The author of the doctoral dissertation: mgr inż. Karolina Pełka

Scientific discipline: Chemical Sciences

# DOCTORAL DISSERTATION

Title of doctoral dissertation: Investigation of the antimicrobial potential and microbiota of bee pollen and bee bread derived from Polish apiaries.

Title of doctoral dissertation (in Polish): Badanie potencjału przeciwdrobnoustrojowego oraz mikrobioty pyłku pszczelego oraz pierzgi pochodzących z polskich pasiek.

| Supervisor<br><br>*signature* |
| --- |
| dr hab. inż. Piotr Szweda, prof. uczelni |

Gdańsk, year 2023

## STATEMENT

The author of the doctoral dissertation: mgr inż. Karolina Pełka

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# DESCRIPTION OF DOCTORAL DISSERTATION

**The Author of the doctoral dissertation**: Karolina Pełka

**Title of doctoral dissertation**: Investigation of the antimicrobial potential and microbiota of bee pollen and bee bread derived from Polish apiaries.

**Title of doctoral dissertation in Polish**: Badanie potencjału przeciwdrobnoustrojowego oraz mikrobioty pyłku pszczelego oraz pierzgi pochodzących z polskich pasiek.

**Language of doctoral dissertation**: English

**Supervisor**: dr hab. inż. Piotr Szweda

**Date of doctoral defense**:

**Keywords of doctoral dissertation in English**: bee pollen, bee bread, antimicrobial activity, *Bacillus*

**Keywords of doctoral dissertation in Polish**: pyłek pszczeli, pierzga, aktywność przeciwdrobnoustrojowa, *Bacillus*

**Summary of doctoral dissertation in English**:

The phenomenon of resistance of microorganisms to antibiotics is becoming one of the greatest challenges of modern medicine. At the same time, the number of new antibiotics introduced into clinical practice is decreasing. This creates the need to search for new, effective, non-antibiotic methods of combating and preventing infectious diseases. Over the last two decades, there has been renewed interest in the antimicrobial potential of natural products, including bee products. The object of my interest were two less known bee products – bee pollen and bee bread, mainly the antimicrobial activity of these products. The results I obtained are very promising. The high activity of ethanolic extracts of bee pollen and bee bread, as well as the products themselves, has been demonstrated to combat dangerous pathogens (e.g., *Staphylococcus aureus*) of humans and animals. The important role of proteinaceous substances (mainly bee-derived peptides and enzymes) in the antimicrobial activity of these products has been confirmed. It was also found that a significant percentage of bacterial strains isolated from pollen and bee bread (mainly *Bacillus* spp.) have a high probiotic potential and the ability to synthesize metabolites that effectively inhibit the development of pathogenic bacteria. The results of the research were presented in the form of four scientific publications.

**Summary of doctoral dissertation in Polish**:

Zjawisko lekooporności drobnoustrojów staje się jednym z największych wyzwań współczesnej medycyny. Jednocześnie maleje liczba nowych antybiotyków wprowadzanych do praktyki klinicznej. Stwarza to konieczność poszukiwania nowych, skutecznych, nieantybiotykowych metod zwalczania i profilaktyki chorób infekcyjnych. W ostatnich dwóch dekadach obserwuje się ponowne zainteresowanie potencjałem przeciwdrobnoustrojowym produktów naturalnych, w tym produktów pszczelich. Obiektem moich zainteresowań były dwa mniej znane produkty pszczele – pyłek i pierzga, a przede wszystkim aktywność przeciwdrobnoustrojowa tych produktów. Uzyskane przeze mnie wyniki są bardzo obiecujące. Wykazano dużą aktywność alkoholowych ekstraktów pyłku i pierzgi, jak i samych produktów, w zwalczaniu groźnych patogenów (np. gronkowca złocistego - *Staphylococcus aureus)* ludzi i zwierząt. Potwierdzono istotną rolę substancji białkowych (głównie peptydów i enzymów pochodzących z organizmu pszczoły) w aktywności przeciwdrobnoustrojowej tych produktów. Stwierdzono także, że znaczący odsetek szczepów bakteryjnych wyizolowanych z pyłku i pierzgi (głównie bakterii z rodzaju *Bacillus* spp.) wykazuje duży potencjał probiotyczny oraz zdolność do syntezy metabolitów skutecznie hamujących rozwój bakterii patogennych. Wyniki badań przedstawiono w postaci czterech publikacji naukowych.

*I would like to express my deepest gratitude to my Supervisor, dr hab. inż. Piotr Szweda, for the invaluable guidance and support you provided throughout my venture as your PhD student.*

*I could not have undertaken this journey without my dearest fiancé, Paweł, 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.*

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

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

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

## TABLE OF CONTENTS

## LIST OF IMPORTANT SYMBOLS AND ABBREVIATIONS

AMR – Antimicrobial Resistance

AOI – Areas of Interest

BP – Bee Pollen

BB – Bee Bread

BHI – Brain Heart Infusion Broth

BLAST – Basic Local Alignment Search Tool

CA-MRSA - community-associated methicillin-resistant Staphylococcus aureus

CFU – Colony Forming Unit

ClfA - clumping factor A

CLSI – Clinical and Laboratory Standards Institute

CMHB2 – Concentrated Mueller Hinton Broth 2

CTAB – Cetyl Trimethylammonium Bromide

DNA – Deoxyribonucleic Acid

ETs - Exfoliative Toxins

EtOH - Ethanol

FAs – Fatty Acids

Fg - Blood Plasma Protein Fibrinogen

GAE – Gallic Acid Equivalent

GBDP – Genome Blast Distance Phylogeny

GIC – Glass Ionomer Cement

GIT – Gastrointestinal Tract

GOx – Glucose Oxidase

GRAS – Generally Recognized as Safe

HA-MRSA - hospital-associated methicillin-resistant Staphylococcus aureus

HPLC – High Pressure Liquid Chromatography

IgE, IgG, IgM – Immunoglobulin E, G, M

iTOL – Interactive Tree of Life

LA – Luria Bertani Agar

LAB – Lactic Acid Bacteria

LB – Luria Bertani Broth

MAFLD - Metabolic Dysfunction-Associated Fatty Liver Disease

MBC – Minimal Bactericidal Concentration

MHB – Mueller Hinton Broth

MIC – Minimal Inhibitory Concentration

MCD peptide – Mast Cell Degranulating peptide

MSCRAMMs - Microbial Surface Components Recognising Adhesive Matrix Molecules

MRSA - methicillin-resistant Staphylococcus aureus

MRJP1 – Major Royal Jelly Protein 1

MUSCLE – Multiple Sequence Comparison by Log- Expectation

MW – Molecular Weight

NAD – Nicotinamide adenine dinucleotide

NCBI – National Center for Biotechnology Information

NK – natural killer cells

$OD_{600}$ – Optical Density at 600nm

ORF – Open Reading Frame

OrthoANI – Orthologous Average Nucleotide Identity

PBPs - Penicillin-Binding Proteins

PBS – Phosphate Buffer Saline

PEG – Polyethylene Glycol

PGAP – Prokaryotic Genome Annotation Pipeline

PSMs - Phenol-Soluble Modulins

PUFAs – Polyunsaturated Fatty Acids

PVL - Panton-Valentine Leukocidin

ROS – Reactive Oxygen Species

RNA – Ribonucleic Acid

SAgs - Superantigens

SCCmec – Staphylococcal cassette chromosome mec

SD – Standard Deviation

SDS-PAGE – Sodium Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis

SEs - staphylococcal enterotoxins

SE*l*s - staphylococcal enterotoxin-*like* proteins

SGIZ – Size of the Growth Inhibition Zone

SPAdes – St. Petersburg Genome Assembler

TAs - teichoic acids

TBST – Tris-Buffered Saline-Tween buffer

TNF – Tumor Necrosis Factor

TSS - Toxic Shock Syndrome

TPC – Total Phenolic Content

TPPC – Total Protein Content

TSST-1 - toxic shock syndrome toxin-1

TYGS – Type Genome Server

WHO – World Health Organization

VISA - Vancomycin-Intermediate S. aureus

VRSA - Vancomycin-Resistant Staphylococcus aureus

YPD – Yeast Extract-Peptone-Dextrose Broth

## ARTICLES PRESENTED IN THE DISSERTATION WITH AUTHOR'S CONTRIBUTION

### 1. ARTICLE 1

Bee Bread Exhibits Higher Antimicrobial Potential Compared to Bee Pollen
by **Karolina Pełka**, Olga Otłowska, Randy W. Worobo and Piotr Szweda
Antibiotics 2021, 10, 125; doi:10.3390/antibiotics10020125
$IF_{2021}$=5.222, Q1, Ministry of Science and Higher Education Points (MHES): 70
Accepted: 25/01/2021
Published: 28/01/2021

Karolina Pelka's contribution to the articles included in the dissertation was participation in the development of the research concept, development and validation of the methodology, performance of analyses, development and visualization of data, participation in the preparation of the manuscript.

### 2. ARTICLE 2

Glucose oxidase as an important yet overlooked factor determining the antibacterial activity of bee pollen and bee bread
**Karolina Pełka**, Marcela Bucekova, Jana Godocikova, Piotr Szweda and Juraj Majtan
European Food Research and Technology 2022; doi:10.1007/s00217-022-04101-z
$IF_{2022}$=3.498, Q1, MHES Points: 70
Accepted: 25/07/2022
Published: 29/07/2022

Karolina Pelka's contribution to the articles included in the dissertation was participation in the development of the research concept, the development and validation of the methodology, performance of analyses, development and visualization of data, participation in the preparation and edition of the manuscript.

### 3. ARTICLE 3

Bee Pollen and Bee Bread as a Source of Bacteria Producing Antimicrobials
**Karolina Pełka**, Randy W. Worobo, Justyna Walkusz and Piotr Szweda
Antibiotics 2021, 10, 713; doi:10.3390/antibiotics10060713
$IF_{2021}$=5.222, Q1, MHES Points: 70
Accepted: 9/06/2021
Published: 13/06/2021

Karolina Pelka's contribution to the articles included in the dissertation was participation in the development of the research concept, the development and validation of the methodology, performance of analyses, development and visualization of data, participation in the preparation and edition of the manuscript.

## 4. ARTICLE 4

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:
$IF_{2023}$=5.000, Q1, MHES Points: 70
Accepted: 12/09/2023
Published: 19/09/2023

Karolina Pelka's contribution to the articles included in the dissertation was participation in the development of the research concept, the development and validation of the methodology, performance of analyses, development and visualization of data, participation in the preparation and edition of the manuscript.

## 5. SUMMARY IMPACT FACTOR (IF)

The summary IF of the articles presented in the dissertation is 18.942.

# INTRODUCTION

## 1. ANTIMICROBIAL RESISTANCE

Antibiotics play a crucial role in the treatment of diseases caused by pathogens (bacteria, yeasts, molds, and some parasites). Nevertheless, the use of antibiotics caused the rapid appearance of resistant strains [1]. Antimicrobial Resistance (AMR) is one of the leading public health threats these days. The analysis of the global burden of AMR in 2019 showed that 4.95 million deaths were associated with AMR, where 1.27 millions of deaths were directly caused by resistant bacteria. Furthermore, ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* spp.) have been classified as priority pathogens that are a global threat to human health. The bacteria belonging to the ESKAPE pathogens were responsible for more than 70% of AMR-related deaths in 2019 according to WHO report [2], [3].

### *1.1. The discovery of antibiotics*

Before the discovery of antibiotics, infectious diseases were treated with plants and animal preparations such as *Scabiosa* L. (a plant used in middle ages that was recognized as universal remedium for many ailments), honey bee products, and even clay derived from special places, for example Lemnos island. Furthermore, mixtures and suspensions of minerals and metals - for example, gold and mercury - were used as alchemy products [4]. Nevertheless, the discovery of antibiotics in the twentieth century was a great milestone in the history of treating infectious diseases. In 1910 Paul Ehrlich, Koch's co-worker and bacteriologist, synthesized the arsenic derivative number 606 – arsphenamine (known by its trade name *salvarsan*) that exhibited high activity against *Treponema pallidum* and was effective in the treatment of syphilis. Thus, humanity received the first chemotherapeutics to fight pathogens [4], [5]. Afterwards, in 1932, the work of Bayer chemists Josef Klarer, Fritz Mietzsch, and Gerhard Domagk led to the discovery of a sulfonamide derivative drug named *sulfonamidochrysoidine* (Prostosil). The discovery of penicillin was one of the most well-known cases in the history of drugs. Alexander Fleming discovered by accident that *Penicillium* molds inhibit the growth of *S. aureus* strain. Subsequently, Howard Florey and Ernst Chain invented the methodology of penicillin purification and obtained the substance soluble in water [4], [5]. The discovery of penicillin brings the new idea of using microbes to treat pathogens. Selman Waksman studied *Actinomycetes* microbes from soil and their ability to produce antimicrobials, which led to the 'Golden Era' of antibiotics (1940-1960s). The examples of discovered and clinically used antibiotics, that were produced using microorganisms, were, for instance: kanamycin A (*Streptomyces kanamyceticus*), tetracycline (*Streptomyces aureofaciens*), chloramphenicol (*Streptomyces venezuelae*), erythromycin (*Saccharopolyspora erythrea*), vancomycin (*Amycolatopsis orientalis*), clindamycin (semi-synthetic derivative of lincomycin from *Streptomyces lincolnensis*), bacitracin A (*Bacillus subtillis*), gramicidin A (*Bacillus brevis*), fusidic acid (*Fusidium coccineum*) or mupirocin (*Pseudomonas fluorescens*) [6].

### *1.2. Development of resistance to antibiotics*

Overuse of antibiotics, inappropriate prescribing of drugs, and extensive agricultural use lead to the development of antibiotic resistance [7]. There are two mechanisms of resistance used by bacteria – intrinsic and acquired resistance. Intrinsic resistance occurs in the genome of bacteria and causes resistance to antibiotics that is not related to horizontal gene transfer or antibiotic selective pressure. This type of resistance can occur due to no affinity of the drug with the molecular target, efflux of the drug by pumps, or deactivation of antibiotic by enzymes produced by the bacterial cell. Whilst, the acquired resistance happens when bacteria obtain resistance to particular antibiotics, that was previously active against this microorganism. It is caused by a gene mutation or horizontal gene transfer [8]. Resistance naturally occurs in some microorganisms, or the presence of resistance to drugs can appear through spontaneous gene mutation and gene transfer between microorganisms [9]. However, the spread of antimicrobial resistance is associated with many processes and involves additional mechanisms [9]–[11]. AMR bacteria can secure the target of antibiotic action by preventing the entry of antibiotics into the cell or removing the antibiotic by efflux pumps [11]. For example, the lack of specific D2 porin in imipenem-resistant *P. aeruginosa* prevents the antibiotic from entering the cell. Additionally, the gene *tet(A)* of *Enterobacteriaceae* encodes an efflux pump that is responsible for resistance to tetracycline by efficient transport of antibiotic molecules out of cells. Bacteria can resist antibiotics by also altering the site of action. The antibiotic can enter the cell and reach the target, but its inhibition is impossible due to structural changes in this molecule [11]. For instance, Penicillin-Binding Proteins (PBPs) of *Enterococci* have low affinity to cephalosporins, therefore, they cannot be inhibited by these antibiotics. It is an example of inherent resistance of bacteria to antibiotics of a specific chemical group [11]. Furthermore, bacteria may produce an alternative target that is resistant to antibiotic inhibition [11], [12]. In **Table 1**., the mode of action of selected antibiotic groups and resistance mechanisms are presented.

**Table 1.** Modes of resistance to selected groups of antibiotics with their mechanism of action. Adapted from [1].

| Group of antibiotics | Antibiotic mode of action | Mechanism of resistance |
|---|---|---|
| β-lactams | Cell wall synthesis inhibition | Hydrolysis and efflux of the antibiotic molecule, production of altered molecular target of antibiotic |
| Aminoglycosides | Proteins synthesis inhibition | Phosphorylation, acetylation, and efflux of the antibiotic molecule, production of an altered molecular target of antibiotic |

| Group of antibiotics | Antibiotic mode of action | Mechanism of resistance |
|---|---|---|
| Glycopeptides | Cell wall synthesis inhibition | Reprogramming peptidoglycan biosynthesis |
| Tetracyclines | Proteins synthesis inhibition | Monooxygenation and efflux of the antibiotic molecule, production of altered molecular target of antibiotic |
| Macrolides | Proteins synthesis inhibition | Hydrolysis, glycosylation, phosphorylation, efflux of the antibiotic molecule, production of an altered molecular target of antibiotic |
| Lincosamides | Proteins synthesis inhibition | Methylation of molecular target of antibiotic, efflux of the antibiotic molecule, production of altered molecular target of antibiotic |
| Phenicols | Proteins synthesis inhibition | Acetylation and efflux of the antibiotic molecule, production of an altered molecular target of antibiotic |
| Oxazolidinones | Proteins synthesis inhibition | Efflux of the antibiotic molecule, production of an altered molecular target of antibiotics |
| Quinolones | DNA synthesis inhibition | Acetylation and efflux of the antibiotic molecule, production of an altered molecular target of antibiotic |
| Pyrimidines | Metabolic pathway inhibition | Efflux of the antibiotic molecule, production of an an altered molecular target of antibiotics |
| Sulfonamides | Metabolic pathway inhibition | Efflux of the antibiotic molecule, production of an altered molecular target of antibiotics |
| Rifamycins | RNA synthesis inhibition | ADP-ribosylation of the antibiotic molecule, efflux of the antibiotic molecule, production of an altered molecular target of antibiotic |

### *1.3. Staphylococcus aureus*

In this dissertation, the antimicrobial activity was tested against broad spectrum of human pathogens, nevertheless, for preliminary studies *S. aureus* reference strains and clinical isolates were applied as model pathogenic bacteria.

#### *1.3.1. General information and clinical significance*

The *Staphylococcus* genera include nearly 50 species [13]. *S. aureus* is Gram-positive spherical approximately 1 μm bacterium [14]. The first suggestion of disease caused by staphylococci appeared in 1880, when Scottish surgeon Alexander Ogston connected abscesses and neonatal disease with these bacteria [15]. Afterwards, in 1884, the German physician Friedrich Julius Rosenbach isolated pure strains and differentiated them according to the color of the colonies – *S. aureus* from the Latin aurum (gold), and *S. albus*, which in Latin means white. However, the *S. albus* species was later renamed *S. epidermidis* due to its omnipresent existence on human skin [16]. *S. aureus* can grow in clusters, pairs, and occasionally in short chains [14]. The cells of *S. aureus* have a thick cell wall (20-80 nm) and consist mainly of peptidoglycan and teichoic acids [17]. Peptidoglycan (murein) is the main component of the cell wall and is made up of long polysaccharide chains, which is composed of repeating molecules of N-acetyl-D-glucosamine and N-acetyl-D-muramic acid connected by a β-1,4-glycosidic bond. Each polysaccharide chain is cross-linked by peptide bridges. *S. aureus* can grow under aerobic and anaerobic conditions (facultatively), at a temperature between 18 and 40 °C, where the optimal temperature for growth is 35-40 °C [13]. Furthermore, differentiation of *S. aureus* from other staphylococci is due to its ability to produce coagulase and its ability to produce the carotenoid pigment staphyloxanthin, that causes yellowish/goldish color of the colonies. This orange-red triterpenoid scavenges free radicals and protects cells from oxidation [18].

*S. aureus* belongs to the human microbiota, and its presence on the skin and mucosa is ubiquitous. However, when it is transferred to the bloodstream or internal soft tissues, it can cause infections that threaten human life [19]. After the discovery of *S. aureus* in the 1880s, it has been considered a major human opportunistic pathogen that causes a multitude of diseases [20]. The discovery of antibiotics impeded the spread of infections caused by *S. aureus,* however, resistant strains emerged very quickly. It is estimated that about 20% of human population is the chronic carrier of *S. aureus*, and 60% - occasional [15]. Among all *Staphylococcus* species, *S. aureus* is the most pathogenic and it can cause many infections such as skin diseases (abscesses, boils), respiratory system (pharyngitis, bronchitis), urinary tract (nephritis, urethritis), gastrointestinal tract (food poisoning, enteritis), as well as sepsis, purulent arthritis, mastitis, osteomyelitis, meningitis, scalded skin syndrome, and toxic shock syndrome [17]. The pathogenesis of *S. aureus* is based on the production of various virulence factors, such as extracellular proteins or toxins [13], [21]. Methicillin-resistant strains of *S. aureus* (MRSA) cause most infections in hospitals, leading to significant morbidity, mortality, length of stay, and cost

burden. Resistance occurs due to the presence of the *mecA* gene sequence that produces PBP2a (also called PBP2') transpeptidase that exhibits a lower affinity for binding the β-lactam antibiotics – in the consequence these antibiotics are not effective against bacteria (they are unable to inhibit the process of synthesis of the cell wall process). However, MRSA infection can be distinguished into community-associated methicillin-resistant *S. aureus* (CA-MRSA) infections and hospital-associated methicillin-resistant *S. aureus* infections (HA-MRSA) [22]. The difference between these two groups not only refers to their origin, but also to biological features – they differ genetically and phenotypically. CA-MRSA infections appears in non-hospitalized individuals and usually they are sensitive to numerous quantities of antibiotics and are non-multidrug-resistant. CA-MRSA strains frequently are able to produce virulence factor named Panton Valentine Leukocidin (PVL) – toxin that eliminates the white blood cells [21], [23]. In comparison, HA-MRSA are linked with risk factors such as surgeries, prolonged hospitalization and immune-suppression [23]. Moreover, HA-MRSA are often resistant to broad spectrum of non-β-lactam antibiotics [24]. Moreover, between CA and HA-MRSA differences in SCCmec types and bacterial growth rate were also found [24], [25].

### *1.3.2. Virulence factors of S. aureus*

Some microorganisms have the potential to infect the host and cause infections, which is called virulence, while molecules that help the microorganism settle in the host are named virulence factors [26]. *S. aureus* produces a wide range of virulence factors that are involved in adhesion, invasion, and host immune evasion, and include structural factors and secreted molecules [13], [27]. The most important virulence factors of *S. aureus* are presented in

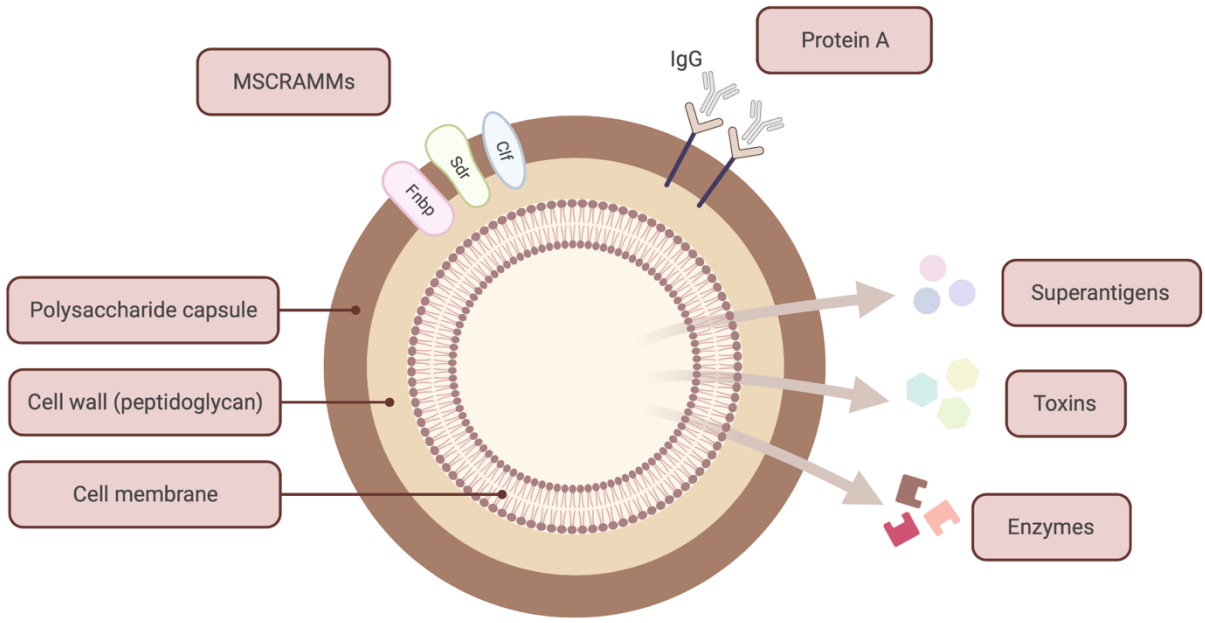


**Figure *1*.**

**Figure 1.** Virulence factors of *S. aureus*. Based on [27], [28]. Designed in BioRender by K. Pełka.

#### *1.3.2.1. Adherence factors*

*S. aureus* can attach to the host cell surface and colonize it, using numerous adhesin proteins. Adhesins are anchored to the cell wall peptidoglycan by the sortase enzyme. These proteins are referred as Microbial Surface Components Recognising Adhesive Matrix Molecules (MSCRAMMs) [27], [28]. The MSCRAMMs presents high affinity to collagen, fibronectin, fibrinogen, elastin, laminin, von Willebrand factor, vitronectin, and thrombospondin [29]. The family of MSCRAMMs includes protein A, fibronectin-binding proteins A and B, serine-aspartate proteins, collagen-binding proteins, and clumping factor A and B proteins [13], [27], and serves several functions. For instance, protein A interacts with the fragment crystallizable region (Fc) of immunoglobulin G (IgG), thus it facilitates opsonization and phagocytosis; fibronectin-binding proteins assist in cell adhesion to the host [27], [30], [31], and clumping factor A (ClfA) enhances the bacterial adhesion to fibrinogen (Fg) - the blood plasma protein [32].

#### *1.3.2.2. Polysaccharide capsule*

Capsular polysaccharides envelope the cell wall of *S. aureus,* protecting the bacterial cell from phagocytosis and bacteriophage infections. Among all polysaccharide capsules, types 5 and 8 are associated with the majority of clinically significant *S. aureus.* [27], [30], [33].

#### *1.3.2.3. Extracellular toxins and enzymes*

*S. aureus* is able to produce and secrete various molecules for host immune system stimulation and to destroy the immune cells of the host. Pore-forming and enzymatic toxins molecules produced by *S. aureus* include hemolysins, leukocidins, phenol-soluble modulins and exfoliative toxins [27], [29]. Hemolysins are molecules that cause the lysis of blood cells by disrupting their cell membrane. They are also cytotoxic to tissue cells and leukocytes [30]. Leukocidins are able to form a hetero-octameric pores in the cytoplasmic membrane. They consist of two components (S and F) that act in synergistic action – they attach in a sequentially to the surface of the host cell [13]. Leukocidins cause lysis of the cells and destroy leukocytes. The group of leukocidins include γ-hemolysin, leukocidin ED, leukocidin AB/GH, and Panton-Valentine leukocidin [27], [30]. Phenol-Soluble Modulins (PSMs) are pore-forming toxins that are responsible for membrane disintegration, which induces several pathological processes, such as red and white blood cells lysis, inflammation and biofilm formation. Exfoliative Toxins (ETs) are highly specific serine proteases that catalyze the reaction of hydrolysis of bonds in keratinocytes and cause cell-to-cell adhesion to the epidermis of the hosts [27].

#### *1.3.2.4. Superantigens*

The Superantigens (SAgs) of *S. aureus* include the staphylococcal enterotoxins (SEs), the staphylococcal enterotoxin-*like* (SE*l*s) proteins and toxic shock syndrome toxin-1 (TSST-1) [34]. SAgs (except TSST-1) are highly resistant to heat and acid, and gastrointestinal enzymes as well.

The mode of action of SAgs is based on attachment to MHC class II molecules in antigen presenting cells leading to excessive production of T cells and cytokine release [35], [36]. SAgs are responsible for several diseases such as Food Posioning and Toxic Shock Syndrome. Food Poisoning cause abdominal cramps, nausea, vomiting and sometimes diarrhea. Intoxication with TSST-1 causes Toxic Shock Syndrome (TSS) that induces high fever, erythematous rash, skin desquamation, and hypotension [37]. The high number of cases caused by TSS is dated to the 1980s and is associated with the use of high absorbency tampons by young women, however, TSS is still a huge problem [34]. Nevertheless, SAgs are also involved in the spread of Kawasaki disease, chronic rhinosinusitis, atopic dermatitis, and guttate psoriasis [34].

### *1.3.3. Resistance to antibiotics*

Nosocomial infections, which are most typically caused by Gram-positive cocci, especially Methicillin-Resistant *Staphylococcus aureus* and Vancomycin-Resistant (VRSA) *S. aureus*, are one of the most difficult challenges in modern epidemiology. Such infections not only endanger individuals, but also place a significant financial burden on medical systems. To reduce its spread, it is critical to understand the causes of staphylococcal resistance, as well as design adequate infection prevention strategies, and implement an acceptable antibiotic strategy [38].

Methicillin is an antibiotic that inhibits the action of penicillin-binding protein (PBP), which is involved in the final phase of bacterial cell wall peptidoglycan production. Methicillin resistance is an indicator of resistance to a wide range of antibiotics, including penicillin and cephalosporin (β-lactam drugs) as well as erythromycin, clindamycin, gentamicin, ciprofloxin, and fusidic acid [39]. Resistance typically refers to the acquisition of a non-native gene encoding a penicillin-binding protein (PBP2a) that has substantially reduced affinity for β-lactams. Hence, cell wall biosynthesis continues even in the presence of inhibitory quantities of antibiotics. PBP2a is encoded by the *mecA* gene, which is located on a separate genetic element (SCC*mec* – Staphylococcal Cassette Chromosome *mec*), and its expression is regulated by a proteolytic signal transduction pathway that includes a sensor (MecR1) and a repressor (MecI) protein [10], [40].

Vancomycin is a glycopeptide antibiotic that is used to treat Gram-positive bacterial infections. It is used particularly against MRSA strains due to the growing resistance of *S. aureus* to methicillin. In 2002, a strain of *S. aureus* with significant glycopeptide resistance was discovered for the first time in Michigan, USA, while the resistance of the strain was acquired from *Enterococcus* spp. VRSA strains can be classified into two types: vanA operon positive and *vanA*-negative. The presence of the *vanH, vanA,* and *vanX* genes indicates VanA-type vancomycin resistance. Non-resistant strains contain murein precursor with D-Ala-D-Ala which is the molecular target of vancomycin. However, the proteins VanA, VanH and VanX are involved in the synthesis of the peptidoglycan precursor with D-Ala-D-Lac residues, thereby making the antibiotic unable to attach to the precursor and inhibit the cell wall synthesis [41]. VRSA *vanA*-negative, which used to be named Vancomycin-Intermediate *S. aureus* (VISA) was identified in 1996 in Japan. The Mu50

strain exhibited in general 213 differences in DNA level compared to vancomycin-sensitive *S. aureus,* which were caused by spontaneous mutations. These mutations led to increased resistance to vancomycin that was associated with modification of membrane charge, cellular transport, and carbohydrates metabolism, as well as thickening of the cell wall, biofilm formation, and modulation of cellular processes such as immune evasion, reduced autolysis, and virulence attenuation [41]–[43]. Due to the continued development of resistant strains, it is crucial to make an attempt to find new antibiotics or non-antibiotic molecules to treat infections caused by this microorganism.

## 2. HONEY BEES *APIS MELLIFERA*

Due to pollination, insects such as honey bees (*Apis mellifera*) play a crucial role in the ecosystem. It is estimated that 87.5% of flowering plants are pollinated by animals [44], and approximately one third of the total human dietary supply is contributed to bee-pollinated crops [44]. Honey bees produce many precious products including honey, propolis, beeswax, venom, royal jelly, bee pollen and bee bread, all of which have been used for ages due to their nutritional value and activities such as antibacterial, anti-inflammatory, or antioxidant [45]. Furthermore, honey bees and their products can serve as bioindicators of environmental pollution [46]. Beeswax, venom and royal jelly are synthesized by the bees themselves, while other bee products are derived from plants and transformed by bees [47].

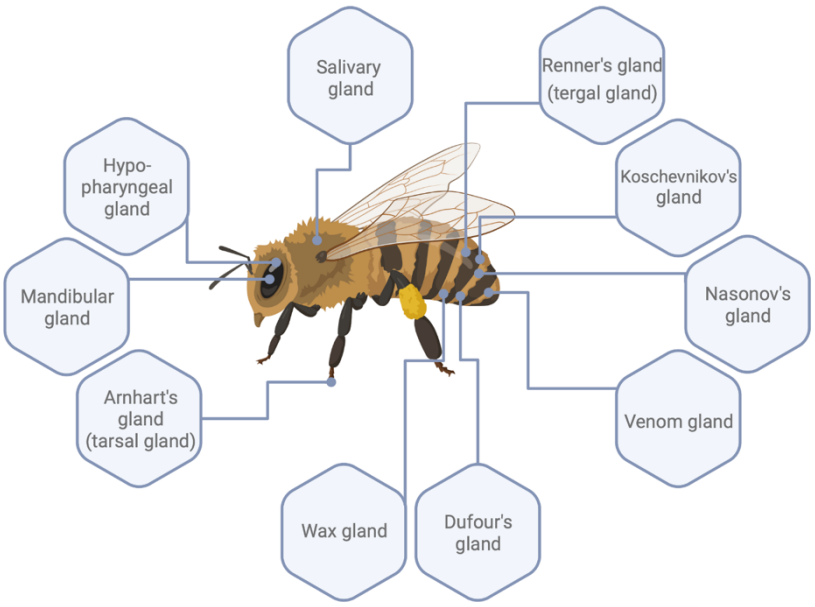


**Figure 2.** *Apis mellifera* (honey bee) glands that are involved in development of bees' family and production of bee products. Created in BioRender by K. Pełka.

Worker bees collect nectar by proboscis. Mandibles are for biting and chewing [48]. The nectar is stored in the special bee's stomach, called the honey crop, without digestion [49]. On the bee's body are developed branched hairs that facilitate pollen collection and pollination of plants. In the hind legs of bees are pollen baskets (corbiculae) that hold the pollen loads, and the pollen grains can be transported to the hive. Bees communicate by pheromones produced in the mandibles, tergites, rectum, and tarsi. The glands involved in the production of pheromones are: Dufour's gland producing the egg marking pheromone, discriminating the eggs of the queen and worker bees, Koschevnikov's gland producing the alarm pheromones, Nasonov's and tarsal glands producing pheromones helping bees with the orientation, tergal glands that generate pheromone responsible for the attraction of drones to the queen on their mating flights, and the mandibular glands, producing foraging-marking pheromone, which helps with communication among colony members. The queen's role in the hive is to regulate the colony and lay the eggs. Young worker bees usually take care of larvae and adult queen by secreting royal jelly, that acts as food for them, using enlarged hypopharyngeal, salivary, and mandibular glands. Bees also contain wax glands for wax production when they need to develop the comb. Furthermore, bees have a stinger at the tip of the abdomen, which is connected to the venom sac, where venom is produced by the venom glands [48], [50], [51]. The location of glands of honey bees are presented in the **Figure 2**.

### *2.1. Honeybee products – general information*

Bee products are valued not only for their unique taste but also for their nutritional value and health-promoting properties, meaning they have a special place in the human diet. It is known that since ancient times, honey bee products (honey, bee pollen, bee bread, royal jelly, propolis, bee venom, and beeswax) have been used as traditional remedies in the treatment of many infections and ailments [52]–[54]. As in many other natural product cases, the investigation of their antibacterial/antifungal potential was impeded by the discovery of antibiotics. However, in the last two decades, there has been a significant renewed interest in the health-promoting properties of honey and other bee products.

#### *2.1.1. Honey*

Honey is one of the most known bee products. It is a supersaturated sugar solution with syrup consistency. Honey bee foragers collect the nectar or honeydew with a specialized organ named proboscis, and then store the nectar/honeydew in the honey crop stomach without digesting of its content [49]. When the bees return to the hive, they pass the content of honey stomach to other bees – the whole process is repeated several times and it is performed to evaporate the water from the nectar. During this procedure, salivary secretions from bees are added to nectar, for instance, enzymes such as invertase, which break the sucrose bonds into glucose and fructose. After the passage, the nectar is collected in honeycombs. The excess water is evaporated until the level of water reaches approximately 17%. Bees use their wings to ventilate the hive and accelerate the evaporation process. When the honey is ready, the bees cover the combs with a thin layer of

wax [55]. Honey composition, as other bee products, is deeply correlated with its botanical origin, geographical location and season, and climate as well [56]. However, honey contains approximately 80-85% carbohydrates (mainly fructose and glucose) and 0.3% proteins, including amino acids and enzymes [57].

Honey exhibits many valuable properties including antimicrobial activity, which is attributed to physical and chemical factors such as low pH, high osmolarity, and presence of hydrogen peroxide produced by glucose oxidase, as well as other additional compounds, such as methylglyoxal, 3-phenyllactic acid, bee defensin, and bacteriocins [58], [59]

### *2.1.2. Propolis*

Propolis, also known as bee glue, consists mainly of resins, which are collected by honey bees from the exudate found in tree buds and/or sap flows. Resins are mixed with bee saliva and beeswax inside the hive, and applied to seal cracks and narrow openings, to protect the hive from invaders or cold weather conditions. The most important use of propolis is protection of the brood and storage food – bees coat the nest walls and the comb cells with the propolis [58]. It has been shown that propolis has a number of properties, including antibacterial, antifungal, antiviral, anti-inflammatory, anaesthetic, antioxidant, antitumoral, antiprotozoal, anticancer, antihypertensive, anticancerogenic, and antihepatotoxic activity. In addition, propolis helps heal wounds and improves the healing of injuries, due to the presence of flavonoids, arginine, vitamin C, provitamin A and B complexes with some minerals [60]–[62].

### *2.1.3. Royal jelly*

Royal jelly is a product of the secretion of the cephalic glands of very young bees, called nurse bees. It plays an important role in caste differentiation due to the presence of protein called royalactin that induces the differentiation of larvae into queens [63] and, furthermore, it is essential for the diet of honey bee larvae for the first three days [64]. Royal jelly was studied *in vitro* models as well as in laboratory and farm animals, and was also tested in clinical trials. This product has many biological activities, including antioxidant, antitumoral, immunomodulatory, anti-inflammatory, and wound healing effects, as well as hypertensive, neurotrophic, and insulin-like actions [65]. Furthermore, some components of royal jelly (trans-10-hydroxy-2-decenoic acid, royalisin, Major Royal Jelly Proteins and glucose oxidase) are responsible for the antimicrobial activity of this product [58].

### *2.1.4. Bee venom*

Bee venom, like other bee products, has long been used as a therapeutic substance and is one of the most well-known natural toxins. It consists of a very diverse set of chemicals, such as peptides (melittin, apamine, adolapin, MCD peptide), enzymes (phospholipase A2, hyaluronidase, acid phosphomonoesterase, lysophosphofolipase) and amines (histamine, dopamine,

norepinephrine) [66]. Bee venom is known for its antimicrobial, antifungal, and antiviral activity and is also used to reduce pain, suppress inflammation in musculoskeletal disorders, and has a therapeutic effect in the treatment of neurological disorders, e.g. chronic neuralgia or Parkinson's disease [58], [66].

