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REVIEW

of the doctoral dissertation of Ahmer Bin Hafeez, MSc

entitled

„Pharmacological potential of bee products microbiota"

(*Potencjał farmakologiczny mikrobioty produktów pszczelich)*

The formal basis for preparing this review is the letter from the Chair of the Council of the Chemical Sciences Discipline at Gdansk University of Technology, Sebastian Demkowicz, Ph.D, D.Sc, Eng.

(letter L. Dz. 226 WCh/Dz/2026)

The doctoral dissertation submitted for evaluation was prepared by Ahmer Bin Hafeez, MSc at the Faculty of Chemistry of the Gdansk University of Technology, at the Department of Pharmaceutical Technology and Biochemistry under the supervision of Piotr Szweda, Ph.D, D.Sc, Eng.

Contemporary medicine and environmental protection face significant challenges associated with the widespread use of pharmaceuticals and their uncontrolled release into the natural environment. At the same time, the increasing resistance of microorganisms to currently available antimicrobial agents represents one of the most pressing challenges in modern medicine and microbiology, which highlights the need to seek new therapeutic solutions. In this context, research regarding natural products, including bee products and their associated microbiota, is of particular importance, as these may constitute a valuable source of novel biologically-active compounds with antimicrobial potential. One particularly promising research direction involves the investigation of the microbial composition of bee products and identification of microorganisms capable of producing substances with antimicrobial activity. Characterizing the properties of these compounds, elucidating their mechanisms of action, and evaluating their potential to inhibit the growth of microorganisms, including drug-resistant strains, may contribute to the development of new strategies for the prevention and treatment of infections. Consequently, research that addresses these issues is of considerable scientific

and practical significance. The doctoral dissertation of Ahmer Bin Hafeez, MSc, which focuses on evaluating the pharmacological potential of microbiota associated with bee products, particularly with regard to their probiotic properties, their ability to produce metabolites with antimicrobial activity, and the identification of the genetic determinants as well as structural characteristics of these compounds, fits exceptionally well within this field of research. In my opinion, the research topic undertaken by the Doctoral Candidate is highly relevant and valuable, not only from a scientific perspective but also in terms of its potential practical applications.

The doctoral dissertation was written in English and comprises a thematically coherent series of five scientific articles published in journals such as *Applied Microbiology and Biotechnology*, *Probiotics and Antimicrobial Proteins*, *International Journal of Molecular Sciences*, *ACS Omega*, and *Molecular Genetics and Genomics*. The Doctoral Candidate is the first author of four publications and the second author of one. In one of the articles, he also serves as the corresponding author. His substantial contribution to the preparation of these publications is confirmed by the statements provided by the co-authors. A detailed description of the Doctoral Candidate's contribution to each publication is presented on pages 47–49 of the dissertation, in the section listing the publications comprising the thesis. This information enables an appropriate assessment of the extent of his independent contribution to the presented research.

The publications comprising the doctoral dissertation are preceded by abstracts in both English and Polish, a list of abbreviations and acronyms, and a 34-page introduction providing background to the research topic. The literature review is well-structured and concise, comprising nine subsections devoted to topics such as the growing problem of bacterial resistance, antibiotic discovery, mechanisms underlying the development of antibiotic resistance, the general characteristics and clinical significance of ESKAPE pathogens, the biology of the honey bee (*Apis mellifera*), the microbiota of honey and other bee products, the functional classification of antimicrobial peptides and specialized metabolites produced by bacteria belonging the genera *Bacillus* and *Lactobacillus*, as well as the characteristics of ribosomally synthesized and post-translationally modified peptides and non-ribosomal peptides. The literature review confirms that Ahmer Bin Hafeez, MSc possesses a substantial theoretical background and is thoroughly prepared to conduct experimental research. In the subsequent part of the dissertation, the Doctoral Candidate formulated the scientific objective of the study, which was to determine the pharmacological potential of microbiota associated

with bee products, with particular emphasis on its probiotic properties and its ability to produce metabolites which exhibit antimicrobial activity. Achieving this objective required conducting a multifaceted research programme involving the assessment of the probiotic potential of the isolated strains and their antimicrobial activity, optimization of cultivation conditions, genomic and bioinformatic analyses of selected isolates, as well as structural identification of metabolites exhibiting the strongest antimicrobial effects.

Subsequently, the Doctoral Candidate included a list of articles constituting the basis of the dissertation, followed by copies of the individual publications, each of which was preceded by a 3–4-page introduction outlining the research conducted and presenting the most important findings obtained from his research. On page 72, he also presented the results of his own research that had not yet been published. The next section of the dissertation comprises an overview of the key research findings presented in the individual publications, together with the relevant conclusions. The dissertation concludes with acknowledgements, a list of the Doctoral Candidate's scientific achievements, a bibliography, and lists of figures and tables.

