

## REVIEW

from Assoc. Dr. Zlatka Miltcheva Alexieva,  
"Stefan Angelov" Institute of Microbiology, BAS

Regarding: The scientific production of ch. assistant professor, Dr. Galina Dinkova Stoyancheva for participation in a competition for awarding the academic position "Associate professor" under direction 4.3. Biological Sciences; scientific specialty Microbiology – Development of new functional foods, for the needs of the Department of "General Microbiology", Laboratory "Microbial Genetics", Institute of microbiology – Bulgarian Academy of Sciences, announced in the State Gazette No. 29 of 12.04.2022 r.

Dr. Galina Dinkova Stoyancheva, Ch. Assistant Professor in the "Microbial Genetics" Laboratory at the Department of "General Microbiology" - the Institute of Microbiology, BAS is the only candidate who submitted documents for the announced competition. The documents for the competition meet the requirements of ZRAS RB and the relevant Regulations.

## PROFESSIONAL BIOGRAPHY

Galina Stoyancheva received her higher education (Master's degree) in 1997 at the Faculty of Biology, SU "St. Kliment Ohridski", majoring in Biotechnological processes, Genetic and cellular engineering. She began working as a specialist at the Institute of Microbiology of the Bulgarian Academy of Sciences in 1998. Since 2000 she has held the position of research assistant - III degree. After defending her doctoral thesis in 2006 and obtaining the scientific and educational degree "PhD" at the "Stefan Angelof" Institute of Microbiology - BAS, from 2007 to the present she holds the position of "Chief Assistant Professor".

Dr. Galina Stoyancheva has conducted two specializations at the Laboratory of General and Food Microbiology, Department of Science and Technology, University of Verona, Verona, Italy, 2007 and 2008. The topics of these specializations had a significant impact on her development, as a scientist with a wide spectrum of views and approaches to research related to the focus of her research activity presented in the current competition.

The candidate's participation in national research projects is also significant. The list contains 17 projects, of which 12 are national and 5 international projects. Galina Stoyancheva is the head of four of these projects.

Dr. Galina Stoyancheva is a member of the Union of Scientists in Bulgaria, Microbiology Section, and the Federation of European Microbiological Societies (FEMS). She participated in the training of students under the "Student Practice" Program of the Ministry of Education.

Dr. Galina Stoyancheva won a FEMS scholarship and the Stefan Angelov Foundation's Annual Award for the best work of a young microbiologist in 2007. She was awarded a certificate from the VAK, SUB and FNS competition for scientific achievements of doctoral students who defended in 2006. A list of 16 participations in international and 14 - in national scientific forums of the candidate is presented.

#### MAIN SCIENTIFIC-METRIC INDICATORS RELATED TO THE COMPETITION

The analysis of the data relating to the indicators under the National Minimum Requirements from the Regulations for the Application of the ZRAS RB, shows the following:

50 points are set for the possession of "PhD". According to indicators from group B, 5 publications are presented: 1 with Q1; 3 - with Q2; and 2 - with Q4, which gives a total of 109 points. According to indicators from group D, within indicator 7, 15 publications are presented, of which 1 - with Q1; 7 post with Q2; 3 post with Q3; 4 posts with Q4. According to indicator G item 8, 2 book chapters are included. These data give a total of 288 points.

From the indicator for citations of publications in world-famous databases with scientific information (Web of Science and Scopus) or in general for indicators D, 300 citations without self-citations are presented, with which 600 points are collected. The management and participation in scientific research projects and the funds raised by them are not described in indicator group E.

The total number of points from the data filled in the Table for the minimum national requirements is 1047 points with a minimum of 430 points required.

The conclusion is that Dr. Galina Stoyancheva more than meets the requirements for each of the groups of indicators necessary for occupying the scientific position "Associate Professor" and in general scores significantly more points than the mandatory minimum.

Regarding "Additional criteria for the growth of the academic staff at IMikB", it can also be noted that the criteria have been exceeded. For the competition in this part, Dr. Galina Stoyancheva has submitted 22 publications in journals referenced in WoS/Scopus and 6 publications in peer-reviewed publications: 2 book chapters and 3 collections of international forums (published in full text). Total IF 27.49, total number of citations – 300 and H-index = 7.

