lactobacilli* in the Gut Microbiome of Honey Bees during Winter. *Sci. Reports* **2025**, *15*, 7409-, doi:10.1038/s41598-025-90763-0.
 194. Abadi, M.E.G.M.; Hosseini-Safa, A.; Habibi, S.; Dehghan, M.; Forouzani-Moghaddam, M.J.; Oshaghi, M. Isolation and Characterization of the *Lactobacillus* Strain from Honey and Its Probiotic Properties. *Iran. J. Microbiol.* **2023**, *15*, 439–447, doi:10.18502/IJM.V15I3.12905.
 195. Luca, L.; Pauliuc, D.; Oroian, M. Honey Microbiota, Methods for Determining the Microbiological Composition and the Antimicrobial Effect of Honey – A Review. *Food Chem. X* **2024**, *23*, 101524, doi:10.1016/J.FOCHX.2024.101524.
 196. Stavropoulou, E.; Remmas, N.; Voidarou, C.; Vrioni, G.; Konstantinidis, T.; Ntougias, S.; Tsakris, A. Microbial Community Structure among Honey Samples of Different Pollen Origin. *Antibiot.* **2023**, *Vol. 12*, **2023**, *12*, doi:10.3390/ANTIBIOTICS12010101.
 197. Hsu, P.S.; Lin, J.A.; Hung, Y.T.; Wu, T.H.; Chen, Y.H.; Wu, M.C. Pollen Beebreads as a Source of Probiotics: A Metagenomic and Biochemical Characterization. *J. Sci. Food Agric.* **2026**, *106*, 842–853, doi:10.1002/JSFA.70209;REQUESTEDJOURNAL:JOURNAL:10970010;WGROU:STRING:PUBLICATION.
 198. Uçar, D.K.A.; Yurt, M.N.Z.; Taşbaşı, B.B.; Acar, E.E.; Yeğin, Z.; Özalp, V.C.; Sudağidan, M. Identification of Bacterial Diversity of Bee Collected Pollen and Bee Bread Microbiota by Metagenomic Analysis. *Acta Vet. Eurasia* **2022**, *48*, 189–199, doi:10.5152/ACTAVET.2022.22031.
 199. Lawrence, B.L.; Custer, G.F.; Underwood, R.M.; Dunn, R.R.; Dini-Andreote, F.; López-Urbe, M.M. The Bee Bread of Honey Bees Is Characterized by a Core Microbiota despite the Application of Miticide Treatments and Variation across Space and Time. *PeerJ* **2025**, *13*, e20366,

doi:10.7717/PEERJ.20366/SUPP-1.

200. Li, F.; Zhou, X.; Zhang, C.; Yang, S.; Xuan, H.; Zhang, Y. Improvement of Nutritional Value and Bioactivity of Bee Pollen by Co-Fermentation Process of Lactobacillus Screened from Bee Bread and Commercial Compound Probiotics. *Process*. 2026, Vol. 14, **2026**, 14, doi:10.3390/PR14040722.
201. Zhang, Y.; Li, L.; Pang, X.; Zhang, S.; Liu, Y.; Wang, Y.; Xie, N.; Li, X. Lactic Acid Bacteria as the Green and Safe Food Preservatives: Their Mechanisms, Applications and Prospects. *Foods* 2026, Vol. 15, **2026**, 15, 241, doi:10.3390/FOODS15020241.
202. Cerqueira, A.E.S.; Lima, H.S.; Silva, L.C.F.; Veloso, T.G.R.; De Paula, S.O.; Santana, W.C.; Da Silva, C.C. Melipona Stingless Bees and Honey Microbiota Reveal the Diversity, Composition, and Modes of Symbionts Transmission. *FEMS Microbiol. Ecol.* **2024**, 100, fiae063, doi:10.1093/FEMSEC/FIAE063.
203. Rosli, F.N.; Hazemi, M.H.F.; Akbar, M.A.; Basir, S.; Kassim, H.; Bunawan, H. Stingless Bee Honey: Evaluating Its Antibacterial Activity and Bacterial Diversity. *Insects* 2020, Vol. 11, **2020**, 11, 1–13, doi:10.3390/INSECTS11080500.
204. Ghosh, S.; Namin, S.M.; Jung, C. Differential Bacterial Community of Bee Bread and Bee Pollen Revealed by 16s rRNA High-Throughput Sequencing. *Insects* **2022**, 13, doi:10.3390/INSECTS13100863.
205. Moran, N.A. Genomics of the Honey Bee Microbiome. *Curr. Opin. Insect Sci.* **2015**, 10, 22–28, doi:10.1016/J.COIS.2015.04.003.
206. Fernandes, K.E.; Frost, E.A.; Kratz, M.; Carter, D.A. Pollen Products Collected from Honey Bee Hives Experiencing Minor Stress Have Altered Fungal Communities and Reduced Antimicrobial Properties. *FEMS Microbiol. Ecol.* **2024**, 100, 91, doi:10.1093/FEMSEC/FIAE091.
207. Sinpoo, C.; Williams, G.R.; Chantawannakul, P. Dynamics of Fungal Communities in Corbicular Pollen and Bee Bread. *Chiang Mai J. Sci* **2017**, 44, 1244–1256.
208. Friedle, C.; D’Alvise, P.; Schweikert, K.; Wallner, K.; Hasselmann, M. Changes of Microorganism Composition in Fresh and Stored Bee Pollen from Southern Germany. *Environ. Sci. Pollut. Res. Int.* **2021**, 28, 47251, doi:10.1007/S11356-021-13932-4.
209. Disayathanoowat, T.; Li, H.; Supapimon, N.; Suwannarach, N.; Lumyong, S.; Chantawannakul, P.; Guo, J. Different Dynamics of Bacterial and Fungal Communities in Hive-Stored Bee Bread and Their Possible Roles: A Case Study from Two Commercial Honey Bees in China. *Microorganisms* **2020**, 8, doi:10.3390/MICROORGANISMS8020264.
210. Bush, D.S.; Calla, B.; Berenbaum, M.R. An Aspergillus Flavus Strain from Bee Bread of the Western Honey Bee (Apis Mellifera) Displays Adaptations to Distinctive Features of the Hive Environment. *Ecol. Evol.* **2024**, 14, e10918, doi:10.1002/ECE3.10918.
211. Dimov, S.G.; Zagorchev, L.; Iliev, M.; Dekova, T.; Ilieva, R.; Kitanova, M.; Georgieva-Miteva, D.; Dimitrov, M.; Peykov, S. A Snapshot Picture of the Fungal Composition of Bee Bread in Four Locations in Bulgaria, Differing in Anthropogenic Influence. *J. fungi (Basel, Switzerland)* **2021**, 7, doi:10.3390/JOF7100845.
212. Ranjan, A.; Rajput, V.D.; Prazdnova, E.V.; Gurnani, M.; Bhardwaj, P.; Sharma, S.; Sushkova, S.; Mandzhieva, S.S.; Minkina, T.; Sudan, J.; et al. Nature’s Antimicrobial Arsenal: Non-Ribosomal Peptides from PGPB for Plant Pathogen Biocontrol. *Ferment.* 2023, Vol. 9, **2023**, 9, doi:10.3390/FERMENTATION9070597.
213. Mihaylova-Garnizova, R.; Davidova, S.; Hodzhev, Y.; Satchanska, G. Antimicrobial Peptides Derived from Bacteria: Classification, Sources, and Mechanism of Action against Multidrug-Resistant Bacteria. *Int. J. Mol. Sci.* 2024, Vol. 25, **2024**, 25, doi:10.3390/IJMS251910788.

