

Review

From: Prof. Iskra Vitanova Ivanova, Doctor Habil.,

Regarding: evaluation of a dissertation work for the acquisition of an educational and scientific degree "doctor" in the field of higher education 4. Natural sciences, mathematics and informatics, professional direction, 4.3 Biological sciences, Scientific specialty Genetics – Bacterial genetics and molecular cloning
on the topic: GENOME TYPING OF PROBIOTIC MICROFLORA ISOLATED FROM NATURAL PRODUCTS

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Scientific supervisors: Prof. Dr. SVETOSLAV DIMOV

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1. RELEVANCE AND SIGNIFICANCE OF THE DEVELOPED PROBLEM

The microbiota is one of the decisive factors favoring human metabolism and health and has been the subject of numerous studies in recent years. The development of molecular techniques now allows the complete identification of individual microorganisms in complex communities. The isolation and taxonomic characterization of lactic acid bacteria, both from traditional for Bulgaria, naturally fermented products, and from bee products harvested in different ecological regions of the country is of particular interest. The application of lactic acid bacteria in the development of new probiotic and functional foods is constantly increasing. Worldwide research is focused on the development of bioinformatic and molecular methods for the analysis and genomic typing of various microorganisms. On the other hand, some of these genomes are derived from lactic acid bacteria used as probiotics or starter cultures in food fermentations. The study of Bulgarian green cheese probably possesses an extremely rich microbiome, as well as the only Bulgarian noble mold. Analysis of lactic acid bacteria, part of the microbiome of naturally fermented and bee products, have a beneficial effect on human and bee health, have a protective effect against pathogenic microorganisms. All of the above gives me reason to evaluate the presented scientific development as current, with the potential for scientific achievements that have a quick practical implementation.

2. SCOPE AND STRUCTURE OF THE DISSERTATION

The dissertation is laid out in 231 standard pages of text. The generally accepted scheme and the recommended ratios between the individual parts of the work were followed, as follows

- ☐ Introduction – 3 pages;
- ☐ Literature review – 64 pages;
- ☐ Purpose and tasks - 2 pages;
- ☐ Materials and methods – 36 pages;
- ☐ Results and discussion – 64 pages;

☐ Conclusions -2 pp. i

☐ Literature – 18 pages.

The obtained results are illustrated with 32 figures and 17 tables.

3. LITERARY AWARENESS AND STATEMENT OF THE GOAL AND TASKS

The present dissertation is complex and implies a good knowledge of the literary sources and the methods for solving it. The doctoral student has made a thorough review of the achievements of other researchers, which she was able to convey and analyze on 64 pages in the literature review. The overview presents the state of the problem in detail and proves the necessity of developing the dissertation thesis. The literature review consists of several sections. The author consistently reviews and offers an extensive description of the main characteristics of the main genera of lactic acid bacteria (type Firmicutes. Actinobacteria.) Aspects of the participation of lactic acid bacteria in food fermentation, in the fermentation of dairy products and meat products, in the fermentation of vegetables are considered and sauerkraut and in the fermentation of bread sourdough. The production of anti-microbial metabolites (bacteriocins and peptides) has been extensively reported. Data are presented on the therapeutic effect of lactic acid bacteria as probiotics. Information on molecular genetic methods for the study of lactic acid bacteria is particularly useful, including standard methods for genus determination, species determination by 16S RNA gene sequencing, Multilocus Sequencing Typing (MLST analysis) and Next-Generation Sequencing (Sequencing). Some unsolved problems are also brought to the attention of the reader. The literature review is concrete, it is structured correctly, following the logical connection of the information. The data from the references served for the clear and correct determination not only of the goal, but also of the formulation of the tasks. 17 well-grounded experimental tasks are set for solving. The literature (both in the overview and in the whole work) is closely related to the topic of the dissertation work. The literary list included the impressive number of 452 titles in Latin. They are mainly from recent years. This speaks of an excellent theoretical awareness of the PhD student and with the aim of finding a new scientific challenge.

