

REVIEW

by Assoc. Prof. Denitsa Rumenova Teofanova, PhD,

SU "St. Kliment Ohridski", Faculty of Biology, Department of Biochemistry, member of the Scientific Jury, appointed by Order No. RD-38-107 of 28.02.2023. of the Rector of SU "St. Kliment Ohridski", Prof. Anastas Gerdzhikov, for awarding the Educational and Scientific Degree "Doctor" in

Field of higher education: 4. Natural sciences, mathematics and informatics,

Professional field: 4.3. Biological Sciences

PhD program: Genetics – Bacterial genetics and molecular cloning

REGARDING:

Dissertation topic: " Genome typing of probiotic microflora isolated from natural products "

Author of the dissertation: Anita Bozhidarova Gyurova, part-time (after transformation from full-time) PhD student at the Genetics Department of the Faculty of Biology, SU "St. Kliment Ohridski"

Scientific supervisors: Assoc. Prof. Svetoslav Dimov, PhD
Acad. Draga Toncheva, DBS

1. General presentation of the procedure and the PhD student

1.1. Documents

The materials submitted by Anita Bozhidarova Gyurova include the following required documents according to the Law for the Development of the Academic Staff in the Republic of Bulgaria and the Regulations for the Terms and Conditions for Acquiring Scientific Degrees and Holding Academic Positions at SU "St. Kliment Ohridski": 1) Dissertation work; 2) Abstract in Bulgarian and English; 3)) List of scientific publications in full text - general and those related to the dissertation; 4) CV; 5) Copy of the Master's degree qualification diploma and its annex; 6) Certificate of passed exams from the individual plan; 7) Report and opinion for checking the originality and absence of plagiarism in the dissertation; 8) Order for enrollment and transformation of PhD program, and of dismissal with the right of defense; 9) Reports from the PhD student and her supervisor on the admission to internal defense procedure; 10) Order for the appointment of a scientific jury and 11) Certificate of compliance with the minimum national requirements for ESD "Doctor" with an appendix containing a list of publications.

The dissertation corresponds to the criteria for acquiring scientific degrees and holding academic positions in SU for professional field 4.3. Biological Sciences.

1.2. Introduction of the PhD student

Anita Gyurova obtained a Master's degree in "Industrial Management and Marketing" and an additional specialization in "Banks and Financial Business" at the Technical University, Sofia, and subsequently an additional Master's degree in "Business Administration" at the University of National and World Economy, Sofia, in the period 1995-2007. In 2018 she also obtained a Master's degree in "Genetics and Genomics" at the Faculty of Biology of the SU "St. Kliment Ohridski". In the period 2018-2022 is a full-time (subsequently transformed into a part-time) PhD student at the Genetics Department of the Faculty of Biology at SU. Until now, her professional work experience has not been in the field of biological sciences, but since 2018 she has acquired additional competencies thanks to a number of courses and workshops. All of the above shows scientific interest combined with upgrading of knowledge, experience and

skills. The attached curriculum vitae is supplemented with the publication activity and PhD student's participation in scientific projects and scientific forums, which speaks of an increase in qualifications and growth both on a scientific and a personal level.

1.3.Plagiarism

Based on the plagiarism check annexes, it is clear that the detected similarity percentages for the three criteria do not affect the original results obtained and are not a consequence of illegal borrowing and copying of foreign text. The percentages reported for the various parameters are below the regulated threshold.

2. Characteristics of the thesis

2.1. Relevance and significance of the dissertation topic

The relevance of the topic of submitted dissertation is indisputable and is determined by a number of factors.

On the one hand, there is the economic and ecological importance of keeping beehives healthy and the associated restrictions on the use of antibiotics. In addition, the topic of the potential of bee products in terms of their benefits for good physical condition and increasing the health status of people is extremely attractive and of wide interest. This sets the base for the current study of the bee microbiome in order to identify bacteriocin-producing microbial strains with the potential to inhibit the growth of the honeybee pathogens, as well as those with probiotic potential.

