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Centrum Bioimmobilizacji i Innowacyjnych
Materiałów Opakowaniowych

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The Assessment of the Doctoral Dissertation of mgr Ahmer Bin Hafeez,

Title of doctoral dissertation:

''Pharmacological Potential of Bee Products Microbiota"
(„Potencjał Farmakologiczny Mikrobioty Produktów Pszczelich"),

The research was conducted under the supervision of Piotr Szweda, PhD, DSc, Eng., Gdańsk Tech Professor, at the Department of Pharmaceutical Technology and Biochemistry, Faculty of Chemistry, Gdańsk University of Technology.

The research addresses important and timely challenges in contemporary microbiology and biotechnology, particularly the increasing need to identify new sources of biologically active compounds and to explore the functional potential of microorganisms originating from natural environments including, raw honey, bee pollen and bee bread. In the context of the global problem of antimicrobial resistance and the growing interest in microorganisms with potentially beneficial biological properties, I consider the research topic both relevant and scientifically justified.

Formal Assessment of the Doctoral Dissertation

The doctoral candidate has prepared a dissertation written in English and structured in accordance with established academic standards. The dissertation displays logical and coherent organization, allowing the reader to progress easily through the successive research topics. The Introduction provides a clear and comprehensive overview of the current state of knowledge in the field, facilitating an understanding of both the theoretical foundations and the scientific

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background of the research undertaken. The dissertation incorporates a series of five thematically related scientific publications supplemented by additional research findings. These components systematically address the clearly defined research objectives and scientific questions, describe the methodologies employed and provide a detailed presentation and analysis of the results obtained. The Summary and Final Conclusions are presented in clearly distinguished sections, facilitating an understanding of the principal findings and their significance within a broader scientific context. This organization enhances the clarity of the dissertation and highlights the interdisciplinary nature of the research.

The dissertation comprises of 221 pages in total. Its principal components include an Introduction, well illustrated with various figures and tables (33 pages); a statement of the research objectives and their implementation across four stages of the research programme (2 pages); a series of five thematically related scientific publications with a combined Impact Factor (IF) of 20 and a total of 480 points according to the Polish ministerial journal evaluation system (123 pages); Additional Unpublished Research data and results (20 pages); a Summary and Final Conclusions (2 pages); a list of References comprised of 295 bibliographic entries (19 pages); and lists of figures and tables (5 pages).

The dissertation is preceded by a Summary, including Abstracts in Polish and English, as well as Acknowledgements, expressing gratitude to the individuals who collaborated with and supported the doctoral candidate at various stages of their research. Towards the end of the dissertation (pp. 120–121), the author also provides a list of scientific conferences in which he participated, together with information on any scholarships and awards received. These achievements further demonstrate his active engagement in scientific activities and his ability to collaborate effectively within research teams.

The scientific publications included in the dissertation provide evidence of the doctoral candidate's advanced research competencies and his ability to undertake and accomplish ambitious scientific tasks. The dissertation as a whole has been prepared with considerable care and a clear attention to detail, resulting in a well-organized, well presented and highly readable scientific document.

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Scientific Coherence of the Research Programme

The doctoral dissertation represents a valuable scientific contribution to the investigation of the antimicrobial and probiotic potential of bacteria associated with bee products. One of its major strengths is the integration of experimental microbiological methods with whole-genome sequencing, comparative genomics, functional annotation and advanced bioinformatic and molecular modelling approaches. This comprehensive methodology has enabled the characterization of selected bacterial isolates and an investigation of genetic determinants potentially associated with their biological activities. The research programme is thematically coherent and develops along two complementary directions. The first focuses on the antimicrobial properties of bacterial isolates, their biosynthetic potential and the possible molecular mechanisms underlying their activity. The second addresses the identification and characterization of potential probiotic candidates, including the assessment of their functional properties and safety. Although the individual publications investigate different bacterial isolates, they pursue closely related scientific objectives and progressively expand our understanding of the biological potential of *Bacillus* strains associated with bee products. In my opinion, this logical progression, together with the methodological diversity of the studies, demonstrates the doctoral candidate's ability to conduct a coherent and multifaceted research programme.