### *2.1.5. Beeswax*

Beeswax is a product secreted by special wax glands in the abdomen of younger worker bees and is initially liquid. It solidifies on scales when it contacts the air. Beeswax is very complex and consists of more than 300 compounds, e.g. hydrocarbons, free fatty acids, esters of fatty acids and fatty alcohol, diesters, and exogenous substances. It has antimicrobial activity and exhibits synergy with other natural bee products against pathogenic bacteria, fungi, and yeast [67].

### *2.1.6. Bee pollen and bee bread*

Bee pollen and bee bread (**Figure 3**.) are the least known bee products, however, they are gaining more attention due to their high nutritional values. Bee pollen (BP) is the product gathered by bees and is the mixture of flower pollen, nectar, and bee secretions and after fermentation in the hive, BP is transformed into bee bread (BB). Both products are rich in various substances, such as carbohydrates, proteins, lipids, fatty acids, amino acids, vitamins, micro and macroelements, and polyphenols. According to their composition, they exhibit many activities: antioxidant, antimicrobial, anticancerogenic, anti-inflammatory, anti-osteoporosis, and hepatoprotective, but the mechanism of action is still barely known.


**Figure 3**. A) multifloral pollen grains, B) bee bread pellet. Courtesy of K. Pełka.

## 3. BEE POLLEN AND BEE BREAD AS AN ALTERNATIVE PHARMACEUTICS

### *3.1. Production of BP and BB by honey bees*

Pollination plays a key role in the natural balance of ecosystems and crop production. It is process of transferring pollen from male anthers to female stigmata within the same flower or between plants. It can be executed by vectors such as animals and the wind [68]. Honey bees are involved in the pollination of approximately 75 % of human food crops, which constitute about 35 % of the total crop production worldwide [69].

Bees harvest flower pollen and mix it with nectar and/or honey and salivary secretions, then merge them, forming pollen loads, which are then transferred to the hive in pollen baskets, located on the hind legs of bees [70]. Bee pollen is stored in the cells of the comb, where it is preserved with wax and honey, which cause an anaerobic environment. Because of the presence of anaerobic microorganisms, including lactic acid bacteria, bee pollen is transformed into bee bread during the solid-state fermentation process, which takes approximately seven days [71]. Fermentation, as well as predigestion of biopolymers present in pollen grains, caused by enzymes added by bees, promotes its nutritional value and preserves bee bread in the hive, where then bee bread serves as the main source of proteins for young bees and larvae [58]. The scheme representing the production of bee bread by honey bees is presented in **Błąd! Nie można odnaleźć źródła odwołania.**.

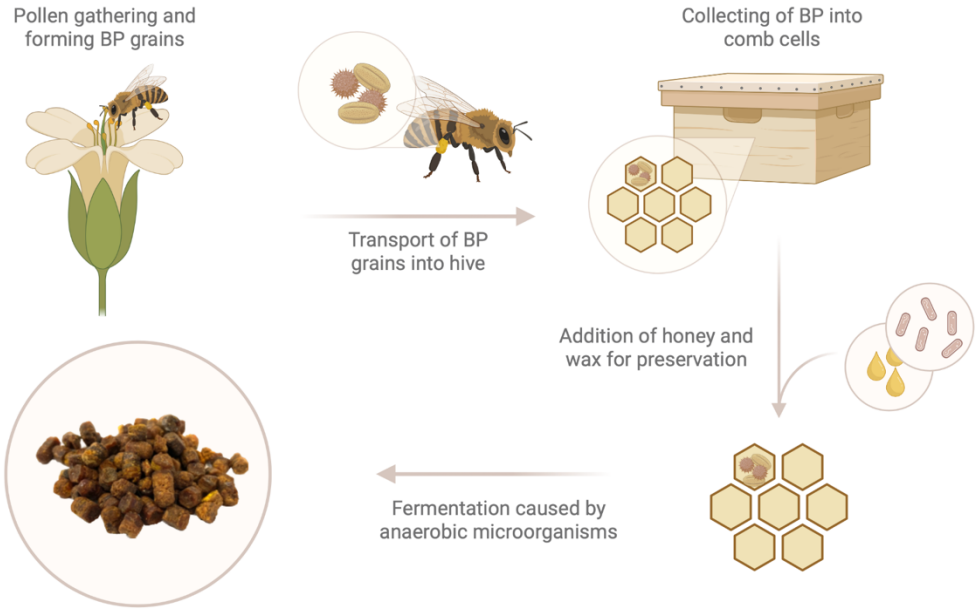


**Figure 4.** Bee bread production by honey bees, based on [73]. Designed in BioRender by K. Pełka

### *3.2. Chemical composition and physicochemical properties of BP and BB*

The chemical composition of bee pollen and bee bread is similar and strongly correlated with their botanical and geographical origin, as well as with the climate, soil type, season, weather conditions during the collection of these products and of the bee race or beekeeping management [58]. More than 250 biologically active compounds were found in bee pollen and bee bread [72], [73]. Bee pollen compared to bee bread is less nutritious, which is the result of the presence of two layers, intine and exine, which affect the bioavailability of bee pollen nutrients. Exine is the outer layer of the pollen grain and is made of sporopollenin. Exine is harder and more resistant than intine. Its surface is not smooth – it has folds, grooves, or spikes, and the interstices are coated with tryphine. This hydrophobic lipid and proteinaceous layer helps in the adhesion of pollen to insects and other vectors and in the initial contact between pollen and the stigmatic surface [74]. Intine is made of cellulose microfibrils and pectin. It contains two or three cells – generative cell or two sperm cells inside the large vegetative cell – that accumulate storage materials, such as lipids and polysaccharides [75]. Microorganisms associated with bees and predigestion caused by enzymes from bee secretions are essential in the release of nutrients from pollen grains, which is important in the nutrition and development of the bee colony [58]. The comparison of chemical composition of bee pollen and bee bread is presented in **Table 2**.

**Table 2.** Comparison of the chemical composition of bee pollen and bee bread. Based on [58], [72], [73].

| Component | Bee pollen | Bee bread |
|---|---|---|
| **Water content** | 21-30 | 7.26-48.54 |
| **Ash content** | 1.5-3.2 | 2.03-4.9 |
| **pH** | 3.8-6.3 | 3.8-4.3 |
| **Carbohydrates** | 24-60 | 24-72.83 |
| **Proteins** | 7-40 | 14-37 |
| **Lipids** | 1-13 g/100g | 6-13 g/100g |
| **Phenolic compounds** | 0.69-213.53 mg GAE/g | 2.5-23.3 mg GAE/g |

### *3.2.1. Moisture and ash content*

The moisture content in bee pollen and bee bread is an important parameter due to the microbial stability and chemical composition of these products. A large amount of water affects the growth of different microorganisms, which could decrease the quality of bee pollen and bee bread. On the other hand, a limited level of water might have an impact on the discoloration of the BP and BB components and can prompt unwanted chemical reactions, such as the Maillard reaction or oxidation of lipids. The amount of water in fresh pollen loads ranges from 21 % to 30 %, but the moisture of dried pollen grains varies from 2 % to 9 % [72]. In the case of bee bread from different regions of the world, water content varies in the range 7.26-48.54 % [73].

In addition, the mean values of the ash content in bee pollen range from 1.5 % to 3.2 % and the ash content in bee bread varies from 2.03 % to 4.9 % [72], [73].

### *3.2.2. Carbohydrates*

The most abundant compounds contained in bee pollen and bee bread are carbohydrates. In bee pollen, the level of carbohydrates starts from 24 % to 60 %, which constitutes up to two thirds of its dry weight. Monosaccharides represent the majority of carbohydrates in bee pollen [72]. A great part of the monosaccharides in this product is composed of fructose followed by glucose. Other carbohydrates present in bee pollen are sucrose, arabinose, isomaltose, melibiose, melezitose, ribose, trehalose, and turanose [76].

The amount of carbohydrates in bee bread is slightly similar to bee pollen and ranges from 24 % to 72.83 % [72], [73]. In this product, mainly fructose, glucose, sucrose, maltose, and trehalose were found. However, the presence of mannitol, sorbitol, cellobiose, isomaltose, maltose, raffinose, and stachyose was also established [73]. The presence of polysaccharides in both products was also confirmed, including arabinoxylans, β-glucan, cellulose and starch that are structural components of pollen grains.

### *3.2.3. Proteins*

Proteins are essential for the human body. They are components of cells and tissues and might be a source of energy [72]. Proteins are the second group of compounds present in the majority in both bee pollen and bee bread. The protein content in bee pollen was estimated between 7% and 40%, whereas in bee bread is in the range of 14-37%. Bee bread constitutes the main source of proteins for bees, and contains easily assimilated proteins, as well as all essential human amino acids [58].

A broad spectrum of amino acids was detected in bee pollen and bee bread. Amino acids play an important role in the nitrogen balance of humans and are necessary for the synthesis of, for example, proteins. The amino acids present in both products are glutamic acid, aspartic acid, proline, arginine, valine, histidine, leucine, isoleucine, lysine, methionine, tryptophan,

phenylalanine, threonine, cysteine, tyrosine, alanine, glycine and serine [58], [77]. It should be noted that the amino acid content is higher in bee pollen, but in bee bread there is a better bioavailability of them [78], [79].

Several enzymes were detected in both products. In bee bread enzymes such as amylase, invertase, phosphatase transferases and glucose oxidase were found, as well as enzymatic cofactors, such as biotine, glutathione and NAD [58]. In the case of bee pollen grains, invertase and diastase were detected [78].

*3.2.4. Lipids*

Lipids are an essential group of diet compounds and are a source of energy and fat-soluble nutrients, such as vitamins [79]. The total lipid content in the case of bee pollen was estimated at 1-13 g/100 g and for bee bread - 6-13 g/100 g [58]. Furthermore, lipids are also a source of fatty acids. Fatty acids (FAs) are essential due to their influence on cell and tissue metabolism, as well as the regulation of hormonal and other signals. In particular, PUFAs (Polyunsaturated Fatty Acids) are important dietary agents due to the function they serve, for example, constitution and modulation of membranes, enhancement of signal transmission and regulation of transcription factors [80].

Approximately 20 fatty acids were found in the bee pollen, where the most abundant were ω-3 fatty acids. Among saturated fatty acids, myristic, stearic and palmitic acids dominate, and, considering unsaturated fatty acids, in the majority are α-linolenic, linoleic and oleic acids [76]. The content of fatty acids in bee bread was reported as 311.09 mg/100 g [81]. Bee bread contains saturated, mono, and polyunsaturated fatty acids. Among saturated fatty acids, the most abundant is myristic, palmitic, margaric, and stearic acid [82]. Furthermore, unsaturated aliphatic acids represent more than half of all fatty acids contained in this product. The most abundant are α-linolenic, linoleic, oleic and 11,14,17-eicosatrienoic acid [83]. Nevertheless, the ratio of unsaturated to saturated fatty acids was estimated from 1.38 to 2.39 [87]. According to the significant amount of fatty acids, especially unsaturated, bee pollen and bee bread are indicated as good sources of these compounds. Examples of the fatty acids found in bee pollen and bee bread are presented in **Figure 5**.

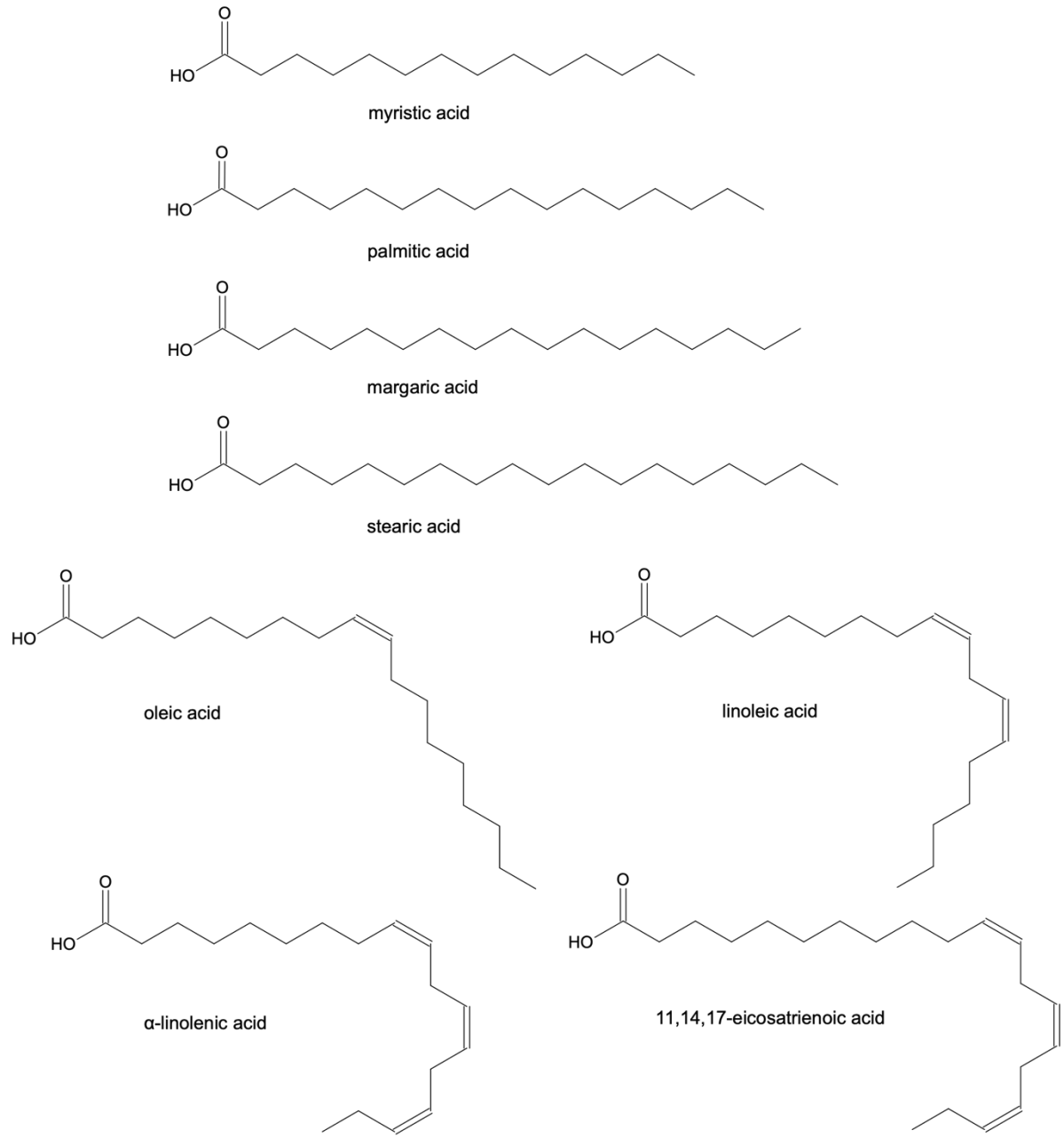


**Figure 5.** Examples of fatty acids structures detected in bee pollen and bee bread. Created in MoleculeSketch by K. Pełka.

### *3.2.5. Organic acids*

The presence of organic acids in bee pollen and bee bread was also reported. The source of organic acids in these products might be plants themselves, the secretions of bees added during bee pollen and bee bread formation, and the microorganisms contained in these products [72], [85], [86]. Organic acids revealed antioxidant and antibacterial activity, and they did not exhibit risks to human health. The most abundant organic acids in bee pollen are gluconic and lactic acid, followed by oxalic, tartaric, malic, citric, succinic, and acetic acid. In the case of bee bread, gluconic, oxalic, formic, butyric, propionic, lactic, acetic, and succinic acids were reported. However, the total content of organic acids determined for bee bread is higher in comparison to bee pollen.

Nevertheless, the variety of organic acids in both products is higher than in bee venom and royal jelly [86], [87].

In addition, the pH of both products is established as 3.8-6.3 for bee pollen and 3.8-4.3 for bee bread. During pollen fermentation, lactic acid bacteria (LAB), e.g. *Lactobacillus* spp. metabolize carbohydrates and produce lactic acid up to the concentration of 3.2% [58]. Moreover, the concentration of lactic acid in bee bread is six times higher than in the case of bee pollen [72].

### *3.2.6. Phenolic compounds*

Polyphenols are secondary metabolites of a plant and their role is to protect the plant from UV radiation as well as to protect it from plant diseases. Furthermore, they also exhibit many beneficial properties for the health of bees and humans that consume bee pollen and/or bee bread, including antioxidant, antimicrobial, and anti-inflammatory activity [88].

The composition of phenolic compounds in bee pollen and bee read is strongly related to their plant origin. The total phenolic content determined for bee pollen ranged from 0.69 to 213.53 mg GAE/g [76] and for bee bread from 2.5 to 21.3 mg GAE/g [73]. Among all phenolic compounds, flavonoids are the group that appears most in bee pollen and bee bread (

**Figure 6**.). The flavonoid content in bee pollen is around 1.4% [72] and the most abundant flavonoids identified in this product were tricetin, luteolin, selagin, myricetin, rhamnetin, isorhamnetin, isoquercetin, catechin, narigenin, apigenin, quercetin and kaempferol. What is more, quercetin and kaempferol and their glycosidic forms are in the majority of determined flavonoids in BP [58], [89]. In the case of bee bread, the presence of naringenin, kaempferol, apigenin, isorhamnetin, quercetin, rutin, myricetin, herbacetin, and their glycoside derivatives were determined.

Except flavonoids, phenolic acids are the most abundant group of non-flavonoid phenolic compounds, that were reported in natural products, including bee products. Mostly, benzoic acid and cinnamic acid derivatives occur in bee pollen and bee bread. In bee pollen, coumaric acids (o-coumaric and p-coumaric), sinapic, ferulic, cinnamic and hydroxycinnamic, chlorogenic, gallic, abscisic, and caffeic acids were found [58]. However, in the case of bee bread, gallic, protocatechuic, caffeic, and p-coumaric acids were detected [73].

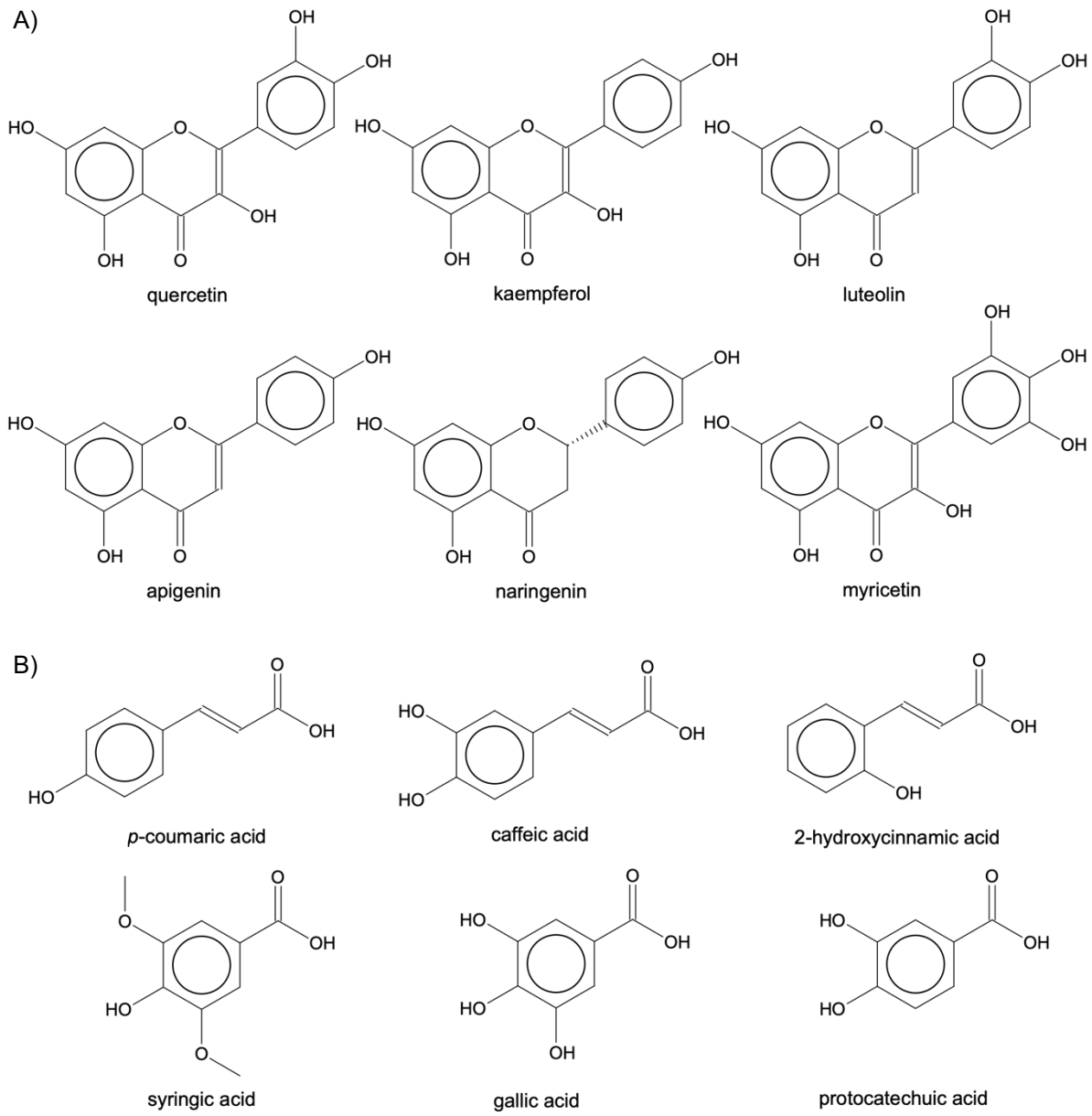


**Figure 6.** Examples of structures of A) flavonoids and B) phenolic acids found in bee pollen and bee bread. Created in MoleculeSketch by K. Pełka.

### *3.2.7. Vitamins*

Vitamins play an essential role as coenzymes or prohormones in many biological processes. Due to their biological and chemical activities, 13 different types of vitamins have been identified. A distinction can be made between fat-soluble vitamins (A, D, E and K) and water-soluble vitamins (other) [90]. The vitamin content in bee pollen is established between 0.02 and 0.7 % [72], where there are present water-soluble vitamins - B complex vitamins, vitamin C, rutin, biotin, inositol, panthotenic, nicotinic and folic acids, and fat-soluble vitamins as well, such as vitamin A (mostly β-carotene), D and E [58], [72]. In the case of bee bread, the presence of B-complex vitamins, C, E, biotin, nicotinic acid, folic acid and vitamin K was reported [89]. It should be noted

that vitamin K is the only one not present in fresh pollen. Additionally, in both products, the presence of coenzyme Q10 was also detected, which is known for its essential role in electron transport in mitochondria and antioxidant activity in cytoplasmic membranes and lipoproteins [58], [77].

#### *3.2.8. Minerals*

The appropriate balance of minerals in the human body is required for normal functioning of the organism. Minerals exhibit plenty of beneficial functions – they are components of the bones, they influence the function of muscles and nerves, and regulate water balance in the body [91]. Bee pollen can be a source of different micro- and macro-elements, where the most predominant are iron, calcium, phosphorus, potassium, copper, zinc, selenium, manganese, and magnesium. In bee bread the highest level of potassium was reported followed by phosphorus, calcium, magnesium, iron, sodium, zinc and manganese [58], [73], [92].

### ***3.3. Biological activities of bee pollen and bee bread***

According to the unique composition of bee pollen and bee bread, both products provide many health benefits, such as antimicrobial, antioxidant, anti-inflammatory, anticancerogenic, cardio and hepatoprotective properties [92] (**Figure 7**).

#### *3.3.1. Antioxidant and anti-inflammatory activity*

Antioxidative effect of bioactive compounds is essential due to the regulation of the redox state of the body. Antioxidants decrease the oxidative damage of cells caused by free radicals and reactive oxygen species (ROS). The antioxidant potential of bee pollen and bee bread is connected with the decreasing level of ROS in cells. The bioactive compounds that are mostly involved in antioxidant activity of mentioned bee products are polyphenols. The phenolic hydroxyl groups of flavonoids capture radicals, leading to the formation of stable products that inhibit further oxidation [93], [94]. Furthermore, flavonoids can bind to metal ions such as iron and copper, which are known to participate in reactions that generate free radicals. By chelating these metal ions, flavonoids can prevent their involvement in free radical production, thus reducing oxidative stress [95]. Flavonoids, phenolic acids and fatty acids can inhibit the arachidonic acid pathway that lowers the arachidonic acid level, which in the end leads to a decrease of prostaglandins. What is more, they can inhibit hyaluronidse and elastase (for example, kaempferol), which suppress the inflammatory response [96]. The anti-inflammatory effect of pollen ingredients is comparable to non-steroidal anti-inflammatory drugs (for example naproxen, analgin, indomethacin) [68], [93].

#### *3.3.2. Cardio- and hepatoprotective activity*

Bee pollen and bee bread extracts can reveal beneficial effects on the cardiovascular and hepatobiliary systems. The cardioprotective activity is associated with bioactive components of bee pollen and bee bread – PUFAs, vitamin E, phytosterols, phospholipids and flavonoids. The mechanism of action is based on inhibition of the enzyme that converts angiotensin I

to angiotensin II, which causes hypotensive effects [68], [97], [98]. Bee pollen and bee bread extracts may reveal anti-atherosclerotic potential, by reducing blood viscosity and decreasing the intensity of atherosclerotic plaque formation, which prevents heart diseases and stroke [72], [97].

Bee pollen can improve the liver functions by detoxification of the liver and lowering pathological levels of enzymes, such as gamma-glutamyl transferase, lactate dehydrogenase, alanine and aspartate transaminase, and acid and alkaline phosphatase as well. Furthermore, bee pollen extracts can lower the bilirubin in the blood caused by poisoning with organic compounds [89], [93], [97]. MAFLD (metabolic dysfunction-associated fatty liver disease) is a new disease that is associated with hepatic lipid accumulation, oxidative stress and abnormal inflammatory response. Bee bread exhibits hepatoprotection against MAFLD in rats and can reduce obesity, hyperlipidemia, liver injury, hyperglycemia, insulin resistance, hepatic steatosis and fibrosis [99].

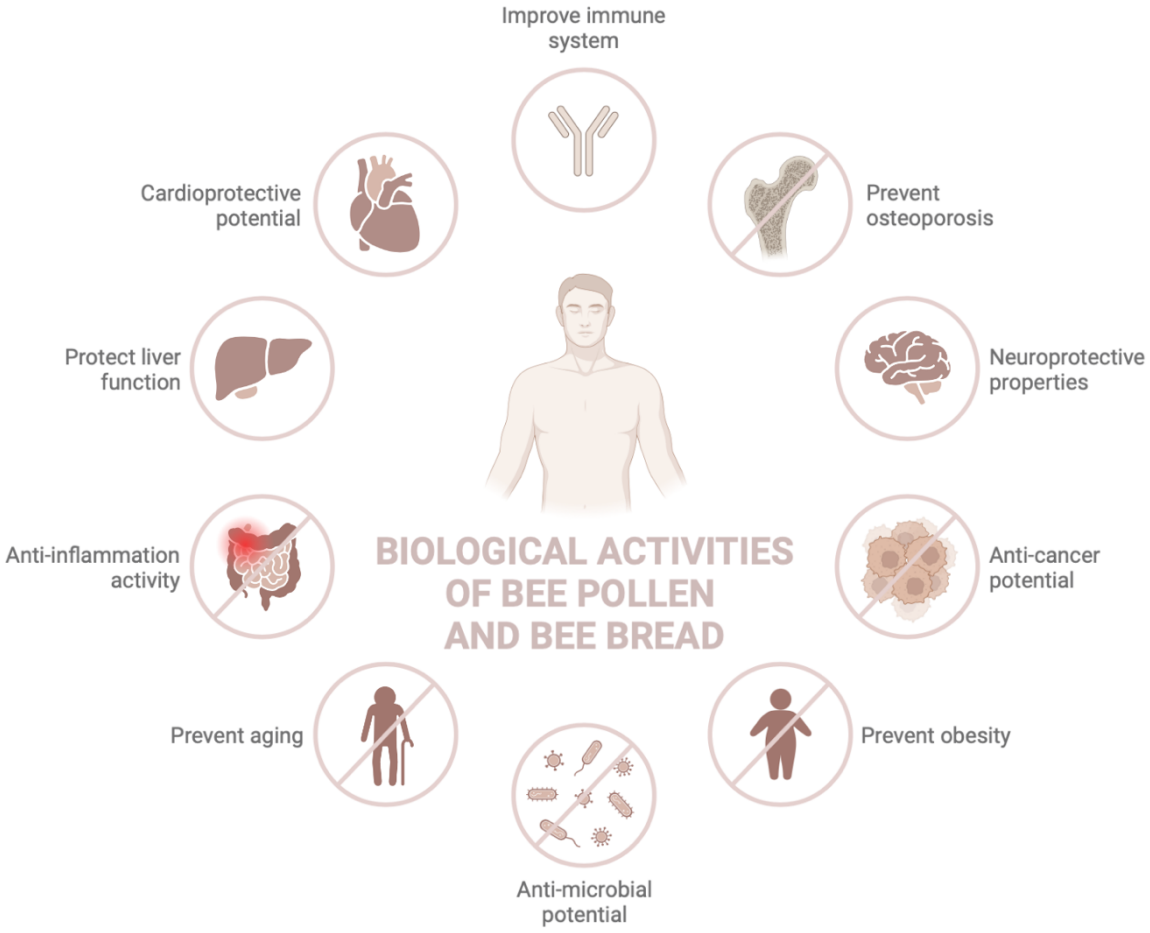


**Figure 7.** Scheme of biological activities of bee pollen and bee bread. Designed in BioRender by K. Pełka.

### *3.3.3. Antimicrobial activity*

For determination of antimicrobial activity, several solvents have been used for the extraction of active compounds from BP and BB. So far, extracts prepared with ethanol or methanol have been best characterized. In general, bee pollen and bee bread revealed antibacterial activity mostly against Gram-positive bacteria such as *S. aureus*, *S. epidermidis*, *Listeria monocytogenes,* and *Bacillus cereus*. The growth inhibition of Gram-negative bacteria including *E. coli* and *P. aeruginosa* is significantly worse compared to Gram-positive bacteria. It can be explained by differences between the structures of Gram-positive and Gram-negative bacteria cells – Gram-negative bacteria has an additional outer cell membrane – and the production of hydrolytic enzymes by Gram-negative bacteria, which can degrade the bioactive components of bee pollen and bee bread [100], [101]. In the case of antifungal activity, both bee pollen and bee bread presented activity against *Candida* strains (*Candida albicans*, *Candida krusei*, *Candida glabrata*) and *Aspergillus* strains (*Aspergillus niger* and *Aspergillus fumigatus*) [58]. The antiviral potential of bee pollen and especially bee bread is the less known topic among antimicrobial activities of bee products. The antiviral activity of bee pollen against Influenza virus was described by Lee et al. [102]. In this study, the polyphenols of Korean bee pollen were investigated against the virus. All of the tested samples showed notably anti-influenza activity, however, the most active was luteolin. In the case of bee bread, it presents antiviral activity against mammalian viruses – Enterovirus-D68 and Influenza A virus [103], [104].

Antimicrobial activity depends on the composition of bee pollen and bee bread, which in turn depends on many factors such as weather, climate, geographic location of apiary and plant species that are available to bees. To date, there is no information that describes the exact mechanism of antimicrobial action of both products. One of the aims of this dissertation was to investigate antimicrobial potential and to examine a mechanism/mechanisms of action of bee pollen and bee bread derived from Polish apiaries against pathogenic bacteria. Phenolic compounds and fatty acids have been shown to be crucial for the antimicrobial potential of BP and BB. There are several mechanisms of action of flavonoids that inhibit microbial infections. For instance, flavonoids can disrupt the integrity of the bacterial cell membrane and cell wall, inhibit the synthesis of nucleic acids in bacterial cells, impede the biofilm formation by interference with the pathway in bacterial quorum sensing [105], [106]. Furthermore, flavonoids can interact with the envelope proteins of viruses, which can block virus entrance to the host cell. Flavonoids can inhibit virus replication, inhibit the secretion of the virus from the cell and bind to viral proteins and enzymes [107]. On the other hand, the antimicrobial potential of saturated and unsaturated fatty acids includes inhibition of enzymes of cytosol or membrane, which inhibits FAs synthesis, where the unsaturated FAs exhibited more effective antimicrobial activity than saturated FAs [108]. FAs are involved in the disruption of an electron transport chain, preventing the energy production by uncoupling oxidative phosphorylation and they can also insert themselves into the cell membrane, which can then cause cell lysis [109]. The mechanisms of action of flavonoids against microorganisms are presented in **Figure 8**.

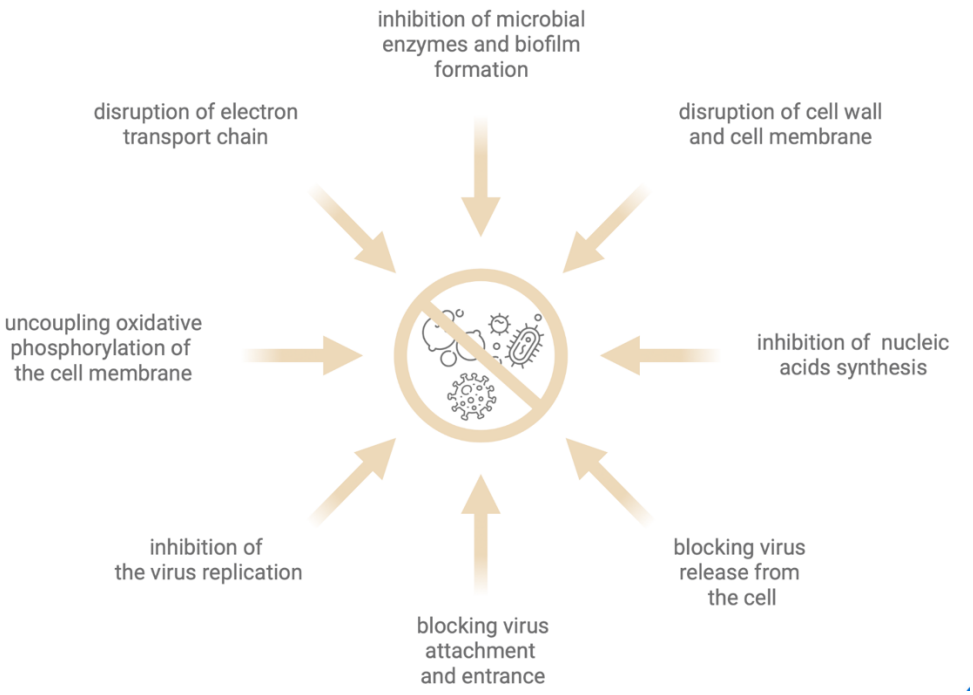


**Figure 8.** Mode of action of flavonoids found in bee pollen and bee bread against bacteria, fungi and viruses. Created in BioRender by K. Pełka.

### *3.3.4. Anti-cancer potential*

According to WHO reports, cancer is one of the leading causes of death worldwide [110], and the most common types are breast, lung, colon, rectum, and prostate cancers. Bee pollen and bee bread reveal an inhibitory effect on several cancer cell lines, for example chronic myelogenous leukemia cells K-562, prostatic cancer cells PC-3, [93], breast cancer cells MCF-7, lung cancer cells NCI-H460, cervix cancer cells HeLa, liver cancer cells HepG2 [111], malignant gliomas cells U87 MG [83] and colorectal cancer cells Caco-2 [112]. Treatment of cancer cells with BP and BB extracts inhibits cancer growth and increases apoptosis [89]. The mechanism of action of the extracts includes increasing the activity of the caspase-3 enzyme, lowering the level of anti-apoptotic proteins Bcl-2, and stimulation of tumor necrosis factor α (TNF-α) [68], [97].

### *3.3.5. Other bioactivities of bee pollen and bee bread*

Bee pollen and bee bread, as well as extracts produced from these products, exhibit many other health-promoting properties. For instance, bee bread may exhibit anxiolytic, neuroprotective and anticonvulsant activity [85]. In the case of bee pollen, the extracts revealed an anti-anemic potential by increasing hemoglobin level and decreasing platelet aggregation [93]. Furthermore, bee pollen extracts can prevent osteoporosis by inhibiting femur resorption, osteoclastic cell formation, and improving the level of alkaline phosphatase [97]. Bee pollen may also stimulate the immune system by increasing the level of specific IgM and IgG and can reveal an antiallergic potential according to inhibition of mast cell activation, thereby affecting the early and late phases

of the allergic reaction. The mechanism of antiallergic action of bee pollen is based on the interception of the connection of IgE with its receptor and the inhibition of histamine secretion [93]. Furthermore, both bee pollen and bee bread may have a positive impact on the obese state. Bee bread can reverse apoptosis caused by activation of the caspase pathway induced by suppression of Bcl-2 and increase the level of Bax [113]. Bee pollen can restore the Nrf-2Keap-1 pathway to decrease oxidative stress and can modulate the intestinal microbiota (reduction of *Firmicutes/Bacterioides* and increase in *Lactobacillus* and *Lactococcus* level) [114]. According to the presence of polyphenols, vitamins, and fatty acids in both bee pollen and bee bread, these products can be used in cosmetics to prevent aging [96], [115].

#### *3.3.6. Preclinical and clinical studies*

Bee pollen has been tested in preclinical studies related to diabetes. Administration of bee pollen or date palm pollen for four weeks reduced body, testis and pancreas weight, as well as insulin resistance in streptozotocin-induced diabetic Wistar male rats. Additionally, bee pollen investigation was performed according to inhibition of angiotensin-converting enzyme. The extract of bee pollen decreased the high level of angiotensin-converting enzyme and angiotensin II-induced by high-fat diets in C57BL6 mice, causing enhancement of endothelial function [116].

Moreover, bee pollen was tested in clinical trials against prostatitis and prostate cancer, breast cancer, and skin diseases. Due to the anti-inflammatory and anti-androgen properties of bee pollen, its efficacy against prostate disorders was found. Cernilton® pollen extracts (Cernitin SA, Switzerland) inhibits prostate cancer cells PC-3 and decreases chronic prostatitis without negative reactions (clinical efficacy was estimated as 85%) [89], [117]. The anticancer activity of bee pollen was the foundation of clinical research against breast cancer. Non-estrogenic bee pollen PCC-100 was used to alleviate expected menopausal symptoms due to antihormonal therapy in patients with breast cancer [117], [118].

### ***3.4. Microflora of bee products***

In insects, as in other animals, there is a large population of microorganisms that live mainly in their digestive system but also in other parts of their body. The microflora of insects, in particular their digestive system, provides benefits to the host, such as protection against parasites and pathogens, assistance in the digestion of food (including pollen grains) or protection against toxic compounds present in the environment of these animals. Due to the decreasing number of pollinating insects, including honeybees (*Apis mellifera* L.) and bumblebees (*Bombus* spp.), their microflora is increasingly being studied to find the cause of this phenomenon [119]. Microorganisms, such as bacteria, molds and yeasts are associated with honey bees and bee products [120] – they are involved, for instance, in the biotransformation of pollen loads into bee bread. In **Figure 9**., selected groups of microorganisms associated with bee pollen and bee bread are presented.