On the other hand, the extremely popular topic in recent years about the distribution and mass use of functional foods containing microorganisms with probiotic potential, in combination with the growing antibiotic resistance of bacteria, determine the demand for bacteriocin producers as an alternative to traditional antibiotics, and producers of other biologically active substances with possible positive effect on people's health, immune system and options for use in therapy and prevention of various diseases.

In third place is the improvement of the quality of wide-used fermented products, which is also mediated by metabolites most often produced by lactic acid bacteria.

And last, but not least, is the fundamentally significant study of the microbiome of the unique for Bulgaria green cheese, the use of which is limited only to domestic use without strict quality control. This traditional product can be considered a national cultural heritage and obtaining detailed information about it could be a prerequisite for its preservation over time.

2.2. Structure of the dissertation, persuasiveness of the obtained results, interpretations, conclusions, and scientific contributions

The dissertation was prepared according to the generally accepted scheme. It is written on 231 standard printed pages and comprises the following sections, including sub-sections: Title Page (1 page), Acknowledgments (1 page), Table of Contents (5 pages), List of Tables (1 page), List of Figures (2 pages), List of abbreviations used (2 pages), Introduction (3 pages), Literature review (65 pages), Aim and objectives (2 pages), Materials and methods (37 pages), Results (40 pages), Discussion (23 pages), Conclusion (2 pages), Summary of the achieved results (1 page), Final Conclusions (2 pages), Statement of contributions (1 page), List of scientific publications in connection with the dissertation (1 p.), Appendices (23 p.), Reference list (19

p.). The information presented in it is illustrated with 32 figures (25 of which in the "Results" section and 1 in the "Discussion" section) and 17 tables (11 of which in the "Results" section), which are of sufficiently good quality.

The dissertation work is written in a good scientific language, with a small number of foreign words, punctuation, technical and stylistic errors, and is of a volume sufficient to acquire the PhD degree, and even exceeds it. There is an imbalance in terms of the optimal ratio between the sections, and it is recommended that the "Results" and "Discussion" sections dominate the "Literature Review", which is not the case here. A positive impression is made by the separation of the "Results" and "Discussion" sections to show the possibility of the PhD student to interpret the obtained data and distinguish the obtained results from their discussion, which is an important acquired skill at this level of education.

The literature used includes 452 sources, all in Latin and, with few exceptions, the same style has been followed in terms of the presentation of the bibliographic reference. About half are from the last 10 years.

The introduction shows the importance of the topic, provides information about the objects of the study and the innovativeness of some of the approaches and outlines the foundations on which the work rests, but its structuring lacks a smooth logical transition between the parts.

The literature review is extremely detailed and covers all aspects of the subject on which the work rests. It includes detailed data on lactic acid bacteria, starting with historical information about them and their general characteristics, and examining the main genera belonging to them. Detailed information is given on the taxonomic and phylogenetic status, morphological, physiological and cultivation characteristics of the main representatives of the individual genera, as well as their role as producers of various biologically active substances and secondary metabolites, and the possibilities for the application of the latter in various spheres of the food and pharmaceutical industries, biotechnological industries, and their effects on human health, the immune system and the potential for use in various diseases. The participation of these bacteria in the fermentation of various foods is indicated and the relationship between the production of metabolites from them and the organoleptic properties of a number of products is given, as well as their potential as probiotic cultures, producers of various bacteriocins, antifungal compounds, etc. In light of the increasing antibiotic resistance of bacteria, special attention is given to bacteriocins as an alternative to antibiotics, and the main characteristics of different classes of them are reviewed. The relationship between the proteolytic activity of certain lactic acid bacteria and its role in the fermentation processes of various foods was also analyzed in terms of improving the quality of the products on the one hand and their impact on human health on the other. The beneficial health effects in various conditions such as allergies, viral infections, etc., of lactic acid bacteria and their probiotic potential, as well as the possibilities of their application and incorporation into pharmaceutical products and functional foods for the purpose of therapy and prevention, are also affected. The role of the lactic acid bacteria of the bee microbiome and the options for its use for a probiotic preparation for increasing the resistance of bee colonies to diseases and the interrelationship with the preservation of bee colonies are added here. Last but not least, the state-of-the-art molecular-genetic approaches that are applied in studies for the determination of taxonomic affiliation, phylogenetic, population and evolutionary analysis, as well as those for the detection

and determination of genetic polymorphisms in bacteria and in particular lactic acid bacteria, and also for the targeted determination of the identity of bacteriocins, are very well described.