214. Montalbán-López, M.; Scott, T.A.; Ramesh, S.; Rahman, I.R.; Van Heel, A.J.; Viel, J.H.; Bandarian, V.; Dittmann, E.; Genilloud, O.; Goto, Y.; et al. New Developments in RiPP Discovery, Enzymology and Engineering. *Nat. Prod. Rep.* **2021**, *38*, 130–239, doi:10.1039/D0NP00027B.
215. Gulick, A.M. Nonribosomal Peptide Synthetase Biosynthetic Clusters of ESKAPE Pathogens. *Nat. Prod. Rep.* **2017**, *34*, 981, doi:10.1039/C7NP00029D.
216. Chen, M.; Wang, H.; Zhang, C.; Zhang, Y.; Sun, H.; Yu, L.; Zhang, Q.; Chen, X.; Xiao, H. Recent Advances in Antimicrobial Lipopeptide Fengycin Secreted by *Bacillus*: Structure, Biosynthesis, Antifungal Mechanisms, and Potential Application in Food Preservation. *Food Chem.* **2025**, *489*, 144937.
217. Zhen, C.; Ge, X.F.; Lu, Y.T.; Liu, W.Z. Chemical Structure, Properties and Potential Applications of Surfactin, as Well as Advanced Strategies for Improving Its Microbial Production. *AIMS Microbiol.* **2023**, *9*, 195, doi:10.3934/MICROBIOL.2023012.
218. Yokoyama, K.; Numakura, M.; Kudo, F.; Ohmori, D.; Eguchi, T. Characterization and Mechanistic Study of a Radical SAM Dehydrogenase in the Biosynthesis of Butirosin. *J. Am. Chem. Soc.* **2007**, *129*, 15147–15155.
219. May, J.J.; Wendrich, T.M.; Marahiel, M.A. The Dhb Operon of *Bacillus Subtilis* Encodes the Biosynthetic Template for the Catecholic Siderophore 2,3-Dihydroxybenzoate-Glycine-Threonine Trimeric Ester Bacillibactin. *J. Biol. Chem.* **2001**, *276*, 7209–7217, doi:10.1074/jbc.M009140200.
220. Chatterjee, C.; Paul, M.; Xie, L.; van der Donk, W.A. Biosynthesis and Mode of Action of Lantibiotics. *Chem. Rev.* **2005**, *105*, 633–683, doi:10.1021/CR030105V.
221. Garcia-Gutierrez, E.; O'Connor, P.M.; Saalbach, G.; Walsh, C.J.; Hegarty, J.W.; Guinane, C.M.; Mayer, M.J.; Narbad, A.; Cotter, P.D. First Evidence of Production of the Lantibiotic Nisin P. *Sci. Reports* **2020**, *10*, 3738–, doi:10.1038/s41598-020-60623-0.
222. Zhang, Q.; Kobras, C.M.; Gebhard, S.; Mascher, T.; Wolf, D. Regulation of Heterologous Subtilin Production in *Bacillus Subtilis* W168. *Microb. Cell Fact.* **2022**, *21*, 57, doi:10.1186/S12934-022-01782-9.
223. Stein, T.; Borchert, S.; Conrad, B.; Feesche, J.; Hofemeister, B.; Hofemeister, J.; Entian, K.-D. Two Different Lantibiotic-like Peptides Originate from the Ericin Gene Cluster of *Bacillus Subtilis* A1/3. *J. Bacteriol.* **2002**, *184*, 1703–1711.
224. Chen, J.; Zhifei, L.U.; An, Z.; Ji, P.; Liu, X. Antibacterial Activities of Sophorolipids and Nisin and Their Combination against Foodborne Pathogen *Staphylococcus Aureus*. *Eur. J. Lipid Sci. Technol.* **2020**, *122*, 1900333, doi:10.1002/EJLT.201900333;WGROUP:STRING:PUBLICATION.
225. Champion, A.; Casey, P.G.; Field, D.; Cotter, P.D.; Hill, C.; Ross, R.P. In Vivo Activity of Nisin A and Nisin V against *Listeria Monocytogenes* in Mice. *BMC Microbiol.* **2013**, *13*, 23–, doi:10.1186/1471-2180-13-23.
226. Gut, I.M.; Blanke, S.R.; Van Der Donk, W.A. Mechanism of Inhibition of *Bacillus Anthracis* Spore Outgrowth by the Lantibiotic Nisin. *ACS Chem. Biol.* **2011**, *6*, 744–752, doi:10.1021/CB1004178.
227. Le Lay, C.; Dridi, L.; Bergeron, M.G.; Ouellette, M.; Fliss, I. Nisin Is an Effective Inhibitor of *Clostridium Difficile* Vegetative Cells and Spore Germination. *J. Med. Microbiol.* **2016**, *65*, 169–175, doi:10.1099/JMM.0.000202/CITE/REFWORKS.
228. Shi, Y.; Wen, T.; Zhao, F.; Hu, J. Bacteriostasis of Nisin against Planktonic and Biofilm Bacteria: Its Mechanism and Application. *J. Food Sci.* **2024**, *89*, 1894–1916, doi:10.1111/1750-3841.17001;WGROUP:STRING:PUBLICATION.
229. Barbosa, J.C.; Gonçalves, S.; Makowski, M.; Silva, Í.C.; Caetano, T.; Schneider, T.; Mösker, E.; Süssmuth, R.D.; Santos, N.C.; Mendo, S. Insights into the Mode of Action of the Two-Peptide