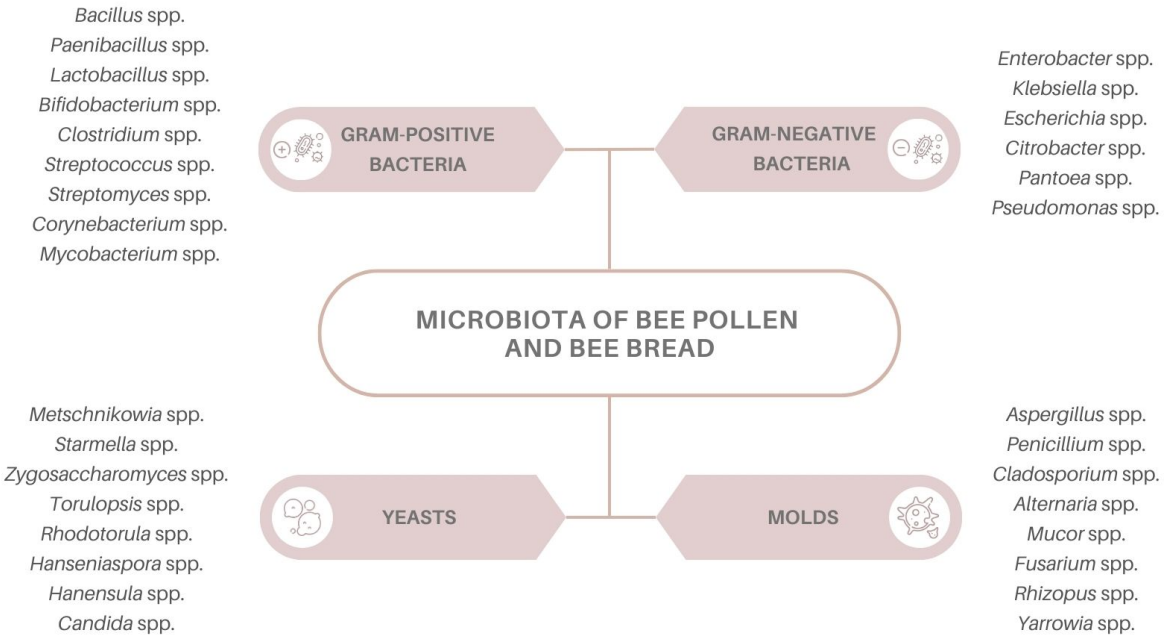


**Figure 9.** Selected genera of microorganisms that was detected in bee pollen and bee bread. Created in Canva by K. Pełka.

There are four phases of microbial development in fermenting pollen. The first phase takes about 12 hours, where mainly the growth heterogenous group of microorganisms (including yeasts) occur. The second phase is characterized by the development of anaerobic lactic acid bacteria (*Streptococcus* spp.). For their growth, they use growth factors such as simple sugars produced by yeasts and putrefactive bacteria. It is essential to lower the pH of pollen in the first phase of its processing. In the third phase, streptococci disappear and the growth of bacteria of the genus *Lactobacillus* is developed. During the fourth phase (after seven days), the disappearance of lactic acid bacteria and some yeasts occurs due to the high level of lactic acid production, which makes bee bread almost sterile - its pH is around 4 [121]. Spore-forming bacteria, e.g. of the *Bacillus* genus, and few other bacteria species, as well as yeasts and molds, can survive in the harsh habitat of bee bread. These microorganisms, derived mainly from the environment, but also from the digestive system of bees, cause that bee bread can be stored in the wells of honeycombs for a long time at a temperature of about 30 - 37°C without symptoms of microbial spoilage [122].

In this dissertation, the second aim was to investigate the microflora of bee pollen and bee bread derived from Polish apiaries. It should be noted that both bee pollen and bee bread were matured, therefore, every consumer has access to these products in stores.

### *3.4.1. Bacteria*

#### *3.4.1.1. Bacillaceae*

The rod-shaped *Bacillaceae* family includes anaerobic spore-forming bacilli of *Clostridium* spp. and aerobic or facultatively anaerobic endospore-forming bacteria of *Bacillus* spp. [123]. Bacteria belonging to the genus *Bacillus* are the most abundant microorganisms found in honey bee products [120], [124]. Due to the harsh environment of bee products (high osmolarity, antimicrobial proteins, phytochemicals, etc.), only highly resistant bacteria can survive under these conditions. Species of *Bacillus* spp. that were detected in honey, bee pollen, and bee bread include *Bacillus megaterium, Bacillus subtillis, Bacillus licheniformis, Bacillus pumilus, Bacillus amyloliquefaciens, Bacillus clausii, Bacillus coagulans, Bacillus cereus, Bacillus altitudinis, Bacillus safensis, Bacillus xiamenensis,* and *Bacillus circulans* [122], [124]–[127].

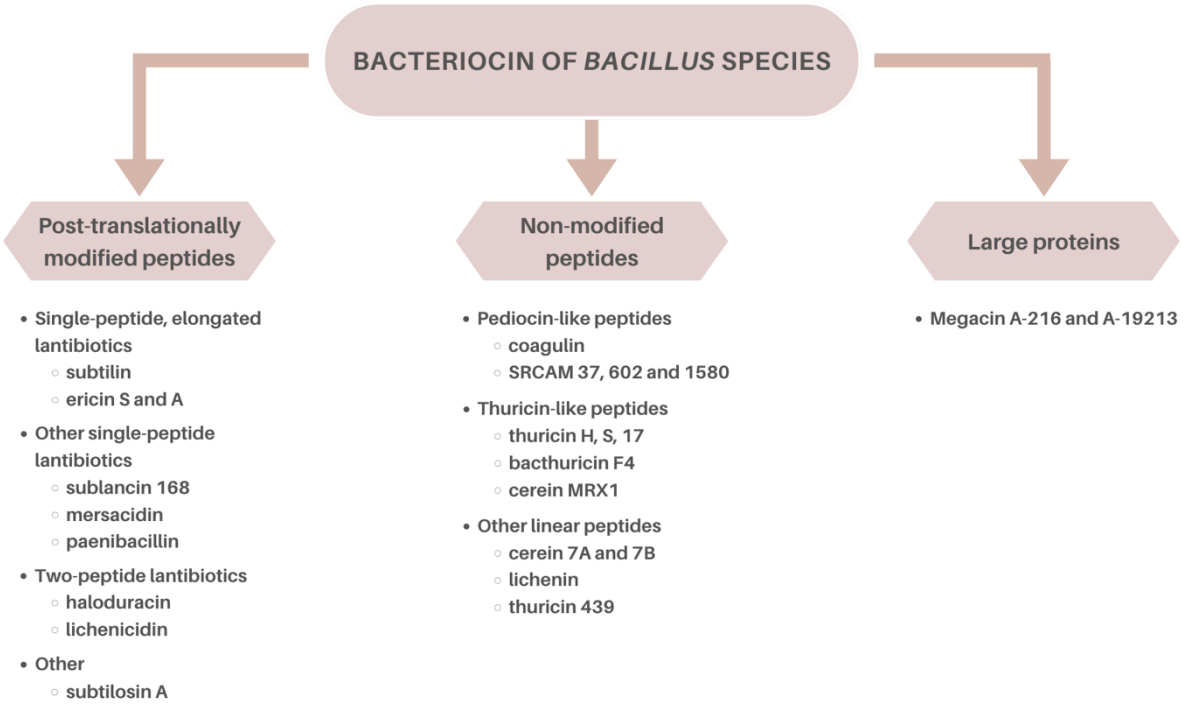


**Figure 10.** Bacteriocins produced by *Bacillus* strains. Based on [131]. Created in Canva by K. Pełka.

Bacteria belonging to the genus *Bacillus* also play a significant role for bees and humans. These microorganisms are known for their ability to produce many antimicrobials, such as peptide and lipopeptide antibiotics and bacteriocins (**Figure 10**.) [128]. *Bacillus* strains are involved in the production of enzymes crucial for bees' health, the biotransformation of bee pollen to bee bread, and the increase of resistance to some diseases such as chalkbrood. Furthermore, *Bacillus* spp. provides many benefits to bees, for example, an increase in the number of bee larvae, a reduction in *Varroa* and *Nosema* levels in the hive and greater accumulation of honey compared to control hives [129]. It should be noted that most *Bacillus* strains are considered safe

for mammals, except *B. cereus* and *Bacillus anthracis* [130]. Moreover, several studies showed that *Bacillus* species isolated from bee products exhibited probiotic potential [130]–[133]. According to the WHO, probiotics are live microorganisms that, when administered in adequate amounts, improve host health. As probiotics, *Bacillus* strains have GRAS status (Generally Recognized as Safe) and exhibit the ability to tolerate gastrointestinal conditions and can survive in the presence of gastric acid and bile salts. They possess the ability to adhere to epithelial cells and reveal antimicrobial activity and a lack of antibiotic-resistant genes [134].

#### *3.4.1.2. Lactic Acid Bacteria (LAB)*

A healthy gut microbiota is essential for bee development, immune function, and protection against pathogens. One of the groups of bacteria that are present in the gastrointestinal tract of *Apis mellifera* are lactic acid bacteria. LAB are group of microorganisms producing lactic acid during homo- or heterofermentative metabolism. The distribution of LAB distribution is not only limited to the guts of bees, but also associated with the hive environment and bee products, such as bee bread, honey, wax and comb [135], [136]. LAB may provide many advantages to the bee colony, for example, they have an impact on decreasing mortality rate, stimulation of queen egg laying, significant increase in honey yield and production, induce immune system stimulation, and enhancement of bee health. They also help alleviate antibiotic-associated microbiota dysbiosis and participate in lignin digestion and promotion of the growth of the gut microbiota [135].

LAB may also produce antimicrobials, such as bacteriocins, hydrogen peroxide, siderophores, lysozymes, proteases, organic acids, and volatile fatty acids, which inhibit the development of many pathogens, causing harmful diseases for bees, including chalkbrood, American and European Foulbrood, Varroosis, and Nosemosis as well [135]. The LAB include families of *Lactobacillus* (for example, *Lactobacillus kunkeei*, *Lactobacillus johnsonii*, *Lactobacillus plantarum*, *Lactobacillus salivarus*, *Lactobacillus acidophilus*, *Lactobacillus delbrueckii, Lactobacillus brevis*, *Lactobacillus rhamnosus*, *Lactobacillus casei*), *Fructobacillus* (for example, *Fructobacillus fructosus, Fructobacillus tropaeoli*), and *Bifidobacterium* (for example, *Bifidobacterium lactis, Bifidobacterium bifidum, Bifidobacterium longum, Bifidobacterium asteroides*).

Lactic Acid Bacteria can also serve as probiotics for humans due to their beneficial health properties. As probiotics, they exhibit good adhesion properties and survivability in the gastrointestinal tract. They are able to produce antioxidant substances, that can scavenge the free radicals from the gastrointestinal tract [137]. In addition, its ability to produce antimicrobials, which can inhibit the growth of pathogens in the gastrointestinal tract, such as *Helicobacter pylori, Clostridium difficile, Salmonella enterica* var. *Typhimurium, P. aeruginosa, Enterococcus faecium, Escherichia coli* and *S. aureus* [137]. During viral infections, for instance, rotavirus, LAB can reduce acute diarrhea. They can also affect the immune system by inducing pro-inflammatory and/or anti-inflammatory cytokines, activation of macrophages, natural killer cells (NK), antigen-specific

cytotoxic T-lymphocytes, and can induce different cytokine responses [138]. Furthermore, LAB can provide the host with a preventive effect on malignant development by binding to mutagens and interacts with heterocyclic amines, production of antimutagenic substances (antioxidants), and scavenging ROS, increase the production of β-glucosidase that release flavonoids, inhibition of procarcinogenic enzymes (e.g. nitroreductase and β-glucuronidase), etc. [137].

#### *3.4.1.3. Other bacterial families*

The family of *Enterobacteriaceae* and *Pseudomonadaceae* are Gram-negative, facultatively anaerobic, rod-shaped bacteria that are associated with the intestinal microbiota of honey bees [120]. In bee pollen and bee bread samples, bacteria belonging to these families was detected as well [139]–[143]. However, the role of *Enterobacteriaceace* and *Pseudomonadaceae* families in the hive is still unknown. Nevertheless, their presence in bee's guts can indicate health problems of honey bees. The growth inhibition of these microorganisms in bees guts is associated with the presence of beneficial bacterial families (*Lactobacillaceae, Orbaceae* and *Neisseriaceae*) that stimulate the production of antimicrobial peptides [144].

### *3.4.2. Yeasts, molds, and fungi*

Yeasts participate in the biotransformation of bee pollen into bee bread with LAB. Their abundance of bee pollen and fresh bee bread decreases rapidly during storage in the comb. Furthermore, the distribution of yeast species is associated with the gathering and storage of pollen [145]. In the pollen loads collected on the legs of bees, mostly *Metschnikowia* strains were detected [121], [145]. However, in bee honey stomachs and in fresh bee bread (up to 7 days in the comb), *Starmella* strains dominated. Except *Starmella* strains, the fresh bee bread microbiota includes *Metschnikowia spp., Zygosaccharomyces* spp., and others. In mature bee bread, yeast strains, such as *Starmella* spp. and *Zygosaccharomyces* strains, were the most abundant [121], [145], [146].

The species of molds that are associated with the intestinal tracts of bees are mainly strains of *Penicillium* and *Aspergillus* [120]. What is more, in bee pollen grains, *Alternaria spp., Mucor* spp., *Aspergillus* spp., *Fusarium* spp., *Penicillium spp.,* and *Cladosporium* spp. strains were detected as well [147], [148]. In bee bread, the species that belonged to the genera *Cladosporium, Penicillium, Alternaria* and *Rhizospus* are the most abundant [148]–[150]. However, strains of *Mucor* spp., *Aspergillus* spp., *Fusarium* spp. and *Trichoderma* spp. were also found in that product [148], [149].

# AIM AND OBJECTIVES OF THE RESEARCH

## 1. AIM

Two main goals of my PhD project were: 1. the determination and the comparison of antimicrobial (mainly antistaphylococcal) activity of bee pollen and bee bread samples collected from Polish apiaries; 2. investigation of the microbiota associated with these two bee products, particularly in terms of pharmacological (ability to produce antimicrobial metabolites and probiotic potential) and industrial (ability to enzyme synthesis) potential of isolated strains of bacteria.

## 2. OBJECTIVES

To fulfill these aims of the research, the following objectives have been set:

A. To investigate the antimicrobial activity of bee pollen and bee bread:

- Preparation of the collection of honey, bee pollen, and bee bread samples from Polish apiaries,
- Determination of the antibacterial activity of bee pollen and bee bread ethanolic and aqueous extracts against reference strains of important pathogenic bacteria: *S. aureus* (ATCC 25923, ATCC 29213, CCM 4223), *S. epidermidis* (ATCC 12228), *E. coli* (ATCC 25922, ZPM90), *P. aeruginosa* (ATCC 27853, CCM 1960), *P. mirabilis* (ZPM82) and *E. fecalis* ZPM118),
- Determination of antistaphylococcal activity of BP and BB extracts against clinical isolates of *S. aureus,* including MSSA and MRSA strains,
- Investigation of the possible mechanism of the antimicrobial action of bee pollen and bee bread extracts;

B. To investigate the microbiota of bee pollen and bee bread and its pharmacological and industrial potential:

- Isolation of a wide number of bacteria from selected bee pollen and bee bread samples,
- Screening of the abilities of isolated strains to produce antimicrobials and enzymes,
- Identification and classification of isolates with the most promising abilities using 16S rRNA sequencing and Whole Genome Sequencing,
- Examination of the probiotic potential of *Bacillus* strains isolated from bee pollen and bee bread samples.

# BEE BREAD EXHIBITS HIGHER ANTIMICROBIAL POTENTIAL COMPARED TO BEE POLLEN

**Karolina Pełka[1], Olga Otłowska[1], Randy W. Worobo[2] and Piotr Szweda[1]**

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The flower pollen collected by bees is called bee pollen (BP). It consists of many valuable ingredients, such as polyphenols, fatty acids, vitamins, and microelements. However, it is significantly susceptible to microbial spoilage. Therefore, to prevent the products before microbiota development, it is fermented under anaerobic conditions, mainly due to the presence of Lactic Acid Bacteria and salivary enzymes from bees. However, the particular mechanism of biotransformation is still unknown. The product after fermentation is named bee bread (BB) and is the main source of proteins and fat for larvae and young bees.

The first publication (Article 1.), which was published in Antibiotics (MDPI), focused on examination and comparison of the antimicrobial potential of ethanolic extracts of bee pollen and bee bread derived from Polish apiaries.

The research material consists of 30 samples of BP and 19 samples of BB that were obtained directly from the beekeepers or purchased at the grocery stores and harvested between the 1st of May and the 15th of September 2019. The antimicrobial activity of the collected samples was investigated against common Gram-positive and Gram-negative human pathogens – *S. aureus, S. epidermidis, P. aeruginosa,* and *E. coli*. Furthermore, to determine the antistaphylococcal activity of the tested samples, the extracts of BP and BB were tested against six methicillin-susceptible *S. aureus* strains (MSSA) and three MRSA strains that were obtained from patients from Medical University of Gdansk.

To determine Total Phenolic Content (TPC) of the selected samples, the Folin-Ciocalteu spectrophotometric method was used. Furthermore, to assess the antibacterial activity of BP and BB ethanolic extracts, the minimal inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and kill-time assays were performed. In the MIC and MBC assays, the final concentrations of the ethanolic extracts were in the range of 0.039% to 20% (v/v). For the identification of the MIC values, after incubation of 96-well plates, the resazurin test was conducted. To determine MBC values, the content of the wells after incubation was transferred to Baird-Parker agar plates using a 48-well microplate replicator. For the investigation of the kinetics of bactericidal effects, two samples (most active) of both products were selected. The kill-time assay was performed in two variants: in the first, the extracts of selected samples were added to the cell suspensions of *S. aureus*, and in the second, the cells were incubated in water suspensions of the products.

The results of the MIC and MBC assays showed that the ethanolic extracts of BB exhibited higher antimicrobial activity compared to the extracts of BP - the MIC values ranged from 2.5

to 10% (v/v) and from 5 to >20% (v/v), respectively. Furthermore, the extracts were more effective against staphylococci compared to other Gram-negative bacteria, which confirms the results obtained in several other studies. It should be noted that the TPC estimated for the extracts was comparable among all BP and BB samples and was not correlated with the antimicrobial potential of BP and BB. Phenolic compounds do not directly cause the antimicrobial effect of tested products. Perhaps other ingredients of BP and BB, such as PUFAs, are involved in the inhibition of bacterial growth. The high antistaphylococcal potential of BB and BP was verified in MIC assay against clinical isolates of *S. aureus*, where the higher antimicrobial potential of BB compared to BP was confirmed (MIC values were in the range of 2.5 – 5.0% (v/v) and 5.0 – 10% (v/v), respectively). Furthermore, the extracts were significantly effective against MSSA and MRSA strains; thus, it supports the idea of the application of bee products in the treatment of bacterial infections, including those caused by resistant bacteria.

The kill-time assay provides results related to inhibition of staphylococcal growth. In the case of ethanolic extracts, the bactericidal effect was obtained when the concentration of the extracts was four times higher compared to the MIC values. Incubation of *S. aureus* in raw BP and BB suspensions also inhibited bacterial growth - the concentration of the samples ranged from 2 to 10% (w/v). In three out of four selected samples, the bactericidal effect was clearly visible in the concentration of 10% (w/v) during 24 hours of incubation.

This research indicated that bee pollen and bee bread extracts and aqueous suspensions of these products exhibited antibacterial, mainly antistaphylococcal activity. Therefore, bee pollen and bee bread deserved to be investigated as therapeutic agents, especially against Gram-positive bacteria.

Article 1.

**Bee Bread Exhibits Higher Antimicrobial Potential Compared to Bee Pollen**

Antibiotics 2021, 10, 125; doi:10.3390/antibiotics10020125

Accepted: 25/01/2021 / Published: 28/01/2021

$IF_{2021}$ = 5.222, Q1, MHES Points: 70

*antibiotics*

MDPI

*Article*

# Bee Bread Exhibits Higher Antimicrobial Potential Compared to Bee Pollen

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**Abstract:** This study aimed at investigation of the antimicrobial potential of ethanolic extracts of bee bread (BB) and bee pollen (BP) and suspensions of these products in MHB (Mueller Hinton Broth). We covered 30 samples of BP and 19 samples of BB harvested in Polish apiaries. Slightly lower activity was observed against Gram-negative bacteria compared to Gram-positive staphylococci. BB extracts exhibited higher inhibitory potential with minimum inhibitory concentration (MIC) values in the range from 2.5 to 10% (*v*/*v*) against *Staphylococcus aureus* ATCC 25923 and ATCC 29213. Most active BB extracts, namely, BB6, BB11 and BB19, effectively inhibited growth of clinical isolates of *S. aureus* ($n = 9$), including MRSA (*methicillin resistant Staphylococcus aureus*) strains ($n = 3$) at concentrations ranging from 2.5 to 5.0% (*v*/*v*). Minimal bactericidal concentration (MBC) values were in the same range of concentrations; however, a shift from 2.5 to 5.0% (*v*/*v*) was observed for some products. The most active BP extracts inhibited the growth of reference strains of *S. aureus* at a concentration of 5% (*v*/*v*). Up to the concentration of 20% (*v*/*v*) three and seven BP extracts were not able to inhibit the growth of *S. aureus* ATCC 29213 and *S. aureus* ATCC 25923 respectively. The growth of staphylococci was also importantly inhibited in suspensions of the products in MHB. No correlation between phenolic content and antimicrobial activity was observed.

**Keywords:** bee pollen; bee bread; *Staphylococcus aureus*; antimicrobial activity



**Citation:** Pełka, K.; Otłowska, O.; Worobo, R.W.; Szweda, P. Bee Bread Exhibits Higher Antimicrobial Potential Compared to Bee Pollen. *Antibiotics* **2021**, *10*, 125. https://doi.org/10.3390/antibiotics10020125

Academic Editor: Jean-Marc Sabatier
Received: 18 December 2020
Accepted: 25 January 2021
Published: 28 January 2021



## 1. Introduction

A healthy microbiota is absolutely crucial for a developing bee colony. The whole surface of the hive, including the combs, is covered with propolis—a highly agglutinative, resinous substance of complex chemical composition that is collected by bees from flower and leaf buds. Some of its ingredients, mainly polyphenols and flavonoids, exhibit high antimicrobial activity and protect the bee colony against dangerous pathogens from the hive environment [1–4]. A high concentration of sugars (high osmotic pressure), bee defensins, enzymatic production of hydrogen peroxide and phytochemicals protect the honey against microbial spoilage and development of pathogenic microorganisms for bees [5–10]. Bees also collect pollen, which is a primary source of protein and fat for larvae and young bees [11]. Pollen is the male reproductive cell of the flower. Beekeepers collect pollen granules and sell it on the market. Recently, this product has gained popularity among consumers. Bee pollen (BP) is considered as a healthy/functional food; it contains all the essential amino acids needed by bees, but also human bodies. It is a rich source of fatty acids, vitamins and microelements [12,13]. Moreover, it exhibits a wide range of therapeutic properties, such us antimicrobial, antioxidant, anti-radiation, anti-inflammatory, anti-tumor, hepatoprotective and chemopreventive/chemoprotective benefits [12–16]. High contents of polyphenols and other ingredients that exhibit antibacterial and antifungal activity only partly inhibit the growth of microorganisms and protect pollen grains against microbial spoilage. The drying process is required to ensure the microbiological safety of BP that

is proposed for consumers. It is important to perform this process in mild conditions, at ambient temperature to protect the health beneficial ingredients of this product.

As mentioned above, the BP collected by bees is susceptible to microbiological deterioration. In fact, for bees the pollen is only the raw material for production of bee bread (BB). In hives, some of the BB is stored in the wells of the honeycomb through the winter and in the spring it is used as food for new populations of bee larvae. Bee workers collect pollen from plant anthers, mix it with a small dose of the secretion from salivary glands and/or nectar and place it in specific baskets (corbiculae) which are situated on the tibia of their hind legs. These pollen loads are transported to the hive. Subsequently, pollen loads are packed in the honeycomb cells, and covered with a thin layer of honey and a waxy lid. In these anaerobic conditions, bee pollen undergoes fermentation and biochemical changes that also constitute a method of preservation for the final product of the process—bee bread [16–19]. The exact mechanism of the biotransformation processes of BP to BB remains not fully understood. However, it is known that different enzymes from bees' glands (e.g., amylases that are responsible for starch hydrolysis) and bacteria that are present in bees' saliva and on the surfaces of pollen loads (mostly lactic acid bacteria—LAB but also bacteria of the *Pseudomonas* genus and yeast of the *Saccharomyces* genus) are crucial for this process [16,18,20,21]. The development of the population of LAB, hydrolysis of triacylglycerols and production of lactic acid and probably other metabolites of antimicrobial activity (e.g., bacteriocins) is certainly of primary importance for preservation of BB [20,21]. Additions of honey and polyphenols that are present in the raw material (BP) enhance the antimicrobial potential of BB and allow for long term storage of this product in the hive.

A healthy microbiota is crucial for bee larvae and young bees that are fed with BB. Another important and interesting aspect is the ability of using the antimicrobial potential of BB and BP for prophylaxis and treatment of bacterial and fungal infections of humans and animals. The primary goals of this study were the assessment and comparison of the antimicrobial abilities of ethanolic extracts of ingredients of BP and BB produced in Polish apiaries.

## 2. Results

The outcomes of this study revealed differences in the antimicrobial activity of ethanolic extracts from BB and BP. Moreover, slight differences in the susceptibility of Gram-positive and Gram-negative bacteria were also observed (Table 1). Gram-negative *P. aeruginosa*, and particularly *Escherichia coli*, exhibited higher levels of resistance compared to Gram-positive staphylococci. In the case of both *Staphylococcus aureus* reference strains, minimum inhibitory concentration (MIC) values of BB extracts were in the range of concentrations from 2.5 to 10% (*v*/*v*), and from 5 to 10% (*v*/*v*) for *Staphylococcus epidermidis* ATCC 12228. The highest activity (MIC = 2.5% (*v*/*v*)) was observed for three extracts (against *S. aureus* ATCC 25923) and for 12 products (against *S. aureus* ATCC 29213). At least 10% and 20% (*v*/*v*) concentrations were required for growth inhibition of *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922, respectively. The highest susceptibility to the activity of extracts produced from BP was observed with *S. epidermidis* ATCC 12228; three out of thirty tested extracts (assigned with numbers 11, 15 and 17) inhibited the growth of this strain at a concentration of 5% (*v*/*v*); MIC values for other extracts were 10 or 20% (*v*/*v*). Two products, with numbers 15 and 20, effectively inhibited the growth of *S. aureus* ATCC 29213 at concentrations equal to 5% (*v*/*v*). However, six BP extracts did not exhibit any activity against this strain up to the concentration of 20% (*v*/*v*). At least 10% (*v*/*v*) concentration of BP extracts was necessary for growth inhibition of *S. aureus* ATCC 25923, and one of these extracts was not active even at the highest investigated concentration of 20% (*v*/*v*). The effectiveness of BP extracts against *P. aeruginosa* ATCC 27853 was comparable to the activity of extracts produced from BB, except one (not active up to the concentration of 20% (*v*/*v*)); these products inhibited the growth of this strain at concentrations equal to 10 or 20% (*v*/*v*). *E. coli* ATCC 25922 exhibited lower susceptibility to BP extracts; 14 products did not exhibit activity up to the concentration of 20% (*v*/*v*); and the MIC for other products ($n$ = 16)

was 20% ($v/v$). As is shown in Table 1, for many of the extracts, particularly produced from BP, a shift of minimal bactericidal concentration (MBC) values compared to MIC was observed—higher concentrations were necessary to achieve a bactericidal effect compared to growth inhibition. Clear differences were observed for *S. aureus* ATCC 25923; MBC values of 23 products were higher compared to MIC. Twenty BP extracts did not exhibit bactericidal activity against *S. epidermidis* ATCC 12228 up to the concentration of 20% ($v/v$) and the same values of MBC and MIC parameters were found for only three extracts. Only four BP extracts effectively killed *E. coli* ATCC 25922 at the highest investigated concentration of 20% ($v/v$). Considering two other reference strains of bacteria, the shift of MBC values compared to MIC was observed for 9 and 12 BP extracts for *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 29213, respectively. The differences in MBC and MIC values for BB extracts were also observed. However, the differences were not so evident. The MBC values for all staphylococci and *P. aeruginosa* ATCC 27853 were $\leq$20% ($v/v$) and only six BB extracts were not capable of killing *E. coli* ATCC 25922 cells at the highest investigated concentration of 20% ($v/v$). Similarly, in the case of BP extracts, the most important differences in MIC and MBC values were found for *S. epidermidis* ATCC 12228—15 out of 19 of the extracts tested. On the other hand, only four extracts exhibited differences in effective inhibitory and bactericidal activity against *P. aeruginosa* ATCC 27853. Considering *S. aureus* reference strains differences between MBC and MIC values were noted for 10 and 8 BB extracts for *S. aureus* ATCC 29213 and *S. aureus* ATCC 25923, respectively.

The high anti-staphylococcal potential of extracts produced from BB and BP collected in Polish apiaries was confirmed for clinical isolates, including six methicillin-susceptible strains (MSSA) and three isolates that were methicillin-resistant (MRSA) (Table 2). The investigation was performed for three BB extracts with numbers: 4, 11 and 14 and three extracts produced from pollen, with numbers 9, 15 and 20. These extracts were found to be highly active against reference strains of staphylococci. The results of the assay confirmed the high anti-staphylococcal potential of the extract produced from both types of raw materials, namely, BP and BB. However, similarly to the case of reference strains of staphylococci, BB extracts exhibited higher inhibitory potential with MIC values in the range from 2.5 to 5.0% ($v/v$) against all strains tested, including MRSA isolates. In addition, the MBC values were in the same range of concentrations. However, in some cases, a two times higher concentration of BB extract was necessary to achieve a bactericidal effect in comparison to MIC value (shift from 2.5 to 5% ($v/v$)). The MIC values of BP extracts against clinical isolates of *S. aureus* were in the range from 5 to 10% ($v/v$); the products with numbers 15 and 20 generally exhibited higher activity compared to BP9. The MBC values were in the range from 5 to 20% ($v/v$). However, in most cases the values of MIC and MBC were exactly the same. For BP9, BP15 and BP20 extracts, the shift of MBC value compared to MIC was observed for only two, one and two strains, respectively.

Using a slightly modified Folin–Ciocalteu method [22], we determined total phenolic content in the produced extracts (Table 1). The content of polyphenols, expressed as milligrams of gallic acid equivalent (GAE) per gram of the product, ranged from 13.94 to 21.054 mg GAE/g for BP extracts and from 16.877 to 20.179 mg GAE/g for BB extracts. Most importantly, no correlation between the antibacterial efficacy and concentrations of polyphenols was observed. In the study of Markiewicz-Żukowska [23], who investigated three samples of BB collected by Polish beekeepers, the total phenolic content (TPC) values ranged from 32.78 to 37.15 mg GAE/g. These differences in concentration for polyphenols can be explained by some differences in extractions procedures that were applied in both studies.

**Table 1.** Antimicrobial activity and total phenolic content (TPC) of extracts produced from bee pollen (BP) and bee bread (BB) samples harvested in Polish apiaries.

| Product | *S. aureus* ATCC 253923 | | *S. aureus* ATCC 29213 | | *S. epidermidis* ATCC 12228 | | *E. coli* ATCC 25922 | | *P. aeruginosa* ATCC 27853 | | TPC [mg GAE/g] | Sample Location |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | MIC (*v*/*w*) [%] | MBC (*v*/*w*) [%] | MIC (*v*/*w*) [%] | MBC (*v*/*w*) [%] | MIC (*v*/*w*) [%] | MBC (*v*/*w*) [%] | MIC (*v*/*w*) [%] | MBC (*v*/*w*) [%] | MIC (*v*/*w*) [%] | MBC (*v*/*w*) [%] | | |
| BP1 | 10 | >20 | 10 | 20 | 10 | >20 | >20 | >20 | 20 | 20 | 21.05 ± 0.09 | Szczytno [a] |
| BP2 | 20 | >20 | 20 | >20 | 20 | >20 | >20 | >20 | 10 | 20 | 20.61 ± 0.16 | Mielec [a] |
| BP3 | 10 | 20 | >20 | >20 | 10 | >20 | >20 | >20 | 10 | 20 | 20.92 ± 0.18 | Mielec [a] |
| BP4 | 20 | >20 | >20 | >20 | 20 | >20 | 20 | >20 | 20 | 20 | 20.96 ± 0.23 | Mielec [a] |
| BP5 | 10 | >20 | 10 | 10 | 10 | >20 | 20 | 20 | 20 | 20 | 20.74 ± 0.06 | Gdańsk [a] |
| BP6 | 10 | 20 | 20 | 20 | 20 | >20 | 20 | >20 | 20 | 20 | 20.37 ± 0.17 | Kozaki |
| BP7 | 10 | 20 | 10 | 20 | 10 | 20 | 20 | 20 | 20 | 20 | 20.81 ± 0.09 | Koryciny |
| BP8 | 20 | >20 | >20 | >20 | 20 | >20 | 20 | >20 | >20 | >20 | 20.76 ± 0.28 | Stróże |
| BP9 | 10 | 20 | 10 | 10 | 10 | 10 | 20 | >20 | 20 | 20 | 20.49 ± 0.43 | Czarne |
| BP10 | 10 | 20 | 20 | 20 | 10 | >20 | 20 | >20 | 20 | 20 | 20.99 ± 0.16 | Brusy |
| BP11 | 10 | 20 | 10 | 20 | 5 | 20 | 20 | >20 | 10 | 20 | 20.40 ± 0.06 | Bielsko-Biała [a] |
| BP12 | 20 | >20 | >20 | >20 | 20 | >20 | >20 | >20 | 10 | 20 | 19.95 ± 0.22 | Gdańsk [a] |
| BP13 | 10 | 20 | 10 | 20 | 10 | 20 | >20 | >20 | 10 | 10 | 20.93 ± 0.19 | Cychry |
| BP14 | 10 | >20 | 20 | >20 | 20 | 20 | >20 | >20 | 10 | 20 | 19.91 ± 0.18 | Malbork [a] |
| BP15 | 10 | 10 | 5 | 5 | 5 | 10 | 20 | 20 | 10 | 10 | 19.32 ± 0.12 | Niżna Łąka |
| BP16 | 20 | 20 | 10 | 20 | 10 | 10 | >20 | >20 | 20 | 20 | 20.39 ± 0.35 | Siedlce [a] |
| BP17 | 20 | 20 | 20 | 20 | 5 | 20 | 20 | >20 | 10 | 20 | 16.38 ± 0.35 | Miłogoszcz |
| BP18 | 20 | >20 | >20 | >20 | 10 | 20 | 20 | >20 | 10 | 20 | 13.95 ± 0.50 | Miłogoszcz |
| BP19 | 20 | >20 | 10 | 20 | 20 | >20 | >20 | >20 | 10 | 20 | 19.81 ± 0.35 | Miłogoszcz |
| BP20 | 10 | 10 | 5 | 5 | 10 | 20 | >20 | >20 | 10 | 10 | 19.60 ± 0.41 | Modzele |
| BP21 | 20 | >20 | >20 | >20 | 20 | >20 | >20 | >20 | 20 | 20 | 16.17 ± 0.81 | Miłogoszcz |
| BP22 | 20 | 20 | 20 | >20 | 10 | >20 | 20 | >20 | 10 | 10 | 20.56 ± 0.34 | Wałcz [a] |
| BP23 | 20 | 20 | 10 | 10 | 10 | >20 | 20 | >20 | 20 | 20 | 14.79 ± 0.20 | Miłogoszcz |
| BP24 | 10 | 20 | 10 | 20 | 10 | >20 | 20 | 20 | 10 | 10 | 20.24 ± 0.18 | Czaplinek |
| BP25 | 10 | 20 | 10 | 20 | 20 | >20 | 20 | >20 | 10 | 10 | 20.95 ± 0.32 | Stanisławowo |
| BP26 | 10 | >20 | 10 | 10 | 20 | >20 | 20 | >20 | 20 | 20 | 19.08 ± 0.14 | Mielec [a] |
| BP27 | >20 | >20 | 20 | >20 | 20 | >20 | >20 | >20 | 20 | 20 | 20.40 ± 0.08 | Miłogoszcz |
| BP28 | 20 | >20 | 20 | >20 | 20 | >20 | >20 | >20 | 20 | 20 | 18.34 ± 0.25 | Mielec [a] |
| BP29 | 10 | 20 | 10 | 10 | 10 | >20 | >20 | >20 | 10 | 20 | 16.64 ± 0.21 | Miłogoszcz |
| BP30 | 10 | 20 | 10 | 10 | 20 | >20 | >20 | >20 | 10 | 10 | 18.94 ± 0.21 | Pelplin [a] |
| BB1 | 10 | 10 | 2.5 | 5 | 5 | 10 | 20 | >20 | 10 | 20 | 20.18 ± 1.22 | Legnica [a] |
| BB2 | 5 | 5 | 2.5 | 5 | 5 | 20 | 20 | 20 | 10 | 10 | 19.47 ± 0.38 | Malbork [a] |
| BB3 | 10 | 20 | 5 | 5 | 5 | 10 | 20 | 20 | 10 | 10 | 16.88 ± 0.52 | Bielsko-Biała [a] |
| BB4 | 5 | 5 | 2.5 | 5 | 5 | 10 | 20 | >20 | 10 | 10 | 19.42 ± 0.31 | Cychry |
| BB5 | 5 | 5 | 2.5 | 5 | 5 | 10 | 20 | 20 | 10 | 10 | 17.65 ± 0.29 | Stanisławowo |
| BB6 | 2.5 | 5 | 2.5 | 5 | 5 | 10 | 20 | >20 | 10 | 10 | 17.03 ± 0.41 | Czaplinek |
| BB7 | 5 | 10 | 2.5 | 2.5 | 5 | 10 | 20 | 20 | 10 | 20 | 18.89 ± 0.43 | Mielec [a] |

**Table 1.** *Cont.*

| Product | *S. aureus* ATCC 253923 | | *S. aureus* ATCC 29213 | | *S. epidermidis* ATCC 12228 | | *E. coli* ATCC 25922 | | *P. aeruginosa* ATCC 27853 | | TPC [mg GAE/g] | Sample Location |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | MIC (*v*/*w*) [%] | MBC (*v*/*w*) [%] | MIC (*v*/*w*) [%] | MBC (*v*/*w*) [%] | MIC (*v*/*w*) [%] | MBC (*v*/*w*) [%] | MIC (*v*/*w*) [%] | MBC (*v*/*w*) [%] | MIC (*v*/*w*) [%] | MBC (*v*/*w*) [%] | | |
| BB8 | 5 | 5 | 5 | 5 | 5 | 10 | 20 | >20 | 10 | 20 | 19.07 ± 0.04 | Mielec [a] |
| BB9 | 5 | 5 | 2.5 | 5 | 5 | 10 | 20 | >20 | 10 | 10 | 17.15 ± 0.25 | nd |
| BB10 | 5 | 10 | 2.5 | 5 | 10 | 10 | 20 | 20 | 10 | 10 | 19.50 ± 0.33 | Brusy |
| BB11 | 2.5 | 2.5 | 2.5 | 2.5 | 5 | 10 | 20 | 20 | 10 | 10 | 18.98 ± 0.18 | Częstochowa [a] |
| BB12 | 5 | 5 | 2.5 | 5 | 10 | 10 | 20 | >20 | 10 | 10 | 18.64 ± 0.13 | Miłogoszcz |
| BB13 | 5 | 5 | 2.5 | 2.5 | 10 | 10 | 20 | 20 | 10 | 10 | 19.66 ± 0.13 | Malbork [a] |
| BB14 | 5 | 5 | 2.5 | 2.5 | 10 | 10 | 20 | 20 | 10 | 10 | 18.54 ± 0.11 | Suchorzew |
| BB15 | 5 | 10 | 5 | 5 | 5 | 10 | 20 | 20 | 10 | 10 | 18.20 ± 0.30 | Miłogoszcz |
| BB16 | 2.5 | 5 | 5 | 5 | 5 | 10 | 20 | 20 | 10 | 10 | 18.49 ± 0.29 | Miłogoszcz |
| BB17 | 10 | 10 | 5 | 10 | 5 | 10 | 20 | 20 | 10 | 10 | 19.04 ± 0.26 | Majdan Starowiejski |
| BB18 | 5 | 5 | 5 | 5 | 5 | 10 | 20 | 20 | 10 | 10 | 18.60 ± 0.46 | Warka |
| BB19 | 10 | 20 | 5 | 10 | 10 | 20 | 20 | 20 | 20 | 20 | 17.70 ± 0.38 | Modzele |

The underlined samples were bought from shops; other samples were provided by beekeepers. "a" indicates that the apiary was located in an area near the presented city (not exactly in the city/urban area).