The subject of the research presented in **Publication No. 1** was the isolation and characterization of bacteria occurring in Polish honey, with regard to their antimicrobial activity, as well as the assessment of their biosynthetic potential using whole-genome sequencing and genome mining approaches. Three samples of multifloral honey collected in northern Poland were examined, yielding a total of 132 bacterial isolates. Among them, three strains exhibited the strongest antistaphylococcal activity against reference strains of *Staphylococcus aureus* (ATCC 25923 and ATCC 29213) and were therefore selected for detailed genomic analysis. Their identification revealed that two strains belonged to the species *Bacillus paralicheniformis* and one to *Bacillus subtilis*. Furthermore, genomic analyses demonstrated the considerable potential of the isolated strains to biosynthesize a variety of antimicrobial metabolites, including lipopeptides, siderophores, ribosomally synthesized and post-translationally modified peptides (RiPPs), and small antimicrobial peptides. The combination of phenotypic studies and genomic analyses enabled the identification of gene clusters associated with the biosynthesis of these metabolites, indicating the potential of the investigated strains to produce novel compounds, including bacteriocins. Moreover, the results also revealed the additional potential of these strains for applications in industrial biocatalytic processes and biomass degradation.

Publication No. 2 was focused on the isolation of bacterial strains belonging to the *Bacillus* genus from flower pollen and bee bread. Their probiotic potential was assessed based on physiological, biochemical, and genomic analyses. Ten *Bacillus* strains were isolated, including

seven from bee pollen and three from bee bread. The *Bacillus* isolates exhibited inhibitory activity against several bacterial pathogens, with the strongest effects observed against staphylococci (*Staphylococcus aureus* and *Staphylococcus epidermidis*) and *Pseudomonas aeruginosa*. A comprehensive assessment, including antimicrobial activity, survival under gastrointestinal conditions, adhesion-related properties, safety, cholesterol assimilation, and metabolic potential, enabled the selection of three bacterial isolates as the most promising candidates for potential probiotic strains. Whole-genome sequencing and phylogenomic analysis enabled their taxonomic identification at the species level: one strain was identified as *Bacillus velezensis*, while the other two belonged to the species *Bacillus subtilis*. Furthermore, genomic analyses revealed the presence of genes associated with the production of antimicrobial compounds, including bacteriocins and other secondary metabolites.

In **Publication No. 3**, the Doctoral Candidate focused on a detailed genomic analysis of previously selected *Bacillus* isolates to assess their probiotic potential, safety profile, antibiotic susceptibility, and the presence of genes involved in beneficial metabolic processes. Genomic analyses revealed that the *Bacillus* isolates obtained from flower pollen and bee bread harboured genes associated with stress tolerance, adhesion, carbohydrate metabolism, vitamin biosynthesis, and the production of antimicrobial compounds. No significant virulence genes or pathogenicity factors were identified, indicating that these isolates may represent promising candidates for probiotic applications, with a favourable safety profile.

Publication No. 4 was focused on the analysis and prediction of the biological functions and structural characterization of hypothetical proteins identified in the genomes of *Bacillus paralicheniformis* and *Bacillus subtilis* strains isolated from honey. A total of 56 hypothetical proteins were selected and subjected to bioinformatic analyses to predict their biological functions. The results indicated their potential involvement in key cellular processes, such as sporulation, biofilm formation, metabolic regulation, motility, and DNA replication. These findings expand current knowledge regarding the biology of bacteria belonging to the *Bacillus* genus; however, further detailed experimental validation is required.

In **Publication No. 5**, the Doctoral Candidate focused on assessing the antimicrobial potential of two *Bacillus subtilis* strains (Miodo8 and Miodo10), isolated from multifloral ginger honey. The study involved a comprehensive set of investigations, including phenotypic characterization, optimization of fermentation conditions, genome sequencing, and *in silico* screening of antimicrobial peptides. This integrated approach enabled a thorough assessment of the bioactive potential of the investigated strains. The results demonstrated that ginger honey may serve as a source of bioactive *Bacillus subtilis* strains exhibiting promising antimicrobial

properties. The observed biological activity was associated with the presence of biosynthetic gene clusters, secretory potential, and sequences encoding putative antimicrobial peptides. Genomic and bioinformatic analyses provided further insight into the biosynthetic potential of the investigated strains, while also highlighting the need for further validation of the predicted metabolites and peptides. Furthermore, the detection of haemolytic activity and antibiotic resistance markers indicates the need for a more comprehensive safety assessment of the investigated strains before their potential therapeutic or biotechnological applications can be considered.