- Lantibiotic Lichenicidin. *Colloids Surfaces B Biointerfaces* **2022**, *211*, 112308, doi:10.1016/J.COLSURFB.2021.112308.
230. McClerren, A.L.; Cooper, L.E.; Quan, C.; Thomas, P.P.; Kelleher, N.L.; Van Der Donk, W.A. Discovery and in Vitro Biosynthesis of Haloduracin, a Two-Component Lantibiotic. *Proc. Natl. Acad. Sci. U. S. A.* **2006**, *103*, 17243–17248, doi:10.1073/PNAS.0606088103;ISSUE:ISSUE:DOI.
 231. Bakhtiary, A.; Cochrane, S.A.; Mercier, P.; McKay, R.T.; Miskolzie, M.; Sit, C.S.; Vederas, J.C. Insights into the Mechanism of Action of the Two-Peptide Lantibiotic Lacticin 3147. *J. Am. Chem. Soc.* **2017**, *139*, 17803–17810.
 232. Holo, H.; Jeknic, Z.; Daeschel, M.; Stevanovic, S.; Nes, I.F. Plantaricin W from *Lactobacillus Plantarum* Belongs to a New Family of Two-Peptide Lantibiotics. *Microbiology* **2001**, *147*, 643–651, doi:10.1099/00221287-147-3-643.
 233. Oman, T.J.; Van Der Donk, W.A. Insights into the Mode of Action of the Two-Peptide Lantibiotic Haloduracin. *ACS Chem. Biol.* **2009**, *4*, 865–874.
 234. Singh, M.; Chaudhary, S.; Sareen, D. Roseocin, a Novel Two-Component Lantibiotic from an Actinomycete. *Mol. Microbiol.* **2020**, *113*, 326–337, doi:10.1111/MMI.14419.
 235. Xin, B.; Zheng, J.; Liu, H.; Li, J.; Ruan, L.; Peng, D.; Sajid, M.; Sun, M. Thusin, a Novel Two-Component Lantibiotic with Potent Antimicrobial Activity against Several Gram-Positive Pathogens. *Front. Microbiol.* **2016**, *7*, 1115, doi:10.3389/FMICB.2016.01115.
 236. Hegemann, J.D.; Zimmermann, M.; Xie, X.; Marahiel, M.A. Lasso Peptides: An Intriguing Class of Bacterial Natural Products. *Acc. Chem. Res.* **2015**, *48*, 1909–1919.
 237. Peerzade, I.J.; Peddha, M.S.; Halami, P.M. The Lasso Peptide Produced by *Bacillus Licheniformis* MCC 2514 Demonstrates Efficacy in Treating In-Vivo *Salmonella Typhimurium* Infection. *Int. J. Biol. Macromol.* **2024**, *281*, 136470, doi:10.1016/J.IJBIOMAC.2024.136470.
 238. Jangra, M.; Travin, D.Y.; Aleksandrova, E. V.; Kaur, M.; Darwish, L.; Koteva, K.; Klepacki, D.; Wang, W.; Tiffany, M.; Sokaribo, A.; et al. A Broad-Spectrum Lasso Peptide Antibiotic Targeting the Bacterial Ribosome. *Nat. 2025 6408060* **2025**, *640*, 1022–1030, doi:10.1038/s41586-025-08723-7.
 239. Chen, Y.; Wang, J.; Li, G.; Yang, Y.; Ding, W. Current Advancements in Sactipeptide Natural Products. *Front. Chem.* **2021**, *9*, 595991, doi:10.3389/FCHEM.2021.595991.
 240. Dinesen, C.; Vertot, M.; Jarmusch, S.A.; Lozano-Andrade, C.N.; Andersen, A.J.C.; Kovács, Á.T. Subtilisin A Production Is Influenced by Surfactin Levels in *Bacillus Subtilis*. *microLife* **2025**, *6*, 29, doi:10.1093/FEMSML/UQAE029.
 241. Thennarasu, S.; Lee, D.K.; Poon, A.; Kawulka, K.E.; Vederas, J.C.; Ramamoorthy, A. Membrane Permeabilization, Orientation, and Antimicrobial Mechanism of Subtilisin A. *Chem. Phys. Lipids* **2005**, *137*, 38–51, doi:10.1016/J.CHEMPHYSLIP.2005.06.003.
 242. Noll, K.S.; Sinko, P.J.; Chikindas, M.L. Elucidation of the Molecular Mechanisms of Action of the Natural Antimicrobial Peptide Subtilisin Against the Bacterial Vaginosis-Associated Pathogen *Gardnerella Vaginalis*. *Probiotics Antimicrob. Proteins* **2011**, *3*, 41, doi:10.1007/S12602-010-9061-4.
 243. Shelburne, C.E.; An, F.Y.; Dholpe, V.; Ramamoorthy, A.; Lopatin, D.E.; Lantz, M.S. The Spectrum of Antimicrobial Activity of the Bacteriocin Subtilisin A. *J. Antimicrob. Chemother.* **2007**, *59*, 297–300, doi:10.1093/JAC/DKL495.
 244. Algburi, A.; Zehm, S.; Natrebov, V.; Bren, A.B.; Chistyakov, V.; Chikindas, M.L. Subtilisin Prevents Biofilm Formation by Inhibiting Bacterial Quorum Sensing. *Probiotics Antimicrob. Proteins* **2017**, *9*, 81–90, doi:10.1007/S12602-016-9242-X.

