

OPINION

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Subject: dissertation on "**Genome typing of probiotic microflora isolated from natural products**" presented by PhD student ANITA BOZHIDAROVA GYUROVA from the Department of Genetics at Sofia University "St. Kliment Ohridski". Kliment Ohridski" for acquiring educational and scientific degree "DOCTOR" in professional field of higher education 4. Natural sciences, mathematics and informatics; professional field 4.3 Biological sciences; scientific specialty in Genetics – Bacterial genetics and molecular cloning.

Received materials: Dissertation, autoreferat, list of publications on the dissertation, materials is an electronic version and dissertation and autoabstracts in Bulgarian and English on paper.

By Order No RD 38-107/28.02.2023 of the Rector of Sofia University "St. Kliment Ohridski" has been appointed a member of a scientific jury for the defense of a dissertation for acquiring educational and scientific degree "Doctor" by PhD student Anita Bozhidarova Gyurova on the topic "Genomic typing of probiotic microflora isolated from natural products". The experimental work on the dissertation was carried out at the Department of Genetics at Sofia University "St. Kliment Ohridski". Kliment Ohridski".

Relevance and significance of the dissertation topic

The human intestinal tract is populated by a rich and dynamic bacterial ecosystem called the gut microbiota, which plays a key role in host homeostasis. Multiple factors can affect this delicate balance, including genetics, age, antibiotics, and environmental factors, especially diet, thereby causing a disturbance in the microbiota (dysbiosis). The maintenance of the health status in modern man requires the use of new functional components to control the microbial population of beneficial microorganisms in the gastrointestinal tract and to be used as a barrier against the entry of pathogenic microorganisms. It is known that the interest on the one hand of science in new probiotic strains of microorganisms and natural BAS with prebiotic properties and on the other hand of the industry for the production of functional and healthy foods have grown significantly over the past decade. Most of them are fermented foods with the participation of lactic acid bacteria. On the other hand, the pharmaceutical industry intensively develops probiotic, prebiotic and symbiotic preparations to be used to control the intestinal microflora in humans, immunomodulation, blood cholesterol reduction and other indicators related to the risk of diseases of the trivascular and gastrointestinal systems. The proposed dissertation work is on topical topics in the field of study of the microbiome of Bulgarian green cheese, which has the potential to include microorganisms with probiotic properties. In addition, the analysis of the probiotic potential of microorganisms from bee products may contribute to the study of their beneficial effects on human and bee health. Although there are various studies on strains of lactic acid bacteria for the production of probiotic preparations, the direction of the research conducted in this

dissertation shows a modern scientific thinking on the problems of genotyping of probiotic strains. In addition, very promising are the studies on the metagenomic study of traditional Bulgarian green cheese.

Analysis of the dissertation

The dissertation is based on a standard model for Bulgaria, written on 231 standard typewritten pages and contains the following main chapters: Contents – 6 pages, Introduction – 3 pages, Literature review – 64 pages, Purpose and tasks – 2 pages, Materials and methods – 36 pages, Results – 40 pages, Discussion – 25 pages, Conclusions – 2 pages, Contributions – 1 p., Publications related to the dissertation – 1 p., Annex – 23 pp.; Literature — 18 pp., The literature review and the results are appropriately illustrated with 32 figures and 17 tables. PhD student Anita Gyurova has used a very large number of literary sources a total of 452 and 6 electronic sources.

The literature review is written on 64 pages and focuses our attention on the latest scientific searches and achievements on the main characteristics of the main genera of lactic acid bacteria (type Firmicutes., Actinobacteria.) Information is presented on the participation of lactic acid bacteria in the fermentation of dairy and meat foods, in the fermentation of vegetables and in the fermentation of a bread starter. The production of anti-microbial metabolites (bacteriocins and peptides) are presented extensively. Here I would like to note that the author, in an effort to present maximum information on the problem, has included information that is not specific on the topic of the dissertation at the expense of missing one on the definition and classification of functional foods and their potential positive actions on the human microbiome. Such information is needed mostly because of the formulated purpose of the study. Separately, I would recommend a significant reduction of the literature review to a maximum of 50 pages in order to focus more on one's own research and achievements. In this way, the accepted requirement in principle for a review ratio: results and discussion 1:2 will be observed.

As a result of the literature review, the author of the dissertation clearly formulated the aim of the study - 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 methods based on new generation sequencing. Achieving the goal requires solving 17 diverse tasks, which the doctoral student has formulated according to the main directions of the study.

In the section "Materials and Methods" are described precisely the main methods that are applied in the implementation of the tasks set in the dissertation. The scale of the set experiment in the development of the dissertation requires the application of a large number of both classical, as well as modern microbiological, molecular-biological, biochemical and bioinformatics methods that the doctorant (PhD student) has mastered. All this is grounds to believe that Anita Gyurova has acquired the necessary methodical experience in deriving a scientific experiment.