Scientific contribution of the individual publications

The first publication provides a foundation for the subsequent research by investigating 132 bacterial isolates obtained from three Polish honey samples. Of the 33 isolates selected for antimicrobial screening, 14 exhibited inhibitory activity against staphylococci, while cell-free culture supernatants from nine isolates demonstrated antimicrobial activity. Three particularly promising strains were selected for whole-genome sequencing and identified as *Bacillus paralicheniformis* SZA14 and SZA16 and *Bacillus subtilis* SZB3. Genomic analysis revealed 14 putative biosynthetic gene clusters in each strain, potentially associated with the production of antimicrobial peptides and other secondary metabolites. I consider the integration of

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experimental antimicrobial screening with detailed genomic characterization a valuable contribution to understanding the biosynthetic potential of honey-derived bacteria.

The second publication extends the research to ten *Bacillus* isolates obtained from bee pollen and bee bread. Their probiotic potential was evaluated through several physiological and functional assays, including tolerance to acidic conditions and bile salts, aggregation properties, cell surface hydrophobicity, antimicrobial activity and cholesterol reduction. Three strains, *B. velezensis* PY2.3 and *B. subtilis* BP20.15 and BB10.1, were selected as the most promising probiotic candidates. Notably, all three reduced cholesterol concentrations in culture-based assays by more than 30% after 24 hours. I particularly appreciate the comprehensive selection strategy, which considered multiple biological characteristics rather than individual favourable properties.

The third publication directly continues the second study through an extensive genomic investigation of these three probiotic candidates. Comparative pan-genome analyses included 180 *B. subtilis* and 204 *B. velezensis* genomes. The author identified genes potentially associated with gastrointestinal survival, stress tolerance, carbohydrate metabolism and vitamin biosynthesis. Particular attention was given to antibiotic resistance determinants, potential virulence factors and mobile genetic elements, providing valuable information for the preliminary assessment of strain safety. Although these analyses do not conclusively establish probiotic safety, the combination of experimental findings and genomic characterization displays a significant strength of the research.

The fourth publication further develops the genomic investigations by focusing on hypothetical proteins encoded in the genomes of *B. paralicheniformis* SZA14 and *B. subtilis* SZB3. Of the 1,007 hypothetical protein sequences examined, 56 were selected for detailed functional characterization, including 36 from SZA14 and 20 from SZB3. Using multiple complementary bioinformatic tools, the author predicted functions associated with sporulation, biofilm formation, cellular transport, metabolism and regulatory processes. The integration of functional annotation, structural modelling and molecular dynamics simulations offers valuable insight into previously uncharacterized genomic components. Although these findings remain

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computational predictions, they constitute an important contribution to the functional characterization of the investigated strains.

The fifth publication returns to antimicrobial investigations through the characterization of two additional *B. subtilis* strains, Miodo8 and Miodo10, isolated from ginger-containing honey. The author examined their physiological characteristics, responses to selected cultivation conditions, antimicrobial activity and genomic potential. Crude extracts obtained from cultures grown in YPD medium demonstrated activity against Gram-positive pathogens, including methicillin-resistant *Staphylococcus aureus* (MRSA) and *Listeria monocytogenes*. Genomic analyses identified approximately 112 carbohydrate-active enzymes and several putative biosynthetic gene clusters associated with the production of compounds such as surfactin, fengycin, plipastatin, bacillaene and subtilosin A. I consider this study a valuable extension of the initial antimicrobial investigations, providing further experimental evidence of the biological potential of bee product-associated bacteria.

Considering the antimicrobial activity demonstrated by cell-free culture supernatants and crude extracts in the first and fifth publications, respectively, together with the identification of putative biosynthetic gene clusters, I would like to ask the doctoral candidate the following question: *Which experimental approaches would you consider the most suitable for identifying the metabolites responsible for the observed antimicrobial activity and determining whether this activity results from individual compounds or their combined effects?*

An additional and particularly interesting component of the dissertation concerns the bioinformatic identification and molecular characterization of a putative two-component lantibiotic biosynthetic system. In this part of the research, the doctoral candidate moves beyond the general identification of biosynthetic gene clusters and undertakes a more focused investigation into a selected genetic system potentially associated with antimicrobial peptide production. The research encompasses an analysis of the organization of the putative biosynthetic gene cluster, comparison of predicted precursor peptide sequences with previously characterized lantibiotics, investigation of possible post-translational modifications, structural prediction and molecular modelling of potential interactions with lipid II.

