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REVIEW OF THE DOCTORAL DISSERTATION BY AHMER BIN HAFEEZ, M.Sc., TITLED: “Pharmacological Potential of Bee Products Microbiota”

The doctoral dissertation submitted for review by Ahmer Bin Hafeez, M.Sc., was prepared under the supervision of Dr hab. inż. Piotr Szweda, prof. PG at the Department of Pharmaceutical Technology and Biochemistry, Faculty of Chemistry, Gdańsk University of Technology. The work was carried out as part of the National Science Centre (NCN) project OPUS UMO-2021/41/B/NZ9/03929, titled "Biotechnological and pharmacological potential of the microbiota of bee products," for which Dr hab. inż. Piotr Szweda, prof. PG served as the principal investigator.

The results of the research presented have been published in five peer-reviewed scientific articles in the following journals: Applied Microbiology and Biotechnology, Probiotics and Antimicrobial Proteins, International Journal of Molecular Sciences, ACS Omega, and Molecular Genetics and Genomics, between 2023 and 2026. In four of these publications, Ahmer Bin Hafeez, M.Sc., is the first and lead author. The cumulative impact factor of the presented work is high, totaling 20.0. The doctoral candidate’s contribution to each of these publications is unquestionable.

The doctoral dissertation opens with abstracts in Polish and English, followed by an extensive Introduction and a list of abbreviations. The table of contents facilitates navigation between the chapters. Each of the five publications included in the dissertation is additionally preceded by a brief outline of the research problem addressed, a summary of the results obtained, and the main conclusions. I highly appreciate the adopted structure of the dissertation, as it allows the reader to directly relate each concise summary to the corresponding publication and facilitates a focused assessment of the individual studies. In my opinion, this approach provides greater clarity than, for example, presenting all the research findings collectively and including the publications as appendices at the end of the dissertation. The dissertation concludes with Chapter 3, which presents unpublished research data, followed by Chapter 4, which provides an overall summary. In my assessment, the dissertation as a whole is presented in a highly coherent, clear, and well-organized manner.

The doctoral candidate demonstrates a sound theoretical understanding of the relevant scientific discipline, is well acquainted with the current state of research, has a thorough understanding of the methodologies employed, and is capable of critically evaluating the scientific literature.

Assessment of the Scientific Content of the Doctoral Dissertation

The research problem outlined by Ahmer Bin Hafeez, M.Sc., could hardly have been presented more convincingly. It is sufficient to note that only 27 new antibiotics were introduced into clinical use between 2000 and 2020, compared with 270 during the earlier period from 1928 to 1970. Against the backdrop of the increasing prevalence of antibiotic resistance among bacteria, combating drug-resistant pathogens has become a major global challenge. Researchers are striving to address this challenge by developing and introducing new antibiotics or identifying alternative agents with antibacterial activity.

Ahmer Bin Hafeez, M.Sc., classifies bacterial resistance to antibiotics into three categories: intrinsic, acquired, and adaptive resistance. He provides a clear and comprehensive introduction to the scientific issues addressed in the dissertation, further enhanced by well-designed and informative figures. Particular emphasis is placed on the ESKAPE group of pathogens (*Enterococcus faecium*,

Staphylococcus aureus, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.), including their mechanisms of antibiotic resistance and strategies for combating these pathogens. The doctoral candidate also highlights the importance of natural products, particularly bee products, as promising sources of novel antibacterial compounds.

The primary objective of the doctoral dissertation under review was to investigate the microbiota of three bee products: honey, bee pollen, and bee bread with particular emphasis on their antimicrobial potential.

The first publication focuses on the preliminary characterization of 132 bacterial isolates obtained from three honey samples collected from apiaries in Sulmin (Pomeranian Voivodeship, Poland). A subset of these isolates was screened for its ability to inhibit the growth of *Staphylococcus aureus*. Three promising strains were selected for whole-genome sequencing: two *Bacillus paralicheniformis* strains (SZA14 and SZA16) and one *Bacillus subtilis* strain (SZB3).

Genome analysis revealed the potential of the isolates to produce a variety of metabolites, including lichenysin, fengycin, bacitracin, and subtilosin A. In addition, the authors identified short peptide sequences with predicted antimicrobial activity: eight in strain SZA14, twelve in SZA16, and nine in SZB3.

The leading contribution of Ahmer Bin Hafeez, M.Sc., to this publication is beyond doubt. He performed the initial screening of the bacterial strains for antibacterial activity, genome sequencing and annotation, as well as homology modelling of putative antimicrobial peptides. He was also responsible for data interpretation and manuscript preparation.

Thus, the authors, including Ahmer Bin Hafeez, M.Sc., provided further evidence supporting the potential health-promoting properties of honey and the *Bacillus* spp. strains present in it.