**Table 2.** Antibacterial activity of selected ethanolic extracts of BP and BB against clinical isolates of *Staphylococcus aureus*. Isolates 1–6 are methicillin-sensitive and strains 7–9 exhibit the methicillin-resistant phenotype.

| Product | BP9 | | BP15 | | BP20 | | BB6 | | BB11 | | BB14 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Strain No. | MIC [*v*/*w*] [%] | MBC [*v*/*w*] [%] | MIC [*v*/*w*] [%] | MBC [*v*/*w*] [%] | MIC [*v*/*w*] [%] | MBC [*v*/*w*] [%] | MIC [*v*/*w*] [%] | MBC [*v*/*w*] [%] | MIC [*v*/*w*] [%] | MBC [*v*/*w*] [%] | MIC [*v*/*w*] [%] | MBC [*v*/*w*] [%] |
| 1 | 10 | 20 | 10 | 10 | 10 | 10 | 5 | 5 | 2.5 | 5 | 5 | 5 |
| 2 | 10 | 10 | 5 | 5 | 5 | 5 | 5 | 5 | 2.5 | 2.5 | 2.5 | 5 |
| 3 | 10 | 10 | 10 | 10 | 10 | 10 | 5 | 5 | 5 | 5 | 2.5 | 2.5 |
| 4 | 10 | 20 | 10 | 10 | 10 | 20 | 2.5 | 5 | 2.5 | 5 | 5 | 5 |
| 5 | 10 | 20 | 5 | 10 | 5 | 10 | 2.5 | 2.5 | 2.5 | 5 | 5 | 5 |
| 6 | 10 | 10 | 5 | 5 | 5 | 5 | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 | 5 |
| 7 | 10 | 10 | 5 | 5 | 5 | 5 | 2.5 | 5 | 2.5 | 5 | 2.5 | 2.5 |
| 8 | 10 | 10 | 5 | 5 | 5 | 5 | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 |
| 9 | 10 | 10 | 5 | 5 | 5 | 5 | 2.5 | 2.5 | 2.5 | 2.5 | 5 | 5 |

**Kinetics of the bactericidal action of BP and BB extracts.**

Four selected extracts, two produced from BP (assigned as 15 and 20) and two produced from BB (assigned with numbers 6 and 11), were used for the determination of the bactericidal effects of ingredients extracted from the raw materials with ethanol (70% *v*/*v*) against *S. aureus* ATCC 25923. As expected, at MICs, all products resulted in only a growth inhibition effect. Slightly higher—though still classified as inhibitory—activity was observed for three products, namely, BB6, BB11 and BP15, when used at concentrations equal to 2 × MIC; extract from bee pollen number 15 exhibited lower antibacterial activity. At concertation 4 × MIC, the extracts from both bee bread samples, and surprisingly BP15, resulted in completely bactericidal effects after 8 h of incubation. Activity of the extract

BP20 was considerably lower. However, complete elimination of living cells of bacteria was achieved after 24 h of incubation (Figure 1).

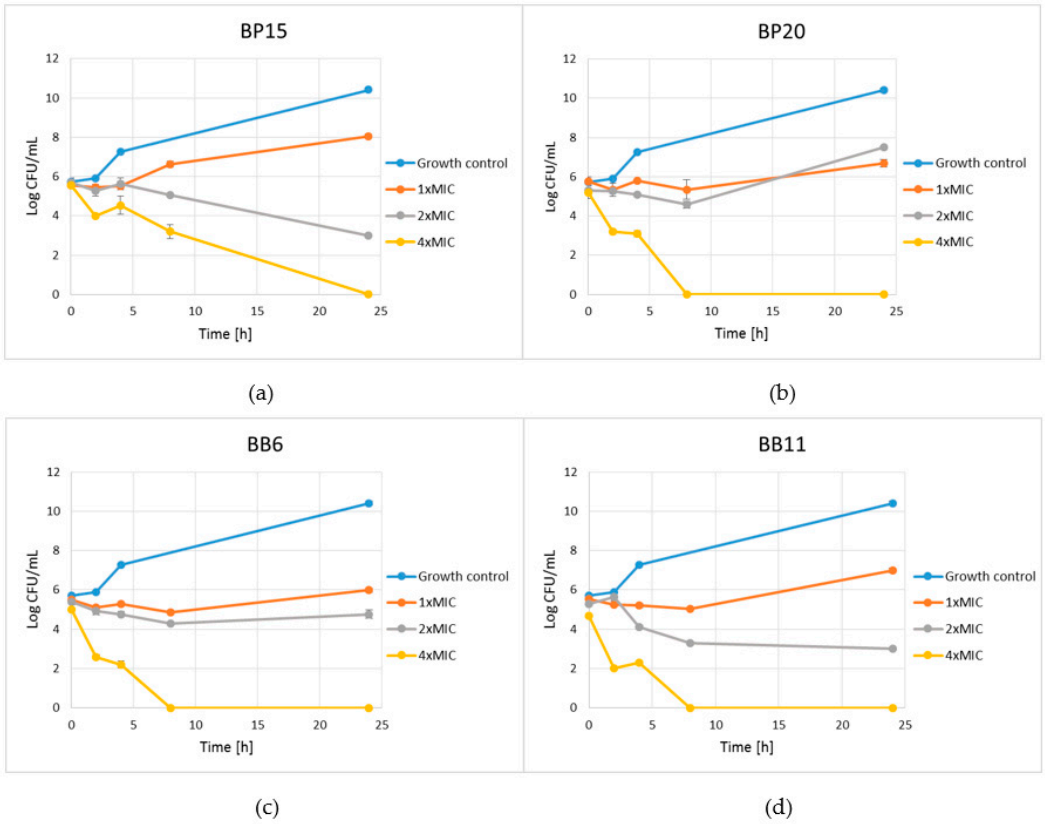


**Figure 1.** Kill-time assay for selected ethanolic extracts of bee pollen and bee bread tested against *S. aureus* ATCC 29213 at or above the MIC. The growth control contained no extracts; (**a**) BP15, (**b**) BP20, (**c**) BB6, (**d**) BB11. The results are presented as means ± SDs ($n = 3$). Data without error bars indicate that the SDs are too small to be observed on the graph.

A promising antimicrobial effect was also observed during incubation of *S. aureus* ATCC 29213 cells in suspensions of selected products in MHB medium (Figure 2). At a concentration of 2.0% ($w/v$), after 24 h of incubation, all products inhibited the growth of the bacterial cells by about 90%, compared to the control sample. In all cases, only a slightly higher growth inhibition effect was observed for suspensions containing 5.0% of the products. Much better effectiveness was achieved in suspensions containing 10% ($w/v$) of the products. After 24 h of incubation, the decreased level of growth inhibition of more than four log cycles was observed for both bee bread samples, from LogCFU/mL 10.1—control sample to 5.7 and 5.2 for BB11 and BB6, respectively. An important growth inhibition level, from LogCFU/mL 10.1—control sample to 6.1—exhibited 10% ($w/v$) suspension of BP20. The efficiency of BP15 was slightly lower. However, even in the case of this product, about 99.9% (about three log cycles, from 10.1 to 7.2.) growth inhibition was observed after 24 h of incubation.

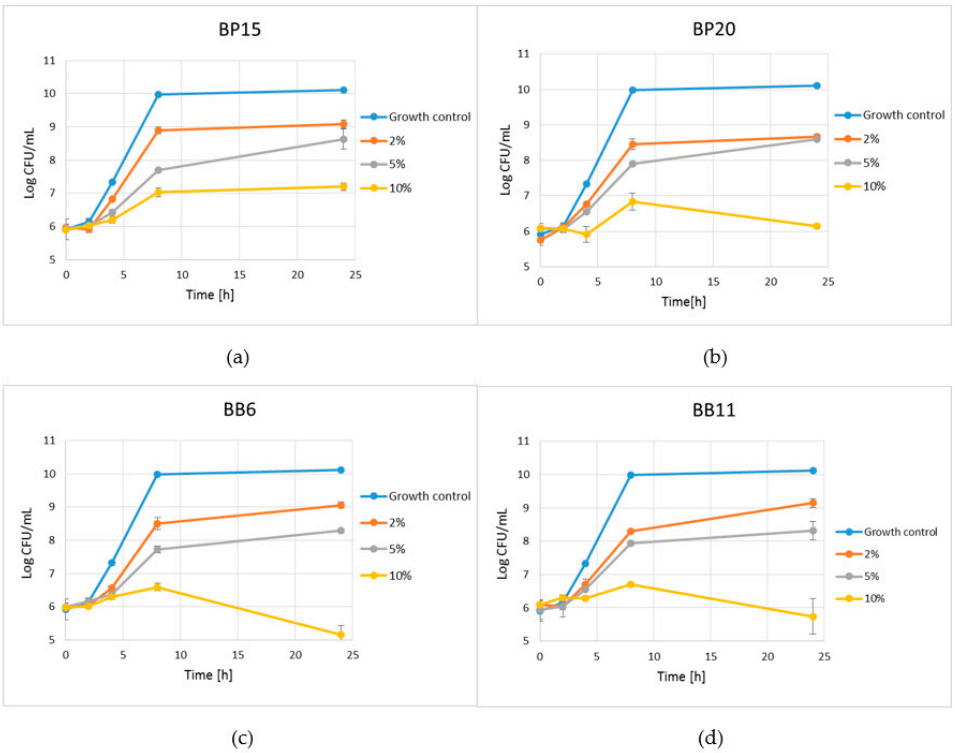


**Figure 2.** Kill-time assay for suspensions of selected products tested against *S. aureus* ATCC 29213 in final concentrations of 2%, 5% and 10% (*v*/*w*). The growth control did not contain any product; (**a**) BP15, (**b**) BP20, (**c**) BB6, (**d**) BB11. The results are presented as means ± SDs ($n$ = 3). Data without error bars indicate that the SDs are too small to be observed on the graph.

## 3. Discussion

Antimicrobial potential of bee honey and propolis has been known since ancient times. These two products belonged to the most important and most common antimicrobial agents of folk medicine that were used for treating of infections, particularly for treatment of difficult to heal infected wounds [1,7,24]. The antimicrobial potential of bee pollen and bee bread is definitely less known and less investigated [16]. We still do know if there are important differences in antibacterial potential of BB and BP, spectrum of activity also remains not clear, some authors suggest that Gram-positive bacteria exhibit higher susceptibility and, which is most important, ingredients that are crucial for antimicrobial activity of these product are not identified. To date, most research has focused on the high nutritional value of these products. Due to high content of health beneficial ingredients, including vitamins [25–27], micro and macro-elements [12,28] fatty acids [23,29–31], amino acids [32,33] and also different groups of phytochemicals—mostly important as antioxidants [16,23,34], BP and BB are considered functional foods. However, the outcomes of recent research showed promising antimicrobial potential of BB and BP produced in apiaries located in different regions of the world. In this study, we have investigated relatively large numbers of samples of both products, namely, 30 samples of BP and 19 samples of BB. To our best knowledge presented to date, reports were based on analysis of lower numbers of samples, in some cases singular products. Analyses of properties of a large group of products led us to conclusion that BB extracts (MIC against staphylococci in the range of

concentrations from 2.5 to 10% $v/v$) exhibit higher antimicrobial potential compared to the activity of BP extracts (MIC values against staphylococci in the range of concentrations from 5 to >20% $v/v$). We assume that it is a consequence of the process of fermentation that is the base for transformation of raw material—BP to the final product—BB. During the fermentation process the enzymes that were added to the raw material by bees and also produced by bacteria that were present on the surface of pollen and in the bees' saliva result in partial digestion of biopolymers (mostly polysaccharides) that cover pollen grains. As a consequence, the ingredients of pollen located inside the grains are more accessible to the solvents and easier to extract. This hypothesis seems to be not supported by the results of investigation of TPC—the extracts produced from both products exhibit comparable values of this parameter. However, Markiewicz-Żukowska and coworkers [23] revealed that BB extracts contain many other components that exhibit antimicrobial activity except for polyphenols. The most important of them seem to be aliphatic acids. Aliphatic acids were found to be the predominant components of the extracts investigated by the group of Markiewicz-Żukowska (62.32 ± 7.0%) and unsaturated, α-linolenic, linoleic, oleic and 11,14,17-eicosatrienoic acids formed more than a half of them (40.63 ± 4.5%). Moreover, Vasquez and Olofsson [21] and Iorizzo [35,36] revealed the presence in BB and BP the presence of Lactic Acid Bacteria (LAB) e.g., *Lactobacillus kunkeei* [35], *L. plantarum* [36] that are able to produce metabolites (e.g., bacteriocins), which exhibit high antimicrobial potential. These bacteria readily grow within the first step of biotransformation of BP to BB—for approximately two weeks [21]. Within this time, they produce and transport to the maturing BB antimicrobial metabolites that include lactic acid and bacteriocins, but also participate in lipid hydrolysis and production of aliphatic acids. All these aspects together are likely the reason for higher antimicrobial activity of BB compared to BP. BB is absolutely necessary for feeding young bee larvae in early spring. Thus high antimicrobial potential (higher than bee pollen) is important benefit of this product, which in fact allows it to be stored in hive during the winter.

In agreement with results presented herein, most of the results presented by other authors confirm higher susceptibility of Gram-positive bacteria to the components of BP or BB extracts. Important higher susceptibility of *S. aureus* compared to *P. aeruginosa* and/or *E.coli* against BP extracts presented for example: Velasquez and coworkers [35] who investigated extract produced from sixteen samples of Chilean pollen samples [37], Pascola and coworkers who investigated eight products from Portugal and Spain [38], Karadal et al. (5 BP samples from Turkey) [39], Abouda et al., (four pollens collected in Morocco) [40], Khider (three Egyptian BP samples) [41] and Graikou who analyzed biological properties of one Greek BP [42]. The same differences in activity against staphylococci and Gram-negative bacteria have been shown for extracts produced form Romanian [43], Malaysian [44], and some of Moroccan samples of BB [40]. However, it should be clearly noticed that currently it is too early to propose a general rule concerning BP or BB extracts against Gram-positive or Gram-negative bacteria. As it is presented above, the number of currently available data is very limited. Moreover, some authors observed contradictory results, e.g., the group of Ivanisova who investigated antibacterial potential of Ukrainian BB [45]. The outcomes of some investigations suggest that method of extraction, such as solvent, can importantly affect different aspects of biological properties of produced extracts, including antimicrobial activity [46–48].

An important advantage of extracts produced from samples of Polish BB and also BP is high efficacy against clinical isolates of *S. aureus*, including MRSA strains. This part of the study additionally confirmed a bit higher inhibitory and also bactericidal activity of BB extracts (MIC and MBC values ranged from 2.5 to 5.0% ($v/v$)) compared to extracts produced from BP (MIC and MBC values ranged from 5.0 to 10.0% ($v/v$)). In our previous study we revealed high activity of honey and propolis produced in Polish apiaries against *S. aureus* isolates that exhibit MRSA phenotype [3,8]. All these results support the idea of application of bee products as alternative antibacterial agents, including treatment of infections caused by resistant strains. Of course, we realize that in clinical scenario potential

application of bee products, similarly as in the case of many other natural products such as essential oils or plant extracts, is limited to topical infections (e.g., treatment of infected wounds or skin infections).

Some important conclusions come also from the analysis of the bactericidal potential of the extracts that was performed with the time-kill kinetic assays. Firstly, it has been shown that achievement of bactericidal effect requires using four times higher concentration compared to the MIC. In microdilution assay the MBC values for most products were two times higher compared to MIC. In our opinion, some differences in the conditions of these two assays should explain the observed differences—shaking (intense aeration) in time-kill kinetic assays is likely the main reason for higher resistance of the staphylococci to the activity of ingredients of the extracts. However, the observed results generally confirm high anti-staphylococcal potential of BP and particularly BB extracts and additionally support the need for more advanced studies focusing on the application of these products for treatment of bacterial infections. Moreover, Olczyk and coworkers revealed that bee pollen ointment may affect the wound healing process of burn wounds, preventing infection of the newly formed tissue [49].

As it was mentioned above we did not observe any correlation between the TPC and antimicrobial activity of produced extracts. However, the values of TPC were quite similar to the results presented by other authors who investigated BP or BB from other different geographical locations e.g., Poland [23], Portugal [50,51], Greece [42], Romania and India [52], or Chile [53].

The last, but not least aspect of our study was to check if growth of staphylococci is affected in water suspension of selected samples of BP and BB (the samples of the products that were used for production of most active extracts were used in this part of the study). In all cases the suspensions containing only 2.5% *w*/*v* of the product efficiently inhibited the growth *S. aureus* ATCC 29213 cells compared to the control. Except for BP20, increase of product concentration resulted in higher growth inhibition activity in concentration dependent manner. At concentration of 10% (*w*/*v*) all products inhibited the growth of bacteria in at least 3 log cycles compared the control. Again, a bit higher activity was observed for BB samples. This part of research clearly indicates that both bee bread and bee pollen contain some antimicrobial components, including polyphenols, fatty acids and bacterial metabolites (produced by endogenous microflora of these raw materials), which are crucial for bee health and also for abilities of storage of some amounts of the BB in the hives during the winter period. It would be very interesting to use this potential to obtain products (extracts) that could be used as antibacterial therapeutic agents.

## 4. Materials and Methods

### *4.1. Bee Pollen (BP) and Bee Bread (BB) Samples*

The study covered 30 samples of BP and 19 samples of BB. The products were harvested between 1 May and 15 September of 2019 in apiaries located in different regions of Poland (Table 1). The pollen loads were collected in special pollen traps that were installed in front of the hive entrance. All samples of BP, even the products that were delivered directly by beekeepers were dried (it protected the product against microbial spoil). The BB was recovered directly from honeycombs in late summer or autumn 2019; thus, only mature bee bread was used for the study. All products were not older than eight months counting form the date of harvesting to the date of preparing the extracts or using them for other assays presented herein. In the case of some products that were bought in shops, we were not able to establish geographic origins—they were not declared by the sellers. All products were stored in the dark, BP was kept at ambient temperature and BB was stored at 4 °C.

### *4.2. Chemicals and Reagents*

All chemicals and reagents were purchased from commercial sources. The Folin–Ciocalteu reagent PBS, methanol, gallic acid and sodium carbonate were purchased from

Merck (Darmstadt, Germany) and ethanol was bought from (POCH, Gliwice, Poland). TheMilli-Q Advantage A10 system (Millipore, Billerica, MA, USA) was used for production of ultrapure $H_2O$ (18.0 MΩ) and Genesys 20 spectrophotometer (Thermo Scientific, Waltham, MA, USA) was used for measurement of absorbance in Folin–Ciocalteu assay.

*4.3. Bacterial Strains and Media*

Five reference strains of bacteria, *S. aureus* ATCC 25923, *S. aureus* ATCC 29213, *S. epidermidis* ATCC 12228, *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922, were applied for preliminary assessments of the antimicrobial potential of all produced BP and BB ethanolic extracts Subsequently anti-staphylococcal activity of selected extracts was evaluated against 6 MSSA (*methicillin-susceptible Staphylococcus aureus*) and 3 MRSA *(methicillin-resistant Staphylococcus aureus*) isolates from patients of the hospital of Medical University of Gdańsk, that suffered with different infections (Table 3). Bacteria were routinely grown on Luria-Bertani Agar (LA, Sigma Aldrich, Schnelldorf, Germany). The determination of the values of Minimum Inhibitory Concentration (MIC) was performed in Mueller-Hinton Broth (MHB, Sigma Aldrich) and for determination of Minimum Bactericidal Concentrations (MBC) the cells were cultivated on the selective Baird Parker Agar plates (Biomaxima, Lublin, Poland). The reference strain *S. aureus* ATCC 29213 was used for antimicrobial potential evaluation of both extracts and suspensions of selected products in kinetic time-kill assay.

**Table 3.** MSSA and MRSA strains used in this work.

| No. | Number/Phenotype | Ward/Material | Antibiogram [1] |
|---|---|---|---|
| 1 | 4471313/MSSA | Intensive care/Nasal swab | Resistant—Pen.<br>Sensitive—Met., Clin., Ery. |
| 2 | 4475564/MSSA | Internal/Nasal swab | Resistant—Pen. Clin. Ery.<br>Sensitive—Met. |
| 3 | 4466686/MSSA | Surgical/Sputum | Resistant—Pen. Clin. Ery.<br>Sensitive—Met. |
| 4 | 4467080/MSSA | Internal/Nasal swab | Resistant—Pen.<br>Sensitive—Met. Clin., Ery. |
| 5 | 4467076/MSSA | Laryngology/A swab from the ear | Resistant—Pen.<br>Sensitive—Met. Clin., Ery.S |
| 6 | 4468505/MSSA | Interna/Nasal swabl | Resistant—Pen. Clin. Ery.<br>Sensitive—Met. |
| 7 | 45300223/MRSA | Pediatrics/Blood | Resistant—Pen. Clin. Ery. Met. |
| 8 | 9935169/MRSA | Dispensary/Wound | Resistant—Pen. Clin. Ery. Met. |
| 9 | 9944662/MRSA | Dermatology/Nasal swab | Resistant—Pen. Clin. Ery. Met. |

[1]—Identification of bacterial isolates and determination of antibiotic susceptibility analysis performed by Laboratory of Clinical Microbiology, University Centre for Laboratory Diagnostics, Medical University of Gdańsk Clinical Centre with Vitek2 Biomerieux system; Pen—penicillin, Met—methicillin, Clin—clindamycin, Ery—erythromycin, R—resistance, S—sensitive.

*4.4. Preparation of BP and BB Ethanolic Extracts*

The rotary platform shaker was used for efficient extraction of active components from raw materials (BP or BB). The suspensions of BP/BB in 70% ethanol at $v/w$ ratio 7:1 were shaken (100 rpm) for 2 h at ambient temperature. Next, the suspensions were centrifuged (2290× $g$, 20 min) and the obtained supernatants were filtered through the sterile, 0.22 μm pore-sized filters (obtained from Millipore, Burlington, MA, USA). Finally, clear (not cloudy) and sterile extracts were obtained and used in subsequent studies.

*4.5. Investigation of Antimicrobial Potential of Alcoholic Extracts of BP and BB—Determination of Values of MIC (Minimum Inhibitory Concentration) and MBC (Minimum Bactericidal Concentration)*

The minimum inhibitory concentrations (MICs) were determined by the two-fold broth microdilution method according to the CLSI standard methodology [54]. All bacterial strains used for the assay (both reference strains and clinical isolates) were cultivated overnight at 37 °C on Luria-Bertani Agar plates. The bacterial suspension of two to three colonies (taken from the L-B Agar plates) in PBS buffer (pH = 7.4) was adjusted to the optical density of $OD_{600}$ = 0.1 and diluted in MHB medium at a ratio of 1:100 $v/v$ to the final cell concentration of approximately $1.0 \times 10^6$ CFU/mL.

Serial two-fold dilutions of the tested extracts of BP or BB (in the range of concentrations from 0.078–40% ($v/v$)) were prepared in 96-well microtitration plates in the final volume of 100 µL of MHB medium (CMHB2). In the next step of the assay, the solutions of the BP and BB extracts in the wells were inoculated with an equal volume (100 µL) of suspension of bacterial cells (prepared as presented above). The final concentrations of inoculated extracts ranged from 0.039% to 20.0% ($v/v$). Ten different concentrations of the extracts were tested: 20.0, 10.0, 5.0, 2.5, 1.25, 0.625, 0.315, 0.156, 0.078 and 0.039% ($v/v$) in the columns 1 to 10 of the microtitration plates. Column 11 contained 200 µL of inoculum (growth control in the medium free of antibacterial agents), and column 12 contained 200 µL of the MHB broth only (as control of sterility of the medium). The plates were incubated 24 h under static conditions at 37 °C. As color and solubility of BP and BB extracts interfered with growth measurement it was necessary to perform the resazurin test. After incubation, resazurin solution (0.015% in PBS buffer) was added to all wells (30 µL), and plates were incubated at 37 °C in the dark for the next two hours. The lowest concentration of the extract with no color change (blue resazurin color remained unchanged) was taken as a MIC value. The same method was applied for determination of activity of the solvent (70% ethanol) that was used for preparing of the extracts and no inhibitory activity was observed even in the wells of column 1, where the concentration of the EtOH was 14%. A sterile 48-well microtiter plate replicator was used for transferring a small volume of each dilution used for MIC assay on Baird-Parker agar plates. Subsequently the plates were incubated for 24 h at 37 °C and growth of characteristic black colonies of *S. aureus* was analyzed. The lowest concentrations of the extracts, where no growth of the colonies was observed, were assigned as MBC.

*4.6. Time-Kill Assay—Determination of Kinetic of Bactericidal Effects of BP and BB Extracts and Suspensions of Raw Materials against Staphylococci*

The kinetic time-kill assay was performed for two most active extracts of BP and BB and also for suspensions of raw materials (two samples of each BP and BB) used for preparing of these extracts. The selected extracts were added to the suspensions of *S. aureus* ATCC 29213 (approx. cell density $1.5 \times 10^6$ CFU/mL) in MHB broth to the final concentration equal to MIC, 2 × MIC and 4 × MIC. In the case of determination of activity of raw materials, the suspension of *S. aureus* ATCC 29213 was supplemented with bee pollen or bee bread to the final concertation of 2.0, 5.0 or 10.0% $w/v$. The treated suspensions of *S. aureus* ATCC 29213 were incubated at 37 °C with shaking. The number of the cells of bacteria that survived treatment for 0, 2, 4, 8 and 24 h was determined by plating 10-fold dilutions of the suspensions on Baird-Parker agar plates and incubating the plates at 37 °C for 24 h. The number of the cells in the control suspension, without extract/product addition, was also determined as a control of growth kinetic of *S. aureus* ATCC 29213.

*4.7. Total Phenolics Determination*

The slightly modified Folin–Ciocalteu method [22] was used for determination of total content of phenolic compounds in produced ethanolic extracts of BP and BB. Briefly, 50 µL of Folin–Ciocalteu reagent diluted 1:10 with de-ionized ultrapure water was mixed with 10 µL of the extract. After 5 min of incubation, 40 µL of $Na_2CO_3$ solution (7.5%) was

added to the mixture. Following shaking 100 µL of ultrapure water was added (to the final volume of 200 µL) and the mixture was incubated for 30 min at ambient temperature. The color intensity—absorbance at 725 nm—was measured using microplate reader (Synergy™ HT BioTek Instruments, Winooski, VT, USA). The calibration curve was prepared with fresh gallic acid standard solutions in the range of concentrations from 1.56 to 50.00 µg GAE/mL. The content of phenolic compounds in BP and BB extracts was expressed as milligrams of gallic acid equivalent (GAE) per gram of the product. All measurements were performed in triplicate.

## 5. Conclusions

The outcomes of the study revealed high antimicrobial potential for ethanolic (70% *v*/*v*) extracts of BP and BB produced in Polish apiaries. Moreover, we observed high growth inhibitory activity of suspensions of BB and BP against *S. aureus*. In both cases (extracts and raw products—suspensions), BB exhibited importantly higher activity and Gram-positive bacteria exhibited higher susceptibility. The extracts exhibited high activity against clinical isolates of *S. aureus*, including MRSA strains, which supports the need for further investigation of possibilities of the applications of BP and BB and products based on these raw materials (extracts, ointments, etc.) as antimicrobial agents.

**Author Contributions:** Conceptualization, K.P., R.W.W. and P.S.; methodology, K.P. and P.S.; validation, K.P.; investigation, K.P., O.O. and P.S.; resources, K.P. and P.S.; data curation, K.P. and P.S.; writing—original draft preparation, K.P., R.W.W. and P.S.; writing—review and editing, R.W.W. and P.S.; visualization, K.P.; supervision, P.S.; project administration, P.S.; funding acquisition, P.S. All authors have read and agreed to the published version of the manuscript.

**Funding:** The studies were financed by the grant2015/18/E/NZ6/00700 from the National Science Centre, Poland.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The data presented in this study are available on request from the corresponding author.

**Acknowledgments:** We are also grateful to the company IGO PHU Maria Borowik from Lublin and beekeepers from the Apiary Hawrn located in Miłogoszcz for some of the BP and BB samples delivered for our research. The authors thank Marek Bronk from Laboratory of Clinical Microbiology, University Centre for Laboratory Diagnostics, Medical University of Gdańsk Clinical Centre for providing MRSA and MSSA clinical isolates.

**Conflicts of Interest:** The authors declare no conflict of interest.

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# GLUCOSE OXIDASE AS AN IMPORTANT YET OVERLOOKED FACTOR DETERMINING THE ANTIBACTERIAL ACTIVITY OF BEE POLLEN AND BEE BREAD

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Honey bee products, including bee pollen and bee bread, have attracted great attention due to their health-promoting properties. Considering the antimicrobial potential of these products to date, mostly methanolic or ethanolic extracts of BP and BB were examined. Whilst mechanism of their activity and active compounds remains unknown. Our previous research revealed a lack of orrelation between polyphenols concentration and antimicrobial potential of investigated extracts. BB is mainly considered as a source of proteins for young bees and bees' larvae, However, only few studies characterized the proteins/peptides from BP and in the case of BB.

The second article (Article 2.) published in European Food Research International (Springer) focused on investigation of antibacterial activity of BP and BB aqueous extracts against selected bacterial pathogens and examination of the mode of action of these products, including the identification of key compound(s) that caused the antimicrobial effect. And at least, but not the last, identification and elucidation of the function of bee proteins, such as major royal jelly protein 1 (MRJP1), glucose oxidase (GOx) and defensin-1 in BP and BB in the pathogen's inhibition.

For the research, eight BB and five BP samples, collected from different geographical locations of Poland and Slovakia, were used. The antimicrobial activity of collected samples were investigated against Gram-positive and Gram-negative bacteria, such as *S. aureus, P. aeruginosa*, *E. coli*, *P. mirabilis* and *E. faecalis*. For the determination of Total Phenolic and Total Protein Content, a Folin-Ciocalteu Phenolic Content Quantification Assay Kit (BioQuoChem, Spain) and Quick Start Bradford protein assay (Bio-Rad, USA) were used, respectively. Detection of MRJP1, GOx and Def-1 was performed by immunoblotting assay. For the determination of GOx enzymatic activity and hydrogen peroxide ($H_2O_2$) concentration, a Megazyme GOX Assay Kit (Megazyme International Ireland Ltd) was applied. The glucose concentration in tested samples was examined by High-Performance Liquid Chromatography (HPLC) analysis. The antibacterial potential of the tested extracts was investigated with minimum inhibitory concentration (MIC) assay.

The assay was conducted using Mueller-Hinton Broth (MHB) medium or MHB medium supplemented with 20 mM glucose. For the investigation of the mode of action of the tested BP and BB extracts, the treatment of the extracts with catalase and proteinase K were performed. Subsequently, the enzyme-treated extracts were used in antibacterial assay against *S. aureus* and *P. aeruginosa*.

The phenolic compounds level varied from 214 to 1506 μg GAE/ml and the Total Protein Content ranged from 434 to 2214 μg/ml. The higher average protein concentration exhibited BB extracts compared to BP extracts. Furthermore, the presence of MRJP1, GOx and defensin-1 in the tested samples was immunodetected. In BB samples the higher amount of MRJP1 was determined compared to BP. GOx was mostly detected in BB extracts, and Def-1 was present in two BB extracts – there was no immunoreaction in any BP samples. To confirm the GOx presence in the tested samples, the enzymatic activity of this enzyme was investigated. The correlation analysis revealed a statistically significant relationship between the protein level in the tested samples and GOx activity ($r=0.571$, $P=0.041$). Furthermore, the $H_2O_2$ concentration was in the range of 0.05 to 6.83 mM after 24 hours of incubation and supplementation with 20 mM glucose. The glucose content in BP and BB samples varied from 5.7 to 9.0 g/100g. These results revealed that there is no correlation between glucose level in tested samples and the enzymatic activity of GOx or the amount of produced $H_2O_2$. The antibacterial activity assay showed that the tested samples were mostly active against *S. aureus*, and *E. faecalis* was the most resistant strain. Furthermore, a significant increase in antibacterial activity was observed after supplementing the medium with glucose. The treatment of selected samples with proteinase K did not have an impact on antibacterial activity; however, samples incubated with catalase showed a decrease in bacterial growth (only in samples where glucose supplementation cause an increase in antibacterial effect).

Taking into consideration obtained results, MRJP1, GOx, and defensin-1 were found primarily in the BB samples. These proteinaceous compounds are probably essential for the antimicrobial activity of BP and BB, where GOx is the key factor. Additionally, this research confirms observation from our previous publication that there is no correlation between the antibacterial activity of BP/BB and the level of phenolic compounds.

Article 2.

**Glucose oxidase as an important yet overlooked factor determining the antibacterial activity of bee pollen and bee bread**

European Food Research and Technology 2022; doi:10.1007/s00217-022-04101-z

Accepted: 25/07/2022 / Published: 29/07/2022

$IF_{2022}$ = 3.498, Q1, MHES Points: 70

European Food Research and Technology
https://doi.org/10.1007/s00217-022-04101-z

ORIGINAL PAPER

# Glucose oxidase as an important yet overlooked factor determining the antibacterial activity of bee pollen and bee bread

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Received: 20 May 2022 / Revised: 25 July 2022 / Accepted: 29 July 2022


**Abstract**
Bee pollen (BP) and bee bread (BB) have attracted great attention due to their biological activities including antibacterial activity. However, the mechanism of antibacterial activity is largely unknown. Therefore, we aimed to characterise the antibacterial effect of BP and BB aqueous extracts against bacterial pathogens (*Staphylococcus aureus*, *Pseudomonas aeruginosa, Escherichia coli*, *Proteus mirabilis* and *Enterococcus faecalis*) and identify the key compound(s) responsible for this effect. Here, we demonstrate that BP and particularly BB extracts display antibacterial activity which is significantly increased in the presence of glucose. Immunoblot analysis of extracts revealed the presence of MRJP1 in all analysed BP and BB samples and the enzyme glucose oxidase (GOX) in the majority of BB samples. Treatment of extracts with catalase resulted in the restoration of bacterial growth but only in those samples where glucose supplementation caused the enhancement of antibacterial activity. Our findings provide a deeper understanding of antibacterial activity of BP/BB which is mediated by the enzymatic activity of bee-derived GOX.

**Keywords** Bee proteins · MRJP · Antibacterial activity · Enzymatic activity · Bacterial pathogen

## Introduction

Honeybee products have attracted great attention and their health-promoting properties have spurred their pre-clinical and clinical testing. Apart from honey and propolis, the most studied and clinically tested bee products, bee pollen (BP) and bee bread (BB) represent a good source of bioactive compounds which can mediate diverse biological effects [1].

Bee pollen, in the form of granules, is formed with flower pollen grains agglutinated by nectar and honeybee secretions. These pollen loads are the major source of proteinous nutrients for bee larvae. The chemical composition of BP is highly variable depending on botanical and geographical origin. A high degree of variability is documented in the content of protein (10–40%) and lipid (1–13%) as well as in the major components–carbohydrates, comprising 13–55% of dry weight [2]. BP is rich in polyphenols including mainly flavonoids and their content is in the range of 0.2–2.5% [3]. Indeed, most of the described biological and pharmacological effects of BP such as antioxidant, antibacterial, immunomodulatory and anti-inflammatory activity are attributed to polyphenols (reviewed in [1, 3–6]).

In hives, BP undergoes the process of fermentation in the honeycomb cells to form the product known as bee bread (BB). Stored pollen loads in honeycomb cells are covered with a thin layer of honey and bee wax. Although BP and BB possess a similar composition, there are notable differences due to biochemical changes of BP. Due to the generation of lactic acid, the pH value of BP changes from 7.2 to 4.2 for BB [7]. Furthermore, it has been suggested that BB has a lower protein content compared to BP due to bee-derived digestive enzymes and it is rich in free amino acids and easily assimilated sugars [1, 3, 8]. Nowadays, BB has become preferred over BP as a dietary supplement due to its higher nutritional value, better bioavailability and reduced

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Published online: 10 August 2022

allergenicity [9]. Moreover, BB is characterised by better digestibility and a higher degree of absorption by humans since the multi-layered wall surrounding the pollen grain is destroyed by natural fermentation, which gives special features to BB [5].

Detailed characterisation of the biological activity of BP and BP and the mechanisms of this activity is essential to provide clear evidence about efficacy and support their further therapeutic use. One of the most important biological activities of BP and BB is antibacterial activity. In recent years, several studies have been conducted to evaluate the antibacterial potential of BP and BB [10–16]. The vast majority of studies have characterised the antibacterial activity of methanolic or ethanolic extracts of BP/BB but have failed to identify the key active compounds or the mechanism of action. Furthermore, considerable variability in the antibacterial activity of BP and BB samples of different botanical and/or geographical origin was observed. Therefore, it is currently difficult to draw a firm conclusion regarding the antibacterial efficacy of BP or BB.

The protein content of BP and BB is an important and key component for bee larvae development. Part of the protein content may represent the bee-secreted proteins and peptides which are mainly products of the hypopharyngeal glands. Interestingly, only a very few studies have attempted to identify bee proteins/peptides in BP [17, 18]. In addition, detailed proteomic data of BB are completely lacking.

In this study, we aimed to (i) characterise the antibacterial effects of BP and BB aqueous extracts against selected bacterial pathogens, (ii) identify the key compound(s) responsible for observed antibacterial activity and finally (iii) identify and elucidate the role of bee-derived compounds (major royal jelly protein 1, glucose oxidase (GOX) and defensin-1) in BP and BB aqueous extracts in inhibiting bacterial growth.