In 8 of the publications, Dr. Stoyancheva is the first/corresponding author. The required values for IF and H-index are significantly exceeded. The additional requirement for participation and management of research projects has also been repeatedly exceeded.

## MAIN SCIENTIFIC CONTRIBUTIONS

The main activity of Dr. Galina Stoyancheva after the defense of her dissertation on the topic "Combined approach for molecular-taxonomic characterization of lactobacilli" is in the field of characterization of newly isolated and established lactic acid bacteria with probiotic action and the production of biologically active substances.

Available data from laboratory research and medical practice clearly show that the use of probiotic microorganisms in our daily life and in healing has great potential, especially with the growing threat of overuse of antibiotics and the spread of resistant microorganisms.

An essential place in the heterogeneous group of lactic acid bacteria is occupied by lactobacilli, which are a natural part of the human microbiome in healthy people. Studies of their physiological and genetic characteristics are the basis of their use in probiotic preparations. For example, lactobacilli are the most common vaginal bacteria in women. They inhibit other bacteria from binding to epithelial cells and produce lactic acid that kills or inhibits the growth of many other microorganisms. The characteristics and benefits of different probiotic strains are generally strain specific and individual strains should be tested for each feature.

In this context, the developments related to the identification of different types of vaginal lactobacilli are an important contribution to the development of this scientific direction.

The application of the method Dot hybridization of total genomic DNA with labeled probes has been evaluated as a promising and relatively easy method for the detection, grouping and initial identification of newly isolated vaginal lactobacilli, which in combination with other highly discriminatory molecular methods of polyphasic taxonomy leads to an accurate determination of the taxonomic belonging to *Lactobacillus* species in mixed cultures (10). A number of subsequent developments have applied taxonomic analysis based on 16S rDNA sequencing.

Thirty strains of different species of *Lactobacillus* have been isolated, identified, and characterized, among which seventeen new vaginal strains with antimicrobial activity against a number of strains of pathogenic microorganisms, including strains of *Klebsiella*, *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus faecalis* and *Candida parapsilosis*, are established. The nature of the active compound with antimicrobial action has been investigated,

and the presence of bacteriocin-like substances has been demonstrated. In three of the newly isolated strains, the genes associated with the production of bacteriocins are identified and sequenced. It is important to emphasize that the presence of an operon for the bacteriocin gassericin A in the genome of the species *Lactobacillus crispatus* is described for the first time (1). An original contribution has made by the results of the genomic analysis of five strains of *L. crispatus*, in which the presence of a gene responsible for the synthesis of the bacteriocin helveticin has confirmed. Two variants of this gene have been described in the genome of two of them. The molecular analysis carried out has shown that the differences in the sequences of this gene can be used to determine the phylogenetic proximity of the different species of the genus *Lactobacillus* (2).

An original strain of *L. lactis* subsp. *lactis*, producing a bacteriocin (bacHV219) and with pronounced antimicrobial activity is isolated, identified and characterized. The optimal conditions for production and the factors affecting the activity of the bacteriocin have been established. The bacteriolytic mechanism of action is established through the application of Atomic force microscopy (7).

For the first time, a molecular genetic study of vaginal lactobacilli isolated from Bulgarian women has been conducted. The study covers the results of an analysis of 48 strains of *Lactobacillus* isolated from vaginal samples collected from 60 Bulgarian women of reproductive age. Six molecular methods (ribotyping, ARDRA, rep-PCR, PCR with species-specific primers, hybridization with species-specific probes and sequence analysis) have applied to reveal the biodiversity of the studied *Lactobacillus* strains. It has found that the predominant species in the studied group is *L. fermentum*. Three strains possessing high antimicrobial activity are investigated by 16S rDNA sequence analysis. Active strains inhibiting the growth of pathogens are selected. The use of probiotics to control certain infections and to restore the human bacterial microbiota is accepted as an alternative to conventional antibiotic therapy (26).