245. Zakharova, A.A.; Efimova, S.S.; Malev, V. V.; Ostroumova, O.S. Fengycin Induces Ion Channels in Lipid Bilayers Mimicking Target Fungal Cell Membranes. *Sci. Reports* 2019 91 **2019**, 9, 1–10, doi:10.1038/s41598-019-52551-5.
246. Buijs, N.P.; Vlaming, H.C.; Kotsogianni, I.; Arts, M.; Willemse, J.; Duan, Y.; Alexander, F.M.; Cochrane, S.A.; Schneider, T.; Martin, N.I. A Classic Antibiotic Reimagined: Rationally Designed Bacitracin Variants Exhibit Potent Activity against Vancomycin-Resistant Pathogens. *Proc. Natl. Acad. Sci. U. S. A.* **2024**, 121, e2315310121, doi:10.1073/PNAS.2315310121;REQUESTEDJOURNAL:JOURNAL:PNAS;CTYPE:STRING:JOURNAL.
247. Zaidi, S.; Singh, S.L.; Khan, A.U. Exploring Antibiofilm Potential of Bacitracin against Streptococcus Mutans. *Microb. Pathog.* **2020**, 149, 104279, doi:10.1016/J.MICPATH.2020.104279.
248. Nerurkar, A.S. Structural and Molecular Characteristics of Lichenysin and Its Relationship with Surface Activity. *Adv. Exp. Med. Biol.* **2010**, 672, 304–315, doi:10.1007/978-1-4419-5979-9_23/COVER.
249. Kumar, A.; Sankhwar, R.; Yadav, S.; Gupta, R.K. Lichenysin Produced by Bacillus Licheniformis RG1002 Inhibit the Biofilm Formation of Pseudomonas Aeruginosa on Various Surfaces. *J. Pure Appl. Microbiol.* **2024**, 18, 1196–1208, doi:10.22207/JPAM.18.2.38.
250. Guo, P.; Yang, F.; Ye, S.; Li, J.; Shen, F.; Ding, Y. Characterization of Lipopeptide Produced by Bacillus Altitudinis Q7 and Inhibitory Effect on Alternaria Alternata. *J. Basic Microbiol.* **2023**, 63, 26–38, doi:10.1002/JOBM.202200530;REQUESTEDJOURNAL:JOURNAL:15214028;WGROU:STRING:PUBLICATION.
251. Dion, H.W.; K Woo, P.W.; Willmer, N.E.; Kern, D.L.; Onaga, J.; Fusari, S.A.; W K Woo, B.P.; Coffey, G.L.; Dion, H.W.; Fusari, S.A.; et al. Butirosin, a New Aminoglycosidic Antibiotic Complex: Isolation and Characterization. *Antimicrob. Agents Chemother.* **1972**, 2, 84–88, doi:10.1128/AAC.2.2.84.
252. Li, Y.; Llewellyn, N.M.; Giri, R.; Huang, F.; Spencer, J.B. Biosynthesis of the Unique Amino Acid Side Chain of Butirosin: Possible Protective-Group Chemistry in an Acyl Carrier Protein-Mediated Pathway. *Chem. Biol.* **2005**, 12, 665–675, doi:10.1016/j.chembiol.2005.04.010.
253. Heifetz, C.L.; Fisher, M.W.; Chodubski, J.A.; DeCarlo, M.O. Butirosin, a New Aminoglycosidic Antibiotic Complex: Antibacterial Activity In Vitro and in Mice. *Antimicrob. Agents Chemother.* **1972**, 2, 89–94, doi:10.1128/AAC.2.2.89.
254. Stannius, R.O.; Kovács, Á.T. Plipastatin Is a Shared Good by Bacillus Subtilis during Combating Fusarium Spp. *FEMS Microbiol. Ecol.* **2025**, 101, doi:10.1093/FEMSEC/FIAF020.
255. Islam, T.; Rabbee, M.F.; Choi, J.; Baek, K.H. Biosynthesis, Molecular Regulation, and Application of Bacilysin Produced by Bacillus Species. *Metabolites* **2022**, 12, 397, doi:10.3390/METABO12050397.
256. Abdulmalek, H.W.; Yazgan-Karataş, A. Improvement of Bacilysin Production in Bacillus Subtilis by CRISPR/Cas9-Mediated Editing of the 5'-Untranslated Region of the Bac Operon. *J. Microbiol. Biotechnol.* **2022**, 33, 410, doi:10.4014/JMB.2209.09035.
257. Nannan, C.; Vu, H.Q.; Gillis, A.; Caulier, S.; Nguyen, T.T.T.; Mahillon, J. Bacilysin within the Bacillus Subtilis Group: Gene Prevalence versus Antagonistic Activity against Gram-Negative Foodborne Pathogens. *J. Biotechnol.* **2021**, 327, 28–35, doi:10.1016/J.JBIOTEC.2020.12.017.
258. Baptista, J.P.; Teixeira, G.M.; de Jesus, M.L.A.; Bertê, R.; Higashi, A.; Mosela, M.; da Silva, D.V.; de Oliveira, J.P.; Sanches, D.S.; Brancher, J.D.; et al. Antifungal Activity and Genomic

Characterization of the Biocontrol Agent *Bacillus Velezensis* CMRP 4489. *Sci. Reports* 2022 **12**, 17401-, doi:10.1038/s41598-022-22380-0.