## Materials and methods

### BB and BP samples

BB ($n=8$) and BP ($n=5$) samples were collected from different regions of Poland and Slovakia (Table 1). Collected samples were multi-floral except for BP2 and BP3 which represent buckwheat and heather BP. Upon receipt, the samples were stored in a dark place at − 20 °C (BB samples) or at room temperature (BP samples) until use. Two BB and BP samples of this collection were purchased from local bee shops and their origin was not exactly specified.

### BB and BP aqueous and methanolic extracts

BB/BP samples (0.5 g) were ground to a powder in liquid nitrogen using a mortar and pestle and subsequently mixed with 5 ml of extraction buffer (0.1 M sodium acetate, 0.1 M NaCl, pH = 5). The suspension was shaken at ambient temperature on a rocker-shaker. After 2 h of extraction, the suspension was centrifuged (14 000 rpm, 5 min) and the obtained supernatant was used for further analysis. BB and BP extracts were stored at − 20 °C until further use.

In the case of the methanolic extracts, 0.5 g of BB/BP sample was mixed with 5 ml of 80% methanol and further processed as described above.

**Table 1** Selected measured parameters of bee bread (BB) and bee pollen (BP) aqueous extracts expressed as mean value with standard deviation

| | Sample origin | Sample no | Glucose (g/100 g) | TPC (µg/ml) | TPPC (µg GAE/ml) | GOX (mU/ml) |
|---|---|---|---|---|---|---|
| Bee bread | Bielsko-Biała (PL) | BB1 | 5.9 ± 0.1 | 1194 ± 20 | 941 ± 64 | 3.9 ± 0.8 |
| | Czaplinek (PL) | BB2 | 5.7 ± 0.0 | 1607 ± 192 | 1072 ± 79 | 12.3 ± 0.3 |
| | Miłogoszcz (PL) | BB3 | 8.1 ± 0.1 | 2214 ± 320 | 1130 ± 139 | 23.1 ± 0.4 |
| | Lublin (PL) | BB4 | 7.2 ± 0.0 | 1306 ± 15 | 1029 ± 161 | ND |
| | Warka (PL) | BB5 | 7.5 ± 0.1 | 1719 ± 90 | 898 ± 246 | 21.8 ± 0.6 |
| | Modzele (PL) | BB6 | 7.5 ± 0.0 | 1848 ± 20 | 817 ± 282 | 22.6 ± 0.5 |
| | unspecified (EU)* | BB7 | 6.8 ± 0.2 | 1065 ± 180 | 411 ± 278 | 6.7 ± 1.2 |
| | unspecified (SK)* | BB8 | 8.9 ± 0.5 | 434 ± 49 | 1135 ± 111 | ND |
| Bee pollen | Czarne (PL) | BP1 | 7.1 ± 0.1 | 538 ± 146 | 1362 ± 203 | ND |
| | Lublin (PL) | BP2 | 7.7 ± 0.1 | 486 ± 98 | 1226 ± 153 | ND |
| | Miłogoszcz (PL) | BP3 | 8.6 ± 0.7 | 615 ± 170 | 542 ± 57 | 17.3 ± 0.2 |
| | unspecified (SK)* | BP4 | 9.0 ± 0.2 | 1758 ± 33 | 863 ± 104 | 0.1 ± 0.8 |
| | unspecified (SK)* | BP5 | 7.9 ± 0.2 | 1392 ± 34 | 711 ± 25 | 1.0 ± 0.9 |

*TPC* total protein content, *TPPC* total polyphenol content, *ND* non-detectable, *PL* Poland, *SK* Slovakia, *EU* European union

*Samples were purchased from local bee shops and the exact origin was not specified

### Microorganisms

The antibacterial activity of BB and BP extracts was assessed against the isolates *Staphylococcus aureus* CCM4223, *Pseudomonas aeruginosa* CCM1960, *Escherichia coli* ZPM90, *Proteus mirabilis* ZMP82 and *Enterococcus faecalis* ZPM118 obtained from the Department of Medical Microbiology, Slovak Medical University (Bratislava, Slovakia).

### Determination of GOX enzymatic activity

The bee-derived GOX activity was determined with a Megazyme GOX assay kit (Megazyme International Ireland Ltd, Bray, Ireland), which is based on the oxidative catalysis of β-D-glucose to D-glucono-δ-lactone, with the concurrent release of $H_2O_2$. The resultant $H_2O_2$ reacts with p-hydroxybenzoic acid and 4-aminoantipyrine in the presence of peroxidase to form a quinoneimine dye complex, which has a strong absorbance at 510 nm. The enzyme activity was determined in 2.5 times diluted BB and BP extracts in a 96-well microplate according to the manufacturer's instructions. In the case of kinetic enzymatic reactions, the absorbance was read every 1 min for a total of 20 min.

### Determination of $H_2O_2$ concentration

The $H_2O_2$ concentration in the honey samples was determined with a Megazyme GOX assay kit (Megazyme International Ireland Ltd), which is based on $H_2O_2$ release. For a standard, $H_2O_2$ diluted to 9.8–312.5 µM was used. Briefly, ten-times diluted BB and BP extracts were incubated at 37 °C for 24 h. Each extract and standard were tested in duplicate in a 96-well microplate and the absorbance was measured at 510 nm using Synergy HT microplate reader (BioTek Instruments, VT, USA).

### Total protein and phenolic content

The total protein content (TPC) of BB and BP extracts was measured using a Quick Start Bradford protein assay (Bio-Rad, USA) as described in the instruction manual.

Total phenolic content (TPPC) was determined with a Folin–Ciocalteu Phenolic Content Quantification Assay Kit (BioQuoChem, Spain) in diluted BB and BP extracts in a 96-well microplate according to the manufacturer's instructions. Gallic acid was used as the reference standard and results were expressed as gallic acid equivalents (GAE, mg/ml). Absorbance was measured at 700 nm at 37 °C.

### Detection of MRJP1, GOX and defensin-1 by immunoblotting

Aliquots (15 µl) of BB and BP extract were resolved by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and 16.5% Tricine-SDS-PAGE using a Mini-Protean II electrophoresis cell (Bio-Rad). The proteins were transferred onto a 0.22 µm nitrocellulose Advantec membrane (Sigma-Aldrich) in 48 mM Tris, 39 mM glycine and 20% methanol using the wet blotting procedure. The membrane was blocked for 1 h in a Tris-buffered saline-Tween (TBST) buffer (50 mM Tris–HCl, pH 7.5, 200 mM NaCl and 0.05% Tween 20) containing 5% non-fat dried milk and incubated overnight with a rabbit polyclonal antibody against honeybee MRJP1, GOX or defensin-1 (1:2000 in TBST) [19, 20]. After washing with TBST, the membranes were incubated for 2 h in blocking buffer containing goat anti-rabbit horseradish peroxidase-linked antibodies (1:2500 in TBST; Promega). Immunoreactive bands were detected in solution containing dissolved SigmaFast 3,3-diaminobenzidine tablets (Sigma-Aldrich).

### Determination of glucose content by HPLC

One gram of BB/BP sample was mixed with 5 ml of deionised ultrapure water and extracted at ambient temperature. After 2 h of incubation, the suspensions were centrifuged (10,000 rpm, 10 min) and supernatants were transferred into new tubes. Cold 96% ethanol was added into 4 ml of supernatants at a ratio of 1:4 (v/v) and incubated overnight at 4 °C to precipitate proteins from the sample. Afterwards, the solution was centrifuged (10,000 rpm, 20 min, 4 °C) and the supernatant was concentrated (by evaporation) to a final volume of 4 ml. Before HPLC determination, the solution was filtered using 0.22 µm filters (Millipore, USA). Glucose content was determined with a Shimadzu HPLC coupled to a refractive index detector. For the separation, a ReproSil Carbohydrate-Plus 5 µm HPLC column 250 × 4.6 mm (Dr. Maisch) was used with acetonitrile/water (70/30) as a mobile phase at a flow rate of 1.4 ml/min.

### Determination of antibacterial activity of BB and BP extracts

The antibacterial efficacy of BB and BP samples was evaluated with a minimum inhibitory concentration (MIC) assay as described by Bugarova et al. [21] with modifications. Briefly, overnight bacterial culture was suspended in phosphate-buffered saline (PBS), pH 7.2, and the turbidity of the suspension was adjusted to $10^8$ colony-forming units (CFU)/ml and diluted with Mueller–Hinton broth (MHB) medium (pH $7.3 \pm 0.1$) or with 20 mM glucose-enriched MHB medium to a final concentration of $10^6$ CFU/ml. Then,

10 µl aliquots of suspension were inoculated into each well of sterile 96-well polystyrene plates (Sarstedt, Germany). The final volume in each well was 100 µl, consisting of 90 µl of sterile medium or diluted BB/BP extracts and 10 µl of bacterial suspension. After 18 h of incubation at 37 °C, bacterial growth inhibition was determined visually. The MIC was defined as the lowest concentration of BB/BP extract inhibiting bacterial growth. All tests were performed in triplicate and repeated three times.

### Enzymatic treatment of BB and BP extracts with catalase and proteinase K

BB and BP extracts were treated with catalase (2000–5000 U/mg protein; Sigma-Aldrich, UK) at a final concentration of 1000 – 2500 U/ml at room temperature for 2 h or proteinase K (30 U/mg; Promega, WI, USA) at a final concentration of 50 µg/ml at 37 °C for 30 min. Catalase- and proteinase K-treated BB and BP extracts were then used in the antibacterial assay to determine MIC values against *S. aureus* and *P. aeruginosa*.

### Statistical analysis

The Shapiro–Wilk test of normality was used to determine the data distribution. The Mann–Whitney test and *t* test were used depending on the calculated normality. The Pearson correlation test was used for correlation analysis between individual parameters. The data are expressed as mean values with the standard deviation. Data with P-values smaller than 0.05 were considered statistically significant. All statistical analyses were performed using GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA).

## Results

### TPC and TPPC in BB and BP extracts

Water-based extraction of disrupted BB and BP samples in liquid nitrogen allowed the acquisition of extracts with a high protein yield. The TPC of each BB and BP extract is shown in Table 1. The highest protein content was determined in extract BB3 with an average value of 2214 ± 320 µg/ml and the lowest in extract of BB8 (434 ± 49 µg/ml). Although the mean protein concentrations in BB extracts were higher (1489 ± 488 µg/ml) than those in BP extracts (1134 ± 571 µg/ml), no statistically significant difference ($P = 0.145$, *t* test) in TPC between the two types of samples was observed.

The protein profile of each extract of BB and BP samples is shown in Fig. 1. The protein profile of BB extracts, except for sample BB8, differed greatly from that of BP extracts. The main difference was associated with the presence of the most dominant protein bands with a MW in the range from 40 to 95 kDa in BB extracts.

Apart from proteinous compounds, polyphenols including flavonoids may contribute to the overall antibacterial effect of BB and BP extracts. The TPPC determined in each BB and BP extract is shown in Table 1. Polyphenol content varied among the samples and was in a range from 214 to 1506 µg GAE/ml. However, no significant difference in TPPC was found when comparing the mean values of TPPC between groups of BB and BP extracts ($P = 0.928$, Mann – Whitney test).

### Content of MRJP1, GOX and defensin-1 in BB and BP extracts

The presence of bee-derived proteinous compounds such as MRJP1, GOX and defensin-1, which may act directly or indirectly as antimicrobials, was analysed in aqueous extracts of BB and BP samples. Indeed, MRJP1 was detected in all analysed BB and BP samples and represents the most abundant bee protein in the samples analysed. As indicated in Fig. 2A, BB samples contained a higher amount of MRJP1 compared to BP samples. Due to the low concentration of GOX and low sensitivity limit of colorimetric detection, we were unable to immunodetect GOX in two BB samples (BB4 and BB8) and in four BP samples. A very faint immunoreactive band corresponding with GOX was found in BP3. Defensin-1 was immunodetected only in two BB samples (BB3 and BB6) (Fig. 2C) and no immunoreaction was visible in any BP extracts.

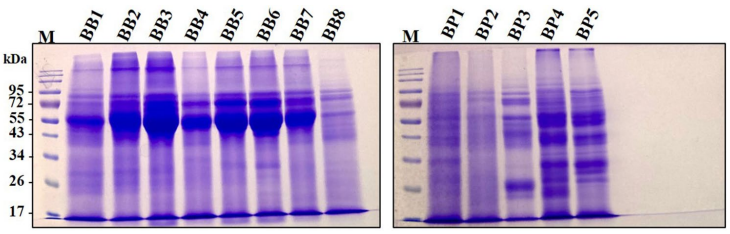


**Fig. 1** Protein profile of bee bread (BB, $n = 8$) and bee pollen (BP, $n = 5$) aqueous extracts. Aliquots (15 µl) of each extract were resolved by 12% SDS-PAGE and protein content assessed after gel staining with Coomassie Brilliant Blue R-250

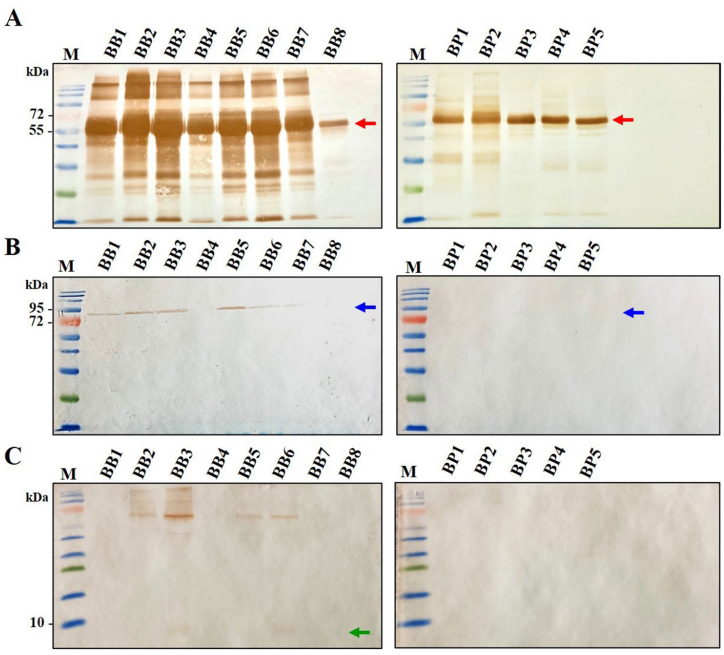


**Fig. 2** Immunodetection of bee-derived proteinous compounds in BB and BP extracts. Aliquots (15 µl) of each extract were resolved by 12% SDS-PAGE and 16.5% Tricine-SDS-PAGE. After the wet blotting procedure, the blocked membrane was incubated overnight with a rabbit polyclonal antibody against honeybee (**A**) MRJP1, (**B**) GOX or (**C**) defensin-1. Immunoreactive bands were detected in solution containing dissolved SigmaFast 3,3-diaminobenzidine tablets. Red, blue and green arrows indicate MRJP1-immunoreactive, GOX-immunoreactive and defensin-1-immunoreacitve band, respectively

## Enzymatic activity of GOX and $H_2O_2$ production in BB and BP extracts

The enzyme GOX was immunodetected in most of the BB extracts and in only one BP extract. In order to prove the presence of GOX, its enzymatic activity was determined in all BB and BP extracts (Table 1). Activity was not detectable in two BB extracts (BB4 and BB8) in which immunodetection of GOX failed. Similarly, no or negligible activity of GOX was documented in those BP extracts where GOX was not immunodetected. The highest GOX activity was found in BB3, BB5, BB6 and BP3 with mean values of 23.1, 21.8, 22.6 and 17.3 mU/ml, respectively. A statistically significant relationship between GOX enzymatic activity and the TPC of all tested samples was revealed by correlation analysis ($r = 0.571$, $P = 0.041$).

The kinetics of the GOX enzymatic reaction and changes in absorbance for each BB and BP extract are shown in Figure S1. The profile of enzymatic curves varied among the analysed samples. GOX enzymatic curves of most of the BP samples remained flat during 20 min and only one sample, BP3, exhibited changes in the absorbance values. Interestingly, the profile of enzymatic curves was not uniform and three BB extracts (BB3, BB5 and BB6) showed a linear increase (Figure S1).

$H_2O_2$, as a product of the GOX enzymatic reaction, was determined after 24 h of incubation at 37 °C in all BB and BP extracts with average values of $0.98 \pm 0.91$ and $1.59 \pm 2.55$ mM for BB and BP group extracts, respectively. The difference in $H_2O_2$ production between the groups of extracts was not statistically significant ($P = 0.622$; Mann – Whitney test).

Although the natural content of glucose, ranging from 5.9 to 9.0 g/100 g, was comparable among the samples, additional glucose supplementation (20 mM) caused an augmentation in $H_2O_2$ generation in BB extracts, excepting BB8 sample (Table 2). On the other hand, no changes in the mean values of $H_2O_2$ were found between BP and glucose-supplemented BP extracts. Statistical analysis revealed a significant correlation between the enzymatic activity of GOX and the level of $H_2O_2$ accumulated in all glucose-supplemented extracts analysed ($r = 0.577$, $P = 0.039$) but no correlation was calculated in the case of unsupplemented samples ($r = 0.465$, $P = 0.110$).

## Antibacterial activity of BB and BP extracts

The antibacterial activity of BB and BP aqueous extracts was expressed as an MIC value and calculated per units of protein content in extracts (Fig. 3). The experiments were

**Table 2** Generation of $H_2O_2$ in BB and BP extracts with/without glucose supplementation at two time points (0 and 24 h). Data are expressed as mean values with standard deviation

| | $H_2O_2$ (mM) | | | |
|---|---|---|---|---|
| | Supplementation | – | – | +20 mM glucose |
| | Sample | $t=0$ h | $t=24$ h | $t=24$ h |
| Bee bread | BB1 | 0.18 ± 0.04 | 0.86 ± 0.09 | 1.59 ± 0.02 |
| | BB2 | 0.76 ± 0.09 | 1.68 ± 0.11 | 2.21 ± 0.02 |
| | BB3 | 0.36 ± 0.05 | 0.49 ± 0.05 | 0.71 ± 0.09 |
| | BB4 | 0.18 ± 0.0 | 0.56 ± 0.20 | 0.63 ± 0.04 |
| | BB5 | 0.39 ± 0.0 | 2.96 ± 0.84 | 6.60 ± 0.57 |
| | BB6 | 0.37 ± 0.06 | 0.52 ± 0.0 | 0.87 ± 0.08 |
| | BB7 | 0.29 ± 0.01 | 0.52 ± 0.10 | 0.63 ± 0.06 |
| | BB8 | 0.22 ± 0.03 | 0.28 ± 0.11 | 0.07 ± 0.03 |
| Bee pollen | BP1 | 0.45 ± 0.0 | 0.75 ± 0.21 | 0.74 ± 0.09 |
| | BP2 | 0.37 ± 0.0 | 0.37 ± 0.04 | 0.27 ± 0.10 |
| | BP3 | 0.21 ± 0.0 | 6.15 ± 0.35 | 6.83 ± 0.25 |
| | BP4 | 0.31 ± 0.08 | 0.30 ± 0.18 | 0.05 ± 0.04 |
| | BP5 | 0.30 ± 0.11 | 0.40 ± 0.10 | 0.45 ± 0.07 |

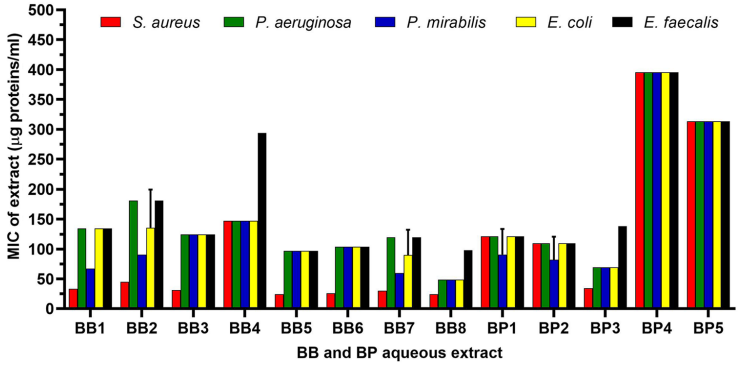


**Fig. 3** Antibacterial activity of bee bread (BB) and bee pollen (BP) aqueous extracts against different bacterial pathogens. Antibacterial activity was determined with a minimum inhibitory concentration (MIC) assay. Antibacterial activity of BB and BP extracts was expressed as an MIC value and calculated per unit of protein content in extracts. Data are expressed as mean values with standard deviation

carried out in glucose-enriched MHB cultivation medium. The overall antibacterial efficacy of BB and BP extracts is shown in Fig. 3. The antibacterial activity of the tested BB extracts was comparable and the highest efficacy was documented against *S. aureus* where MIC values ranged from 24.1 to 147.0 µg proteins/ml. On the other hand, *E. faecalis* was shown to be the most resistant to BB and BP extracts among the tested bacterial strains. Sample BB4 exhibited the weakest antibacterial activity against all bacteria. On the other hand, BB8 was the most effective, in particular against Gram-negative bacteria, where MIC values were around 50 µg proteins/ml.

Regarding BP extracts, the antibacterial efficacy was not uniform and two samples, namely BP4 and BP5, were about four and three-times less effective against *S. aureus* when compared with the average MIC values of the other three BP samples. Moreover, low efficacy of both BP samples was documented against all bacteria tested (Fig. 3).

$H_2O_2$ was found at a higher concentration in three BB extracts and one BP extract (Table 2). However, no correlation was found between the values of MIC and $H_2O_2$ concentrations among all BB and BP samples, regardless of bacterial strain.

## Effect of glucose, catalase and proteinase K on antibacterial activity of BB and BP extracts

As mentioned in the previous section, all MIC-determining experiments were carried out in the presence of glucose artificially added to cultivation medium at a final concentration of 20 mM. Artificially added glucose caused a significant increase in the antibacterial activity of BB extracts against

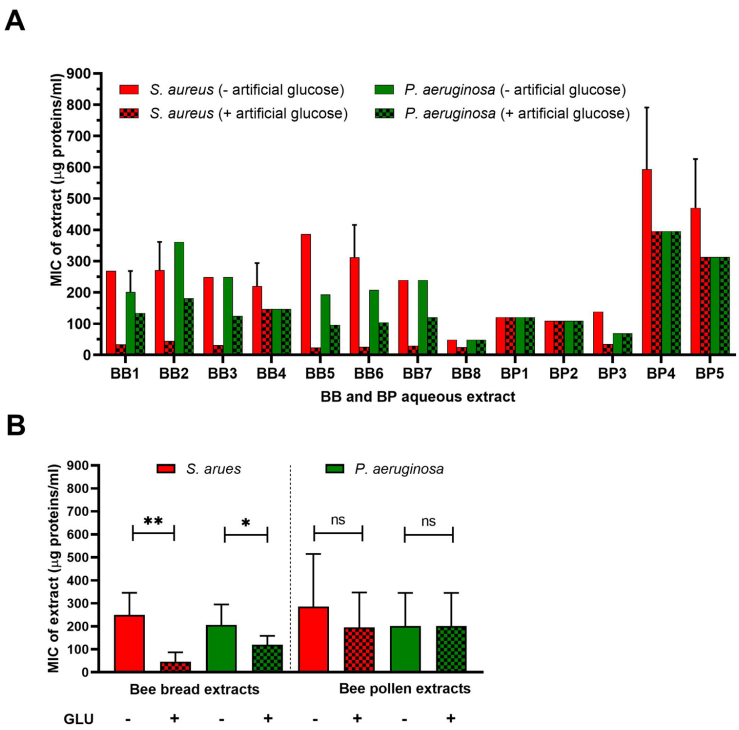


**Fig. 4** Effect of glucose supplementation on antibacterial activity of bee bread (BB) and bee pollen (BP) aqueous extracts against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. **A** Antibacterial activity of individual BB and BP samples was determined with a minimum inhibitory concentration (MIC) assay in MHB medium with or without 20 mM glucose. **B** Comparison of overall antibacterial activity of BB and BP extracts in MHB medium with or without 20 mM glucose (GLU). Antibacterial activity was expressed as an MIC value and calculated per unit of protein content in extracts. Data are expressed as mean values with standard deviation. $*P < 0.05$; $**P < 0.01$

*S. aureus* ($P = 0.008$, Wilcoxon matched-pairs test) and *P. aeruginosa* ($P = 0.016$, Wilcoxon matched-pairs test). On the other hand, glucose supplementation did not result in enhancement of the antibacterial activity of BP extracts against either *S. aureus* or *P. aeruginosa.*

Taking into account all the results obtained, two different BB (BB5 and BB8) and two different BP (BP1 and BP3) samples were selected for further mechanistic analysis. The role of glucose supplementation in the overall antibacterial efficacy of the selected samples was investigated against *S. aureus* and *P. aeruginosa* (Figs. 4 and 5). Four glucose-supplemented extracts were further enzymatically treated with catalase and proteinase K to clarify the role of $H_2O_2$ and proteinous components, respectively (Fig. 5).

Supplementation of glucose in MHB medium augmented the antibacterial activity of BB5, BB8 and BP3 extracts that was documented by 16-, 2- and 4-times lower MIC values against *S. aureus*, respectively. Treatment of BB/BP extracts with Proteinase K did not change the antibacterial efficacy of any extract, whereas incubation of extracts with catalase resulted in a decrease of antibacterial activity in BB5 and BP3 against *S. aureus* and *P. aeruginosa*.

In the case of extracts BB8 and BP1, adding glucose as well as catalase and proteinase K treatment had no effect on their antibacterial activity and it remained stable. This suggests that the antibacterial activity of BB8 and BP1 is not mediated through the $H_2O_2$ generated nor proteinous compounds. In addition, the methanolic extract of selected BB and BP samples was more active against *S. aureus* compared to *P. aeruginosa*. The antibacterial activity of methanolic extracts may largely depend on the botanical and geographical origin of particular BP samples.

## Discussion

A multifactorial antibacterial effect is one of the important advantages of honeybee products, in particular honey, and is being suggested as valuable honey quality parameter [22]. It is, therefore, not surprising to characterise the antibacterial potential of BP and BB, highly attractive nutritional supplements with ideal compositions, allowing their consideration as superfoods.

In the present study, we determined the antibacterial activity of aqueous extracts of BP and BB samples against

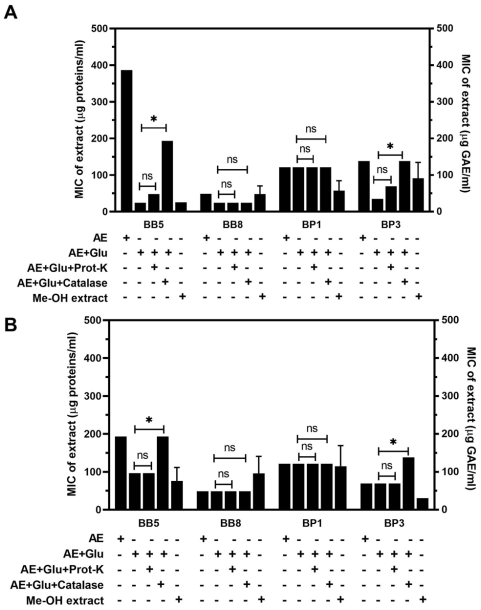


**Fig. 5** Analysis of mechanistic antibacterial effect of selected BB and BP aqueous extracts against (**A**) *Staphylococcus aureus* and (**B**) *Pseudomonas aeruginosa*. BB and BP extracts were treated with catalase (2000 – 5000 U/mg protein) at a final concentration of 1000 – 2500 U/ml at room temperature for 2 h or proteinase K (30 U/mg) at a final concentration of 50 µg/ml at 37 °C for 30 min. Antibacterial activity was determined with a minimum inhibitory concentration (MIC) assay. Antibacterial activity of BB and BP aqueous extracts was expressed as an MIC value and calculated per unit of protein content in extracts. *AE* aqueous extract, *Glu* glucose, *Prot-K* proteinase K. $^{*}P < 0.05$, *ns* non-significant

various bacterial pathogens and identified the compound which could be responsible for the observed antibacterial effect. Similar to honey, BP and particularly BB contains identical compounds from secretions of hypopharyngeal glands and, therefore, honey and BP/BB share the same bee-derived antibacterial compounds.

The vast majority of studies have reported that the antibacterial activity of BP and/or BB is attributed mainly to their polyphenolic and free fatty acid content (reviewed in [23]). In fact, polyphenols including flavonoids undoubtedly take part in inhibiting or killing bacteria; however, their direct antibacterial effect is rather controversial and their effective concentrations in extracts are low. The antibacterial activity of BP and BB extracts depends on the solvents used for extraction. Most studies used methanol and ethanol as solvents followed by water [23]. When comparing the antibacterial efficacy of BP/BB samples, the following factors should be taken into account: type of sample (fresh/dried; monofloral/multi-floral), type of solvent used for extraction, methodology (diffusion/dilution) and type of bacteria. Due to these circumstances, it is difficult to compare the MIC values of BP/BB extracts from particular studies.

The protein/peptide content of honeybee products has biological and pharmacological properties beyond its nutritional value. This is in particular valid for BP and BB. The protein profile of BB extracts was quite different to that of BP extracts, suggesting that partial proteolysis of pollen proteins can occur during the BP fermentation process and, most importantly, a higher content of hypopharyngeal gland secretions is present in BB. Indeed, the MRJP1 content was substantially higher in BB extracts. Immunodetection of MRJP1 revealed that it is a dominant protein in BB extracts and represents regular but quantitatively variable component of BP and BB extracts. A rabbit polyclonal antibody against MRJP1 also immunoreacted with other protein bands suggesting that different post-translational modifications of MRJP1 may contribute to MRJP1 mass heterogeneity [24] and/or contaminant proteases in BB and BP samples can partially cleave MRJP1 into immunoreactive fragments. Thus, MRJP1 could be used as a biomarker of BP and BB authentication, similarly to honey [25] and royal jelly [26, 27].

On the other hand, under the conditions used, we were able neither able to immunodetect GOX nor to determine its enzymatic activity in some samples analysed, in particular BP samples. Likewise, defensin-1 was found only in two BB samples. GOX and defensin-1 are rather variable components and their concentrations in honey, royal jelly and BP/BB depends on multifactorial aspects, such as honeybee genetic/epigenetic factors [28].

The final $H_2O_2$ content in BP and BB extracts depends on the ratio between GOX (producing $H_2O_2$) and catalase (degrading $H_2O_2$) and also on the presence of phytochemicals (*e.g.* polyphenols) which may enhance the enzymatic activity of GOX or, alternatively, may exhibit pro-oxidative activity [20]. We found that the level of $H_2O_2$ did not correlate with the antibacterial activity of any of the samples analysed regardless of bacterial species. It appears that $H_2O_2$ is itself not antibacterial and antibacterial activity depends on the generation of hydroxyl radical from $H_2O_2$ via a Fenton reaction [29]. An increase the number of hydroxyl radicals is determined by the ferrous ion ($Fe^{2+}$) and $H_2O_2$, the concentration of which should be maintained at a relatively high level [30]. We demonstrated that glucose supplementation of BB extracts increased the concentration of $H_2O_2$ accumulated within 24 h. Analysis of trace elements in BP and BB showed that iron is one of the most abundant trace elements [10, 31]. However, the Fenton reaction depends on pH value and the system needs to be acidic. Due to low pH (~ 4.0), BB aqueous extracts seem to present a favourable environment for the Fenton reaction.

To investigate the role of $H_2O_2$ in the antibacterial activity of BB and BP, selected samples were treated with catalase. Catalase was able to restore bacterial growth but only in those samples where glucose supplementation resulted in the enhancement of antibacterial activity. In the samples where catalase and proteinase K treatment did not change the antibacterial activity (BB8 and BP1), no enzymatic GOX activity was observed. It is obvious that the relatively strong antibacterial activity of these samples is not mediated through the action of $H_2O_2$ but through an unknown different mechanism. Recently, bacteria isolated from BB and BP samples were shown to be a suitable and promising source of antimicrobials [32]. Bacteriocins, thermo-resistant antimicrobial peptides of bacterial origin, exhibit potent antibacterial activity against a broad spectrum of pathogens [33]. To date, one antimicrobial peptide, a bacteriocin produced by *Enterococcus avium*, has been found in BB [34]. This peptide inhibits growth of *Listeria monocytogenes* but is ineffective against Gram-negative bacteria. Its antibacterial activity is completely abolished by proteolytic treatment but unaffected by the action of catalase. Therefore, it is unlikely that bacteriocins are responsible for the antibacterial effect of samples BB8 and BP1, because their assumed antibacterial activity was not changed after proteinase K treatment. Proteinase K is a very effective and stable serine protease and whole honey protein content including GOX, MRJP1 and defensin-1 was completely digested [20].On the other hand, the results presented in this study, together with our previously published results [20, 35], indicate that although GOX is digested by proteinase K, it is able to generate substantial amount of $H_2O_2$ due to its fast mechanism of action. This initial level of $H_2O_2$ and ability to form hydroxyl radicals seem to be effective enough to inhibit bacterial growth. However, this hypothesis needs to be verified by further research.

It needs to be emphasised here that fresh BP and BB undergo a drying process due to higher humidity which represents a favourable environment for microbial contamination [36]. However, thermal treatment of BP/BB can have a negative effect on biological properties, in particular those which are mediated by thermolabile compounds. Indeed, controlled-heat drying (50 °C, 8 h) of fresh BP induces detrimental changes in the content of volatile and free fatty acids, proteins and lipids and negatively affects the overall nutritional value [37]. The authors of another study concluded that the most appropriate temperature for drying fresh BP to ensure the reduction of microorganisms is 60 °C [38]. However, there is only scarce information about the effect of thermal processing on BP/BB antibacterial activity and enzyme activity in these products. It has been found that freshly collected BP has higher antibacterial activity compared to processed (dried) BP [39]. Since we used dried BP and BB with unknown processing conditions, it is likely that the antibacterial activity and GOX activity of fresh BP/BB samples are at a higher level. In fact, two BP samples (BP4 and BP5), representing the commercially available samples with unspecified exact origin exhibited the lowest antibacterial activity against all tested bacteria. We assumed that the harsh conditions of technological processing (*e.g.* thermal treatment) of commercially available BP samples could negatively affect biological activity including antibacterial and enzymatic activities.

## Conclusion

In summary, BP and particularly BB aqueous extracts exhibited antibacterial activity which was significantly increased in the presence of 20 mM glucose. The highest activity of extracts was documented against *S. aureus* and the lowest efficacy against *E. faecalis*. Immunoblot analysis of extracts revealed the presence of MRJP1 in all BP and BB aqueous extracts analysed, GOX enzyme in the majority of BB samples and defensin-1 in two out of eight BB samples. A statistically significant relationship between the enzymatic activity of GOX and the TPC of all tested samples was revealed by correlation analysis. Similarly, a significant correlation was found between the enzymatic activity of GOX and the level of $H_2O_2$ accumulated in all glucose-supplemented extracts analysed. Incubation of extracts with proteinase K did not affect the overall antibacterial activity. On the other hand, treatment of extracts with catalase resulted in the restoration of bacterial growth but only in those samples where glucose supplementation caused the enhancement of antibacterial activity. However, the level of $H_2O_2$ did not correlate with the antibacterial activity of any of the samples analysed regardless of bacterial species. Our findings provide a deeper understanding of the antibacterial activity of BP/BB which is mediated, for the most part, by the enzymatic activity of bee-derived GOX. It is obvious that honey, royal jelly and BP/BB share the same mechanism of antibacterial effect based on proteinous content secreted from bee hypopharyngeal glands.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s00217-022-04101-z.

**Funding** This work was supported by the Scientific Grant Agency of the Ministry of Education of the Slovak Republic and the Slovak Academy of Sciences VEGA 2/0022/22 and POWR.03.05.00-00-Z044/17 and Grant No. 2015/18/E/NZ6/00700 from the "National Science Centre, Poland".

## Declarations

**Conflict of interest** The authors declare that they have no conflict of interest.

**Compliance with ethics requirements** This study does not contain any experiment with human participants or animals.

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## BEE POLLEN AND BEE BREAD AS A SOURCE OF BACTERIA PRODUCING ANTIMICROBIALS

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The discovery of antibiotics slowed the investigation of the antimicrobial potential of natural products, including bee products such as honey, propolis, BP, and BB as well. BP and BB are highly nutritious products with many health-promoting properties. In the process of transformation of BP into BB, the key role is played by enzymes and the microbiota of the bee glands, including LAB. Nevertheless, still there is a lack of information concerning role of bee-connected microorganisms. They are involved in the biotransformation BP into BB and preservation of these products from spoilage, however, the mechanism of action is still unknown. The microbiota associated with bees are able to produce secondary metabolites such as antimicrobial peptides, bacteriocins, surfactants, and several types of enzymes.

The third article (Article 3.) published in Antibiotics (MDPI) focused on the investigation of bacteria isolated from bee pollen and bee bread, particularly their ability to produce antimicrobials against different human pathogens, and also their ability to produce enzymes with possible industrial use.

The research material consists of four bee pollen samples and five bee bread samples that were obtained from Polish apiaries. The products suspensions were cultivated on Luria-Bertani (LB) Agar plates, and afterwards, the grown isolates were collected on new LB agar plates. All isolates were investigated in the growth inhibitory assay against reference strains of *S. aureus, S. epidermidis, E. coli* and *P. aeruginosa*. For determination of the ability to produce enzymes such as proteases, cellulases, amylases, esterases, lipases, laccases and β-galactosidases, the following media were used: LB agar with skimmed milk, LB agar with carboxymethylcellulose, LB agar with starch, Tributyrin agar, Spirit Blue Agar, LB agar with guaiacol and LB agar with X-gal, respectively. The most promising strains were identified using sequencing of the 16S rRNA gene.

From four bee pollen samples and five bee bread samples, 81 strains were obtained. The presence of microorganisms in the selected products was in the range of $1.0x10^2$ to $2.1x10^3$. Investigation of antagonistic interactions with five human pathogens revealed that 33.3%, 35.8% and 27.2% of the strains exhibited activity against *S. aureus* ATCC 25923, *S. aureus* ATCC 29213 and *S. epidermidis* ATCC 12228 strains, respectively. In the case of Gram-negative bacteria, 39.5% of the tested isolates demonstrated an antagonistic interaction with *P. aeruginosa* ATCC 27853 and only 18.5% of the tested strains – against *E. coli* ATCC 25922. According to these results, of the 81 strains, 34 isolates exhibited activity against at least one indicator strain were selected

for further analysis. The results of bioinformatic analysis of the 16S rRNA genes of these isolates provide the information that they all belonged to the genus *Bacillus*. Furthermore, five strains were preliminary classified as *B. pumilus, B. altitudinis, B. licheniformis, B. subtilis*, and *B. safensis*. Evaluation of the enzymatic activity of selected isolates revealed that the tested strains are able to produce lipolytic (85.1% of strains), cellulolytic (67%), proteolytic (48%), esterolytic (36%) and amylolytic (3.7%) enzymes. However, none of the isolates exhibited laccase activity.

This study revealed that both products, bee pollen and bee bread, contain a relatively low number of microorganisms. Additionally, isolates that presented antagonistic interactions with human pathogens were identified as *Bacillus* strains. Bee pollen and bee bread products may be considered as a source of microorganisms, particularly bacteria with the ability to produce antimicrobials against human/animal pathogens and enzymes of industrial importance.

Article 3.