For the purpose of selection of probiotic strains, ten vaginal *Lactobacilli* strains are isolated and recognized as "probiotics". As a result of the research, it has been concluded that, along with the accumulation of biomass, the production of bacteriocins and H<sub>2</sub>O<sub>2</sub>, the amount of lactic acid produced and internal acidification are the main mechanism for the antibacterial activity, and accordingly, maintenance of the normal vaginal ecosystem. The results show that the studied groups of vaginal lactobacilli have the potential to develop an alternative treatment for urogenital diseases (6).

The first comprehensive study of plasmid content of 20 Bulgarian endemic *Streptococcus thermophilus* strains used for industrial milk fermentation is presented. *S. thermophilus* belongs to a diverse group of industrial microbes known as lactic acid bacteria. In many countries, it is accepted that industrial strains are plasmid-free, since the presence of non-plasmids can cause problems in their selection. It has found that a total of 6 of the 20 analyzed strains of *S. thermophilus* from the best Bulgarian sourdough starters for yogurt contain different plasmids that do not show homology with plasmids characteristic of *Lactobacillus*. Plasmid pt38 belonging to the pC194 plasmid family has been further characterized and the specificity of the resulting restriction map suggests that it may serve as a shuttle vector for heterologous gene expression in gram-positive and gram-negative hosts (9).

In another development with the LBB.A strain of *S. thermophilus*, a component of an industrial sourdough starter, the wide genetic diversity of phages that are at the same time highly specific for their host has been demonstrated. The strain has been infected by eight genetically unrelated cos-type phages. After precise molecular analysis, a two-step selection procedure involving sequential treatment of the bacterial culture with  $\phi$ A1 and  $\phi$ A7 is applied and bacteriophage-insensitive mutants of technological interest resistant to all eight phages are obtained. Strain LBB.A has been identified as a very good model for phage-host interaction studies in *S. thermophilus* (13).

A large-scale survey of 57 LAB - strains from the genera *Lactobacillus*, *Streptococcus*, *Enterococcus* and *Leuconostoc* isolated from different sources and tested for antimicrobial activity is reported. All strains are taxonomically identified using 16S rDNA sequencing analysis. It has found that 60% of them produce bacteriocin substances active against various Gram (+) and Gram (-) microorganisms and *Candida*. A phylogenetic tree created demonstrates the evolutionary relationships of some of the isolated strains based on 16S rRNA gene sequences. Analyses have been performed in MEGA4 software. The physicochemical nature of the active substances has been investigated. The presence of genes for the bacteriocins: gassericin A, gassericin T, acidocin LF221A and helveticin in the genome of some active isolates has been demonstrated. The genes involved in bacteriocin production from five strains are sequenced. Most inhibitory spectra of isolates are specific at the strain level, suggesting the use of a combination of bacteriocinogenic strains to inhibit different unwanted microorganisms (24).

The study of the microbial content of home-made and market-bought milk products can be added to the topic covering studies of lactic acid bacteria. In the course of the study, twenty-six pure cultures have been isolated and examined by a set of physiological and molecular genetic methods for accurate species identification and genotyping. It is found that among the

microorganisms involved in the fermentation and ripening of dairy products, strains of *L. delbrueckii*, *L. helveticus* and *L. plantarum* predominate. Contaminants such as *Kluyveromyces*, *Rhodotorula* and *Candida* strains have also been found. The obtained results again raise the question of the efficacy of microbiological control in the production and storage of dairy products (5).

Dr. Galina Stoyancheva is a co-author in a scientific review "Starch-modifying enzymes of lactic acid bacteria - structures, properties, and applications", which for the first time summarizes the data available in the literature on starch-modifying enzymes from amylolytic lactic acid bacteria (ALAB), as the sole carbon source. Amino acid sequence comparison and gene expression analysis of genes encoding amylase enzymes in ALAB cells has performed. It has been concluded that ALAB strains can be used for the direct conversion of starch to lactic acid, as well as their involvement in food production (14).

A significant part of the developments presented in the current competition are dedicated to the study of the biodiversity of microorganisms (bacteria, yeasts and fungi) in different ecosystems. The conducted research is not an end in itself and always has results that are a contribution to solving problems of an important applied nature. A major part of these studies is aimed at the taxonomic determination of fungi.