259. Prazdnova, E.; Zaikina, A.; Neurov, A.; Mazanko, M.; Ranjan, A.; Rudoy, D. Bacillibactin, a Potential *Bacillus*-Based Antibacterial Non-Ribosomal Peptide: In Silico Studies for Targeting Common Fish Pathogens. *Int. J. Mol. Sci.* **2025**, *26*, 5811, doi:10.3390/IJMS26125811/S1.
260. Dimopoulou, A.; Theologidis, I.; Benaki, D.; Koukounia, M.; Zervakou, A.; Tzima, A.; Diallinas, G.; Hatzinikolaou, D.G.; Skandalis, N. Direct Antibiotic Activity of Bacillibactin Broadens the Biocontrol Range of *Bacillus Amyloliquefaciens* MBI600. *mSphere* **2021**, *6*, doi:10.1128/msphere.00376-21.
261. Chakraborty, K.; Kizhakkekalam, V.K.; Joy, M.; Chakraborty, R.D. Bacillibactin Class of Siderophore Antibiotics from a Marine Symbiotic *Bacillus* as Promising Antibacterial Agents. *Appl. Microbiol. Biotechnol.* **2022**, *106*, 329–340, doi:10.1007/s00253-021-11632-0.
262. Bochynek, M.; Lewińska, A.; Witwicki, M.; Dębczak, A.; Łukaszewicz, M. Formation and Structural Features of Micelles Formed by Surfactin Homologues. *Front. Bioeng. Biotechnol.* **2023**, *11*, 1211319, doi:10.3389/FBIOE.2023.1211319/FULL.
263. Chen, X.; Lu, Y.; Shan, M.; Zhao, H.; Lu, Z.; Lu, Y. A Mini-Review: Mechanism of Antimicrobial Action and Application of Surfactin. *World J. Microbiol. Biotechnol.* **2022**, *38*, doi:10.1007/S11274-022-03323-3.
264. Crovella, S.; de Freitas, L.C.; Zupin, L.; Fontana, F.; Ruscio, M.; Pena, E.P.N.; Pinheiro, I.O.; Calsa Junior, T. Surfactin Bacterial Antiviral Lipopeptide Blocks In Vitro Replication of SARS-CoV-2. *Appl. Microbiol.* **2022**, Vol. 2, Pages 680-687 **2022**, 2, 680–687, doi:10.3390/APPLMICROBIOL2030052.
265. Hernández-Morales, A.; Martínez-Peniche, R.A.; Arvizu-Gómez, J. lizzeta; Arvizu-Medrano, S.M.; Rodríguez-Ontiveros, A.; Ramos-López, M.A.; Pacheco-Aguilar, J.R. Production of a Mixture of Fengycins with Surfactant and Antifungal Activities by *Bacillus* Sp. MA04, a Versatile PGPR. *Indian J. Microbiol.* **2018**, *58*, 208, doi:10.1007/S12088-018-0711-7.
266. Van Heel, A.J.; De Jong, A.; Song, C.; Viel, J.H.; Kok, J.; Kuipers, O.P. BAGEL4: A User-Friendly Web Server to Thoroughly Mine RiPPs and Bacteriocins. *Nucleic Acids Res.* **2018**, *46*, W278–W281, doi:10.1093/NAR/GKY383.
267. Blin, K.; Shaw, S.; Vader, L.; Szenei, J.; Reitz, Z.L.; Augustijn, H.E.; Cediël-Becerra, J.D.D.; De Crécy-Lagard, V.; Koetsier, R.A.; Williams, S.E.; et al. AntiSMASH 8.0: Extended Gene Cluster Detection Capabilities and Analyses of Chemistry, Enzymology, and Regulation. *Nucleic Acids Res.* **2025**, *53*, W32–W38, doi:10.1093/NAR/GKAF334.
268. Shenkarev, Z.O.; Finkina, E.I.; Nurmukhamedova, E.K.; Balandin, S. V.; Mineev, K.S.; Nadezhdin, K.D.; Yakimenko, Z.A.; Tagaev, A.A.; Temirov, Y. V.; Arseniev, A.S.; et al. Isolation, Structure Elucidation, and Synergistic Antibacterial Activity of a Novel Two-Component Lantibiotic Lichenicidin from *Bacillus Licheniformis* VK21. *Biochemistry* **2010**, *49*, 6462–6472, doi:10.1021/BI100871B/SUPPL_FILE/BI100871B_SI_001.PDF.
269. Dischinger, J.; Josten, M.; Szekat, C.; Sahl, H.G.; Bierbaum, G. Production of the Novel Two-Peptide Lantibiotic Lichenicidin by *Bacillus Licheniformis* DSM 13. *PLoS One* **2009**, *4*, e6788, doi:10.1371/JOURNAL.PONE.0006788.
270. Cooper, L.E.; McClerren, A.L.; Chary, A.; van der Donk, W.A. Structure-Activity Relationship Studies of the Two-Component Lantibiotic Haloduracin. *Chem. Biol.* **2008**, *15*, 1035, doi:10.1016/J.CHEMBIOL.2008.07.020.
271. Smits, S.H.J.; Schmitt, L.; Beis, K. Self-Immunity to Antibacterial Peptides by ABC Transporters. *FEBS Lett.* **2020**, *594*, 3920–3942, doi:10.1002/1873-

3468.13953;JOURNAL:JOURNAL:18733468.