**Bee Pollen and Bee Bread as a Source of Bacteria Producing Antimicrobials**

Antibiotics 2021, 10, 713; doi:10.3390/antibiotics10060713

Accepted: 9/06/2021 / Published: 13/06/2021

$IF_{2021}$ = 5.222, Q1, MHES Points: 70

*antibiotics*

MDPI

*Article*

# Bee Pollen and Bee Bread as a Source of Bacteria Producing Antimicrobials

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**Abstract:** The principal objective of the study was the isolation and identification of bacteria that are present in mature bee bread (BB) and dried (ready for selling and consumption) bee pollen (BP). Obtained isolates were screened for their potential to inhibit select human pathogenic bacteria and their ability to produce enzymes of particular industrial importance. Four and five samples of BP and BB, respectively, were used for the study. In total, 81 strains of bacteria were isolated, and 34 (42%) of them exhibited antagonistic interactions with at least one reference strain of pathogenic bacteria, namely *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* 12228, *Pseudomonas aeruginosa* ATCC 27857, and *Escherichia coli* ATCC 25922. The sequencing of the 16S rRNA gene revealed that all strains producing antimicrobials belong to the genus *Bacillus* spp., and among them, five species were identified: *B. pumilus* ($n = 17$), *B. altitudinis* ($n = 9$), *B. licheniformis* ($n = 4$), *B. subtilis* ($n = 2$), and *B. safensis* ($n = 1$). Furthermore, 69, 54, 39, and 29 of the strains exhibited lipolytic, proteolytic, cellulolytic, and esterolytic activity, respectively. Alpha amylase and beta galactosidase activity were rarely observed, and none of the strains produced laccase. The outcomes of the study revealed that BP and BB can be considered potential sources of bacteria producing antimicrobial agents and/or enzymes of particular industrial importance. Of course, additional research is required to verify this hypothesis, but the results of preliminary studies are promising.

**Keywords:** bee bread; bee pollen; *Bacillus* spp.; enzymes



**Citation:** Pełka, K.; Worobo, R.W.; Walkusz, J.; Szweda, P. Bee Pollen and Bee Bread as a Source of Bacteria Producing Antimicrobials. *Antibiotics* **2021**, *10*, 713. https://doi.org/10.3390/antibiotics10060713

Academic Editor: Tetsuo Asai

Received: 21 April 2021
Accepted: 9 June 2021
Published: 13 June 2021



## 1. Introduction

Since ancient times, honey bee products, particularly honey and propolis, have been used as traditional remedies. Both these product exhibit high antimicrobial activity and have mostly been applied for treatment of infected and difficult to heal wounds. As in the case of many other natural products, the investigation of their antibacterial/antifungal potential was impeded by the discovery of antibiotics. However, renewed interest in the investigation and use of the pharmacological (not only antimicrobial) potential of bee products has been observed within the last couple of decades. Recent studies performed with modern analytical techniques and using in vitro and in vivo models have proved that the chemical components of bee products exhibit a broad range of health-beneficial properties including antimicrobial, antioxidant, anti-inflammatory, anti-cancer, and immunomodulatory activity [1–5]. It has been found that the enzymatic production (by glucose oxidase (GOx)) of hydrogen peroxide is a dominant mechanism by which honey collected by bees from most plant sources exert bacteriostatic and bactericidal activity. The physiochemical properties of these products, namely high osmotic pressure and low pH, as well as some of their components, e.g., polyphenols and bee defensin−1, only support the antimicrobial effect of $H_2O_2$ [1,6,7]. Thus, these honeys are called peroxide honeys. However, interesting results presented by Brudzynski and coworkers [7–10], Bucekova et al. [11] and

Grecka and colleagues [12] suggested an important role of plant-derived phytochemicals (mostly polyphenols) for the level of production of hydrogen peroxide in some honey types and probably also the transformation of $H_2O_2$ for most active radical products, e.g., $OH^{\cdot}$ [7–12]. A unique mechanism of antimicrobial activity has been identified for New Zealand's manuka honey and several Australian and Malaysian honeys. High efficiency in the inhibition of bacterial growth by these product is attributed to a non-peroxide component—methylglyoxal [13–15].

Propolis is a highly agglutinative, resinous substance of complex chemical composition that is collected by bees from flower and leaf buds. Propolis-containing extracts exhibit a broad spectrum of biological activities, among which antimicrobial potential has been the most intensively investigated [3,4,16]. The research carried out in our research group revealed the high antibacterial—particularly anti-staphylococcal [17] and antifungal [18]—activity of propolis collected in Polish apiaries, and flavonoids (flavonols, flavones, and flavanones) have been identified as components crucial for the antimicrobial activity of these products. Many trials have confirmed usefulness of propolis-containing products (e.g., extracts, ointments, wound materials, and dental materials such as toothpaste, glass-ionomer cement (GIC), and dental varnish) for treatment and prophylaxis against bacterial and fungal infections [4,19]. Interesting health-associated properties including antimicrobial potential have also been identified and described for lesser known and less popular bee products, namely royal jelly [20,21], bee wax [22], and bee venom [23].

The bee products that have recently gained particular popularity are bee pollen (BP) and bee bread (BB) [5]. Because of their high nutrition values, both of them are classified as functional foods [24] and both exhibit a wide range of therapeutic properties, such as antimicrobial, antioxidant, anti-radiation, anti-inflammatory, anti-tumor, hepatoprotective, and chemopreventive/chemoprotective benefits [5,24–27]. The term "bee bread" refers to the collected pollen that is processed by bees and fermented [5]. The exact mechanism of the biotransformation of BP to BB is still not fully elucidated. However, it known that enzymes from bees' glands (e.g., amylases that are responsible for starch hydrolysis), as well as bacteria (mostly lactic acid bacteria—LAB) and some yeasts sourced from bees' saliva and surfaces of pollen loads, play crucial roles in BP fermentation and BB production [5,28–30]. Some of the BB is stored in the wells of the honeycomb through the winter, and in the spring it is used as a main source of proteins for the new populations of bee larvae. It has been also found that ethanolic or methanolic extracts of components of both BB and BP exhibit antimicrobial potential. The outcomes of our recent study revealed a considerably higher antimicrobial potential of extracts produced from BB compared to BP extracts [31]. We also found the efficient inhibition of growth of *Staphylococcus aureus* in water suspensions of both products [31].

Important gaps in our knowledge remain regarding the microbial ecosystem of bee products, including both bacteria and fungi. Still very little is known about species composition and the role of these microorganisms in maturing bee products (e.g., the biotransformation of BP to BB) and in the protection of honey and bee bread against microbial spoilage, which is crucial for the health of bees (both mature and larvae) and humans who consume these products. There is mounting evidence implicating microbial ecosystem of the bee raw materials (nectar and pollen)–bee products (honey and bee bread)–honey bee axis involved in the production of a range of antimicrobial agents. These agents are used as weaponry in competitive interspecies interactions to effectively kill competing microorganisms in the fight for nutrients and space in each of these niches (nectar, pollen, honey, bee bread, and honey bee). Among the secondary metabolites produced by microorganisms that constitute the microflora of bee products are antimicrobial peptides, bacteriocins, surfactants, siderophores, proteolytic enzymes, and cell wall-degrading enzymes [32]. The main goal of this study was to investigate the ability of bacteria that constitute the microbiome of BP and BB for the growth inhibition of selected pathogenic microorganisms. Most of isolated strains of bacteria were identified as *Bacillus* spp., and some of them exhibited high antagonistic activity against important clinical human pathogens including staphylococci,

*E. coli*, and *P. aeruginosa*. Our future studies will be focused on the identification of the molecular mechanism or metabolites that are responsible for these antagonistic interactions. We believe this could lead to the identification of producers of new antimicrobial agents. Moreover, most of isolates derived from both raw materials revealed high proteolytic, lipolytic, esterolytic, and cellulolytic activity. The outcomes of the study revealed that bacteria isolated from BP and BB can be considered a possible source of novel antimicrobial compounds and enzymes of particular industrial importance.

## 2. Results

As is shown in Table 1, the investigated samples of BP and BB presented different, though generally low, levels of microbial contamination—only aerobic and facultative aerobic were considered in this study. Four products (44%), two samples of each BB and BP, exhibited a level of contamination of above $10^3$ CFU (colony forming units) per gram of the raw material. The other five samples contained less bacteria, from 100 to 600 CFU per gram of the raw material. No evident differences in the level of microbial contamination between BP and BB were observed in this study.

**Table 1.** Level of microbial contamination of investigated BP and BB samples and the antagonistic activity of isolates against reference strains of pathogenic bacteria.

| Sample | No. of Colonies | CFU/g of Product | Activity against *S. aureus* ATCC 25923 | | Activity against *S. aureus* ATCC 29213 | | Activity against *S. epidermidis* ATCC 12228 | | Activity against *E. coli* ATCC 25922 | | Activity against *P. aeruginosa* ATCC 27853 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | No. of Colonies | % | No. of Colonies | % | No. of Colonies | % | No. of Colonies | % | No. of Colonies | % |
| BP3 | 14 | 1400 | 6 | 42.86 | 6 | 42.86 | 5 | 35.71 | 5 | 35.71 | 7 | 50.00 |
| BP15 | 5 | 500 | 2 | 40.00 | 2 | 40.00 | 2 | 40.00 | 2 | 40.00 | 3 | 60.00 |
| BP12 | 1 | 100 | 0 | 0.00 | 0 | 0.00 | 0 | 0.00 | 0 | 0.00 | 0 | 0.00 |
| BP20 | 15 | 1500 | 6 | 40.00 | 7 | 46.67 | 3 | 20.00 | 1 | 6.67 | 7 | 46.67 |
| BB3 | 2 | 200 | 0 | 0.00 | 0 | 0.00 | 0 | 0.00 | 0 | 0.00 | 0 | 0.00 |
| BB6 | 13 | 1300 | 1 | 7.69 | 2 | 15.38 | 1 | 7.69 | 1 | 7.69 | 2 | 15.38 |
| BB10 | 6 | 600 | 3 | 50.00 | 3 | 50.00 | 3 | 50.00 | 1 | 16.67 | 3 | 50.00 |
| BB15 | 4 | 400 | 1 | 25.00 | 1 | 25.00 | 0 | 0.00 | 0 | 0.00 | 1 | 25.00 |
| BB19 | 21 | 2100 | 8 | 38.10 | 8 | 38.10 | 8 | 38.10 | 5 | 23.81 | 9 | 42.86 |
| TOTAL | 81 | | 27 | 33.33 | 29 | 35.80 | 22 | 27.20 | 15 | 18.52 | 32 | 39.51 |

In total, 81 strains of bacteria were recovered from nine tested products (Tables 1 and 2). In each case, the bacteria were cultivated from a 0.1 mL suspension of the raw material in sterile water (1:10 *w/v*). All these isolates were screened for antagonistic interactions with pathogenic bacteria and the production of select essential hydrolytic enzymes. The antagonistic relationship was investigated through the observation of growth inhibition zones (GIZs) of indicator strains of bacteria around the growing colonies of tested strains—isolates from BB or BP (Figure 1).

Considering Gram-positive staphylococci, the antagonistic activity was observed for 27 (33.3%), 29 (35.8%), and 22 (27.2%) strains against *S. aureus* ATCC 25923, *S. aureus* ATCC 29213, and *S. epidermidis* ATCC 12228, respectively. A considerable number of strains, $n = 32$ (39.5%), inhibited the growth of *Pseudomonas aeruginosa* ATCC 27853, while activity against *E. coli* ATCC 25922 was not so common and was confirmed for 15 isolates (18.5%). The largest number of active isolates was recovered from bee bread assigned as BB19. Eight strains inhibited the growth of staphylococci, and an antagonistic relationship with *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 was confirmed for five (23.8%) and nine (42.9%) strains, respectively. Forty percent or more of isolates derived from products BP3, BP15, BP20, and BB10 exhibited antagonistic potential against both strains of *S. aureus* and *P. aeruginosa* ATCC 27853. On the other hand, the only strain recovered from the raw material was assigned as BP12, and both isolates from BB3 did not exhibit any antimicrobial properties. Relatively low percentage levels of active strains were also found in the cases of BB6 and BB15.

**Table 2.** Species classification based on the BLAST analysis of 16S rRNA gene sequences and antagonistic activity against reference strains of pathogenic bacteria.

| Sample | Species Classification * | Exhibited Activity | | | | |
|---|---|---|---|---|---|---|
| | | ***S. aureus*** **ATCC 25923** | ***S. aureus*** **ATCC 29213** | ***S. epidermidis*** **ATCC 12228** | ***E. coli*** **ATCC 25922** | ***P. aeruginosa*** **ATCC 27853** |
| BP3.2 | *Bacillus* spp. (*pumilus*, *zhangzhouensis*) | ++ | +++ | +++ | +++ | +++ |
| BP3.3 | *Bacillus* spp. (*pumilus*, *zhangzhouensis*) | + | ++ | ++ | ++ | ++ |
| BP3.7 | *Bacillus* spp. (*pumilus*, *zhangzhouensis*) | + | + | + | ++ | ++ |
| BP3.10 | *Bacillus* spp. (*pumilus*, *zhangzhouensis*) | +++ | +++ | +++ | +++ | +++ |
| BP3.11 | *Bacillus* spp. (*altitudinis*, *stratosphericus*) | + | + | – | – | +++ |
| BP3.12 | *Bacillus* spp. (*licheniformis*, *aerius*) | – | – | – | – | ++ |
| BP3.13 | *Bacillus* spp. (*pumilus*, *zhangzhouensis*) | +++ | +++ | +++ | +++ | +++ |
| BP15.1 | *Bacillus* spp. (*lichemiformis*) | – | – | – | – | +++ |
| BP15.3 | *Bacillus* spp. (*pumilus*, *zhangzhouensis*) | ++ | ++ | +++ | +++ | +++ |
| BP15.4 | *Bacillus* spp. (*altitudinis*, *stratosphericus*) | +++ | +++ | +++ | +++ | ++ |
| BP20.1 | *Bacillus* spp. (*safensis*, *pumilus*) | + | + | – | – | ++ |
| BP20.3 | *Bacillus* spp. (*altitudinis*, *stratosphericus*) | + | + | – | – | ++ |
| BP20.4 | *Bacillus* spp. (*altitudinis*, *stratosphericus*) | + | + | – | – | + |
| BP20.6 | *Bacillus* spp. (*altitudinis*, *stratosphericus*) | – | + | – | – | +++ |
| BP20.7 | *Bacillus* spp. (*altitudinis*, *stratosphericus*) | + | + | +++ | – | ++ |
| BP20.9 | *Bacillus* spp. (*altitudinis*, *stratosphericus*) | +++ | ++ | ++ | ++ | ++ |
| BP20.15 | *Bacillus* spp. (*subtilis*) | +++ | ++ | + | – | +++ |
| BB6.2 | *Bacillus* spp. (*pumilus*, *zhangzhouensis*) | +++ | ++ | +++ | ++ | ++ |
| BB6.5 | *Bacillus* spp. (*altitudinis*, *stratosphericus*) | – | ++ | – | – | + |
| BB10.1 | *Bacillus* spp. (*subtilis*) | +++ | +++ | +++ | – | +++ |
| BB10.3 | *Bacillus* spp. (*pumilus*, *zhangzhouensis*) | + | + | + | – | ++ |
| BB10.6 | *Bacillus* spp. (*pumilus*, *zhangzhouensis*) | +++ | +++ | +++ | +++ | +++ |
| BB15.3 | *Bacillus* spp. (*altitudinis*, *stratosphericus*) | + | ++ | – | – | +++ |
| BB19.2 | *Bacillus* spp. (*pumilus*, *zhangzhouensis*) | – | – | ++ | – | – |
| BB19.7 | *Bacillus* spp. (*pumilus*, *zhangzhouensis*) | +++ | ++ | +++ | +++ | +++ |
| BB19.9 | *Bacillus* spp. (*licheniformis*, *aerius*) | – | – | – | – | ++ |
| BB19.10 | Bacillus spp. (*altitudinis*, *aerius*) | + | + | – | – | + |
| BB19.11 | *Bacillus* spp. (*licheniformis*, *paralicheniformis*) | – | – | – | – | +++ |

**Table 2.** *Cont.*

| Sample | Species Classification * | Exhibited Activity | | | | |
|---|---|---|---|---|---|---|
| | | *S. aureus* ATCC 25923 | *S. aureus* ATCC 29213 | *S. epidermidis* ATCC 12228 | *E. coli* ATCC 25922 | *P. aeruginosa* ATCC 27853 |
| BB19.12 | *Bacillus* spp. (*pumilus*, *zhangzhouensis*) | ++ | ++ | +++ | + | +++ |
| BB19.13 | *Bacillus* spp. (*pumilus*, *zhangzhouensis*) | +++ | ++ | +++ | – | ++ |
| BB19.15 | *Bacillus* spp. (*pumilus*, *zhangzhouensis*) | ++ | +++ | +++ | +++ | +++ |
| BB19.17 | *Bacillus* spp. (*pumilus*, *zhangzhouensis*) | +++ | +++ | ++ | – | +++ |
| BB19.19 | *Bacillus* spp. (*pumilus*, *zhangzhouensis*) | +++ | +++ | ++ | ++ | – |
| BB19.21 | *Bacillus* spp. (*pumilus*, *zhangzhouensis*) | +++ | +++ | +++ | +++ | +++ |

*—sequences of gene coding for 16S rRNA of different species of the genus *Bacillus* exhibit high level of similarity. Thus, in most cases, two most possible species are proposed. The classification of antagonistic interaction as S—strong (+++); M—moderate (++); W—weak (+); or L—lack (−) was based on the measurement of the size of the growth inhibition zone (SGIZ) of indicatory strain counted from the edge of the colony of the investigated isolate. The following scale was used for the classification of antagonistic interactions: strong—SGIZ > 3 mm; moderate—SGIZ in the range from 1 to 3 mm; and weak—SGIZ ≤ 1 mm. The mean value of this parameter from three independent experiments was used for final classification of each strain tested.

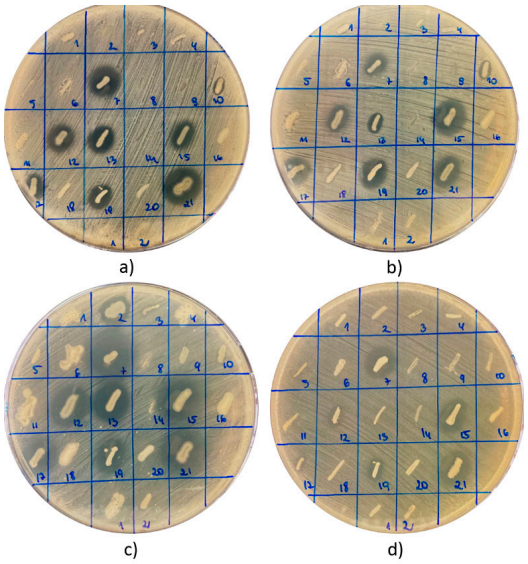


**Figure 1.** An example of antagonistic interaction between isolated strains (strains 1–21 were isolated from the product BB19, and strains 1 and 2 were derived from the product BB3) and indicatory/reference strains of pathogenic bacteria: (**a**) *S. aureus* ATCC 25923, (**b**) *S. aureus* ATCC 29213, (**c**) *S. epidermidis* ATCC 12228, and (**d**) *E. coli* ATCC 25922. Interactions with *P. aeruginosa* ATCC 27853 were a separately analyzed (results not presented).

The 34 out of 81 isolated strains that exhibited antagonistic activity against at least one indicator strain were selected for species identification and amplification and sequencing of the gene coding for 16S rRNA (Table 2). The bioinformatics analysis of 16S rRNA gene sequences (carried out with the BLAST software) revealed that all tested strains belong to the genus *Bacillus*. In general, five species were distinguished among the isolates. Most

of the strains (eighteen) were classified as *B. pumilus.* Nine isolates were identified as a second most common species—*B. altitudinis*. Four, two, and one strains were recognized as *B. licheniformis*, *subtilis,* and *safensis*, respectively. However, it is necessary to remember that sequences of the 16S rRNA gene of different species of *Bacillus* spp. are characterized by a high level of similarity or even identity. Thus, further analysis, e.g., whole genome sequencing or mass spectrometry, would be required for final species identification. The phylogenetic analysis based on results of comparative analysis of the sequences of 16S rRNA genes revealed some diversity between the tested strains (Figure 2) and generally confirmed results of the classification of the species (Figure 2).

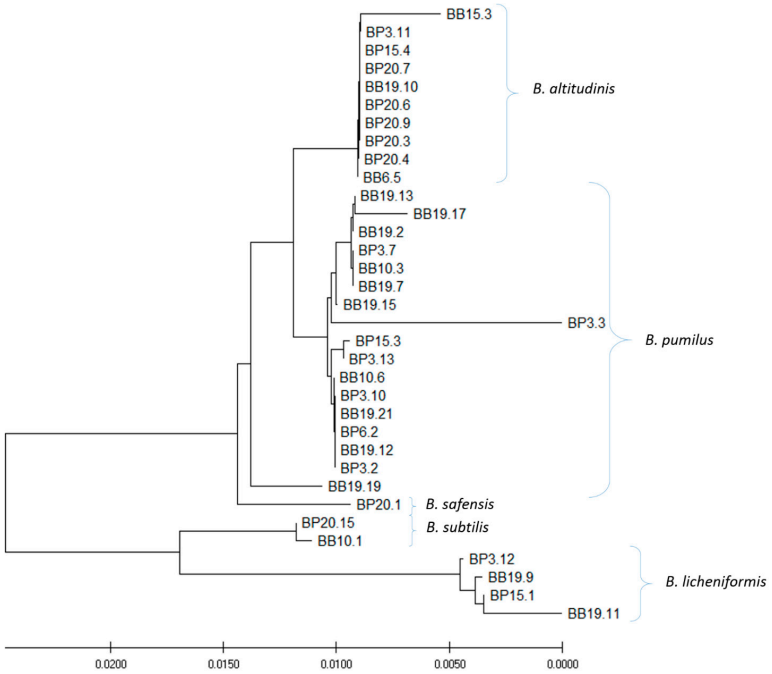


**Figure 2.** Phylogenetic tree of 34 strains that exhibited antagonistic potential against at least of one reference strain of pathogenic bacteria. MUSCLE multiple alignment/construction was done using the neighbor−joining method.

Five clusters of the strains could be distinguished, which was in agreement with species identification.

Some interesting observations were also made regarding the assessment of the enzymatic activity of isolated strains of bacteria (Table 3 and Figure 3). None of the strains were able to produce laccase. No discoloration of the bacterial colonies was observed on the agar medium supplemented with guaiacol. Furthermore, a small number of strains ($n$ = 3; 3.7%) exhibited amylolytic activity. Flooding the active strains with Lugol's solution resulted in the appearance of clear halos around the colonies (Figure 3b). Eleven strains (13.58%) formed lightly blue colonies on the LA agar medium supplemented with X−gal, which probably confirmed β−galactosidase production, though with a low efficiency (data not shown). Almost half of the isolates presented esterolytic ($n$ = 29; 36%) and proteolytic ($n$ = 39; 48%) activity. In the case of strains isolated from BP and BB that are able to produce proteases and esterases, clear halos around the grown bacterial colonies were observed on the media supplemented with skimmed milk and tributyrin, respectively (Figure 3a,d).

Moreover, a significant number of strains ($n$ = 54; 67%) displayed cellulolytic activity, and 18 of them were classified as strong producers. In this case, the halos zones appeared around the colonies grown on LA agar medium supplemented with carboxymethylcellulose and flooded with Congo red solution (Figure 3e). Interestingly, the largest number of isolates ($n$ = 69; 85.1%) exhibited lipolytic activity. The isolates contributed to the formation of bright halos around the colonies on Spirit Blue Agar (Figure 3c).

**Table 3.** Enzymatic activity of isolates.

| | Isolates | Proteolytic Activity | Amylolytic Activity | Lipolytic Activity | Esterolytic Activity | Cellulolytic Activity | Presence of Beta−Galactosidase | Presence of Laccase |
|---|---|---|---|---|---|---|---|---|
| 1 | BP3.1 | − | − | − | − | − | − | − |
| 2 | BP3.2 | +++ | − | + | − | ++ | − | − |
| 3 | BP3.3 | ++ | − | ++ | − | ++ | − | − |
| 4 | BP3.4 | − | − | − | − | − | − | − |
| 5 | BP3.5 | +++ | − | ++ | ++ | +++ | − | − |
| 6 | BP3.6 | + | − | + | − | + | − | − |
| 7 | BP3.7 | + | − | ++ | ++ | − | − | − |
| 8 | BP3.8 | − | − | − | − | − | − | − |
| 9 | BP3.9 | − | +++ | ++ | ++ | +++ | + | − |
| 10 | BP3.10 | +++ | − | ++ | − | ++ | − | − |
| 11 | BP3.11 | + | − | + | − | + | − | − |
| 12 | BP3.12 | − | − | +++ | + | +++ | + | − |
| 13 | BP3.13 | ++ | − | + | − | ++ | − | − |
| 14 | BP3.14 | + | − | + | − | + | − | − |
| 15 | BP12.1 | ++ | − | +++ | − | ++ | + | − |
| 16 | BP15.1 | − | − | +++ | − | ++ | − | − |
| 17 | BP15.2 | ++ | − | + | + | ++ | − | − |
| 18 | BP15.3 | +++ | − | ++ | − | ++ | − | − |
| 19 | BP15.4 | + | − | ++ | − | +++ | − | − |
| 20 | BP15.5 | − | − | ++ | − | − | − | − |
| 21 | BP20.1 | ++ | − | ++ | − | − | − | − |
| 22 | BP20.2 | ++ | − | +++ | − | + | + | − |
| 23 | BP20.3 | ++ | − | ++ | + | +++ | − | − |
| 24 | BP20.4 | ++ | − | ++ | + | ++ | − | − |
| 25 | BP20.5 | − | − | +++ | − | + | − | − |
| 26 | BP20.6 | +++ | − | ++ | + | ++ | − | − |
| 27 | BP20.7 | ++ | − | ++ | + | ++ | − | − |
| 28 | BP20.8 | ++ | − | +++ | − | − | + | − |
| 29 | BP20.9 | ++ | − | ++ | + | +++ | − | − |
| 30 | BP20.10 | ++ | − | − | − | − | − | − |
| 31 | BP20.11 | − | − | + | − | + | + | − |
| 32 | BP20.12 | − | − | + | ++ | − | − | − |
| 33 | BP20.13 | − | − | − | − | − | − | − |
| 34 | BP20.14 | − | − | − | − | − | − | − |
| 35 | BP20.15 | +++ | +++ | +++ | + | +++ | − | − |
| 36 | BB3.1 | − | − | ++ | − | +++ | − | − |
| 37 | BB3.2 | − | − | − | − | − | − | − |
| 38 | BB6.1 | − | − | ++ | − | − | + | − |
| 39 | BB6.2 | +++ | − | ++ | + | ++ | − | − |
| 40 | BB6.3 | − | − | ++ | − | − | + | − |
| 41 | BB6.4 | − | − | − | − | − | − | − |
| 42 | BB6.5 | − | − | +++ | +++ | ++ | − | − |
| 43 | BB6.6 | − | − | − | − | − | − | − |
| 44 | BB6.7 | − | − | + | − | − | − | − |
| 45 | BB6.8 | ++ | − | ++ | − | + | + | − |
| 46 | BB6.9 | − | − | +++ | − | − | − | − |
| 47 | BB6.10 | − | − | − | − | − | − | − |

**Table 3.** *Cont.*

| | Isolates | Proteolytic Activity | Amylolytic Activity | Lipolytic Activity | Esterolytic Activity | Cellulolytic Activity | Presence of Beta−Galactosidase | Presence of Laccase |
|---|---|---|---|---|---|---|---|---|
| 48 | BB6.11 | − | − | + | ++ | +++ | − | − |
| 49 | BB6.12 | − | − | +++ | − | +++ | − | − |
| 50 | BB6.13 | − | − | ++ | + | ++ | − | − |
| 51 | BB10.1 | + | +++ | + | ++ | +++ | − | − |
| 52 | BB10.2 | − | − | +++ | − | ++ | − | − |
| 53 | BB10.3 | + | − | ++ | ++ | + | − | − |
| 54 | BB10.4 | − | − | +++ | − | ++ | − | − |
| 55 | BB10.5 | − | − | +++ | − | +++ | + | − |
| 56 | BB10.6 | ++ | − | + | ++ | ++ | − | − |
| 57 | BB15.1 | − | − | + | + | + | − | − |
| 58 | BB15.2 | − | − | + | − | + | − | − |
| 59 | BB15.3 | + | − | + | + | ++ | − | − |
| 60 | BB15.4 | − | − | +++ | − | +++ | − | − |
| 61 | BB19.1 | − | − | +++ | − | ++ | − | − |
| 62 | BB19.2 | +++ | − | ++ | + | + | − | − |
| 63 | BB19.3 | − | − | ++ | − | − | − | − |
| 64 | BB19.4 | − | − | ++ | − | − | − | − |
| 65 | BB19.5 | − | − | ++ | + | − | − | − |
| 66 | BB19.6 | + | − | ++ | − | ++ | − | − |
| 67 | BB19.7 | + | − | +++ | + | ++ | − | − |
| 68 | BB19.8 | − | − | ++ | − | − | − | − |
| 69 | BB19.9 | − | − | − | + | + | − | − |
| 70 | BB19.10 | ++ | − | ++ | − | +++ | − | − |
| 71 | BB19.11 | − | − | + | − | + | + | − |
| 72 | BB19.12 | +++ | − | ++ | + | +++ | − | − |
| 73 | BB19.13 | ++ | − | ++ | + | ++ | − | − |
| 74 | BB19.14 | − | − | + | − | − | − | − |
| 75 | BB19.15 | + | − | ++ | − | +++ | − | − |
| 76 | BB19.16 | − | − | − | − | − | − | − |
| 77 | BB19.17 | ++ | − | +++ | − | +++ | − | − |
| 78 | BB19.18 | − | − | ++ | − | − | − | − |
| 79 | BB19.19 | ++ | − | +++ | + | − | − | − |
| 80 | BB19.20 | − | − | ++ | − | ++ | − | − |
| 81 | BB19.21 | +++ | − | +++ | + | +++ | − | − |
| | TOTAL | | | | | | | |
| | S | 10 (12%) | 3 (4%) | 19 (23%) | 1 (1%) | 18 (22%) | 0 (0%) | 0 |
| | M | 18 (22%) | 0 | 33 (41%) | 8 (10%) | 23 (28%) | 0 (0%) | 0 |
| | W | 11 (14%) | 0 | 17 (21%) | 20 (25%) | 13 (16%) | 11 (14%) | 0 |
| | L | 42 (52%) | 78 (96%) | 12 (15%) | 52 (64%) | 27 (33%) | 70 (86%) | 81 (100%) |

S—strong (+++); M—moderate (++); W—weak (+); L—lack (−).

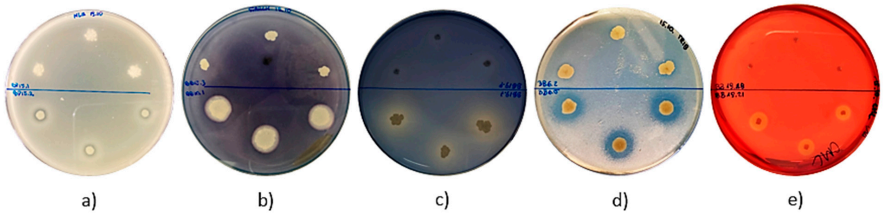


**Figure 3.** Enzymatic activity of isolates. Selected agar plates are presented with observed (**a**) proteolytic, (**b**) amylolytic, (**c**) lipolytic, (**d**) esterolytic, and (**e**) cellulolytic activity. In the upper part of the plates, there are strains that did not exhibit enzymatic activity, while in the lower part of the plates, there are strains capable of producing hydrolases.

## 3. Discussion

The recently observed increase in the popularity and consumption level of BP and BB is mainly a consequence of the high nutritional value and health benefit properties of these products, including the high contents of vitamins, minerals, amino acids, some fatty acids, and polyphenols (antioxidants) that seem to be the most important [5,24–26]. However, very little is known about the microbiome of both these products. Both bacterial and fungal communities associated with BP and BB are important in at least four different aspects: (1) the process of biotransformation of BP to BB; (2) the stability of BB in the hive (in the cells of honey combs) during long term storage in the winter season; (3) the microbial safety of bees, particularly bee larvae that are fed with the BP/BB; and (4) the influence on the health of people who are consumers of BP/BB. The collected pollen loads by bee workers are prone to microbial deterioration—mostly due to molds. The BP that is sold in markets must be dried, which inhibits the growth of molds and other pathogenic microorganisms. For bees, the BP is, in fact, the only raw material for BB production. The BP loads collected by bee workers are mixed with small amounts of the secretion from the bee's saliva, tightly packed in honeycomb cells, and finally covered with a thin layer of honey and a wax lid. Subsequently, under these anaerobic conditions, the BP undergoes the biotransformation process to BB. The exact biochemical mechanism of the biotransformation processes remains not fully understood. However, it is known that different enzymes from bees' glandular secretion, as well as bacteria that are present in bees' saliva and on the surface of pollen loads, are crucial for this process [5,33,34]. It still remains unclear which species of bacteria participate in BB maturing. However, the outcomes of several investigations have suggested that lactic acid bacteria are of primary importance. Vasquez and Olofsson (2009) observed the intense growth of these bacteria within maturing BB for about two weeks—the first step of BP biotransformation [30]. The LAB are important part of *Apis mellifera* gut community and are probably introduced to the raw material—BP from bees' saliva [35]. The presence of LAB seems to be particularly important from the point of view of the microbial stability and preservation of the final product—BB. These bacteria produce lactic acid, bacteriocins, and aliphatic acids (products of lipids hydrolysis) that efficiently inhibit the growth of not only pathogenic (for both bees and humans, the consumers of BB) microorganisms but also bacteria, yeasts, and molds that could cause microbial deterioration or undesirable sensory changes [35]. Iorizzo and coworkers (2020) revealed the high inhibitory activity of LAB, namely *Lactobacillus kunkeei* and *Lactiplantibacillus plantarum* (isolated from bees' gastrointestinal tract and bee products) against the important bee pathogens *Ascosphaera apis* and *Paenibacillus larvae*, respectively [36,37]. It also has been found that yeasts and molds participate in BP biotransformation. Detry et al. (2020) identified *Starmerella*, *Metschnikowia*, and *Zygosaccharomyces* as the most common yeast species in bee bread. However, the high abundance of yeasts in fresh bee bread decreased rapidly with the storage duration. *Starmerella* species dominated fresh bee bread, while mostly *Zygosaccharomyces* members were isolated from aged bee bread [38]. Disayathanoowat et al. (2020) investigated dynamic of bacterial and fungal community structures in corbicula pollen and hive−stored BB collected in China. They found that corbicula pollen was colonized by the *Enterobacteriaceae* bacterium (*Escherichia−Shigella*, *Panteoa*, and *Pseudomonas*) group; however, the number of bacteria significantly decreased in hive−stored bee bread in less than 72 h. In contrast, *Acinetobacter* was highly abundant and could utilize protein sources. In terms of the fungal community, the genus *Cladosporium* remained abundant in both corbicula pollen and hive−stored bee bread. The authors also concluded that filamentous fungus might encourage honey bees to reserve pollen by releasing organic acids [29].

Both mature BB and dried BP—ready for sale in markets—are considered microbial—safe and free from dangerous pathogenic microorganisms. However, none of these products are sterile, and very little is known about the microbiota of these products, including species composition and the metabolic and enzymatic properties of bacterial and fungal communities present in BP and BB. The herein presented results confirmed the generally

low level of microbial contamination of samples of both products, with a maximum level of contamination of approximately 2.1 × $10^3$ CFU/g. Interestingly, all of 34 isolates that exhibited antimicrobial activity were classified into the genus *Bacillus* spp., and five different species were identified: *B. subtilis*, *B. licheniformis*, *B. pumilus*, *B. altitudinis*, and *B. safensis*. The above−mentioned studies did not show the presence of *Bacillus* spp. in BB samples [29,30,38]. In our opinion, this difference could be explained by the fact that only mature BB samples harvested from honeycomb cells and stored for about four months (under refrigeration) and dried BP samples were used in our study. The phytochemicals present in the raw material and in honey added to the BP, as well as the metabolites of bacteria growing in the maturing product, formed an unfavorable environment for the growth and development of most microorganisms. Thus, only highly resistant bacteria, e.g., spore−forming *Bacillus* spp. bacteria, can survive under these conditions. The outcomes of our previous investigation revealed very similar species composition and properties of microorganisms isolated from honey samples [39]. Most of these isolates were classified as *Bacillus* spp., and most of them exhibited the ability to produce metabolites of antibacterial activity [39]. One of these strains, namely *Paenibacillus alvei* MP1, was found to be an efficient producer of proteinaceous agent that exhibited promising activity against a broad spectrum of pathogenic bacteria [40]. Moreover, several genes responsible for antimicrobial activity have been identified in the genome of *P. alvei* MP1 [41]. Some other research groups have also reported the isolation of antimicrobials producing bacteria from honey. Lee et al. (2008) screened six US honeys and two manuka honeys originating from New Zealand. The researchers reported that 92.5% of a total of 2398 strains exhibited antimicrobial activity [42]. One of the isolates, identified as *Paenibacillus polymyxa*, showed a broad range of antibacterial activity against Gram−positive and −negative bacteria including *P. larvae* ssp. larvae ATCC 25747 and foodborne pathogens such as *Bacillus cereus* F4552 and *Escherichia coli* O157:H7 ATCC 43895 [43]. Zulkhairi Amin and coworkers (2020) revealed probiotic properties, including the production of antibacterial metabolites, of *Bacillus* spp. strains isolated from honey of the stingless bee *Heterotrigona itama* [44]. Khalili Samani et al. (2021) isolated several bacteriocin−producing strains of *Bacillus* spp. and Gram−positive cocci from the samples of Iranian honey. In contrast to the bacteriocins produced by these isolates, most of produced metabolites characterized in this study that were BP− and BB−derived strains that exhibited activity against both Gram− positive and Gram−negative bacteria [45]. However, to date, *Bacillus* spp.producing antimicrobial agents have not been isolated from BB or BP. A small amount of honey is added to BP before biotransformation, and this could be the source of *Bacillus* spp. in the final product—BB. On the other hand, bacteria of the genus *Bacillus* are common in the environment and could be present on the surfaces of pollen grains collected by bee workers.

Important and interesting information provided in this study included the investigation of enzymatic potential of the isolates. The production of lipases, cellulases, and proteinases were most common among tested strains. It can be assumed that these activities were essential for the "extraction" of basic food ingredients: amino acids, fatty acids, and glucose from components of pollen grains—proteins, lipids, and cellulose. These activities also improve the nutritional value of BB through the pre−digestion of biopolymers (e.g., cellulose and proteins), which is important for bees and human consumers. Moreover, the release of aliphatic acids from lipids can be important for the preservation of BB. Markiewicz−Żukowska and coworkers (2013) identified aliphatic acids as important antimicrobial components of BB, and unsaturated, α−linolenic, linoleic, oleic, and 11,14,17−eicosatrienoic acids formed more than a half of them (40.63 ± 4.5%) [46]. Neither β−galactosidase nor laccase are crucial for surviving in BB or BP, so these activities are rarely observed or not observed at all. Surprisingly, a relatively low percentage level of alpha amylase positive isolates was identified. Starch is important component of BP, and only 3 out of 81 tested strains exhibited strong potential for the hydrolysis of this polysaccharide. To our knowledge, the enzymatic potential of bacteria, other than LAB, isolated from BB or BP had not been investigated to date. Most of bacteria that belong to

the genus *Bacillus* are not harmful to mammalians, with the exception of *B. cereus* and *B. anthracis*. Thus, the strains isolated from bee products, including BB or BP, can be considered sources of antimicrobials or enzymes. Moreover, they are also suitable candidates for probiotic bacteria [44].