Since the publication was largely based on *in silico* analyses, I would like to ask Ahmer Bin Hafeez, M.Sc., what experimental studies he would propose as the next step to validate and extend the computational findings presented in this work.

The second publication in the series includes a considerably more extensive experimental component. However, according to the doctoral candidate's statement of contribution, his involvement was primarily focused on genome sequencing and bioinformatic analyses. Nevertheless, the data generated through these analyses provide valuable support for and complement the experimental findings. One limitation of the first publication was that the antibacterial activity of the honey-derived isolates was assessed against only a single bacterial species, *Staphylococcus aureus*, rather than a broader panel of bacterial pathogens.

In the publication entitled "Probiotic Potential of Bacillus Isolates from Polish Bee Pollen and Bee Bread," published in Probiotics and Antimicrobial Proteins, Ahmer Bin Hafeez, M.Sc., and his co-authors evaluated the probiotic potential of ten *Bacillus* strains isolated from bee pollen and bee bread. Notably, eight of the ten isolates inhibited the growth of the Gram-negative bacterium *Pseudomonas aeruginosa*, whereas the Gram-positive bacterium *Listeria monocytogenes* exhibited the highest resistance to the tested isolates. The strains also demonstrated moderate antifungal activity against *Candida albicans*, while no inhibitory activity was observed against *Candida glabrata*.

In general, the ability to inhibit pathogenic microorganisms is a desirable characteristic supporting the potential suitability of a bacterial strain as a probiotic candidate. Interestingly, strain BP20.9, the only isolate exhibiting inhibitory activity against *Candida krusei*, also displayed β-haemolytic activity. This observation highlights the importance of a comprehensive assessment of candidate probiotic strains, demonstrating that antimicrobial activity alone is insufficient to establish their suitability for probiotic applications. In particular, potential safety concerns must be carefully evaluated alongside beneficial functional properties.

Based on the results obtained, three strains were selected as promising probiotic candidates: BP20.15 and BB10.1, both identified as *Bacillus subtilis*, and PY2.3, classified as *Bacillus velezensis*. These strains exhibited no haemolytic activity, were resistant to penicillin but susceptible to the other nine antibiotics tested, and demonstrated the ability to assimilate more than 30% of cholesterol following 24 hours of incubation. Given the established association between elevated cholesterol levels and an increased risk of cardiovascular disease, cholesterol assimilation represents a potentially

beneficial characteristic of probiotic candidates. Nevertheless, the physiological relevance of this property requires further validation beyond in vitro assays.

As previously indicated, Ahmer Bin Hafeez, M.Sc., was primarily responsible for whole-genome sequencing of the selected strains, their taxonomic classification, and bioinformatic analyses aimed at identifying genes encoding bacteriocins and enzymes involved in the biosynthesis of secondary metabolites.

The bioinformatic analyses were appropriately conducted and thoroughly described. In my opinion, the identification of putative bacteriocin biosynthetic gene clusters using the BAGEL4 tool is a particularly interesting aspect of this study and clearly warrants further experimental investigation. Although the findings provide evidence supporting the potential probiotic properties of the selected strains, they do not establish the expression of the identified genes, the production of mature antimicrobial peptides, or their contribution to the observed antimicrobial activity. An important next step would be to determine whether the antimicrobial activity is retained following the removal of the producer cells, with appropriate negative controls included. The active cell-free samples should subsequently be subjected to pH adjustment, catalase treatment, and exposure to selected proteolytic enzymes to assess the nature of the inhibitory compounds. A loss of antimicrobial activity following proteolytic treatment would support the involvement of a proteinaceous component but would not, in itself, establish the identity of a specific bacteriocin.

In this context, I would like to ask the doctoral candidate which experimental approaches he would propose for the purification and identification of selected antimicrobial peptides.

My scientific curiosity has prompted several questions concerning the experimental aspects of the studies under review. How can one conclusively demonstrate that a bacterial strain possesses probiotic properties without conducting appropriate *in vivo* studies? This is, of course, a rhetorical question. Nevertheless, the importance of integrating computational and experimental approaches in scientific research is increasingly being recognized. Modern research frequently generates large datasets, the analysis of which using computational tools enables the identification and prioritization of promising candidates for further investigation, such as bacterial strains with potential health-promoting properties or selected genes and their products.

In this context, I particularly appreciated the third publication. The three previously described bacterial isolates (BP20.15, BB10.1, and PY2.3) were subjected to extensive analyses using a range of bioinformatic tools to assess genome completeness and identify potential virulence factors and antimicrobial resistance genes. The analyses were comprehensive, and, in my assessment, the doctoral candidate employed an impressive range of computational algorithms and analytical approaches.