272. Tang, W.; Dong, S.H.; Repka, L.M.; He, C.; Nair, S.K.; Van Der Donk, W.A. Applications of the Class II Lanthipeptide Protease LicP for Sequence-Specific, Traceless Peptide Bond Cleavage. *Chem. Sci.* **2015**, *6*, 6270–6279, doi:10.1039/C5SC02329G.
273. Soliman, W.; Wang, L.; Bhattacharjee, S.; Kaur, K. Structure–Activity Relationships of an Antimicrobial Peptide Plantaricin S from Two-Peptide Class IIb Bacteriocins. *J. Med. Chem.* **2011**, *54*, 2399–2408, doi:10.1021/JM101540E.
274. Brötz, H.; Bierbaum, G.; Reynolds, P.E.; Sahl, H.G. The Lantibiotic Mersacidin Inhibits Peptidoglycan Biosynthesis at the Level of Transglycosylation. *Eur. J. Biochem.* **1997**, *246*, 193–199, doi:10.1111/j.1432-1033.1997.t01-1-00193.x.
275. Katoh, K.; Misawa, K.; Kuma, K.I.; Miyata, T. MAFFT: A Novel Method for Rapid Multiple Sequence Alignment Based on Fast Fourier Transform. *Nucleic Acids Res.* **2002**, *30*, 3059, doi:10.1093/NAR/GKF436.
276. Dereeper, A.; Guignon, V.; Blanc, G.; Audic, S.; Buffet, S.; Chevenet, F.; Dufayard, J.F.; Guindon, S.; Lefort, V.; Lescot, M.; et al. Phylogeny.Fr: Robust Phylogenetic Analysis for the Non-Specialist. *Nucleic Acids Res.* **2008**, *36*, doi:10.1093/NAR/GKN180.
277. Letunic, I.; Bork, P. Interactive Tree Of Life (ITOL) v5: An Online Tool for Phylogenetic Tree Display and Annotation. *Nucleic Acids Res.* **2021**, *49*, W293–W296, doi:10.1093/NAR/GKAB301.
278. Altschul, S.F.; Gish, W.; Miller, W.; Myers, E.W.; Lipman, D.J. Basic Local Alignment Search Tool. *J. Mol. Biol.* **1990**, *215*, 403–410, doi:10.1016/S0022-2836(05)80360-2.
279. Panina, I.S.; Balandin, S. V.; Tsarev, A. V.; Chugunov, A.O.; Tagaev, A.A.; Finkina, E.I.; Antoshina, D. V.; Sheremeteva, E. V.; Paramonov, A.S.; Rickmeyer, J.; et al. Specific Binding of the α -Component of the Lantibiotic Lichenicidin to the Peptidoglycan Precursor Lipid II Predetermines Its Antimicrobial Activity. *Int. J. Mol. Sci.* **2023**, *Vol. 24*, **2023**, *24*, doi:10.3390/IJMS24021332.
280. Abramson, J.; Adler, J.; Dunger, J.; Evans, R.; Green, T.; Pritzel, A.; Ronneberger, O.; Willmore, L.; Ballard, A.J.; Bambrick, J.; et al. Accurate Structure Prediction of Biomolecular Interactions with AlphaFold 3. *Nature* **2024**, *630*, 493–500, doi:10.1038/S41586-024-07487-W.
281. Turpin, E.R.; Mulholland, S.; Teale, A.M.; Bonev, B.B.; Hirst, J.D. New CHARMM Force Field Parameters for Dehydrated Amino Acid Residues, the Key to Lantibiotic Molecular Dynamics Simulations. *RSC Adv.* **2014**, *4*, 48621–48631, doi:10.1039/C4RA09897H.
282. Huang, J.; Mackerell, A.D. CHARMM36 All-Atom Additive Protein Force Field: Validation Based on Comparison to NMR Data. *J. Comput. Chem.* **2013**, *34*, 2135–2145, doi:10.1002/JCC.23354.
283. Jo, S.; Lim, J.B.; Klauda, J.B.; Im, W. CHARMM-GUI Membrane Builder for Mixed Bilayers and Its Application to Yeast Membranes. *Biophys. J.* **2009**, *97*, 50, doi:10.1016/J.BPJ.2009.04.013.
284. Sheridan, M.S.; Pandey, P.; Hansmann, U.H.E. In Bacterial Membranes Lipid II Changes the Stability of Pores Formed by the Antimicrobial Peptide Nisin. *J. Phys. Chem. B* **2024**, *128*, 4741–4750, doi:10.1021/ACS.JPCB.4C01249.
285. Abraham, M.J.; Murtola, T.; Schulz, R.; Páll, S.; Smith, J.C.; Hess, B.; Lindah, E. GROMACS: High Performance Molecular Simulations through Multi-Level Parallelism from Laptops to Supercomputers. *SoftwareX* **2015**, *1–2*, 19–25, doi:10.1016/J.SOFTX.2015.06.001.
286. Wu, E.L.; Cheng, X.; Jo, S.; Rui, H.; Song, K.C.; Dávila-Contreras, E.M.; Qi, Y.; Lee, J.; Monje-Galvan, V.; Venable, R.M.; et al. CHARMM-GUI Membrane Builder toward Realistic Biological Membrane Simulations. *J. Comput. Chem.* **2014**, *35*, 1997–2004, doi:10.1002/JCC.23702.