A study of fungal contamination in seven Egyptian tombs and mosques has conducted. This study is the first of its kind and covers 30 fungal isolates from 13 samples of 6 different materials in historical tombs and mosques in Egypt. Sixteen strains from groups Ascomycota and Basidiomycota are isolated and identified by sequencing and comparative analysis of ITS regions, LSU and SSU ribosomal RNA, *tef1- $\alpha$*  gene (translation elongation factor 1 alpha) and *tub2* gene (beta-tubulin). Strains of the genera *Penicillium*, *Aspergillus*, *Cladosporium* and *Bjerkandera* have been shown to inhabit stone surfaces in ancient Egyptian tombs. In the conducted research, the dominance of representatives of *Penicillium* and *Aspergillus* is established. The study of microbial diversity in historical monuments and their role in the destructive processes is a problem of global interest and an important contribution in finding suitable antifungal agents to eliminate microbial growth (4).

Extreme conditions, such as low temperatures, are known to lead to the accumulation of free oxygen radicals in the cell. The main defense mechanism of cells is provided by hydroperoxidases, which include several types of catalase enzymes, reducing oxidative stress by detoxifying cellular hydrogen peroxide. Filamentous fungi often possess a large number of catalases, which are the subject of increasing interest and especially catalases induced under low temperature oxidative stress, for which data are scarce.

19 Antarctic fungal strains that possess significant extracellular and intracellular catalase activity have been isolated. Representatives of *Penicillium chrisogenum* and *Aspergillus fumigatus* are promising producers of cold-active catalase enzymes (16). In initial studies, an Antarctic strain of *Penicillium griseofulvum* P29 has reported to actively produce cold-active catalase (17). With the leading participation of Dr. Galina Stoyancheva, the identification, sequencing and expression analysis of catalase genes in strain *P. griseofulvum* P29 is carried out. In the presented development, with the help of originally created oligonucleotide primers, PCR products have been obtained and by the "genome walking" method, and the processing of the obtained sequences with the program CAP3 (Sequence Assembly Program), the complete sequences of the five catalase genes in the genome of the strain are obtained.

Quantitative PCR (qPCR), more commonly referred to as "real-time" PCR, is a widely used method for the quantitative assessment of gene expression. The method is called reverse transcription (RT)-qPCR because amplification requires conversion of an RNA template to cDNA as a preliminary step. The results of the gene expression study of the 5 catalase genes by the applied method at two different temperatures: 10°C and 25°C, has showed that four of the genes are induced by low temperature as a response to oxidative stress. Their participation in the mechanism neutralizing free oxygen radicals, assists and allow the growth of *P. griseofulvum* P29 under conditions of oxidative stress induced by low temperature. Most significantly, the expression of the *cat1* gene encoding an enzyme with catalase-peroxidase activity has increased (3).

Of interest is the development in which a strain of *Trametes trogii* has isolated and characterized an efficient producer of ligninolytic enzymes, which can be used in optimizing the delignification process and biotechnological production of laccase (15).

The role of the antioxidant response in the tolerance of fungi to different concentrations of a mixture of metal ions has investigated. Experiments with two strains, *A. fumigatus* G and *A. fumigatus* 3-2 isolated from soil heavily contaminated with heavy metals in Bulgaria (around the town of Pazardzhik) and Ukraine (around the town of Kryvyi Rih) show that thanks to its more active antioxidant protection, demonstrate better tolerance to the presence of mixtures of Cu, Cd, Ni and Zn in the culture medium. These results indicate a good bioremediation potential of the studied filamentous fungal species (18).

Isolated, characterized and identified by physiological, biochemical and molecular taxonomic methods is a bacterium *Microbacterium* sp. capable of inhibiting the growth and causing death of laboratory cultures of Antarctic microalgae strains: cyanobacteria

*Synechocystis salina* and green microalgae *Choricistis minor*, illustrated by transmission electron microscopy. The study of such strains shows the possibility of their use as biological agents in excessive algal blooms in polluted waters and contributes to the optimization of the use of algae in biotechnological productions (8).