4. EVALUATION OF USED METHODS AND MATERIALS

The Materials and Methods section demonstrates an impressive array of methods tailored to the specific requirements of the experiments. They are modern and adequate for the realization of the dissertation work. They are described precisely and in detail, fully covering the multifaceted fields of work: from classical to modern molecular genetic studies. In the individual stages of the work, the doctoral student skilfully combines the used approaches. All this allows me to give a high assessment of the scientific level and the excellent preparation of the doctoral student, who manages to correctly combine a variety of classical and modern methods for the purposes of the dissertation, successfully solving the set experimental tasks.

5. EVALUATION OF THE RESULTS OBTAINED

The main goal of the present study is the molecular genetic and genomic characterization of strains of microorganisms isolated from functional foods and bee products, as well as the study of the microbiota of functional foods and bee products using next-generation sequencing-based methods.

The "Results and discussion" section is well structured, supported by tabular and graphic material, with appropriate interpretation of results obtained by foreign scientific teams. The author consistently presents evidence for her scientific thesis, thereby logically finalizing an experimental work. Extensive and varied experimental work was carried out within the framework of complex microbiological and molecular biochemical research.

The present study is the first metagenomic study of the traditional Bulgarian green cheese produced in the village of Cherni Vit. This unique dairy product is the only Bulgarian cheese with noble mold. As a result of the experiments carried out, it was found that green cheese has a rich microbiome, mainly containing microorganisms that are not starter cultures by nature and which in turn contribute to its distinctive characteristics, including olfactory and organoleptic. In this study, aiming to characterize Bulgarian green cheese, a next-generation sequencing-based metagenomic study was the first step. The study covered regions V3-V4 of the 16S RNA gene in bacterial species and ITS2 in fungi. The author found that Firmicutes occupy the first place with more than 50% presence, followed by Actinobacteria with slightly more than 40% and in third place are Proteobacteria with about 6%. The remaining bacterial types are represented in insignificant quantities. Within the phylum Firmicutes, at the genus and species level, gram-positive lactic acid bacteria (LCB) from the genera *Streptococcus*, *Lactobacillus* and *Lactococcus* rank first, with more than 30%. They are followed by representatives of the genus *Staphylococcus* (about 18%). The species from the *Lactococcus* river are the third main ICD group with 3.7% and three species. Less represented ICD genera are *Leuconostoc*, *Weissella*, *Marinilactibacillus* and *Enterococcus*. Gram-negative bacteria are in insignificant amounts and among them the most represented genera are *Veillonella* and *Selenomonas*. While the various species of *Lactobacillus*, *Leuconostoc* and *Enterococcus*, as well as *Lactococcus lactis* and *Streptococcus salivarius* subsp. *thermophilus* have a major role in cheese ripening and their presence is expected, the presence of some species of *Staphylococcus*, and the presence of *Lactococcus garvieae* raises some concerns. The third bacterial phylum that was significantly represented was Proteobacteria with two families, Halomonadaceae and Moraxellaceae. *Chryseobacterium* is the only representative of the genus *Bacteroides*, which ranks among the 35 genera. Almost all (99.99%) of the discovered fungal species belong to the Ascomycota, represented almost equally by three classes: Eurotiomycetes, Saccharomycetes and Sordariomycetes. Among them, the first class is represented by one genus and one species - *Penicillium roqueforti*, which is also the most prevalent fungal species. Sordariomycetes is the second most abundant class of Ascomycota, represented almost entirely by *Scopulariopsis flava*, as well as minor amounts of *Fusarium oxysporum* and *Trichothecium roseum*. Among the class Saccharomycetes, *Debaryomyces hansenii* was the most abundant, followed in descending order by *Pichia membranifaciens*, *Candida zeylanoides*, *Kluyveromyces lactis* and *Torulasporea delbrueckii*. The composition of the microflora of green cheese, although showing a general structure, varies among the different samples studied, which can be explained by the fact that cheese fermentation depends almost entirely on microorganisms coming from the environment, as well as the type of milk used (sheep or goat). The rich microbiome of Bulgarian green cheese was further characterized by examining its alpha diversity. Shannon and Simpson indices were used to estimate diversity based on observed OTEs, with the former giving greater weight to species richness and the latter to species evenness. Dilution curves and species diversity of bacterial and fungal species of the green cheese batches were calculated based on the alpha diversity analyses. The information they contain allows for a primary assessment of the microbiome. In this study, all batches showed fairly similar curves for bacterial and fungal species. . In order to explore and compare the sample communities in the four batches of cheese, beta-diversity indices were analyzed using QIIME software using UniFrac distances. Similarly, UPGMA trees constructed for the weighted