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I consider this research direction scientifically of interest and consistent with the progression of the earlier genomic investigations. It demonstrates an attempt to connect information derived from bacterial genome sequences with hypotheses concerning the structural characteristics and the possible action mechanisms of their predicted peptide products.

Of particular interest is the proposed involvement of two peptide components that may perform complementary roles in the interaction with a bacterial cell envelope target. The application of comparative sequence analysis and molecular simulation techniques provides a theoretical framework for investigating these kind of interactions and identifying structural features that may be relevant to the proposed antimicrobial mechanism.

The originality in this part of the dissertation may lie in the identification and detailed computational characterization of a previously insufficiently described biosynthetic system and its predicted peptide products. However, the precise nature of this contribution should be defined carefully. The identification of a potentially novel biosynthetic gene cluster or peptide sequence is not equivalent to the experimental discovery and characterization of a new antimicrobial compound. Similarly, the proposed post-translational modifications, three-dimensional structures and molecular interactions remain simply predictions that require experimental verification. Their reliability depends on the quality of the underlying sequence analyses, the chemical correctness of the proposed peptide structures and the assumptions employed during molecular modelling. These considerations are particularly relevant to lantibiotics, whose biological properties are greatly influenced by specific post-translational modifications and the resulting spatial organization of their peptide structures.

Consequently, I regard the additional findings as a promising contribution to the molecular characterization of potential antimicrobial peptides and as a valuable source of hypotheses for subsequent experimental work. The scientific merit of this research should be assessed in relation to the quality and methodological rigor of the computational investigations rather than through expectations of experimental validation beyond the scope of the presented study.

Summarizing, the additional research presented in the dissertation provides interesting insights into a putative two-component lantibiotic biosynthetic system. In this context, I would like to ask the doctoral candidate the following question: *Based on your molecular modelling*

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results, how might the predicted lantibiotic peptide components interact with lipid II and what is the potential significance of this interaction for their antimicrobial activity?

Methodological quality and interpretation of the findings

From a methodological perspective, I would assess the broad range of research techniques applied by the PhD student positively throughout the dissertation and the integration of experimental microbiological approaches with modern genomic and computational methods.

The doctoral candidate demonstrates familiarity with whole-genome analysis, comparative genomics, prediction of biosynthetic gene clusters, functional annotation, protein structure modelling and computational investigation of molecular interactions. The progressive use of these approaches across the publication series illustrates the development of his scientific competence and his ability to address increasingly specific biological questions.

An important strength of the research is the attempt to interpret the experimentally observed properties of bacterial isolates in the context of their genetic characteristics. Such an approach is particularly valuable when investigating microorganisms whose metabolic and biosynthetic potential remains incompletely understood. At the same time, the distinction between experimentally confirmed findings and bioinformatically predicted functions is fundamental to the interpretation of the dissertation. The identification of a biosynthetic gene cluster provides evidence of genetic potential but does not necessarily demonstrate the expression of the corresponding genes or the production of the predicted metabolites under the investigated cultivation conditions. Likewise, functional annotation of hypothetical proteins and molecular modelling of peptide interactions generate scientifically valuable predictions, but these do not independently establish the corresponding biological functions or mechanisms.

In my assessment, these limitations do not diminish the importance of the research programme. Rather, they define the level at which particular scientific conclusions can be formulated. It should also be emphasized that not every genomic or computational investigation requires immediate experimental verification of all predicted functions to constitute a meaningful scientific contribution. The identification of previously unrecognized genetic features, the improvement of functional annotations and development of mechanistic hypotheses can

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represent valuable research outcomes in their own right, provided that the conclusions are supported by the applied methods and appropriately reflect their predictive character. The dissertation should therefore be evaluated primarily in the context of its actual scientific objectives and the candidate's demonstrated expertise in microbiology, genomics and bioinformatics.