287. Hess, B. P-LINCS: A Parallel Linear Constraint Solver for Molecular Simulation. *J. Chem. Theory Comput.* **2007**, *4*, 116–122, doi:10.1021/CT700200B.
288. Miyamoto, S.; Kollman, P.A. Settle: An Analytical Version of the SHAKE and RATTLE Algorithm for Rigid Water Models. *J. Comput. Chem.* **1992**, *13*, 952–962, doi:10.1002/JCC.540130805.
289. Darden, T.; York, D.; Pedersen, L. Particle Mesh Ewald: An N·log(N) Method for Ewald Sums in Large Systems. *J. Chem. Phys.* **1993**, *98*, 10089–10092, doi:10.1063/1.464397.
290. Nosé, S. A Unified Formulation of the Constant Temperature Molecular Dynamics Methods. *J. Chem. Phys.* **1984**, *81*, 511–519, doi:10.1063/1.447334.
291. Martoňák, R.; Laio, A.; Parrinello, M. Predicting Crystal Structures: The Parrinello-Rahman Method Revisited. *Phys. Rev. Lett.* **2003**, *90*, 4, doi:10.1103/PHYSREVLETT.90.075503.
292. Passaro, S.; Corso, G.; Wohlwend, J.; Reveiz, M.; Thaler, S.; Somnath, V.R.; Getz, N.; Portnoi, T.; Roy, J.; Stark, H.; et al. Boltz-2: Towards Accurate and Efficient Binding Affinity Prediction. *bioRxiv* **2025**, 2025.06.14.659707, doi:10.1101/2025.06.14.659707.
293. Tribello, G.A.; Bonomi, M.; Branduardi, D.; Camilloni, C.; Bussi, G. PLUMED 2: New Feathers for an Old Bird. *Comput. Phys. Commun.* **2014**, *185*, 604–613, doi:10.1016/J.CPC.2013.09.018.
294. Kumar, S.; Rosenberg, J.M.; Bouzida, D.; Swendsen, R.H.; Kollman, P.A. THE Weighted Histogram Analysis Method for Free-energy Calculations on Biomolecules. I. The Method. *J. Comput. Chem.* **1992**, *13*, 1011–1021, doi:10.1002/JCC.540130812;CTYPE:STRING:JOURNAL.
295. Wiedemann, I.; Breukink, E.; Van Kraaij, C.; Kuipers, O.P.; Bierbaum, G.; De Kruijff, B.; Sahl, H.G. Specific Binding of Nisin to the Peptidoglycan Precursor Lipid II Combines Pore Formation and Inhibition of Cell Wall Biosynthesis for Potent Antibiotic Activity. *J. Biol. Chem.* **2001**, *276*, 1772–1779, doi:10.1074/jbc.M006770200.

List of Figures

Figure 1. The evolution of antimicrobial innovation and resistance milestones. A vertical chronology from 1910 to 2026, highlighting the "Golden Era" of discovery, the subsequent "Discovery Void," and the modern "Renaissance" era. Figure Created in Adobe Illustrator.

Figure 2. Systematic classification of antibacterial drug resistance. The schematic illustrates the major categories of antibacterial drug resistance based on two complementary criteria: **(Left Panel)** the type of resistance, comprising natural (intrinsic and induced), acquired, and adaptive resistance; and **(Right Panel)** the underlying mechanisms of resistance, including modification of drug target sites, enzymatic inactivation of antimicrobial agents, and reduced intracellular drug accumulation through decreased membrane permeability (reduced influx) or enhanced efflux pump activity. Together, these mechanisms enable bacterial survival and persistence under antimicrobial stress. Figure Created in Adobe Illustrator.

Figure 3. Mechanisms of antibacterial drug resistance and dissemination of antimicrobial resistance determinants.

Schematic representation of the major molecular, physiological, and genetic mechanisms by which bacteria resist antibacterial agents. **(1)** Modification (alteration) of the drug target reduces antibacterial binding through mutations or enzymatic modification of cellular targets, including ribosomal RNA, DNA gyrase, RNA polymerase, and penicillin-binding proteins. **(2)** Target replacement or target bypass involves the acquisition of alternative proteins or metabolic pathways that maintain essential cellular functions despite antibacterial exposure, such as PBP2a-mediated methicillin resistance and resistant variants of DHFR and DHPS. **(3)** Target site protection occurs when bacterial proteins shield or mimic the antibacterial target, preventing effective drug binding, as observed with ribosomal protection proteins and the DNA gyrase-protecting protein MfpA. **(4)** Decreased accumulation results from a combination of reduced drug uptake and enhanced drug efflux, leading to subinhibitory intracellular antibacterial concentrations. **(5)** Enhanced efflux is mediated by energy-dependent transport systems that actively export antibacterials from the bacterial cell, including members of the ABC, MFS, RND, SMR, MATE, and DMT transporter families. **(6)** Enzymatic inactivation involves degradation or chemical modification of antibacterial molecules through mechanisms such as β -lactam hydrolysis, aminoglycoside acetylation, phosphorylation, adenylation, and chloramphenicol acetylation. **(7)** Decreased entry (influx) occurs through reduced membrane permeability caused by loss or alteration of porins, changes in outer membrane composition, or other permeability barriers that limit antibacterial penetration. **(8)** Increased expression of efflux pumps further reduces intracellular drug concentrations and contributes substantially to multidrug resistance, particularly in Gram-negative pathogens. **(9)** Enhanced secretion or production of antibacterial-inactivating enzymes enables extracellular or periplasmic degradation of antibacterials before they reach their targets,

with β -lactamases representing the most clinically significant example. **(10)** Biofilm formation protects bacterial populations through the production of an extracellular polymeric substance (EPS) matrix, reduced drug penetration, altered metabolic activity, quorum-sensing-regulated responses, and the persistence of dormant cells. The lower section illustrates the principal mobile genetic elements (MGEs) responsible for the acquisition and dissemination of antimicrobial resistance genes, including insertion sequences (ISs), transposons (Tn), plasmids, integrative and conjugative elements (ICEs), and genomic islands (GIs). These elements facilitate the mobilization and spread of resistance determinants, including genes encoding extended-spectrum β -lactamases (ESBLs), carbapenemases, aminoglycoside-modifying enzymes, and colistin resistance factors. Dissemination of antimicrobial resistance is further driven by horizontal gene transfer (HGT) through conjugation, transduction, transformation, and plasmid mobilization. Collectively, these interconnected mechanisms contribute to the emergence, persistence, and global spread of multidrug-resistant bacteria. Figure Created in Adobe Illustrator.

Figure 4. The ESKAPE pathogen consortium and its role in biofilm-associated infections. Figure Created in Adobe Illustrator.

Figure 5. Schematic representation of the anatomical distribution of *Apis mellifera* (Honey bee) exocrine glands associated with colonial ontogeny and the synthesis of bee-derived products. Figure Created in Adobe Illustrator.

Figure 6. Schematic representation of the bioactive properties and therapeutic potential of honey, bee pollen, and bee bread. Figure Created in Adobe Illustrator.