Unifrac and for the unweighted Unifrac distances in bacteria and fungi did not provide conclusive information about the microbiome connectivity of the four cheese lots analyzed. The four batches of cheese are produced from different types of milk (sheep and goat) and at different times of the year, meaning that different climatic conditions also affect the environmental microbiome. The presence of potential pathogenic microorganisms, typical of all fermented raw milk products, is very insignificant and most likely does not pose a risk for consumption - both because of the extremely low OTE count and because they are suppressed and non-viable, as has been described for other famous cheeses. However, the results obtained in this first study raise new questions and ideas for further analyses—the most important of which is the genomic characterization of the predominant species. The eventual sequencing of whole microbiomes would allow a more precise quantitative characterization of microbiome communities.

A collection of 45 strains obtained as a result of isolation from bee families from 15 hives of apiaries located in the city of Sofia, the city of Vidin, the village of Dushantsi and the village of Momchilovtsi were attributed to *E. Durans*. Molecular genetic studies proved that the same strain was present in four of the six hives, identified as *E. durans* and named EDD2. By performing PCR with primers targeting antibiotic resistance genes, the study strain was found to lack genetic determinants for vancomycin resistance *vanA*, *vanB*, *vanC1* and *vanC2*. A collection of 45 strains obtained as a result of isolation from bee families from 15 hives of apiaries located in the city of Sofia, the city of Vidin, the village of Dushantsi and the village of Momchilovtsi were attributed to *E. Durans*. Molecular genetic studies proved that the same strain was present in four of the six hives, identified as *E. durans* and named EDD2. By performing PCR with primers targeting antibiotic resistance genes, the study strain was found to lack genetic determinants for vancomycin resistance *vanA*, *vanB*, *vanC1* and *vanC2*. An approach based on bioinformatic analysis of its genome sequence was used to study bacteriocins produced by *Enterococcus durans* EDD2. This analysis identified two putative clusters similar to enterocin L50A/L50B and enterocin P. The focus of the study was the identification of bacteriocin-producing enterococcal strains with the potential to inhibit the growth of the disease agent American foulbrood, as well as strains with probiotic potential. The thirteen isolates demonstrated inhibitory activity against *Paenibacillus* larvae. From the collected collection of isolates of the genus *Enterococcus*, a study was conducted for the presence of proteolytic and bacteriocin activity, as a result of which 90 nos. of the isolates showed bacteriocin activity and proteolytic activity. After 16S RNA gene sequencing, the isolates belonged to: *Lactiplantibacillus plantarum*, *Enterococcus faecium*, *Enterococcus faecalis*, *Pediococcus pentosaceus*, *Levilactobacillus brevis*, *Rosenbergia*, *Leuconostoc mesenteroides*, *Serratia*, *Staphylococcus equorum* and *Staphylococcus saprophyticus*. From the research done, it is clear that amplicon-based metagenomic sequencing of samples containing complex microbiota, combined with bioinformatic analysis on online-based platforms, is a convenient, cost-effective, reliable and rapid way for detailed analysis and characterization of fermented functional foods and some other natural products possessing their own microbiomes.