My only complaint about this section is related to observing some fragmentation in its structuring and the presence of unnecessary excessive detail in some of its parts. However, this does not in any way reduce its high level and the opportunity to clearly show that the PhD student is well acquainted in the subject matter and in addition to good theoretical background, her ability to work with scientific literature to systematize the available information is proven.

The aim of the dissertation is clearly formulated and hindered by the relevance of the topic. **The tasks** related to the planned scientific research activities to achieve the stated aim are also adequately set.

In the "**Materials and methods**" section, the methods used are described in detail, it is evident that they are adequately selected and meet the set tasks. They are presented in a manner suitable for reproducing the experiments and include a range of microbiological, biochemical, sequencing and other techniques. They reflect the interdisciplinarity of the study. However, for some of them (albeit a small part of them) there are no corresponding citations. In places, more used volumes are observed, and it is recommended everywhere to use final concentrations of the reagents used. Some deficiencies are observed, e.g. regarding antibiotic resistance studies, it is good to give specifics, not just "a certain concentration of the antimicrobial agents tested" - which they are and what the concentrations are. The same applies to more information about the indicator bacterial strains - where they were obtained from, what ensures their species identity and why they were selected. I believe that "Collection of 368 strains of lactic acid bacteria was isolated from 31 naturally fermented dairy products" is part of the 'Results' section. I consider "Growth kinetics of bacteriocins at low temperature" to be incorrectly used - it is not the bacteriocins that grow, but the bacteria that produce them.

Despite my remarks, the large range and variety of microbiological and molecular genetic techniques learned, as well as, last but not least, the extremely labor-intensive bioinformatic processing of a large amount of data, which speaks of good methodical preparation of the doctoral student, are clearly visible.

The results obtained from the conducted research are described consistently and in accordance with the tasks set in the "**Results**" section. An extremely detailed and comprehensive characterization was made of the collection of bacterial isolates of 45 strains belonging to the genus *Enterococcus* obtained from honey bees. It includes preliminary species identification with selective media and PCR analysis, as well as demonstration of activity against *Paenibacillus larvae* and strain affiliation. Further detailing of the information was done on strain EDD2 belonging to *Enterococcus durans*, including sequence confirmation of its taxonomic status, checking for virulence factors, antimicrobial activity and antibiotic resistance, latter shown to be lacking. From the representatives of the genus *Enterococcus*, it was found that 90 of the isolates showed bacteriocin activity and 21 - proteolytic activity against 9 indicator microorganisms. By bioinformatic analysis of the genome sequence of this strain, with regard to the bacteriocin production potential, the presence of the clusters similar to enterocin L50A/L50B and enterocin P was established. Almost identical characteristic analyzes were also made with *Enterococcus faecium* strain EFD. They show the presence of antibacterial activity probably due to the activity of one or more of the five bacteriocins detected. In terms of antibiotic resistance, this strain was resistant only to erythromycin and susceptible to the

other tested substances. Comprehensive virulence analysis of the *Enterococcus faecium* EFD isolate indicated that it lacked functional pathogenic traits and therefore should not be considered a potential pathogen.