Figure 7. Diagrammatic overview of the shared and unique microbiota of honey, bee pollen, and bee bread. The central node delineates the Core Microbial Consortia shared across all three matrices, systematically categorized into distinct bacterial, yeast, and mold genera (e.g., *Lactobacillus* spp., *Zygosaccharomyces* spp., and *Aspergillus* spp.). **Left:** The Honey Reservoir highlights taxa unique to honey or selectively shared with bee bread (e.g., *Cronobacter* spp., *Pseudomonas* spp.). **Right:** The Bee Pollen source traces the introduction of environmental fungi and yeasts (e.g., *Torulopsis* spp., *Ascosphaera* spp.). Figure Created in Adobe Illustrator.

Figure 8. Classification and biosynthetic pathways of antimicrobial secondary metabolites in *Bacillus* and *Lactobacillus*. The pathways are color-coded into four primary categories: Ribosomally synthesized and post-translationally modified peptides (RiPPs) and bacteriocins (**blue**); Non-ribosomal peptide synthetase (NRPS)-derived peptides (**green**); Polyketides (**orange**); and Hybrid NRPS/PKS metabolites (**purple**). Subclasses and representative antimicrobial compounds, such as lantibiotics, lipopeptides, macrolactins, and hybrid structures are delineated hierarchically to illustrate structural and biosynthetic diversity. Figure Created in Adobe Illustrator.

Figure 9. Proposed biosynthetic gene cluster of Paralichenidin. Bacteriocin homologues are identified using the accepted nomenclature for describing lantibiotics. For Paralichenidin, *PldA1* and *PldA2* encode the putative bacteriocins, *PldM1* and *PldM2* encode the accompanying modification enzymes, and *PldT* and *PldP* are involved in bacteriocin transport and leader cleavage. Genes are color-coded as per antiSMASH, indicating the putative role of each protein. Figure Created in Adobe Illustrator.

Figure 10. Multiple sequence alignment and phylogenetic analysis of Pld α and β peptides with representative two-component lantibiotics. **(A)** Multiple sequence alignment of the Pld α prepropeptide with Amyloliqecidin α , Haloduracin α , Lichenicidin α , Plantaricin α , Lacticin α , and Mersacidin. **(B)** Multiple sequence alignment of the Pld α mature core peptide with the same set of α peptides; red dots indicate positions of amino acid variation among Pld α and its closest homologues, flanked by conserved cysteine residues involved in lanthionine and methyllanthionine bridge formation. **(C)** Maximum-likelihood phylogenetic tree of α prepropeptide sequences, rooted at the midpoint, showing Pld α clustering with Amyloliqecidin α . **(D)** Maximum-likelihood phylogenetic tree of α core peptide sequences, rooted at the midpoint, showing Pld α within a tightly supported clade alongside Haloduracin α , Amyloliqecidin α , and Plantaricin α . **(E)** Multiple sequence alignment of the Pld β prepropeptide with Amyloliqecidin β , Haloduracin β , Lichenicidin β , Plantaricin β , and Lacticin β . **(F)** Multiple sequence alignment of the Pld β mature core peptide with the same set of β peptides; red dots indicate positions of amino acid variation among Pld β and its closest homologues. **(G)** Maximum-likelihood phylogenetic tree of β prepropeptide sequences, rooted at the midpoint, showing Pld β clustering with Amyloliqecidin β . **(H)** Maximum-likelihood phylogenetic tree of β core peptide sequences, rooted at the midpoint, showing Pld β clustering closely and consistently with Amyloliqecidin β . Figure Created in Adobe Illustrator.

Figure 11. Predicted structure of the **(A)** Paralichenidin α and **(B)** Paralichenidin β peptides. The structures of Pld α and Pld β were predicted using GF610 α and Hal β , respectively, as structural templates. Conservation of key amino acid residues suggests a high degree of structural homology between Pld and previously characterized two-component lantibiotics. Predicted lanthionine and methyllanthionine rings are also presented. The corresponding prepropeptide sequences are shown beneath each structure, with putative dehydrated serine (Dha) and threonine (Dhb) residues indicated. The predicted proteolytic cleavage site separating the leader peptide from the mature core peptide is highlighted in red. Figure Created in Adobe Illustrator.

Figure 12. The structures of Pld α and Pld β peptides, obtained from equilibrium simulations based on their post translational modification sequences. Figure Created in VMD

Figure 13. Pld α - and Pld β -peptide interaction models generated using the Boltz-2 predictor, subsequently used as a framework for free energy simulations. The peptide fragments that interact with pyrophosphate

are highlighted in purple. Lipid II is represented by pyrophosphate, GlcNAc, and an isoprenoid fragment. Figure Created in VMD

Figure 14. (A) Definition of the reaction coordinate used in free energy simulations. The reaction coordinate was defined as the center-of-mass (COM) distance between the Lipid II pyrophosphate moiety and the peptide Lipid II-binding region. **(B)** Free energy profiles of Pld α - and Pld β -peptide binding to Lipid II. The profiles were calculated along the center-of-mass (COM) distance between the peptide binding region and the Lipid II pyrophosphate moiety. Figure Created in VMD

Figure 15. Structural models predicted by Boltz-2 for Pld α - and Pld β -peptide binding to Lipid II. The primary Lipid II-interacting motifs are highlighted in purple, while the Pld α peptide is blue and the Pld β peptide is shown in green. Figure Created in VMD

List of Tables

Table 1. Classification of selected antibiotics: mechanisms of action, sources, and antimicrobial spectra.

Table 2. Profiles of bioactive secondary metabolites produced by *Bacillus* and Lactic Acid Bacteria (LAB): Comparative analysis of molecular weight, producing species, and primary mechanisms of action

Table 3. BLAST results of the α and β components of Paralichenidin, including both prepropeptide and core peptide sequences, alongside their closest characterized homologues, producer strains, percentage identity, alignment length, and E-value.