**TO THE CHAIRMAN OF THE SCIENTIFIC JURY APPOINTED BY ORDER No. I-141/01.10.2025 of the Director of the Institute of Microbiology at the Bulgarian Academy of Sciences**

# STATEMENT

**Regarding** procedure for acquiring the PhD Dgree with candidate Ivan Dimitrov Ivanov, a full-time doctoral student at the The Stephan Angeloff Institute of Microbiology – BAS (IMicB – BAS) at the Bulgarian Academy of Sciences, in the field of higher education 4. Natural Sciences, Mathematics and Informatics, professional field 4.3 Biological Sciences, in the scientific specialty "Microbiology"

**Topic of the dissertation:**"NEW PRODUCERS OF PREBIOTIC CARBOHYDRATES FROM BULGARIAN YOGURT"
**Scientific supervisors:** PROF. DSc PENKA PETROVA, PROF. DSc KALOYAN PETROV
**Written by:** Assoc. Prof. Ivan Nikolaev Ivanov, Deputy Head of the National Reference Laboratory for Control and Monitoring of Antimicrobial Resistance, Department of Microbiology, National Center for Infectious and Parasitic Diseases

I declare that I have no common publications or any conflict of interest with the doctoral student as per the meaning of para. 1, items 3 and 5 of the Act on development of the academic staff in the republic of Bulgaria .

The application documents have been prepared correctly and in accordance with the ADRB and the regulations for its implementation in the IMicB – BAS.

**Relevance of the topic.** Ivan Ivanov's thesis deals with an extremely topical issue, namely the study of biologically active compounds produced by lactic acid bacteria and in particular *L. delbruecki ssp bulgaricus*. With the growing importance of the problem of antimicrobial resistance, over the last decade, new therapeutic and prophylactic approaches have been increasingly sought to reduce the antibiotic pressure on pathogenic bacteria. In this sense, the role of lactic acid bacteria in the therapy of gastroenteritis has long been proven, and they are increasingly used in the treatment of infections with extraintestinal localization (e.g. rhinosinusitis, vaginosis and UTIs). However, existing problems related to the lack of generally accepted international standards for

good manufacturing practices and safety measures still hinder the clinical trials of these preparations. That is why the detailed molecular characterization of the metabolome of probiotic strains is of key importance. Overall, I find the topic very interesting and significant.

**Structure of the thesis and bibliography.** The text is 121 pages long and is structured as follows: Introduction - 1 page; Literature review - 44 pages; Aim and objectives - 2 pages: Materials and methods 12 pages; Results and discussion - 31 pages, Conclusions and Achievements 1 page each. It is illustrated with 23 figures and 13 tables as well as 216 references, 2 of which are in Cyrillic. The bibliography indicates that over 80% of the cited articles have been published in the last 10 years, which once again emphasizes the relevance of the work.

**Literature review**. The doctoral student presents a competently written, modern and informative review. All aspects of the problem are comprehensively considered. The taxonomy is presented in accordance with the modern genotypic classification of lactobacilli. Attention is paid to the physiology, cultural features and antimicrobial properties with an emphasis on *L. bulgaricus*. The composition and characteristics of regional dairy products are examined, and here I believe that the text is at times too detailed, given that the work is essentially not technological. Further, metabolites with proven beneficial effects on health are described (amino acids, antioxidants, peptides, short-chain fatty acids, etc.), in order to reach the central subject of the thesis, the galactooligosaccharides or GOS. The biosynthesis of GOS and the role of lactose reduction mechanisms in dairy products are examined and the Bulgarian studies on the topic are reviewed. Here it may be necessary to point out the advantages of GOS over FOS, as the latter being largely established as nutritional supplements.

Apart from prebiotics, I did not find any clear definitions of the terms probiotic, postbiotic, and the newer concept of synbiotic, which I think is important, given the known contradictions in the scientific literature.

In the conclusion of the review, the candidate brings out and systematizes the most important contemporary debatable topics, emphasizing the need for up-to-date, scientifically based knowledge to serve as the basis for the creation of new and improved food products. On page 48, I found a duplicate paragraph.

**Goal and objectives.** The goal and six tasks are precisely formulated, reflecting the main aspects and essence of the dissertation work.

**Materials and methods** are described in detail and with precision, which clearly shows the high level of competence of the candidate. The skillful use of a significant set of traditional microbiological, biochemical, molecular genetic and statistical methods, that have been mastered and applied, is impressive. All microbiological and biochemical techniques are performed according to modern recommendations and standards. Molecular methods are adequately described with the required positive control strains and negative controls. The experiments involving gene cloning are presented in sufficient detail with the characteristics of the relevant vectors, selection and verification of the clones. In almost all places in the text I find $MgSO_4$ incorrectly written as "$MrSO_4$" and $MgCl_2$ as "$MrCl_2$", which is too unusual to be a technical error.

**Results and discussion.** In the first part, a targeted 16S rRNA metagenomic analysis of four yogurt samples from different regions of the country was performed. The NGS methods and pre-analytical preparation for the metagenomic analysis are described in Results and Discussion, and not in Materials and Methods, as it should be. There is also no description of the applications used for processing and filtering the reads, as well as the algorithms and databases for taxonomic profiling. I recommend that in future studies, the 16S rRNA gene alone should not be used as a taxonomic marker, since it is already known that it is not sufficiently informative in lactobacilli.

I find the analysis of species diversity, and especially the discovery of conditionally pathogenic strains, interesting and significant with implications for potential health risks upon consuming homemade products.

**Here I have the following question: Have experiments been done to isolate Lb strains with β-galactosidase or other desired activity from these four samples and what is their role besides for metagenomic analysis?**

The following analyses describe the characteristics of the *lacZ* gene, as well as the amino acid composition and 3D structure of the protein in *L. bulgaricus* strain 43, which has shown high transgalactosidase activity in previous studies. The gene was successfully cloned and expressed, and the characteristics and activity of the recombinant β-galactosidase towards various factors and

cofactors were analyzed and established. The structure of the products (GOTs) of the enzyme activity was precisely studied by HPLC and mass spectrometry, which I consider to be a significant contribution and acquired skills. Overall, the discussion is presented analytically and comprehensively.

**The conclusions, are precisely formulated and adequately reflect the implementation of the goal and the objectives set,** while simultaneously emphasizing the most significant findings from the thesis work.

**The work presents significant original scientific and practical contributions, which I fully accept.**

Ivanov presents three refereed publications with an impact factor related to the work and cited nearly 100 times. In one of them he is the first author, while in two he is the second, which testifies to his leading and significant contribution to the research. The number of credits he has accumulated during his doctoral studies is 463 and exceeds by two and a half times the required for defense (200). I believe that the scientometric indicators exceed the criteria for acquiring "PhD" Degree set out in the ADRB regulations and IMicB – BAS

**Conclusion.** The work has undeniable scientific value, the research was carried out accurately using modern methods and approaches. The data obtained expand our understanding of the topic and are presented in a manner worthy of a scientific work of this nature.

**Based on the above, I believe that the doctoral student fully meets the criteria for acquiring the educational and scientific degree "PhD" according to the Act on the Development of the Academic Staff in the Republic of Bulgaria (ADRB) and the regulations for its implementation at the Institute of Microbiology at the Bulgarian Academy of Sciences , and I strongly suggest that the esteemed members of the jury and the scientific council approve his candidacy.**

Date: 19/11/2025 Written by :.....

/ Assoc. prof. Ivan Ivanov/