## 4. Materials and Methods

### *4.1. Essential Chemical Reagents and Growth Media*

All chemicals and growth media were purchased from commercial sources. LB broth and LB agar medium were bought from A&A Biotechnology (Gdynia; Poland). Mannitol salt phenol−red agar, Spirit blue agar, tributyrin agar, skimmed milk, starch, carboxymethylcellulose, guaiacol, X−gal (5−bromo−4−chloro−3−indolyl−β−D−galactopyranoside), Tween 80, cottonseed oil, Lugol's solution, Congo red dye, and PBS tablets (pH 7.4) were purchased from Merck (Darmstadt, Germany). Ultrapure $H_2O$ (18.0 MΩ) was produced with the Milli−Q Advantage A10 system (Millipore, Billerica, MA, USA).

### *4.2. Bee Pollen and Bee Bread Samples and Isolation of Bacterial Strains*

The samples of bee pollen ($n$ = 4) and bee bread ($n$ = 5) were provided by Polish apiaries. All samples of BP were dried (to protect the product against microbial spoilage). The BB samples were directly recovered from honeycombs in late summer or autumn 2019; thus, only mature bee bread was used for the study. All products were not older than six months counting from the date of harvesting to the date of using them for the experiment. The samples of BP were stored in dark conditions at ambient temperature, and BB was kept refrigerated at 4 °C. The suspensions of BP and BB in sterile deionized water at a 1:10 (*w/v*) ratio were performed for sample preparation. Subsequently, 100 µL of each suspension were streaked on the LB (Luria–Bertani) agar medium. The plates with inoculated agar medium were incubated at 37 °C for 24 h. Thereafter, the growing colonies were enumerated, and the level of microbial contamination of BB and BP samples (CFU/g of the product) was calculated. Each colony was individually transferred onto new Petri dish with an LB agar medium and incubated overnight at 37 °C. Then, a collection of isolates from BP and BB was obtained for further investigation.

### *4.3. Growth Inhibitory Assay*

For assessing the antimicrobial activity of isolated bacteria, the colonies from the collection were transferred with sterile pipette tips onto LB agar plates inoculated with reference strains: *S. aureus* ATCC 253923, *S. aureus* ATCC 29213, *S. epidermidis* ATCC 12228, *E. coli* ATCC 25922, and *P. aeruginosa* ATCC 27853. Reference indicator strains were inoculated using a sterile cotton swab soaked in a diluted suspension of each tested strain prepared in a phosphate buffered solution (final optical density of each solution $OD_{600}$ = 0.1; approximately 1–5 × $10^8$ CFU/mL). Agar plates were incubated for 24 h at 37 °C. Thereafter, the presence and size of the GIZs of indicator strains were observed and recorded. The antimicrobial activity of each isolate was determined on the basis of the sizes of growth inhibition zones of indicatory strains observed around the colonies of bacteria isolated from BP or BB. All tests were performed in at least triplicate. Isolated bacteria with antimicrobial activity against reference strains were cataloged for further investigation.

### *4.4. Investigation of Enzymatic Activity of Isolated Strains*

The bacterial isolates from BP and BB were tested to confirm or exclude their ability to produce enzymes from the group of hydrolases such as proteases, cellulases, amylases, esterases, lipases, laccases, and β−galactosidases. Two aspects were taken into account for the selection of the set of enzymatic activities that were investigated: the chemical composition of the BP (and therefore the substances that are available for the bacteria) and the industrial relevance of the enzymes regarding their application in industry. In order to determine the individual hydrolytic activities of isolates, the following media were applied: LB agar with skimmed milk (1.5% *w/v*) for proteolytic activity, LB agar with

carboxymethylcellulose (2% *w/v*) for cellulolytic activity, LB agar with starch (2% *w/v*) for amylases activity, LB agar with guaiacol (100 µL/L) for laccase activity, LB agar with X−gal (20 mg/L) for β−galactosidase activity, a Tributyrin agar with neutral tributyrin (10 g/L) for esterolytic activity and a Spirit blue agar supplemented with 30 mL/L of lipase substrate (400 mL of warm distilled water, 1 mL of Tween 80, and 100 mL of cottonseed oil) for lipase activity. Using sterile pipette tips, each of isolates was applied on the appropriate agar medium in triplicate and incubated for 24 h at 37 °C. Following incubation, the appearance of halos around the colonies was observed for the confirmation of proteinase, esterase, and lipase activities. Halo zones around colonies indicated amylases and cellulolytic activities. However, the confirmation of the production of these enzymes required the flooding of the agar medium with Lugol's solution or Congo red solution, respectively. Staining the growing colonies with blue or brownish dye was able to confirm the production of beta−galactosidase or laccase, respectively.

### *4.5. Identification of Bacterial Species of Isolates That Exhibited Antagonistic Activity against Selected Pathogenic Microorganisms*

The identification of the isolates that exhibited antagonistic activity against selected pathogenic bacteria was executed by sequencing of the 16S rRNA gene. The DNA was isolated using Genomic Mini AX Bacteria+ (A&A Biotechnology, Gdynia, Poland) according to the protocol purchased from the manufacturer of the kit.

The PCR amplification of the targeted gene was determined with a pair of primers:
rP1 5′ CCCGGGATCCAAGCTTAGAGTTTGATCCTGGCTCAG 3′
Fd2 5′ CCCAATTCGTCGACAACACGGCTACCTTGTTACGACTT 3′

The amplified products sequencing was carried out by Macrogen (Amsterdam, the Netherlands). The purification of the amplified gene coding for 16S rRNA was executed using the enzymatic Post−PCR Immediate Cleanup (EPPiC) purification kit (A&A Biotechnology, Gdynia, Poland) following the protocol provided by the producer.

### *4.6. DNA Sequence Analysis*

BLAST (Basic Local Alignment Search Tool) was used to the sequence analyses. Multiple sequence alignment in the MEGA X software was performed using the MUSCLE algorithm. The phylogenetic tree was constructed from the 16S rRNA sequences from previously generated FASTA sequence using the MEGA X software. The phylogenetic tree was assembled using the neighbor−joining method and sorting by distance.

## 5. Conclusions

Bacteria of the genus *Bacillus* have been identified as most important component of mature and stored BB, as well as dried BP. Moreover, the outcomes of the study revealed that BP and BB can be considered to be potential sources of bacteria producing antimicrobial agents and/or enzymes of particular industrial importance. Of course, additional research is required to verify this hypothesis, but the results of preliminary studies are promising.

**Author Contributions:** Conceptualization, P.S., R.W.W. and K.P.; methodology, K.P. and P.S.; formal analysis, P.S., K.P. and R.W.W.; investigation, K.P., J.W. and P.S.; data curation, K.P. and P.S.; software, K.P. and P.S.; writing—original draft preparation, P.S., K.P. and R.W.W.; writing—review and editing, P.S. and R.W.W.; visualization, K.P.; supervision, P.S. and R.W.W.; project administration, P.S.; funding acquisition, P.S. All authors have read and agreed to the published version of the manuscript.

**Funding:** The studies were financed by the grant2015/18/E/NZ6/00700 from the National Science Centre, Poland.

**Data Availability Statement:** The data presented in this study are available on request from the corresponding author.

**Acknowledgments:** We are grateful to the company IGO PHU Maria Borowik from Lublin and beekeepers from the Apiary Hawran located in Miłogoszcz for some of the BP and BB samples delivered for our research.

**Conflicts of Interest:** The authors declare no conflict of interest.

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# PROBIOTIC POTENTIAL OF *BACILLUS* ISOLATES FROM POLISH BEE POLLEN AND BEE BREAD

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Bee products have been known since ancient times because of their beneficial health properties. Bee pollen and bee bread are the least known among all bee products; however, they are gaining more attention due to their high nutritional value – they consist of significant amounts of amino acids, fatty acids, vitamins, and microelements. The health-promoting properties of BP and BB were also investigated and both products exhibit, for instance, antimicrobial, antioxidant, anti-radiation, anti-inflammatory, antitumor, hepatoprotective, and chemoprotective abilities. Except for bioactive compounds present in BP and BB, the microbiota of these products can exhibit health-promoting properties for bees and humans. Among all *Bacillus* strains, only two (*B. cereus* and *B. anthracis*) are considered harmful to humans. In addition, some of them are used commercially as probiotics.

The fourth paper (Article 4.) published in Probiotics and Antimicrobial Peptides (Springer) focused on the investigation of ten *Bacillus* isolates of bee pollen and bee bread according to their probiotic potential: survivability under simulated gastrointestinal conditions, adhesion properties, and lack of antibiotic resistant genes. Simultaneously, whole genome sequencing and phylogenomic analysis were conducted. Additionally, the ability to assimilate cholesterol and carbohydrates metabolism of the tested strains was also examined.

In this research, seven strains obtained from bee pollen and three obtained from bee bread were investigated. All of them were identified as *Bacillus* strains using 16S rRNA sequencing analysis. The antagonistic interactions of the tested isolates with bacterial (*S. aureus, S. epidermidis, E. coli, L. monocytogenes, Salmonella enterica*) and yeast (*C. albicans, C. krusei, C. glabrata*) strains were performed. To investigate the probiotic potential of the tested isolates, first, tolerance to bile salts and acidic condition were examined. The adhesion properties of the isolates were studied using auto-aggregation assay, co-aggregation assay with four pathogenic bacteria (*S. aureus, E. coli, L. monocyotgenes* and *S. enterica*) and cell surface hydrophobicity assay. To determine the safety of the tested *Bacillus* strains, an antibiotic disk susceptibility test with ten different antibiotics was performed, as well as the hemolytic and DNase activity of the isolates. To examine cholesterol assimilation ability, a colorimetric method that includes o-phtalaldehyde reagent was conducted. Carbohydrate metabolism was studied using API® 50 CH kits. The species identification of selected potential probiotic isolates was also investigated using whole genome sequencing.

The results presented in this article indicate that all strains tested showed tolerance to 0.3% bile salt solution and acidic conditions (pH=2). The survivability of strains varied from 84.63% to 110.15% and from 35.5% to 68.23% respectively. Additionally, the results of the hydrophobicity assay differ among the isolates tested and the affinity to xylene ranged from 5.69% to 61.08%. Auto-aggregation of isolates was also determined - after 4 hours of incubation it was in the range from 21.55% to 46.75%; after 24 hours, the values increased and the level of auto-aggregation was from 47.08% to 88.52%. Furthermore, the tested isolates showed the strongest co-aggregation with *L. monocytogenes*, followed by co-aggregation with *S. aureus* and *S. enterica*. In the antagonistic interactions assay, the most sensitive strains to isolated *Bacillus* strains were staphylococci and *P. aeruginosa*, where the most resistant were *L. monocytogenes* and *E. coli*. The strains revealed weak activity against *C. albicans* strains. Furthermore, the safety assessment provides information that the tested isolates were sensitive to almost all antibiotics, except penicillin (six strains were resistant) and clindamycin (two strains resistant). None of the tested strains produces DNase, and five exhibited no hemolytic activity. Furthermore, all isolates were able to assimilate cholesterol from the broth – the level of assimilation was in the range of 10.74% to 36.45%. What is more, *Bacillus* isolates had ability to metabolize 30 out of 49 carbohydrates, including mono-, di-, polisaccharides, glycosides and triols. According to obtained results, from ten tested strains three – PY2.3, BP20.15 and BB10.1 were selected as potential probiotic strains and for these strains whole-genome sequencing and phylogenomic analyses were performed. The isolate PY2.3 was identified as *B. velezensis* and isolates BP20.15 and BB10.1 – as *B. subtilis*. Whole genome investigation revealed that these three strains are potential producers of various antimicrobial compounds, for example, bacteriocins and secondary metabolites.

Taking into consideration the results of this preliminary study, bacteria isolated from bee pollen and bee bread can exhibit a probiotic potential that may provide beneficial properties to the host, including activity against pathogens, lowering the cholesterol level, and metabolizing carbohydrates that can have a positive impact on the gut microbiome and the host. However, additional in vitro and in vivo studies are necessary to confirm the beneficial effect of these three strains on human organism.

Article 4.

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

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

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

$IF_{2023}$ = 5.000, Q1, MHES Points: 70

Probiotics and Antimicrobial Proteins
https://doi.org/10.1007/s12602-023-10157-4

# Probiotic potential of *Bacillus* Isolates from Polish Bee Pollen and Bee Bread

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Accepted: 12 September 2023


**Abstract**
The main goal of this study was the evaluation of the probiotic potential of 10 *Bacillus* spp. strains isolated from 5 bee bread and 3 bee pollen samples. The antagonistic interaction with *Staphylococcus aureus* and *Escherichia coli* was a primary criterion for the preliminary selection of the isolates. Three out of ten strains—PY2.3 (isolated from pollen), BP20.15 and BB10.1 (both isolated from bee bread)—were found to be possible probiotic strains. All these strains are safe for humans (exhibiting $\gamma$-hemolytic activity) and meet all essential requirements for probiotics in terms of viability in the presence of bile salts and acid conditions, hydrophobicity, auto-aggregation, and co-aggregation with the cells of important human pathogenic bacteria. They also assimilate more than 30% of cholesterol after 24 h of incubation. These three isolates are resistant to penicillin but sensitive (or exhibit moderate resistance) to the other nine antibiotics tested herein. On the basis of whole-genome sequencing, BP20.15 and BB10.1 were classified as *B. subtilis* and PY2.3 as *B. velezensis*. Moreover, genomic analyses revealed that all these isolates are potential producers of different antimicrobial compounds, including bacteriocins and secondary metabolites. The outcomes of this study have proven that some of the *Bacillus* strains isolated from bee pollen or bee bread are potential probiotics.

**Keywords** *Bacillus* · Probiotic · Bee bread · Bee pollen

## Introduction

Bee products, such as honey, propolis, or royal jelly, are known since ancient times and have been used as traditional remedies in folk medicine. Bee pollen, particularly bee bread, is still less known and less popular among consumers. However, the research conducted during the last decade indicates that both of them deserve special attention due to their wide array of health-beneficial properties such as antimicrobial, antioxidant, anti-radiation, anti-inflammatory, antitumor, hepatoprotective, and chemoprotective activity [1–5]. They are also rich sources of essential amino acids, fatty acids vitamins and microelements. Bee pollen is made up of 7–17% water, 36–37% carbohydrates (fructose and glucose), 20–23% proteins (with all necessary amino acids: methionine, lysine, threonine, histidine, leucine, isoleucine, valine, phenylalanine, and tryptophan), 5.1% fat, 2.2–3% ash content, and 1.6% phenolic compounds (flavonoids, leukotrienes, catechins, and phenolic acids) [6]. Bee workers collect pollen during plant pollination. However, it must be noted that for bees, pollen is only the raw material for preparing the final product — bee bread, which in fact is the main source of proteins for young bees and bee larvae. The pollen grains gathered from plants are mixed with a small dose of the secretion from bee workers' salivary glands and/or nectar and placed in specific baskets (corbiculae) that are situated on the tibia of their hind legs. The bee workers transport the pollen loads to the hive, pack them in the honeycomb cells, and cover them with a thin layer of honey and a waxy lid. In these anaerobic conditions, bee pollen undergoes solid-state fermentation and biochemical changes [2, 7]. Endogenic enzymes and microflora of pollen grains, as well as enzymes and microorganisms present in bees saliva, are crucial for the biotransformation of bee pollen to bee bread [2, 3]. However, the exact mechanism of this process is

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Published online: 19 September 2023

not fully recognized. The most important benefits (for both bees and human consumers) of this process are (1) improvement of nutritional value of the final product (importantly higher availability of food ingredients) by partial hydrolysis of biopolymers that covers each grain of bee pollen: sporopollenin, cellulose, and pectins [8] and subsequently partial hydrolysis of proteins, lipids, and poli/oligo saccharides [8–10]; (2) stabilization of the final product against microbial spoilage that is a consequence of supplementation of bee pollen with bees glandular secretions (contains glucose oxidase and major royal jelly protein) [11], release from pollen grains polyphenols and other natural compounds that exhibit antimicrobial potential [1, 3] and first of all development of microflora producing metabolites (e.g. lactic acid and bacteriocins) that exhibit high antagonistic activity against pathogenic microorganisms [1, 2, 12–15]. Thus, the final product — bee bread is stable and safe for young bees and is a source of readily available food ingredients for bee larvae.

As mentioned above, microorganisms play a crucial role in the biotransformation of bee pollen into bee bread. Except for LAB, bacteria belonging to the genus *Bacillus* are one of the most abundant species found in bee pollen and bee bread, but also in honey [12, 16–18]. *Bacillus* spp. are widespread, spore-forming, rod-shaped bacteria that produce metabolites with biotechnological applications, including enzymes, amino acids, and antimicrobial agents [19]. In the hive, these bacteria are involved in the production of enzymes beneficial for bees' health, the biotransformation of bee pollen into bee bread, and increasing resistance to some diseases such as chalkbrood. Sabate and colleagues (2012) reported that *B. subtilis* subsp. *subtilis* Mori2 exhibited a few benefits for bee colonies: an increase in the number of bees' larvae, a reduction of *Varroa* and *Nosema* levels in the hive and higher honey accumulation compared to the control hives [20]. The majority of the bacterial species that belong to the genus *Bacillus* spp. are considered safe, and only a few of them, including *B. cereus* and *B. anthracis*, are human and animal pathogens [21].

The intestinal microflora plays a key role in the host body and is associated with the regulation of nutritional, immunologic, and physiological functions [22]. Disproportion of the gut microbiota can cause many gastrointestinal diseases, such as inflammatory bowel disease, obesity, or type 2 diabetes [23]. According to the WHO, probiotics are live microorganisms that, when delivered in adequate amounts, improve the host's health. The characteristics of probiotics include the ability to tolerate gastrointestinal conditions, survivability in the presence of gastric acid and bile salts, ability to adhesion to epithelial cells, antimicrobial activity, and a lack of antibiotic-resistant genes [24]. Several strains of the genus *Bacillus* are used commercially as probiotics: *B. subtilis*, *B. polyfermenticus*, *B. clausii*, some *B. cereus*, *B. coagulans*, *B. pumilus*, and *B. licheniformis* [25]. Recent studies revealed that bee products are a good reservoir of *Bacillus* strains that were investigated in terms of probiotic potential [21, 26, 27]. Moreover, our latest study revealed that *Bacillus* strains obtained from bee pollen and bee bread demonstrated high antibacterial activity, especially against Gram-positive *staphylococci,* and produced many essential enzymes such as lipases, esterases, cellulases, and proteases [12]. In the present study, we aimed to evaluate the probiotic characteristics of selected *Bacillus* strains isolated from Polish bee pollen and bee bread.

## Materials and Methods

### Chemicals and Materials

All chemicals and reagents were purchased from commercial sources. Luria–Bertani broth (LB) was purchased from Biomaxima (Lublin, Poland); Columbia Blood Agar was bought from Graso Biotech (Starogard Gdanski, Poland); and Yeast Extract–Peptone–Dextrose (YPD) was obtained from A&A Biotechnology (Gdynia, Poland). Bile salts, phosphate-buffered saline (PBS), phenol, xylene, Mueller–Hinton Agar (MHA), DNase agar with toluidine blue, cholesterol-PEG600, o-phtalaldehyde, and Brain Heart Infusion (BHI) broth were purchased from Merck (Darmstadt, Germany). Hydrochloric acid, acetic acid, absolute ethanol, n-hexane, and sulfuric acid were bought from POCH (Gliwice, Poland), and potassium hydroxide was purchased from Chempur (Piekary Slaskie, Poland). Antibiotic disks (chloramphenicol (30 µg), azithromycin (15 µg), linezolid (30 µg), rifampin (5 µg), penicillin (10 units), trimethoprim (5 µg), clindamycin (2 µg), ciprofloxacin (5 µg), gentamycin (10 µg), and kanamycin (30 µg)) were obtained from Oxoid (Basingstoke, UK). API® CH 50 kit was bought from Biomerieux (Marcy-l'Étoile, France). Ultrapure water (18.0 Ω) was obtained with the Milli-Q Advantage A10 System (Millipore, Billerica, MA, USA).

### Bacterial and Yeasts Cultures

In this study, ten isolates from bee pollen ($n = 5$) and bee bread ($n = 3$) samples derived from Polish apiaries were isolated, cultivated, and characterized according to the protocol presented in Pełka et al. 2021 [23]. Six isolates from this study (BP20.9, BP20.15, BP15.4, BP15.1, BB10.1, and BB19.21) and four other strains isolated in our laboratory from Polish bee pollen and bee bread with interesting antimicrobial activities were selected for probiotic potential investigation. All four additional strains — namely PY2.3, PY5.3, PY6.4, and PG10.5 — effectively inhibited the growth of *S. aureus* ATCC 29213 and *E. coli* ATCC 25922 and were identified as *Bacillus* spp. based on 16S rRNA

sequencing (data not published). The isolated strains were cultured on LB agar plates at 37 °C.

The frequent human pathogens, such as *Staphylococcus aureus* ATCC 29213, *S. epidermidis* ATCC 35984, *Escherichia coli* ATCC 25922, *Listeria monocytogenes* ATCC 35152, *Salmonella enterica* PCM 2266, and yeasts *Candida albicans* ATCC 10231, *C. albicans* SC 5314, *C. glabrata* DSM 6128, and *C. krusei* DSM 11226, were used as indicator strains for the assays aimed at determining the antimicrobial potential of the isolates. Indicator strains were cultivated on BHI agar plates (bacterial strains) and YPD agar plates (yeasts) at 37 °C.

### Acid and Bile Tolerance

To determine the survivability of potential probiotic strains in the human gastrointestinal tract (GIT), the isolates were tested in artificial gastrointestinal juices. The tolerance to low pH and the presence of bile salts were tested as described by Zulkhairi Amin et al. (2020), with slight modifications [21]. Bacterial strains were incubated in LB broth at 37 °C for 18 h at 180 rpm. The cells of the strains tested were then centrifuged (1500 s g, 10 min), washed once, and resuspended in PBS solution. Acid tolerance was tested in LB broth with pH = 2 adjusted with 0.1 M HCl, and bile salt tolerance was tested in LB broth supplemented with 0.3% bile salts. The control sample was conducted in LB broth. Into 5 ml of broth (LB, LB with pH = 2, LB with 0.3% bile salts) 50 µl of bacterial cell suspensions were added. Samples were withdrawn after time intervals (0 h and 3 h) and serially tenfold diluted using PBS. Then, the dilutions were applied to LB agar plates in triplicate. The colonies growing on the plates were counted after 24 h of incubation at 37 °C. During the 3-h interval, the samples in the particular broths were incubated at 37 °C and 100 rpm.

### Auto-Aggregation and Co-Aggregation

The adhesive properties of isolates were tested using slightly modified auto-aggregation and co-aggregation assays described by Jeon et al. (2017) [28]. Bacterial strains were incubated in LB broth at 37 °C for 18 h at 180 rpm. The isolates were then centrifuged (1500 s g, 10 min), washed once, and resuspended in PBS solution to reach an optical density of 0.1–0.2 at 600 nm ($OD_{600}$).

To determine the auto-aggregation ability of isolates, 5 ml of bacterial suspensions were incubated at 37 °C for 4 and 24 h, and after that time, absorbance was measured at 600 nm. The percentage of auto-aggregation was calculated using the formula:

$$[\%] = (1 - A_t/A_0 \times 100\%$$

where $A_0$ and $A_t$ represented the $OD_{600}$ at 0 h and at the indicated incubation time (4 h and 24 h, respectively).

To determine the percentage of co-aggregation, isolated strains as well as pathogen strains (*S. aureus* ATCC 29213, *E. coli* ATCC 25922, *L. monocytogenes* ATCC 35152, and *S. enterica* PCM 2266) were used. 2.5 ml of bacterial isolate suspensions were mixed with 2.5 ml of each pathogen cell suspension. The mixture was incubated at 37 °C for 4 and 24 h, and after that time, absorbance was measured at 600 nm. The percentage of co-aggregation was expressed as follows:

$$[\%] = (((A_P + A_B)/2) - A_{MIX}/((A_P + A_B/2 \times 100\%$$

where $A_P$ and $A_B$ represent the absorbance of the pathogen and isolated *Bacillus* strain at 0 h, respectively, and $A_{MIX}$ represents the absorbance of the mixed culture after 4 and 24 h intervals.

### Cell Surface Hydrophobicity

To determine the potential ability of the strains to adhere to epithelial cells of the gut, the adherence to the surface of hydrocarbons (hydrophobicity assay) was measured as described by Yadav et al. (2016) [29]. Bacterial strains were incubated in LB broth at 37 °C for 18 h at 180 rpm. The cultures of the isolates were then centrifuged (1500 s g, 10 min), washed once, and resuspended in PBS solution to reach an optical density of 0.1–0.2 at 600 nm ($OD_{600}$). 3 ml of the prepared cell suspension was mixed with 1 ml of p-xylene and incubated at 37 °C for 1 h. After aqueous and organic phase separation, 1 ml of the aqueous phase was carefully taken from the test tube, and the absorbance was measured at 600 nm. The percentage of hydrophobicity was calculated using the formula:

$$[\%] = (1 - A_t/A_0) \times 100\%$$

where $A_0$ and $A_t$ represent the absorbance of the aqueous phase after 0 and 1 h, respectively.

### Antimicrobial Activity of Isolates

Agar diffusion method was performed to investigate the antimicrobial potential of isolated *Bacillus* strains. For this assay, six bacterial and four yeast reference strains were used: *S. aureus* ATCC 29213, *S. epidermidis* ATCC 35984, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *L. monocytogenes* ATCC 35152, *S. enterica* PCM 2266, *C. albicans* ATCC 10231, *C. albicans* SC 5314, *C. glabrata* DSM 6128, and *C. krusei* DSM 11226. Inoculation of agar media LB (for bacteria) and YPD (for yeasts) was performed by streaking with a sterile cotton swab soaked in a suspension of each tested indicatory strain (final optical density of each

suspension $OD_{600} = 0.1$). Subsequently, the colonies of isolates from the collection were transferred with sterile pipette tips in the form of dots onto LB agar plates (for bacteria) and YPD agar plates (for yeasts). Afterward, plates were incubated at 37 °C for 24 h. After incubation, growth inhibition zones of indicatory strains around colonies (dots) of isolates were observed and measured.

### Antibiotic Disk Susceptibility Test

According to the Performance Standards for Antibiotic Disk Susceptibility Tests (CLSI), the antibiotic susceptibility assay was performed on MHA plates using the antibiotic disk diffusion method. The different groups of antibiotics were selected according to their different modes of action: protein synthesis inhibition (chloramphenicol, azithromycin, linezolid, clindamycin, gentamicin, kanamycin), cell wall synthesis inhibition (penicillin), and DNA or RNA synthesis inhibition (rifampin, ciprofloxacin, trimethoprim). The following antibiotic disks were used: chloramphenicol 30 µg, azithromycin 15 µg, linezolid 30 µg, rifampin 5 µg, penicillin 10 units, trimethoprim 5 µg, clindamycin 2 µg, ciprofloxacin 5 µg, gentamicin 10 µg, and kanamycin 30 µg. Bacterial cell suspensions (0.5 McFarland) were prepared, spread on MHA plates, and allowed to dry. Then, the antibiotic disks were placed on plates and incubated at 37 °C for 18 h. After incubation, the diameters of the inhibition zones were measured. The results were compared with interpretative zone diameters described by the Performance Standards for Antibiotic Disk Susceptibility Tests (CLSI). The criteria used for the interpretation of the results were adapted from the M100 Performance Standards determined for *Staphylococcus* spp. [30].

### Hemolytic Activity

To examine the safety of isolates, the hemolytic activity test was performed using Columbia Agar plates with 5% sheep blood. Overnight-grown isolate cultures were streaked onto blood agar plates and incubated at 37 °C for 24 h. Thereafter, the zones of hemolysis around the grown colonies were observed.

### DNase Activity

For examination of DNase production by isolates, DNase medium with toluidine blue was used. Overnight-grown isolate cultures were streaked onto DNase agar plates and incubated at 37 °C for 24 h. After incubation, the change in color of the DNase medium around the grown colonies was observed.

### In vitro Cholesterol Assimilation

Cholesterol assimilation analyses were performed according to the protocol described by Tomaro-Duchesneau and colleagues (2014) [31], with some modifications. Filter-sterilized water-soluble cholesterol solution was added to LB broth with 0.2% ox gall bile at a final concentration of 100 µg/ml and inoculated with 1% (*v*/v) bacterial overnight culture. Afterward, the suspension was incubated at 37 °C for 24 h. After incubation, the suspension was centrifuged (5500 rpm, 7 min, 4 °C), and the supernatant was collected. 0.5 ml of supernatant was mixed with 1 ml of absolute ethanol and 0.5 ml of 33% (w/v) KOH. The mixture was vortexed and incubated at 37 °C for 15 min. After cooling the samples to room temperature, 1 ml of deionized water and 1.5 ml of n-hexane was added to the solutions and vortexed for 1 min. Afterward, the mixture was left at ambient temperature for phase separation. Subsequently, 0.5 ml of the upper n-hexane phase was removed and transferred into a new test tube, and then evaporated at 45 °C using a rotary concentrator (Eppendorf Concentrator Plus, Hamburg, Germany). Subsequently, 1 ml of o-phtalaldehyde reagent (50 mg/dl in acetic acid) was added into tubes and mixed. Then, 250 µl of concentrated sulfuric acid was added, vortexed for 1 min and allowed to stand for 20 min at room temperature. The absorbance of the final mixture was measured using TECAN Multiplate Reader (Spark 10M Grödig, Austria) at 570 nm. For calculations, a standard curve of cholesterol was prepared for cholesterol concentrations from 0 to 500 µg/ml in LB broth. Cholesterol reduction was calculated as follows:

$$\text{assimilatedcholesterol}[\%] = ((CH_{NC} - CH_s)/CH_{NC}) \times 100$$

where $CH_{NC}$ represents the amount of cholesterol present in the negative control (non-inoculated broth) and $CH_S$ represents the amount of cholesterol present in samples.

### Carbohydrates Metabolism by Isolated Strains

For determining the metabolism of carbohydrates, API® ZYM and API® 50 CH kits were used, respectively. Performance of the assays and analysis of the results were conducted according to the protocols described by the manufacturer.

#### DNA Extraction and Whole-Genome Sequencing Analysis

DNA Sequencing and Oligonucleotide Synthesis Laboratory, Institute of Biochemistry and Biophysics, Polish Academy of Science (Warsaw, Poland), performed the genomic DNA extraction and sequencing. The CTAB/lysozyme method was used, cell pellets from the overnight LB culture

were treated, and the quality and quantity of template DNA were checked on the agarose gel. Following the manufacturer's instructions, KAPA Library Preparation Kit (KAPA/Roche, Basel, Switzerland), the genomic DNA was sheared to an appropriate size for Paired-End TruSeq-like library construction. The bacterial genomes were sequenced in paired-end mode (V3,600 cycle chemistry kit) using MiSeq (Illumina, San Diego, CA, USA).

FASTQC was used to assess the quality of raw reads. The reads were trimmed and paired using Trimmomatic (version 0.38.0) [32] with the following parameters: LEADING: 3 TRAILING: 3 SLIDINGWINDOW: 4:20 MINLEN: 28. To ensure normal results for "per base sequence quality," "per base N content," "sequence duplication levels," and "adapter content," a secondary read quality check was performed. De novo assembly was performed with SPAdes (3.15.5) with the parameters: -k 33, 55, 77, 99, 127 -careful. Scaffolds less than 500 bp were removed, and assembly statistics [e.g., number of contigs, N50 (widely used to assess the contiguity of an assembly), G + C content] were assessed using QUAST (Version 5.0.2) [33]. The final assemblies were BLASTed in the National Center for Biotechnology Information (NCBI) database, and the genome assemblies of closely related group-type strains were downloaded from the assembly database. The orthoANI method using OAT (version 0.93.1) and BLAST + (version 2.13.0) was used to calculate the average nucleotide identity (ANI) of the isolates [34]. The genomes of strains BB10.1, BP20.15, and PY2.3 were compared to the respective type strains with Mauve (v20150226) using progressive alignment and seed-families options [35]. Rapid genome annotation was performed using Prokka (version 1.14.6) [36]. Additional genome annotation was performed with the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (https://github.com/ncbi/pgap) on the local machine [37].

Assembled genomes of the strains BB10.1, BP20.15, and PY2.3 were submitted to the Sequence Read Archive (SRA) and GenBank under the BioProject IDs PRJNA949953, PRJNA949979, and PRJNA949984, respectively. SRA accession numbers for 10.1, 20.15, and PY2.3 are SRR24003043, SRR24003725, and SRR24005238, respectively.

Moreover, the bacteriocin-encoding gene clusters were identified with the BAGEL4 server.

### Phylogenomics Analysis

The whole-genome-based taxonomic analysis was performed by the Type (strain) Genome Server (TYGS) (https://tygs.dsmz.De) [38]. The draft genomes of isolates BB10.1, BP20.15 and PY2.3 sequenced in this study and 22 other closely related genomes of the *B. velezensis* and *B. subtilis* groups, as well as the complete genome of *B. cereus* ATCC 14579 strain as an outgroup, were extracted from NCBI and submitted to the TYGS server, settings: restricted genome mode. A phylogenomic tree was constructed with FastME (based on balanced minimum evolution and renders distance algorithms to infer phylogenies) [39] using the genome blast distance phylogeny (GBDP) method and annotated using Interactive Tree of Life (iTOL) v5, an online tool for phylogenetic tree display and annotation [40]. All pair-wise genome comparisons were carried out with GBDP and inter-genomic distances inferred under the algorithm "trimming" and distance formula d5 [38]. The tree was rooted at the midpoint [38]. Branch supports were inferred from 100 pseudo-bootstrap replicates.

### Statistical Analysis

All experiments were performed in triplicate. The results are expressed as means ± standard deviation (SD) of the mean and checked for normality using D'Agostino-Pearson normality test. Linear regression was carried out for standard curve formation. The data were analyzed using GraphPad Prism (ver. 9.4.1). A statistical comparison was conducted using a two-way ANOVA followed by Tukey's multiple comparison tests. $p < 0.05$ was considered significant.

## Results

### Strains Survivability in the Presence of Acid and Bile Salts

The ability to survive in simulated gastrointestinal conditions is presented in Table 1. After exposure of bacterial cells to acidic conditions and to a 0.3% bile salt solution, the survivability of isolates was in the range of 35.50–68.23% and 84.63–110.15%, respectively. BB19.21 and BP20.9 had the highest survival rates in the harsh conditions of GIT among all strains tested, whereas strains BP5.3 and PG10.5 demonstrated the weakest survivability in the tested conditions.

### Surface Properties of Isolated Strains

The possible adhesive properties of isolates are presented in Table 2. To determine the hydrophobicity of isolates, the adhesion of bacterial cells to xylene was examined. Hydrophobicity varied from 5.69 to 61.08%, while the maximum affinity toward xylene was exhibited by strain PG10.5 and the minimum by BB19.21.

Auto-aggregation of isolates was in the range of 21.55–46.75% after 4 h of incubation and 47.08–88.52% after 24 h of incubation. Strain BP20.15 showed the highest ability to aggregate, followed by BB10.1. The lowest

**Table 1** Comparison of percentage viability of strains in LB broth (growth control), and LB broth with 0.3% bile salts and acid (pH = 2) after the indicated time of incubation. The assay was performed in triplicate and the results are presented as mean values ± SD. $^{a-d}$ – different superscript letters represent statistical differences between strains at the level of $p < 0.05$ measured by Tukey's test. The superscript letter (a) describes the highest significance, (d) – the lowest significance, and (e) – no significant difference between samples

| | Growth control (3h) [%] | Bile salts tolerance (3h) [%] | Acid tolerance (3h) [%] |
|---|---|---|---|
| PY2.3 | $122.33 \pm 4.46^{c,e}$ | $87.10 \pm 2.17^{a,d,e}$ | $68.23 \pm 0.68^{a,d,e}$ |
| PY5.3 | $108.31 \pm 0.79^{b,d,e}$ | $84.63 \pm 0.44^{a,c,d,e}$ | $50.14 \pm 3.06^{a,b,c,e}$ |
| PY6.4 | $116.42 \pm 0.86^{e}$ | $90.51 \pm 0.80^{a,b,e}$ | $56.97 \pm 2.08^{a,d,e}$ |
| PG10.5 | $115.77 \pm 1.83^{e}$ | $99.28 \pm 1.30^{a,c,d}$ | $35.50 \pm 4.24^{a,c,d}$ |
| BP20.9 | $116.01 \pm 1.43^{e}$ | $108.87 \pm 0.95^{a,c,d,e}$ | $64.02 \pm 2.75^{a,b,c,e}$ |
| BP20.15 | $119.88 \pm 0.06^{d,e}$ | $95.63 \pm 1.22^{c,d,e}$ | $47.89 \pm 4.94^{a,b,d,e}$ |
| BP15.4 | $119.96 \pm 1.90^{d,e}$ | $97.25 \pm 0.67^{c,d,e}$ | $58.04 \pm 2.28^{a,e}$ |
| BP15.1 | $116.79 \pm 5.42^{e}$ | $95.53 \pm 0.28^{c,d,e}$ | $65.89 \pm 1.72^{a,b}$ |
| BB10.1 | $120.73 \pm 3.86^{d,e}$ | $107.81 \pm 0.31^{a,b,d,e}$ | $57.53 \pm 1.58^{a,e}$ |
| BB19.21 | $124.27 \pm 5.42^{b,e}$ | $110.15 \pm 1.39^{a,c,d,e}$ | $65.88 \pm 3.07^{a,b,e}$ |

percentage of auto-aggregation was exhibited by strains BP6.4 and BB19.21. A co-aggregation (Fig. 1.) assay was performed using four pathogenic bacterial strains: *S. aureus* ATCC 29231, *E. coli* ATCC 25922, *L. monocytogenes* ATCC 35152, and *S. enterica* PCM 2266. All strains tested exhibited the best co-aggregation ability with *L. monocytogenes*; it ranged from 60.20% (BP15.1) to 89.54% (PY2.3) after 24 h of incubation. Slightly worse co-aggregation was observed for *S. enterica* and *S. aureus*. The percentage of co-aggregation of *Bacillus* isolates with these strains was from 48.47% (BP20.9) to 84.91 (PY2.3) and from 46.76% (PY6.4) to 73.63 (PY2.3), respectively. Tested isolates demonstrated the weakest co-aggregation ability with *E. coli*, from 39.94% (BP15.1) to 59.59% (BP20.15).