The Benchmarking Universal Single-Copy Orthologs (BUSCO) tool was used to assess genome completeness, with high BUSCO scores indicating a high degree of completeness based on the recovery of conserved single-copy orthologous genes. This approach, combined with the previously performed genome assembly quality assessment using QUAST (the results of which were presented in Publication 2), as well as pangenome and synteny analyses, provides a robust framework for comparative genomic investigations.

D-GENIES is a suitable tool for visualizing whole-genome alignments through dot plots, while pangenome analysis enables the genomes of strains BB10.1, BP20.15, and PY2.3 to be examined in the broader genomic context of other *Bacillus subtilis* and *Bacillus velezensis* strains.

According to the guidelines of the World Health Organization (WHO), the key criteria for evaluating a strain as an effective probiotic microorganism include, in particular, the ability to survive in the acidic environment of the stomach and in the presence of bile, the ability to adhere to intestinal epithelial cells, the demonstration of antimicrobial activity, and the absence of antibiotic resistance genes. All these aspects were thoroughly investigated in the study using a variety of bioinformatic tools. I will not list all the programs used in the presented study: KEGG/BlastKOALA and eggNOG-mapper v2, CRISPR-CasFinder, VirulenceFinder, PlasmidFinder, and OriTfinder are just some of them. Furthermore, the Generally Recognized As Safe (GRAS) status of bacterial strains such as *B. subtilis* and *B. velezensis* supports their probiotic potential.

I have a few minor comments regarding Table 2. In the table presenting stress response genes, there is an inconsistency in the section concerning adhesion factors: sortase A (*srt*A) is mentioned in the text, whereas sortase D (*srt*D) is listed in Table 2. Similarly, *hsl*O is described in the text as a gene encoding a protease, while in the table it is classified as a chaperone protein. The putative YtnI protein is listed twice in Table 2 as an antioxidant, and *atp*B and *atp*D are assigned the same UniProt identifier (P37809), whereas their respective identifiers are P37813 and P37809. Such minor errors are entirely understandable given the considerable volume of data presented.

At this point, however, I would like to emphasize that, for example, the identification of adhesion proteins does not demonstrate intestinal colonization, i.e. adhesion to the intestinal epithelium, and additional *in vivo* studies would be required. In the previous study, indirect assays were performed, including aggregation, co-aggregation, and bacterial cell affinity for p-xylene. Similarly, the classification of a prophage region as "intact" by the PHASTER software does not demonstrate its ability to produce active phage particles.

A good example illustrating the need for experimental verification of theoretical predictions is the detection of the haemolysin gene (*hly*III) in the genomes of all three strains investigated, despite the absence of observable haemolytic activity (the strains exhibited so-called γ-haemolysis). Similarly, ResFinder 4.1 predicted streptomycin resistance, which was not confirmed by laboratory testing.

In the publications submitted for evaluation, Ahmer Bin Hafeez, M.Sc., repeatedly emphasizes the need for additional laboratory experiments. This approach deserves recognition, as it demonstrates that the doctoral candidate is aware of the limitations of theoretical predictions.

The next publication submitted for evaluation also concerns the *in silico* characterization of proteins derived from *Bacillus paralicheniformis* and *Bacillus subtilis* bacteria isolated from honey samples. These strains (SZA14, SZA16, and SZB3) were previously described in Publication 1. Analysis of the sequenced genomes of the above-mentioned bacteria revealed that approximately 10% of the open reading frames encode proteins of unknown function. Ahmer Bin Hafeez M.Sc., devoted an enormous amount of effort to determining their three-dimensional structures and biological functions. A range of bioinformatic tools was used to select 56 proteins for further analysis and subsequently to characterize them.

I have the impression that Ahmer M.Sc., is familiar with virtually every bioinformatic tool used in the molecular biology of microorganisms. Do we want to determine the intracellular localization of a protein? The answer is "Certainly!". Do we want to know whether a protein contains a signal peptide? "Of course!". Mr Ahmer's research provides extremely valuable material for further analyses. In databases such as NCBI GenBank, many protein products have been assigned incorrect functions, and once a particular activity has been assigned, it is often cited in subsequent scientific publications, thereby perpetuating the error. A thorough preliminary analysis, similar to the investigations presented by Mr Ahmer, helps to minimize such errors, which deserves positive recognition.

Ahmer Bin Hafeez, M.Sc., successfully predicted the functions of 56 hypothetical proteins and identified their involvement in key cellular processes, such as sporulation, biofilm formation, metabolic regulation, motility, and DNA replication.