Originality and scientific significance

In my opinion, the originality of the dissertation resides primarily in the comprehensive characterization of selected bacterial isolates associated with bee products, the generation and interpretation of genomic information, the identification of their potential biosynthetic capabilities and the development of hypotheses concerning the biological functions of selected proteins and antimicrobial peptides. The characterization of isolates exhibiting antimicrobial activity contributes new information concerning the diversity and functional properties of bacteria originating from bee-associated environments. The investigation of potential probiotic candidates, supported by both physiological testing and genomic analyses, broadens the relevance of the research by identifying microorganisms whose properties merit further assessment for possible beneficial applications.

The functional characterization of hypothetical proteins represents an additional contribution to bacterial genome annotation and provides new predictions concerning proteins whose biological roles had not been clearly established. Furthermore, the focused investigation of a putative two-component lantibiotic biosynthetic system offers an interesting extension of the genomic findings and may provide a foundation for future studies on antimicrobial peptide structure and function.

The scientific relevance of these results is strengthened by the continuing need to explore alternative sources of antimicrobial substances and to evaluate the potential applications and safety of microorganisms with desirable biological properties. The dissertation is therefore primarily of fundamental scientific significance, although its findings also have potential implications for future biotechnological applications.

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Overall evaluation

When considering the dissertation as a whole, I regard its thematic coherence, extensive experimental computational scope and progressive development of scientific questions as particularly noteworthy. The individual publications constitute complementary components of a broader research programme rather than a collection of unrelated studies. Together, they provide information concerning antimicrobial activity, probiotic-related properties, bacterial physiology, genomic diversity, biosynthetic potential, safety-associated genetic characteristics and possible molecular functions of selected proteins and peptides. The dissertation demonstrates the doctoral candidate's ability to investigate complex microbiological problems, apply contemporary genomic and bioinformatic techniques, integrate information derived from different analytical approaches and develop further research questions on the basis of previously obtained results. I also appreciate the substantial scientific effort involved in the generation, analysis and interpretation of the extensive datasets presented throughout the dissertation.

The findings presented in the dissertation provide a valuable foundation for further investigations into the functional and biosynthetic potential of the selected bacterial strains, including their possible biotechnological applications. The integration of experimental microbiology with advanced genomic and computational approaches opens promising perspectives for future research.

In conclusion, I assess the doctoral dissertation very positively. The five publications, together with the additional research findings, constitute a coherent, substantial and original scientific achievement of considerable scientific value. The interdisciplinary nature of the research, combining experimental microbiology with advanced genomic and bioinformatic approaches, contributes significantly to our understanding of the antimicrobial and potential probiotic properties of bacteria associated with bee products. The findings also highlight the importance of exploring natural sources of novel antimicrobial compounds, particularly in the context of the growing need for alternative strategies to combat antimicrobial resistance. The comprehensive research approach, the originality of the results and the doctoral candidate's demonstrated scientific competence deserve particular recognition. In my opinion, the

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dissertation makes a meaningful contribution to microbiology and biotechnology and opens promising perspectives for further research and potential practical applications.

The dissertation under review meets the academic and scientific requirements for doctoral dissertations, particularly with regard to its original contribution to scientific knowledge, the research methodology employed and compliance with established formal and substantive academic standards. Furthermore, the dissertation provides a sound basis for awarding the doctoral degree in the field of Exact and Natural Sciences, in the discipline of Chemical Sciences.

Therefore, I conclude that the doctoral dissertation submitted by Ahmer Bin Hafeez, MSc, entitled "Pharmacological Potential of Bee Products Microbiota", meets the requirements specified in Article 187, paragraphs 1 and 2, of the Act of 20 July 2018 – Law on Higher Education and Science (Journal of Laws of 2026, item 1362). **Accordingly, I recommend that the dissertation be accepted and that the doctoral candidate be admitted to the subsequent stages of the doctoral degree proceedings.**

Szczecin, 17 listopada 2026 r.

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