A similar scheme was followed for the characterization of microbial isolates from foodstuffs. The prepared collection includes 303 strains, for which their morphological and physiological features were initially studied on selective media. These assays were followed by those for the presence of bacteriocin and/or proteolytic activity, with a relatively small percentage of strains exhibiting such, and tested against which and how many bacterial strains could exhibit it. A scheme has been developed for the activities and inhibition of the development of pathogenic microorganisms observed in different groups of products. The taxonomic affiliation of 27 strains was proven by 16S sequencing analysis. Phylogenetic relationships between isolates belonging to *Enterococcus faecalis* and *Lactiplantibacillus plantarum* with pronounced proteolytic and/or bacteriocin activity are also indicated. An additional metagenomic analysis with taxonomic annotation for the bacterial and fungal species (20 bacterial species and 10 fungal species) and the relative abundance of taxon types in green cheese microbial isolates was also performed. Biodiversity in bacteria and fungi in batches of green cheese was also characterized and assessed.

In the "**Discussion**" section, the interpretation of the obtained data is presented. It is preferable to make the connection between the results and explanations for them in more detail. However, the discussion made is fully justified based on the available data in the literature of recent years. Reasonable assumptions have been made regarding selective protection against pathogens of the studied bee hives containing *Enterococcus* strains and sharing of protective strains between hives. The extremely detailed analysis of the microbial isolates from the unique for Bulgaria green cheese gives reason to make assumptions about the role of the established microbes both for the quality and the production process of the product itself, as well as for the potential for probiotic activity. The examined batches of cheese were characterized by 5 to 25 specific bacterial species, and with the exception of one batch, they were characterized by 1 to 2 specific fungal species compared to the other cheeses. In general, green cheese has a complex microbiome that is influenced by different climatic conditions, and in the ripening process, microorganisms from the local environment play a major role. Green cheese has also been found to have a barely noticeable presence of potential pathogenic microorganisms, and most likely does not pose a risk for consumption. There is some confusion between "Results" and "Discussion" and some of the results reported in this section could fit in the previous one where they are missing.

Based on the obtained results, 11 **conclusions** and 5 **contributions** were formulated. The conclusions follow logically from the results of the conducted research, but could be more concisely, clearly and definitively formulated, while some overlap with the results is observed in them. I fully accept the contributions presented in the dissertation.

2.3. Correspondence between the Abstract and the dissertation

The abstract is prepared in accordance with the requirements and presents in an abridged version the study, the experimental work, the results obtained and the conclusions and contributions made on their basis.

3. Questions for the PhD student

I have the following questions for the PhD student:

1. When analyzing the bee microbiome, why did you prefer to analyze in detail the properties of only the species of the genus *Enterococcus* and not other microbial isolates?
2. What were the specific basis and criteria for selecting only this group of "most interesting strains" when performing species determination of selected isolates using 16S sequencing (p. 140) and why the phylogenetic studies of isolates by RAPD (p. 141 -144) includes the selected isolates of only two species?
3. Is it possible that microbial isolates with demonstrated proteolytic activity, capable of hydrolyzing milk proteins, do not have a positive effect on the hypoallergenicity of dairy products, but on the contrary? Why?
4. Based on what has been achieved so far and bearing in mind the need for additional analyses, in what direction do you think future research should be directed?

3. Conclusion

In conclusion, I believe that the presented dissertation represents an in-depth study on an undeniably relevant topic, incorporating modern and innovative techniques and bringing with it corresponding contributions to the scientific field. It demonstrates the PhD student's sound theoretical background and the acquired wide range of methodological skills in the analysis and solving of problems. Regardless of the remarks made, I believe that the dissertation fully complies with the Law on the Development of the Academic Staff in the Republic of Bulgaria, as well as with the Regulations for the Terms and Conditions for Acquiring Scientific Degrees and Holding Academic Positions at SU "St.Kliment Ohridski", for awarding the educational and scientific degree "Doctor". All of the above gives me grounds to positively evaluate the dissertation work of Anita Gyurova and to recommend to the other members of the esteemed Scientific Jury to support the awarding of the educational and scientific degree "Doctor" in the professional field: 4.3 Biological Sciences (Genetics – Bacterial Genetics and Molecular Cloning) to Anita Bozhidarova Gyurova.

31. 03. 2023.

Sofia

Prepared the review:

/Assoc. Prof. Denitsa Teofanova, PhD /