The research topic addressed in the dissertation is scientifically relevant and corresponds well with the recent research trends. Achieving the objectives of the doctoral dissertation required a series of experimental studies, which were carefully planned and carried out. The application of a diverse range of research and analytical methods enabled a comprehensive verification of the underlying research assumptions. Throughout the research, the Doctoral Candidate demonstrated a high degree of independence in planning and conducting experiments, the ability to select appropriate research methods, and sound judgement in interpreting the obtained results. In my opinion, the research objective was fully achieved, and the doctoral dissertation under review presents original and valuable research findings that make a significant contribution to expanding the current knowledge regarding the biological and pharmacological potential of microbiota associated with bee products. The studies provide new insights into the microbial diversity of these products, the biological properties of the isolated strains, and their ability to produce compounds with potential antimicrobial activity. Particular attention should be drawn to the use of advanced genomic and bioinformatic approaches, which enabled an in-depth characterization of the investigated microorganisms and the identification of their biosynthetic potential. The findings not only expand current knowledge regarding the microbiota associated with bee products but also indicate the possibility of its future exploitation as a source of novel bioactive compounds with potential pharmacological and biotechnological applications. Thus, the dissertation makes a significant contribution to advancing the research regarding microorganisms which inhabit bee products and their potential practical applications.

Nevertheless, in fulfilling my duties as a reviewer, I would like to raise several points for discussion.

1. What environmental factors and cultivation conditions can influence the expression of genes involved in the biosynthesis of secondary metabolites in bacteria of the *Bacillus* genus?

2. Genomic analyses identified numerous biosynthetic gene clusters potentially involved in the production of antimicrobial compounds. What experimental approaches should be employed to confirm that these clusters are indeed active, and that the compounds they encode are produced by the investigated strains and exhibit antimicrobial activity?

3. What additional studies would be necessary to comprehensively assess the safety of the investigated strains in the context of their potential biotechnological applications?
4. Genes associated with environmental stress tolerance, adhesion, and survival under the harsh conditions of the gastrointestinal tract were identified in the genomes of the investigated *Bacillus* strains. What molecular mechanisms enable these bacteria to survive in the gastrointestinal tract, and what is the significance of these mechanisms in terms of their probiotic potential?

The questions and comments presented above are intended solely to stimulate scientific discussion and do not affect my positive assessment of the doctoral dissertation.

In assessing the Doctoral Candidate's overall scientific achievements, it should be emphasized that the Candidate is a co-author of five publications indexed in the Web of Science database, all of which are thematically related to the subject of the doctoral dissertation. This is a very good achievement, which demonstrates the Candidate's scientific activity and commitment, particularly at this stage of their academic career. The Candidate also actively participated in three scientific conferences, at which research findings were presented in the form of oral presentations and posters. The Doctoral Candidate's scientific achievements have been recognized through numerous scholarships and research grants. These include mini-grants awarded to doctoral students and young researchers, as well as the international doctoral fellowships Francium and Polonium. Furthermore, the Candidate received a scholarship from the Polish National Agency for Academic Exchange (NAWA), awarded to outstanding doctoral students conducting research projects in international collaboration. The completion of three research internships is also worth highlighting: the first at the Department of Chemistry, Faculty of Natural, Mathematical and Engineering Sciences, King's College London, United Kingdom, under the academic supervision of Professor Sarah M. Barry; the second at the Department of Biotechnology and Biomedicine, Technical University of Denmark, under the academic supervision of Professor Ling Ding; and the third at the Department of Biochemistry and

Biotechnology, University of Thessaly, Greece, under the academic supervision of Professor Grigorios Amoutzias. It is also worth mentioning that the doctoral dissertation was carried out as part of the research project funded by the National Science Centre (NCN) under the OPUS programme, grant No. UMO-2021/41/B/NZ9/03929, entitled "Biotechnological and Pharmacological Potential of the Microbiota of Bee Products", which was led by the doctoral supervisor Piotr Szweda, Ph.D, D.Sc, Eng.

In summary, based on the evaluation of the doctoral dissertation presented in the form of a series of thematically related original publications entitled **„Pharmacological potential of bee products microbiota" (*Potencjał farmakologiczny mikrobioty produktów pszczelich*)** authored by Ahmer Bin Hafeez, MSc as well as the scientific achievements documented in the dissertation, I unequivocally conclude that the reviewed doctoral dissertation Ahmer Bin Hafeez, MSc fully meets the requirements for doctoral theses set forth in the Act of 20 July 2018 – Law on Higher Education and Science (Dz. U. z 2018 r. poz. 1668).

Therefore, I hereby request that the Council of the Discipline of Chemical Sciences at Gdansk University of Technology admits Ahmer Bin Hafeez, MSc to the subsequent stages of the doctoral proceedings.