A natural continuation of these studies would be another publication experimentally validating the predicted functions of several proteins selected from the 56 characterized *in silico*. It is possible that such a study will be published in the future. **Which of these 56 proteins would the doctoral candidate select as a priority for further functional analysis, and why?**

In Publication 5, authored by Ahmer Bin Hafeez, M.Sc., and Professor Piotr Szweda, the antimicrobial potential of two *Bacillus subtilis* strains (Miodo8 and Miodo10) isolated from multifloral ginger honey was validated. My attention was drawn to the incorrect formatting of the ATCC and DSM strain numbers used in the study. The correct designation is *S. aureus* ATCC 25923, not ATCC 25,923. ATCC numbers are unique strain identifiers rather than numerical values; therefore, thousands

separators (commas, full stops, or spaces) should not be used. However, this minor comment does not diminish the high scientific value of the publication.

Reading Publication 5 together with the four other papers submitted for evaluation reveals the doctoral candidate's gradual development, his increasingly proficient use of the relevant scientific literature, and his ability to conduct a well-founded and scientifically mature discussion. In Publication 5, the doctoral candidate correctly concludes that the presence of a particular gene does not necessarily indicate, for example, the functional antibacterial activity of the strain under investigation. The presence of gene clusters responsible for the biosynthesis of specific compounds, such as surfactin, in bacterial genomes does not mean that the bacterium actually produces the compound. The *srf* operon is regulated through *quorum sensing* and the ComP-ComA system. Mutations disrupting the function of this system may result in the genes remaining inactive despite their presence in the genome. Similarly, standard testing under aerobic conditions may fail to reveal the bacterium's potential to produce subtilosin A. I assess the discussion presented in this publication very positively and look forward with great interest to the doctoral candidate's future publications.

In the final part of the dissertation, the doctoral candidate describes the discovery of paralichenidin (Pld), a lantibiotic composed of peptide α (Pld α) and peptide β (Pld β), synthesized by *B. paralicheniformis* strains SZA14 and SZA16. The doctoral candidate performed sequence analyses of both peptides, structural analysis of paralichenidin, and phylogenetic analyses of the prepropeptides and core peptides. Subsequently, the results of molecular dynamics simulations showed that Pld α has a high affinity for lipid II, in contrast to the marginal binding observed for peptide β.

And here is my final question to the doctoral candidate: **How could the binding of paralichenidin to lipid II be demonstrated experimentally?**

Summary and Final Recommendation

The results presented in the dissertation confirm that bee products, such as honey, bee pollen, and bee bread, contain *Bacillus* species with antimicrobial and probiotic potential. The analyses also confirm the need for further experimental validation of the data obtained *in silico*. The publications submitted for evaluation are thematically coherent, and the studies on, among others, the newly identified lantibiotic paralichenidin appear very promising and make an original contribution to scientific knowledge. The doctoral candidate consistently develops his scientific interests, demonstrating diligence, commitment, and increasing independence in research.

It is worth emphasizing that Ahmer Bin Hafeez, M.Sc., completed three research internships abroad: at the Department of Chemistry, Faculty of Natural, Mathematical and Engineering Sciences, King's College London, United Kingdom; at the Department of Biotechnology and Biomedicine, Technical University of Denmark; and at the Department of Biochemistry and Biotechnology, University of Thessaly, Greece.

His scientific activity is also reflected in the scholarships and grants he has received. In 2023–2024, he was awarded mini-grants intended for doctoral candidates and early-career researchers, as well as funding to attend the Trends in Natural Products Research Meeting conference in Paris. He also received the international doctoral scholarships Francium (2022–2026) and Polonium (2021–2022). In 2024, the doctoral candidate was awarded a scholarship from the Polish National Agency for Academic Exchange (NAWA) for outstanding doctoral candidates conducting projects involving international collaboration. In 2025, he received a scientific exchange grant from the European Molecular Biology Organization (EMBO) (although more detailed information regarding the scientific exchange supported by this grant was not provided).

In summary, Ahmer Bin Hafeez, M.Sc., consistently pursues research in the fields of microbiology and bioinformatics, focusing on the genomic characterization of bacteria, the identification of compounds with antimicrobial activity, and the assessment of the biotechnological potential of microorganisms isolated from bee products. The doctoral dissertation of Ahmer Bin Hafeez, M.Sc., meets the requirements set out in Article 187(1)-(2) of the Act of 20 July 2018, Law on Higher Education and Science.

I therefore strongly recommend to the Scientific Discipline Council for Chemical Sciences of Gdańsk University of Technology that Ahmer Bin Hafeez, M.Sc., be admitted to the subsequent stages of the doctoral degree procedure.

Yours sincerely,

Magdalena Plotka



Signed by / Podpisano przez:

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