The results of the studies are presented in detail in the "Results" section and further commented on in the "Discussion" section. The PhD student started with a metagenomic

study of the traditional Bulgarian green cheese produced in the village of Cherni Vit. The study covered V3-V4 regions of the 16S RNA gene in bacterial species and ITS2 in fungi. The author found that Firmicutes microorganisms with over 50% presence predominated, followed by Actinobacteria with slightly more than 40% and in third place are Proteobacteria with about 6%. The remaining bacterial types are presented in negligible quantities. In additional taxonomic studies of the Firmicutes type at the generic and species levels, Gyurova found that over 30% are representatives of gram-positive lactic acid bacteria (ICD) of the genera *Streptococcus*, *Lactobacillus* and *Lactococcus*. Representatives of the genus *Staphylococcus* are about 18%). Underrepresented are ICDs of genera *Leuconostoc*, *Weissella*, *Lactobacillus* and *Enterococcus*. Gram-negative bacteria are in negligible quantities and among them the most represented genera are *Veillonella* and *Selenomonas*. The author proves that Bulgarian green cheese has a rich microbiome, which is further characterized by the study of its alpha diversity. In the next stage of the study, PhD student Anita Gyurova examined the intestinal microbiome in bees and collected a collection of 45 strains obtained as a result of isolation from bee colonies from 15 hives from apiaries located in different ecological niches - Plovdiv, Sofia, gr. Vidin, Dushantsi and Momchilovtsi. They all refer to *Enterococcus durans*. Through molecular genetic studies, the author proved that the same strain was present in four of the six hives designated as *E. durans* and name EDD2.

The doctoral student has studied the ability of the isolated strain *Enterococcus durans* EDD2 to produce bacteriocins through bioinformatics analysis of its genomic sequence. With this analysis, two suspected clusters similar to enterocin L50A/L50B and enterocin P were identified. The focus of the study is the identification of bacteriocin-producing enterococcal strains that have the potential to inhibit the growth of the causative agent of the disease American foulbrood, as well as strains possessing probiotic potential. The thirteen isolates demonstrated inhibitory activity against *Paenibacillus* larvae.

In the following experiments, the PhD student examined the isolated strains for the presence of proteolytic and bacteriocin activity, as a result of which 90 pcs. of the isolates showed bacteriocin activity and proteolytic activity. After sequencing the genes for 16S RNA the doctoral student found that they belonged to: *Lactiplantibacillus plantarum*, *Enterococcus faecium*, *Enterococcus faecalis*, *Pediococcus pentosaceus*, *Levilactobacillus brevis*, *Rosenbergia*, *Leuconostoc mesenteroides*, *Serratia*, *Staphylococcus equorum* and *Staphylococcus saprophyticus*. It is clear from the studies made that amplicon-based metagenomic sequencing of samples containing complex microbiotes, combined with bioinformatics analysis of online-based platforms, it is a convenient, cost-justified, reliable and fast way to analyze and characterize fermented functional foods and some other natural products with their own microbiomes.

As a result of the study, the doctoral student has formulated 11 conclusions, which generally follow the course of the study and the solution of the tasks.

I accept the contributions formulated by the PhD student.

The most significant of them are 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 bee-probiotic strains of the Enterococcus River have been sequenced.

Short PhD thesis presentation and publications

The PhD thesis, regardless of its volume, is written in clear and concise scientific language. The short thesis fully corresponds to the goals, tasks and results achieved in the dissertation.

The results of the dissertation have been published in 5 scientific publications, in one of which the doctoral student is the first author. All publications are printed in reputable scientific journals. The experimental work in the dissertation is part of the implementation of two scientific projects.

Questions, remarks and recommendations

I have the following questions for the PhD student:

1. How would you explain the fact that on the one hand you find a wide variety of microflora in Bulgarian green cheese with predominant lactic acid bacteria, and on the other hand, no strains with pronounced probiotic strains have been identified?

2. How would you explain the fact of the presence of the same strain *E. durans* EDD2 in 4 of the six hives examined?

Conclusions:

My assessment of the dissertation, short thesis, scientific publications and scientific contributions of the PhD student Anita Bozhidarova Gyurova is completely positive. Despite the recommendations made, I would like to note once again its merits – first of all a large-scale research work, far beyond the necessary minimum for a dissertation, containing a huge amount of results, properly interpreted and summarized. The original nature of the data obtained on the microbiome of Bulgarian green cheese and the isolated bacteriocin-producing strains of microorganisms have both scientific and scientifically applied character with potential for application in the preparation of new functional foods.

The presented PhD thesis fully meets the requirements of the Law on Combating Organized Crime and the Regulations for its implementation. The results achieved give me grounds to propose to award the educational and scientific degree "Doctor" to Anita Bozhidarova Gyurova in the professional field of higher education 4.Natural sciences, mathematics and informatics; professional field 4.3 Biological sciences; scientific specialty in Genetics – Bacterial genetics and molecular cloning.

23.03.2023

Plovdiv

Reviewer:

Prof. Dr. Ilia Iliev