**Table 2** Adhesive properties of isolated strains from BP and BB. The assay was conducted in triplicate. The percentages of hydrophobicity and auto-aggregation are expressed as mean values ± SD. $^{a-d}$ – different superscript letters represent statistical differences between strains at the level of $p < 0.05$ measured by Tukey's test. The superscript letter (a) describes the highest significance, (d) – the lowest significance, and (e) – no significant difference between samples

| | Hydrophobicity [%] | Auto-aggregation | |
|---|---|---|---|
| | | After 4 h [%] | After 24 h [%] |
| **PY2.3** | $22.72 \pm 1.37^{a,b,c,d,e}$ | $38.62 \pm 2.14^{a,b,e}$ | $87.30 \pm 3.37^{a,e}$ |
| **PY5.3** | $22.66 \pm 1.09^{a,b,c,d,e}$ | $38.66 \pm 2.77^{a,b,e}$ | $87.42 \pm 2.89^{a,e}$ |
| **PY6.4** | $8.36 \pm 2.11^{a,b,e}$ | $21.55 \pm 0.04^{a,c,e}$ | $61.70 \pm 3.66^{a,b,e}$ |
| **PG10.5** | $61.08 \pm 2.19^{a}$ | $27.34 \pm 2.00^{a,b,c,e}$ | $50.06 \pm 2.92^{a,b,c,e}$ |
| **BP20.9** | $10.71 \pm 1.62^{a,c,e}$ | $25.02 \pm 1.86^{a,b,e}$ | $47.08 \pm 1.32^{a,b,e}$ |
| **BP20.15** | $33.18 \pm 1.65^{a,d,e}$ | $46.75 \pm 0.28^{a,e}$ | $88.52 \pm 0.95^{a,e}$ |
| **BP15.4** | $17.40 \pm 1.38^{a,c,e}$ | $25.13 \pm 0.89^{a,b,c,e}$ | $65.12 \pm 2.16^{a,b,c,e}$ |
| **BP15.1** | $10.63 \pm 2.59^{a,c,e}$ | $31.62 \pm 3.16^{a,c,d,e}$ | $68.74 \pm 3.09^{a,c,d,e}$ |
| **BB10.1** | $26.19 \pm 0.87^{a,e}$ | $38.83 \pm 1.05^{a,b,c,e}$ | $86.30 \pm 1.30^{a,b,c,e}$ |
| **BB19.21** | $5.69 \pm 0.81^{a,c,e}$ | $22.82 \pm 1.16^{a,c,d,e}$ | $48.55 \pm 0.84^{a,c,d,e}$ |

## Antimicrobial Activity of Bacillus Isolates

Isolated strains were tested for antimicrobial activity against bacterial and yeast pathogens, results presented in Table 3. The antimicrobial activity against pathogens cultivated on agar plates was determined by measuring the diameter of inhibition zones around the growing colonies of isolates. The most sensitive bacterial strains were Gram-positive *staphylococci*. Nine and ten out of ten tested isolated strains inhibited the growth of *S. aureus* and *S. epidermidis*, respectively. On the other hand, the most resistant Gram-positive bacteria tested was *L. monocytogenes*. Among Gram-negative bacterial pathogens, *P. aeruginosa* was the most sensitive strain; eight out of 10 isolates inhibited the growth of this strain. Moderate sensitivity was exhibited by *E. coli* and *S. enterica*. Five and six isolates, respectively, inhibited the growth of these pathogens on agar plates. Furthermore, isolated *Bacillus* strains showed reasonable activity against two *C. albicans* strains. Growth of *C. albicans* SC 5314 was inhibited by seven out of ten tested strains, and growth of *C. albicans* ATCC 10231 was inhibited by five strains. Only one strain, BP20.9, was able to slightly inhibit *C. krusei* growth.. None of the tested isolates demonstrated antifungal activity against *C. glabrata*.

## Antibiogram of Isolated Strains from BP and BB

The sensitivity of isolates to particular antibiotics is presented in Table 4. The high susceptibility of all isolated strains was observed against chloramphenicol, azithromycin, linezolid, trimethoprim, ciprofloxacin, gentamicin, and kanamycin. However, six strains were resistant to penicillin and two to clindamycin. The rest of the tested antibiotics presented moderate activity against isolates.

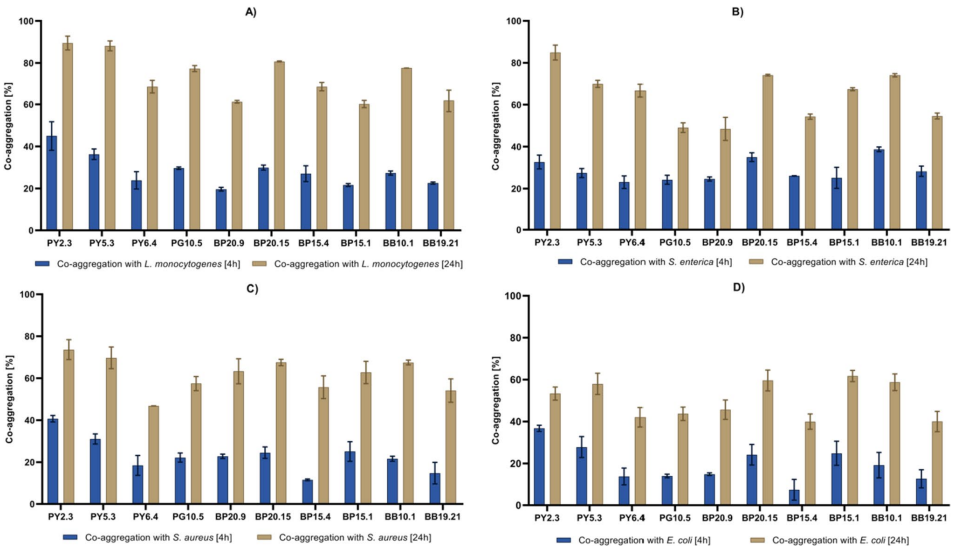


**Fig. 1** Percentage of co-aggregation of *Bacillus* isolates with **A** *L. monocytogenes* ATCC 35152, **B** *S. enterica* PCM 2266, **C** *S. aureus* ATCC 29231, and **D** *E. coli* ATCC 25922 after 4 and 24 h of incubation. The results are presented as means ± SD ($n = 3$). Data without error bars indicates that SD is too small to be observed on the graph

### Hemolytic and DNase Activity of Isolates

Hemolytic and DNase activities of the tested strains were examined. There were no pinkish zones around growing colonies. Thus, none of them demonstrated DNase activity. In terms of ability to hemolyze, two strains (BP20.9 and BP15.4) presented β-hemolytic activity, three showed α-hemolytic activity (PY5.3, BB19.21, and PG10.5), and the rest of the tested isolates exhibited γ-hemolytic activity, which is considered safe for humans.

### Cholesterol Assimilation of Isolates

The ability to assimilate cholesterol in LB broth supplemented with 0.2% of bile salts was determined. The mean value of cholesterol concentration assimilated by strains after 24 h was 27.99 ± 3.80%. The highest cholesterol absorption was exhibited by strain PG10.5 (36.45 ± 4.65%) and the lowest by strain BP15.4 (10.74 ± 0.240%) Fig. 2.

### Carbohydrates Metabolism and Profile of Enzymes Produced by Strains

Isolates were also investigated according to their ability to metabolize carbohydrates using the API CH50 kit.

Tested strains were able to metabolize 30 out of 49 carbohydrates (Supplementary information, Table 1). The most abundant carbohydrates processed by tested strains were glycerol, L-arabinose, D-ribose, D-xylose, D-glucose, D-fructose, D-mannose, D-mannitol, methyl-α-D-glucopyranoside, amygdalin, arbutin, esculin, salicin, D-cellobiose, D-maltose, D-saccharose, D-trehalose, amidon, glycogen, and D-tagatose. On the other hand, none of the investigated strains metabolized erythritol, D-arabinose, L-xylose, D-adonitol, methyl-β-D-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, methyl-α-D-mannopyranoside, D-melezitose, xylitol, D-lyxose, D-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium 2-ketogluconate, and potassium 5-ketogluconate.

### Whole-genome Sequencing Analysis

The 4,051,332 bp genome of isolate BB10.1 was assembled into 24 contigs with a GC content of 43.67% and an N50 of 1,041,549 bp (Supplementary Table 2, Quast report). The 4,022,011 bp genome of isolate BP20.15 was assembled into 53 contigs with a GC content of 43.71% and an N50 of 185,768 bp (Supplementary Table 2, Quast report). The genome of isolate PY2.3 (3,913,508 bp) was assembled into 35 contigs with a GC content of 46.52% and an N50 of

**Table 3** Antagonistic interactions of isolates with pathogenic bacteria and yeasts. (+) – inhibition zone with diameter 1–2 mm, (++) – 3–4 mm, (+++)—≥5 mm, (-) – no inhibition zones around growing colonies

| | *S. aureus* ATCC 29213 | *S. epidermidis* ATCC 35984 | *E. coli* ATCC 25922 | *P. aeruginosa* ATCC 27853 | *S. enterica* PCM 2266 | *L. monocytogenes* ATCC 35152 | *C. albicans* SC 5314 | *C. albicans* ATCC 10231 | *C. krusei* DSM 11226 | *C. glabrata* DSM 6128 |
|---|---|---|---|---|---|---|---|---|---|---|
| PY2.3 | ++ | ++ | + | +++ | + | - | - | - | - | - |
| PY5.3 | ++ | ++ | + | +++ | + | - | ++ | + | - | - |
| PY6.4 | + | + | - | ++ | + | - | - | - | - | - |
| PG10.5 | + | + | - | - | + | ++ | - | - | - | - |
| BP20.9 | +++* | ++ | ++* | ++* | - | - | + | + | + | - |
| BP20.15 | ++* | + | -* | +++* | - | - | + | - | - | - |
| BP15.4 | +++* | ++ | +++* | ++* | + | + | + | + | - | - |
| BP15.1 | -* | + | -* | +* | + | - | + | + | - | - |
| BB10.1 | +++* | ++ | -* | +++* | - | - | + | - | - | - |
| BB19.21 | +++* | ++ | +++* | +++* | - | - | + | + | - | - |

*—results presented in our previous paper (Pełka et al., 2021b)

353,859 bp (Supplementary Table 2, Quast report). Isolate BB10.1 contained an estimated 4241 genes and 4138 coding sequences (CDSs), 103 RNAs, 14 rRNAs (12; 5S, 1; 16S, and 1; 23S) and 84 tRNAs. Isolate BP20.15 consisted of an estimated 4207 genes, 4122 CDSs, 85 RNAs, one rRNA (5S) and 79 tRNAs. On the other hand, isolate PY2.3 had 3891 genes, 3807 CDSs, 84 RNAs, three rRNAs (5S, 16S and 23S) and 76 tRNA.

Average nucleotide identity (ANI) classified the isolates at the species level. Isolates BB10.1 and BP20.15 were most closely related to type strains *B. subtilis* Strain168 *and B. subtilis* 75, with orthoANI values of 99.97% and 99.99%, respectively. Isolate PY2.3 was closely related to *Bacillus velezensis* S4 with an orthoANI value of 98.69% (Table 5). The ANI between isolates 10.1 and 20.15 was 98.77%. Based on the proposed species boundary of 95–96% orthoANI value, 10.1 and 20.15 were classified as *B. subtilis* and PY2.3 as *B. velezensis* [41, 42].

## Bacteriocin-encoding Genes

The blast results of the BAGEL4 webserver for isolate BB10.1 genome predicted four bacteriocin clusters as areas of interest (AOIs) at (i) AOI 7.0 (start at 27,755 and end at 48,325), (ii) AOI 3.5 (start at 67,414 and end at 87,576), (iii) AOI 3.5 (start at 647,654, end at 668,287), and (iv) Node 4.7 (start at 67,715 and end at 87,715). The AOI 7.0 encodes the sporulation-killing factor *skfA*, which resides beside the *bmbF* gene, ABC transporter, ATP-binding proteins, and several ORFs (Open Reading Frames) (Fig. 3). The AOI 3.5, encodes a competence peptide that is found in several ORFs. Another AOI 3.5 was found to code for subtilosin A and subtilosin (*sboX*), which reside near the *bmbF* gene, ABC genes, and several ORFs. The AOI 4.7 encodes a bacteriocin belonging to the sactipeptide class (ribosomally synthesized and post-translationally modified peptides), which consists of a *bmbF* gene and several ORFs. Similarly, in the isolate BP20.15 genome, the same four clusters as in isolate 10.1 were observed: (i) AOI 7.1 Sporulation-Killing factor *skfA* (start: 163,457, end: 184,029); (ii) AOI 3.25 sactipeptides (start: 13,277, end: 33,277); (iii) AOI 8.37 competence (start: 123,287, end: 143,407); (iv) AOI Subtilosin (*sboX*) (start: 196,376, end: 217,009) (supplementary figure S1). In the isolate PY2.3 genome, four bacteriocin clusters were predicted as areas of interest (AOIs) at (i) AOI 5.11 (start: 122,123, end: 142,258), (ii) AOI 7.12 (start: 109,859, end: 130,189), (iii) AOI 7.12 (start: 59,723, end: 79,891), and (iv) AOI 6.5 (start: 180,338, end: 200,338). The AOI 5.11 encodes the antimicrobial peptide LCI, which resides alongside several ORFs. The AOI 7.12 encodes for amylocyclicin, which is located near a lantibiotic ABC transporter ATP-binding protein with several ORFs. Another AOI, 7.12,

**Table 4** Results of Antibiotic Disk Susceptibility Test. In the table below, the diameter of inhibition zones around disks are presented (in mm). The superscript letter (S) represents high sensitivity to antibiotics, (M) – moderate susceptibility, and (R) – resistance to tested antibiotics (according to the CLSI Standard). C30—chloramphenicol (30 µg), AZM15—azithromycin (15 µg), LZD30—linezolid (30 µg), RD5—rifampin (5 µg), P10—penicillin (10 units), W5—trimethoprim (5 µg), DA2—clindamycin (2 µg), CIP5—ciprofloxacin (5 µg), CN10—gentamicin (10 µg), and K30—kanamycin (30 µg)

| | C30 | AZM15 | LZD30 | RD5 | P10 | W5 | DA2 | CIP5 | CN10 | K30 |
|---|---|---|---|---|---|---|---|---|---|---|
| PY2.3 | $24^{S}$ | $17^{M}$ | $28^{S}$ | $18^{M}$ | $21^{R}$ | $23^{S}$ | $20^{M}$ | $27^{S}$ | $18^{S}$ | $21^{S}$ |
| PY5.3 | $25^{S}$ | $19^{S}$ | $26^{S}$ | $18^{M}$ | $22^{R}$ | $24^{S}$ | $21^{S}$ | $30^{S}$ | $20^{S}$ | $26^{S}$ |
| PY6.4 | $14^{M}$ | $19^{S}$ | $30^{S}$ | $30^{S}$ | $22^{R}$ | $29^{S}$ | $0^{R}$ | $35^{S}$ | $19^{S}$ | $23^{S}$ |
| PG10.5 | $22^{S}$ | $19^{S}$ | $29^{S}$ | $22^{S}$ | $34^{S}$ | $36^{S}$ | $19^{M}$ | $33^{S}$ | $18^{S}$ | $21^{S}$ |
| BP20.9 | $22^{S}$ | $20^{S}$ | $31^{S}$ | $23^{S}$ | $35^{S}$ | $35^{S}$ | $17^{M}$ | $35^{S}$ | $19^{S}$ | $21^{S}$ |
| BP20.15 | $29^{S}$ | $23^{S}$ | $29^{S}$ | $17^{M}$ | $23^{R}$ | $26^{S}$ | $21^{S}$ | $33^{S}$ | $19^{S}$ | $23^{S}$ |
| BP15.4 | $20^{S}$ | $19^{S}$ | $30^{S}$ | $19^{M}$ | $33^{S}$ | $33^{S}$ | $17^{M}$ | $30^{S}$ | $19^{S}$ | $22^{S}$ |
| BP15.1 | $24^{S}$ | $27^{S}$ | $33^{S}$ | $22^{S}$ | $22^{R}$ | $27^{S}$ | $0^{R}$ | $35^{S}$ | $19^{S}$ | $22^{S}$ |
| BB10.1 | $27^{S}$ | $18^{S}$ | $30^{S}$ | $17^{M}$ | $24^{R}$ | $30^{S}$ | $18^{M}$ | $29^{S}$ | $28^{S}$ | $20^{S}$ |
| BB19.21 | $23^{S}$ | $21^{S}$ | $28^{S}$ | $21^{S}$ | $37^{S}$ | $33^{S}$ | $18^{M}$ | $32^{S}$ | $17^{S}$ | $20^{S}$ |

coded for a competence pheromone and is located between several ORFs. AOI 6.5 encodes a bacteriocin belonging to the sactipeptide class (ribosomally synthesized and post-translationally modified peptides), which consists of the *bmbF* gene and several ORFs (supplementary figure S2).

### Phylogenomics Analysis

To elucidate the phylogenetic relationships between our isolates and the closely related *Bacillus* species, a total of 23 genomes were downloaded from the NCBI database. Seven *B. velezensis* group isolates, 15 *B. subtilis* isolates, and one *B. cereus* ATCC 14579 as an outgroup were included in the phylogenetic analysis. The phylogenetic tree based on the entire genome revealed that the isolates from this study have relatedness to the type strains *B. subtilis* (isolates BB10.1 and BP20.15) and *B. velezensis* (isolate PY2.3) (Fig. 4).

## Discussion

Diet plays a key role in our physical and mental health. It is not only a source of essential chemical ingredients such as proteins, carbohydrates, fatty acids, vitamins, and many other micro- and macroelements but also of different strains of microorganisms. Some of the food-associated bacteria (e.g., *Salmonella, Listeria,* and *Staphylococci*) and fungi (e.g., molds of the genus *Aspergillus* and Fusarium) are dangerous pathogens. Different technological approaches are available and proposed for growth inhibition (e.g., cooling and freezing) and elimination (sterilization, high pressure, smoking, and acidification) of pathogenic microorganisms from food products. Other food-associated microorganisms called probiotics are beneficial for different aspects of our health. The best-known group of probiotics are lactic acid bacteria (LAB) that are provided to our body mostly with

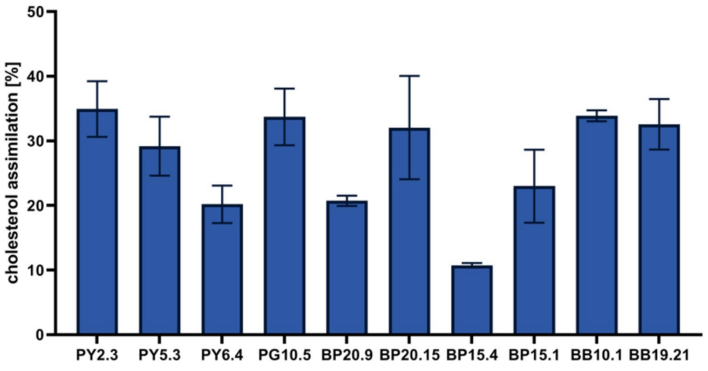


**Fig. 2** The amount of cholesterol assimilated by isolates [%] after 24 h of incubation. The results are presented as means ± SD ($n = 3$)

**Table 5** Average nucleotide identity (ANI) calculated by OAT with BLAST +

| Query genome | Reference genome | OrthoANI value (%) |
|---|---|---|
| Isolate 10.1 | *Bacillus subtilis* Strain168 | 99.97 |
| Isolate 20.15 | *Bacillus subtilis* 75 | 99.99 |
| Isolate 10.1 | Isolate 20.15 | 98.77 |
| Isolate PY2.3 | *Bacillus velezensis* S4 | 98.69 |
| Isolate PY2.3 | *Bacillus velezensis* NZ4 | 98.23 |

fermented milk products (yogurt and kefir) and fermented vegetables (e.g., cabbage and cucumbers). This study aimed to investigate the probiotic potential of bacterial strains isolated from bee pollen and bee bread. Bee bread is also an example of a fermented food product. It is generated in the wells of honeycombs during the fermentation process of bee pollen. Bacteria sourced from raw materials (pollen grains) and bee saliva play a crucial role in the process of biotransformation (fermentation) of bee pollen into bee bread. Still, very little is known about the microbiota of bee bread and pollen grains and their potential influence on the health of consumers. However, several authors reported that the microbial composition of maturing bee bread is dynamic and is changing over time. Vasquez and Olofsson (2015) observed the intense growth of LAB within maturing BB for about two weeks—the first step of BP biotransformation [13]. Disayathanoowat and coworkers (2020) found a significant decrease in the number of pathogenic Enterobacteriaceae (*Escherichia, Shigella, Panteoa,* and *Pseudomonas*)


**Fig. 3** The organization of bacteriocin gene clusters in the isolate *B. subtilis* 10.1 genome predicted through the BAGEL4 webserver. The area of interests represents (a) sporulation killing factor (b) competence pheromone (c) subtilosin (d) sactipeptide classes. The color schemes represent the specific gene clusters identified in BB10.1 genome

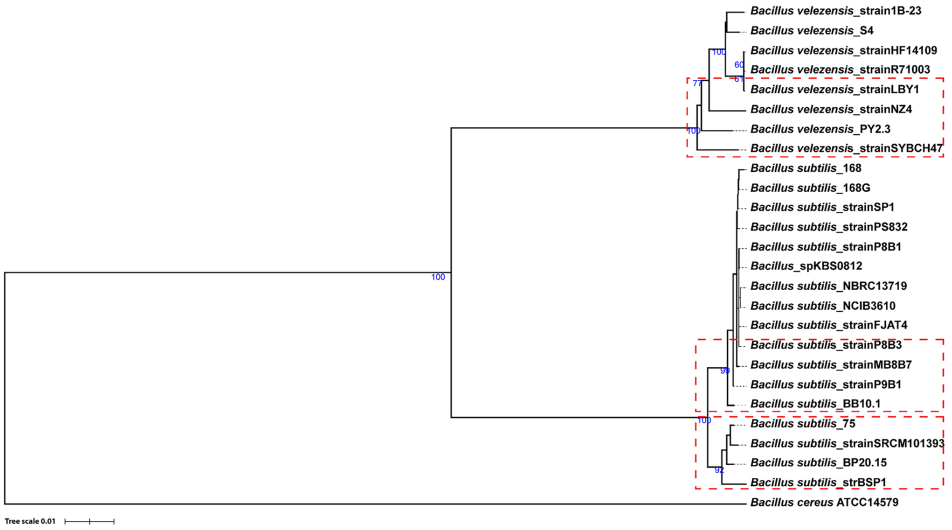


**Fig. 4** (A). TYGS Genome tree inferred with FastME 2.1.6.1 [39] from GBDP distances calculated from genome sequences. The branch lengths are scaled in terms of the GBDP distance formula d5. The numbers above branches are GBDP pseudo-bootstrap support values > 60% from 100 replications, with an average branch support of 21.2%. The tree was rooted at the midpoint [43]

in maturing bee bread in less than 72 h from the time of pollen grain collection in honeycomb wells [44]. This observation is important not only from the point of view of the health of the bee colony but also from the perspective of the possibility of using bee pollen and/or bee bread as a food product for humans. Our previous report revealed that most of the bacteria isolated from bee pollen (partially dried) and mature bee bread belonged to the genus *Bacillu*s spp., and many of them exhibit promising antimicrobial potential [12]. Herein, we investigated the probiotic potential of selected *Bacillus* spp. strains derived from bee pollen and bee bread produced in Polish apiaries.

The probiotic bacteria are expected to improve the health of consumers mostly by regulating the microflora of the gastrointestinal tract and eliminating pathogenic bacteria. The gastrointestinal tract can be a hostile environment for microorganisms. Thus, it was important to examine the resistance of isolated strains (potential probiotics) to the bile salts and low pH. All tested isolates exhibited significant tolerance to 0.3% of bile salt with a viability level of about 80%, a concentration that is considered similar to human bile juice [45]. A slightly higher inhibitory effect was observed in acidic conditions. About 65% of the cells of the most sensitive PG10.5, were eliminated from the suspension within 3 h of incubation. However, for 8 out of 10 strains tested, the survival rate was higher than 50%, with the highest value of 68.23% for PY2.3. The properties of these strains are similar to those of bee products-derived bacteria investigated by other authors. For instance, a low influence of bile salts (at a concentration of 0.3%) on *Bacillus* spp. strains isolated from honey was observed by Toutiaee et al. (2022) [26] and Zulkhairi Amin et al. (2019) [21]. However, both strains investigated by Zulkhairi Amin et al., (2019) and three out of five strains investigated by Toutiaee et al. (2022) exhibited a bit better tolerance to acidic conditions (pH 2.0 or 3.0, respectively), with a survival rate of about 90%. Other essential abilities for potential probiotics are hydrophobicity and auto-aggregation, which reflect cell adhesion to intestinal epithelial cells [46]. The auto-aggregation ability of the strains tested herein was similar to that observed by Toutiaee et al. (2022) [26] and Zulkhairi Amin et al., (2019) [21], where the values of this parameter were in the range of 42 to 84%. Interestingly, the hydrophobicity of our nine isolates was in the range of 5.65 to 33.18%, and only one strain was above 60%, whereas the hydrophobicity of strains tested in the above-mentioned studies was in the range of 48 to 68%, thus being significantly higher. However, a similar level of hydrophobicity was observed for *B. subtilis* Bn1 by Nithya and colleagues (2013) [47]. Considering these "parameters," we can conclude that the *Bacillus* spp. strains investigated in this study meet the basic criteria of probiotics. Moreover, interesting and beneficial results were observed for

co-aggregation ability with selected pathogenic bacteria. All tested strains exhibited the highest level of co-aggregation with *L. monocytogenes,* which can cause listeriosis [48]. The values of this parameter, when tested against other pathogenic strains, were also at a satisfactory level.

One of the most important requirements for bacteria to be considered probiotics is that they are safe for the human body. Antibiotic susceptibility tests showed that all strains tested were sensitive to five out of ten antibiotics with different mechanisms of action. Chloramphenicol and azithromycin also exhibited good activity; in both cases, only one isolate with moderate susceptibility was identified (nine were sensitive). Five strains exhibited moderate resistance to rifampicin; six, namely, PY2.3, PY5.3, PY6.4, BP20.15, BP15.1, and BB10.1, were resistant to penicillin (10 u), and two isolates (PY6.4 and BP15.1) were resistant to clindamycin (2 µg). Six strains exhibited moderate susceptibility to this antibiotic. In the case of probiotics, resistance to antibiotics should be rather considered an advantage; e.g., some commercially available probiotic strains, e.g., *Lactobacillus rhamnosus* GG, also exhibit resistance to some antibiotics (teicoplanin and vancomycin) [21]. It enables the use of these bacterial strains or even bee pollen/bee bread (carriers of probiotic strains) for the regulation of the microbiota of our gastrointestinal tract during therapies conducted with these antibiotics. Another important aspect investigated was hemolytic and DNase activity. Only β-hemolysis is considered harmful; as a result, two of the strains, namely BP20.9 and BP15.4, must not be considered potential probiotics. In this study, five strains (Py2.3, PY6.4, BP20.15, BP15.1, and BB10.1) showed γ-hemolytic activity, which indicates their safety for humans. Furthermore, none of the tested strains exhibited DNase activity.

An important advantage of probiotics is their potential to lower the cholesterol level in the plasma, which can reduce the risk of cardiovascular diseases. Manson et al. (1992) estimated that reducing cholesterol by 1% can decrease the risk of coronary artery disease by 2–3% [49]. All of the tested strains assimilated cholesterol from the broth during the 24 h of incubation, but only 5 strains showed the ability to absorb cholesterol at over 30% (PY2.3, PG10.5, BP20.15, BB10.1, and BB19.21). Thus, regular, long-term consumption of probiotics containing bee pollen and/or bee bread or supplementation of our diet with pure cultures of probiotic strains isolated from these products can positively affect the level of cholesterol in the plasma of consumers.

A significant amount of metabolized carbohydrates (mono-, di- and polysaccharides, glycosides, and triols) by tested *Bacillus* isolates prove that the investigated isolates can easily obtain carbon from various sources and can easily develop in the gut environment; moreover, the robust metabolism of carbohydrates can increase the viability of probiotics and can provide many different benefits to the host that include a positive effect on metabolic disease, improvement of the gut microbiome, and strengthening of the immune system [50, 51]. Taking into account the presence of probiotic bacteria in bee bread and bee pollen and the chemical composition of these products (high concentrations of saccharides), we suggest that both of them should be considered as symbiotics. According to the obtained results, three out of ten strains—PY2.3, BP20.15, and BB10.1—were considered possible probiotic strains. For these strains, whole-genome sequencing was performed. Two strains, BP20.15 and BB10.1, were classified as *B. subtilis* and PY2.3 as *B. velezensis*. Several studies showed that both species have great probiotic potential due to their survivability in the GIT, non-toxicity to the organism, and improvement of the health of the host [52–56]. All three isolates are potential producers of different antimicrobial compounds, including bacteriocins and secondary metabolites, which is an important benefit from the point of view of using these strains as probiotics.

## Conclusions

The preliminary outcomes of this study confirm the probiotic potential of some *Bacillus* spp. strains isolated from bee pollen or bee bread. On the other hand, we have found that some strains exhibit highly unfavorable properties, e.g., the ability of beta hemolysis or non-tolerance gut environment conditions, mostly low pH. Evidently, additional in vitro and in vivo tests are necessary to verify the possibility of using these isolates as probiotics (e.g., as ingredients in food products or dietary supplements) and to determine their positive impact on consumers' health.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s12602-023-10157-4.

**Author contributions** Conceptualization, K.P. and P.S.; methodology, K.P., A.B.H. and P.S.; validation, K.P.; investigation, K.P., A.B.H. and P.S.; resources, K.P., A.B.H. and P.S.; data curation, K.P. and P.S.; writing—original draft preparation, K.P., A.B.H. R.W.W. and P.S.; writing—review and editing, R.W.W. and P.S.; visualization, K.P.; supervision, P.S.; project administration, P.S.; funding acquisition, P.S. All authors have read and agreed to the published version of the manuscript.

**Funding** The studies were financed by the grant UMO-2021/41/B/NZ9/03929 from the National Science Centre, Poland.

**Data Availability** The data presented in this study are available on request from the corresponding author.

## Declarations

**Competing interests** The authors declare no competing interests.

**Conflict of Interest** The authors declare no competing interests.

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## CONCLUSIONS

The development of resistant strains causes the need of investigation of new antibiotics or non-antibiotic antimicrobial compounds. Honey bee products have been known for ages for their health-promoting properties, including antimicrobial potential against a wide variety of pathogenic microorganisms. Bee pollen and bee bread are the least known among all bee products; however, they are gaining more and more attention these days due to their high nutritional value and health-promoting properties. Nevertheless, the antimicrobial activity of these products and their mechanism of action are still barely known.

Research on bee pollen and bee bread constitutes a composition of four articles:

- Article 1. Pełka K., Otłowska O., Worobo R., Szweda P. Bee Bread Exhibits Higher Antimicrobial Potential Compared to Bee Pollen, Antibiotics, vol. 10, issue 2 (2021), 125
- Article 2. Pełka, K., Bucekova, M., Godocikova, J., Szweda, P., Majtan, J. Glucose oxidase as an important yet overlooked factor determining the antibacterial activity of bee pollen and bee bread. European Food Research and Technology (2022)
- Article 3. Pełka K., Otłowska O., Worobo R., Szweda P. Bee Pollen and Bee Bread as a Source of Bacteria Producing Antimicrobials, Antibiotics, vol. 10, issue 6 (2021), 713
- Article 4. Pełka 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).

Articles 1 and 2 focused on the investigation of antimicrobial potential and the examination of the mode of action of bee pollen and bee bread extracts. Both products revealed significant activity against bacteria, mostly Gram-positive staphylococci. Interestingly, bee bread presented higher antibacterial potential compared to bee pollen. To date, the antimicrobial potential of both products has been associated with the presence of phenolic compounds, however, my research provided some evidence that bee-derived proteins play a crucial role in inhibition of pathogens as well. The results of the research indicate that bee pollen and bee bread have a significant potential to be used as an alternative to known therapeutic agents or as a functional food.

The third and fourth articles concentrated on the examination of the microbiota of bee pollen and bee bread and its ability to produce compounds with biotechnological and pharmaceutical potential. The *Bacillus* strains isolated from these products exhibited antagonistic interactions with common human pathogens. Furthermore, the isolates that were identified as *B. velezensis* and *B. subtilis*. The isolated bacteria also possessed the probiotic potential that may bring many benefits to the host, for example, decrease the cholesterol level in the plasma. In addition, BP and BB isolates produce numerous enzymes of industrial significance. Research on the microbiota of bee pollen and bee bread provided the conclusion that these products can be a reservoir of microorganisms with multiple applications.

## ACKNOWLEDGMENTS

The research was funded from the grants of National Science Centre No. 2015/18/E/NZ6/00700 “Assessment of the possibility of using bee products and bacteriocins synthetized by the microflora of these products as an alternative or supplement to classic antibiotic therapy” and No. 2021/41/B/NZ9/03929 “Biotechnological and pharmacological potential of the microbiota of bee products” - the manager of the projects – dr hab. inż. Piotr Szweda.

Part of the research was conducted during scientific internship at Laboratory of Apidology and Apitherapy, Department of Microbial Genetics, Institute of Molecular Biology, Slovak Academy of Sciences, Bratislava, Slovakia (01/09/2021-27/11/2021). Supervisor – prof. Juraj Majtan, DSc. The internship was funded from Integrated Programme of Development of Gdansk University of Technology (POWR.03.05.00-00-Z044/17).

## SCIENTIFIC ACHIEVEMENTS

### 1. ARTICLES PUBLISHED IN SCIENTIFIC JOURNALS

1. Grecka K., Xiong Z.R., Chen H., **Pełka K.,** Worobo R.W., Szweda P. Effect of Ethanol Extracts of Propolis (EEPs) against Staphylococcal Biofim – Microscopic Studies, Pathogens, vol. 9, issue 8 (2020), 169.
doi:10.3390/pathogens9080646

2. **Pełka K.,** Otłowska O., Worobo R., Szweda P. Bee Bread Exhibits Higher Antimicrobial Potential Compared to Bee Pollen, Antibiotics, 10, 2 (2021), 125
doi:10.3390/antibiotics10020125

3. **Pełka K.,** Otłowska O., Worobo R., Szweda P. Bee Pollen and Bee Bread as a Source of Bacteria Producing Antimicrobials, Antibiotics, 10, 6 (2021), 713
doi:10.3390/antibiotics10060713

4. **Pełka, K.,** Bucekova, M., Godocikova, J., Szweda, P., Majtan, J. Glucose oxidase as an important yet overlooked factor determining the antibacterial activity of bee pollen and bee bread. European Food Research and Technology, 248 (2022), 2929-2939
doi:10.1007/s00217-022-04101-z

5. Hafeez A.B., **Pełka 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

6. **Pełka 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

## 2. SCIENTIFIC CONFERENCES

1. **28/10/2020 – 31/10/2020** - FEMS (Federation of Microbiological Societies) Conference on Microbiology, Online
Presentation of the e-poster entitled: “Bee pollen and bee bread as a source of bacteria producing antimicrobial metabolites.”
Authors: **Pełka K.,** Otłowska O., Walkusz J., Szweda P.

2. **01/07/2021 – 02/07/2021** - XLV Sesja Naukowa Komitetu Nauk o Żywności i Żywieniu PAN, Gdańsk, Poland
Presentation of the poster entitled: „Właściwości przeciwdrobnoustrojowe pyłku pszczelego oraz pierzgi pozyskiwanych w polskich pasiekach. (Antimicrobial activity of bee pollen and bee bread derived from polish apiaries)”.
Authors: **Pełka K.,** Otłowska O., Walkusz J., Szweda P.

3. **30/06/2022 – 02/07/2022** - FEMS (Federation of Microbiological Societies) Online Conference on Microbiology, Belgrade, Serbia
Presentation of the poster entitled: “Probiotic properties of bacteria isolated from bee pollen and bee bread.”
Authors: **Pełka K.,** Szweda P.

4. **28/06/2023 – 30/06/2023** - PSE (Phytochemical Society of Europe) Trends in Natural Products, Young Scientists’ Meeting, Paris, France
Poster entitled: Antibacterial, antifungal and antiviral activity of bee bread derived from Polish apiaries.
Authors: **Pełka K.,** Dimitriou T.G., Szweda P.

## 3. SCIENTIFIC INTERNSHIPS

1. **01/09/2021 – 27/11/2021** - Internship at Laboratory of Apidology and Apitherapy, Department of Microbial Genetics, Institute of Molecular Biology, Slovak Academy of Sciences, Bratislava, Slovakia.
Supervisor: Juraj Majtan, DSc

2. **07/11/2022 – 06/12/2022** - Internship at Laboratory of Microbial Biotechnology – Molecular Bacteriology – Virology, Department of Biochemistry and Biotechnology, University of Thessaly.
Supervisor: Dimitrios Mossialos, DSc

3. 

**30/01/2023 – 28/02/2023** - Internship at Laboratory of Microbial Biotechnology – Molecular Bacteriology – Virology, Department of Biochemistry and Biotechnology, University of Thessaly.
Supervisor: Dimitrios Mossialos, DSc

## 4. PARTICIPATION IN PROJECTS

1. 

**15/12/2021 – 15/09/2022** – Participation in OPUS 20 project from Polish National Science Centre (No 2020/39/B/NZ7/02901).
Title of the project: "Propolis and polyphenols derived from this product as potential antifungal agents".
Gdansk University of Technology, Faculty of Chemistry.
Project investigator: Piotr Szweda, DSc

2. 

**08/01/2023 – present** – Project investigator of Preludium 21 grant from Polish National Science Centre (No 2022/45/N/NZ9/02710).
Title of the project: "Antiviral and cell-protective potential of bee bread derived from Polish apiaries".

## 5. SCHOLARSHIPS

| | | |
|---|---|---|
| 1. | **05/2020** | Scholar of Integrated Programme of Development of Gdansk University of Technology – 3-month internship grant (POWR.03.05.00-00-Z044/17). |
| 2. | **10/2020 – 09/2021** | Francium Supporting Outstanding Doctoral Candidates – scholarship of the IDUB program |
| 3. | **10/2021 – 09/2022** | Francium Supporting Outstanding Doctoral Candidates – scholarship of the IDUB program |
| 4. | **30/06/2022 – 02/07/2022** | Conference Attendance Grant – FEMS Conference on Microbiology 2022, Belgrad, Serbia |
| 5. | **04/2022** | Scholar of International Scholarship Exchange of PhD Candidates and Academic Staff (PROM Programme) – 1-month internship grant |
| 5. | **10/2022 – 09/2023** | Francium Supporting Outstanding Doctoral Candidates – scholarship of the IDUB program |

## 6. MEMBERSHIP

| | | |
|---|---|---|
| 1. | **12/2022 – present** | Member of Phytochemical Society of Europe |

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# LIST OF FIGURES

## INTRODUCTION



## ARTICLE 1



## ARTICLE 2

## ARTICLE 3



## ARTICLE 4

# LIST OF TABLES

## INTRODUCTION



## ARTICLE 1



## ARTICLE 2



## ARTICLE 3



## ARTICLE 4