Twenty yeast strains belonging to different genera and capable of degrading starch as a sole carbon source and possessing varying degrees of amylolytic activity have also been isolated and taxonomically identified (11). A review on yeast identification presents major modern genomic tools used in yeast taxonomy (12).

To the original contributions of important scientific importance analyzed above, it should be added that with the leading participation of Dr. Galina Stoyancheva in terms of molecular-taxonomic identification, more than 250 strains of filamentous fungi are identified, among them isolated from soil samples from the national reserve Denali, Alaska, isolated from "Magurata" Cave, enzyme-producing strains, etc. (15-22).

A number of specific oligonucleotide primers necessary for the analysis of bacteriocin genes, catalase genes and reference ("housekeeping") genes are designed; the creation of a phylogenetic tree that represents the evolutionary relationships among 35 *Lactobacillus* taxa; the phylogenetic tree created on the basis of sequenced ITS regions of 28 strains of filamentous fungi isolated in Denali National Preserve, Alaska, including 46 taxa, etc.

187 of the obtained sequences were registered in the NCBI GenBank database.

The scientific contributions of Dr. G. Stoyancheva examined in this section can be summarized and presented in the following formulations, which correspond to the scientific field and scientific direction of the current competition:

1. A number of original *Lactobacillus* strains, with high probiotic activity, producing bacteriocins, active against various pathogenic microorganisms, were isolated, selected and fully characterized.

- An operon for the bacteriocin "gasericin A" was identified for the first time in the genome of *Lactobacillus crispatus*.

- *Lactococcus lactis* HV219 strain producing bacteriocin HV219 was isolated

- A molecular biological analysis of genes encoding the synthesis of bacteriocins, such as gasericin A, helveticin, bacteriocin HV219, was conducted. The growth conditions and media composition required for optimal bacteriocin production have been determined.



2. A polyphasic taxonomic analysis was performed and more than 250 strains of filamentous fungi isolated from Antarctic and Alaskan soil samples, isolates from Magura Cave, from historical monuments in Egypt were identified.

- For the first time, a study was conducted on the biodiversity of fungi inhabiting different materials from historical monuments in Egypt.

- For the first time, a detailed molecular-biological study of a temperature-sensitive catalase from the Antarctic strain *Penicillium griseofulvum* P29 was carried out.

- For the first time, the influence of temperature as a factor in the regulation of the expression of catalase genes in filamentous fungi was investigated.

## CONCLUSION

The developments included for evaluation in this competition are distinguished by originality, relevance, and public significance. They are united by the direct connection between fundamental and applied research and by the skillful application and logical combination of a number of the most modern research methodological approaches. Dr. Galina Stoyancheva has established herself as a qualified specialist in the implementation of modern molecular biological research on microorganisms, based on 16S rDNA sequencing, ITS, identification and sequencing of genes (SSU rRNA, LSU rRNA, *tef1- $\alpha$* ), gene analysis expression using Real-Time PCR (RT-PCR) (catalase genes), bacteriocin mechanism of action by atomic force microscopy (AFM) and other methodological approaches from the field of microbial physiology and biochemistry and electron microscopy.

The achievements of Dr. Galina Stoyancheva have been published in authoritative scientific journals and have found a wide positive response in the scientific community around the world; they have been reflected in a number of funded scientific research projects, which indisputably confirms the significance of the developed tasks and the applicability of the obtained results.

The scientometric data of Dr. Galina Stoyancheva significantly exceed the minimum national and additional requirements of ZRAS RB and the relevant Rules of the IMikB for occupying the academic position "Associate Professor" in the Professional direction 4.3. Biological Sciences.

Dr. Stoyancheva's future research is aimed at a comprehensive study of the expression and application of bacteriocins produced by lactic acid bacteria, as well as of recombinant peptides and their heterologous expression, selection, and study of strains of microorganisms for use in foods with specific health benefits - functional foods.

All this gives me a reason to support the candidate and to confidently recommend to the respected members of the Scientific Jury to evaluate positively and propose to the SC of Institute of Microbiology to award Ch. Ass. Professor Galina Dinkova Stoyancheva, PhD, the academic position "Associated professor".

Sofia, 27/07/2022

REVIEWER:

(Assoc. prof. Dr. Zlatka Alexieva)