The obtained results presented in "Results and discussion" logically follow the course of solving the set tasks. They are summarized and discussed in the light of published data from recent years. Both the idea and the volume of research carried out on the implementation of this task and in the entire work deserve high praise. The discussion made on each experiment, the comparison of the results for the individual strains and experiments, and the comparison with the literature data, once again emphasizes the qualities of the doctoral student in the mastery of the experimental theory. By this she proves that she has fully mastered the third degree of her training and is an accomplished experimenter.

6. CONTRIBUTIONS AND SIGNIFICANCE OF THE DEVELOPMENT FOR SCIENCE AND PRACTICE, NOTES AND QUESTIONS

I accept the contributions made.

I consider it particularly significant that for the first time in Bulgaria an amplicon-based metagenomic analysis of a fermented dairy food product - green cheese - was performed.

For the first time in Bulgaria, genomes of probiotic strains from the *Enterococcus* have been sequenced for bees.

Gyurova is the author of 5 scientific publications, and in two of them she is the leading researcher, which shows the creative and research activity in their creation and shaping. The interest in these developments is also the established citation (Scopus):

Gyurova, A. Vladimirova, A., Peykov, S., Dimitrov, M., Strateva T.& Dimov, S.G. (2021) Characterization of *Enterococcus durans* EDD2, a strain from beehives with inhibitory activity against *Paenibacillus larvae*, Journal of Apicultural Research, doi: [10.1080/00218839.2021.1936915](https://doi.org/10.1080/00218839.2021.1936915);

Dimov, S.G, **Gyurova, A.**, Zagorchev, L., Dimitrov, T., Georgieva-Miteva, D., Peykov, S. NGS-Based Metagenomic Study of Four Traditional Bulgarian Green Cheeses from Tcherni Vit, LWT, Vol. 152, (2021), 112278, ISSN 0023-6438, doi: [10.1016/j.lwt.2021.112278](https://doi.org/10.1016/j.lwt.2021.112278);

Dimov, S.G., **Gyurova, A.**, Vladimirova, A., Dimitrov M., Peykov S., Strateva T., WGS-based characterization of the potentially beneficial *Enterococcus faecium* EFD from a beehive. *Mol Biol Rep* 47, 6445–6449 (2020). doi: [10.1007/s11033-020-05663-5](https://doi.org/10.1007/s11033-020-05663-5);

Peykov S., Vladimirova A., **Gyurova A.**, Dimitrov M., Strateva T., Dimov, S., Draft genome sequences of *Enterococcus durans* EDD2 strain associated with honeybees, *AIMS Agriculture and food*, Volume 5, Issue 2: 288-291 (2020). doi: [10.3934/agrfood.2020.2.288](https://doi.org/10.3934/agrfood.2020.2.288);

Dimov S. G., S. Peykov, A. Vladimirova, M. Balinska, **A. Gyurova**, M. Dimitrov, T. Strateva, Molecular genetic study of potentially bacteriocinogenic and non-virulent *Enterococcus* spp. isolates from beehives in Bulgaria, *Genetics and Plant Physiology*, 8(3–4): 129–137, (2018).

- The dissertation student complied with all the remarks made during the pre-defense.

CONCLUSION

The topic is current, the doctoral student has mastered modern methods, the experiments are set methodically correctly, the results obtained are reliable and are a solid basis for further scientific and

applied developments. Exceptionally original scientific and applied contributions stand out. Based on the above, I can confidently state that the peer-reviewed dissertation is an original scientific work with theoretical and applied significance.

The proposed dissertation is proof that Anita Gyurova has developed competencies necessary for the doctoral degree, including theoretical preparation, methodological knowledge, independence and experience in planning experiments and the ability to analyze the results. Based on the collected credits, the planned educational scientific program was successfully completed.

Based on the presented arguments for the topicality of the issues and the original contributions reflected in the dissertation work, I give my high evaluation for its defense and recommend the members of the scientific jury to award the doctoral student Anita Gyurova the educational and scientific degree "Doctor" in professional direction 4.3. Biological Sciences, specialty Genetics – Bacterial genetics and molecular cloning.

30..03.2023

Signature: