REVIEW

by Prof. Velizar Kostadinov Gochev, PhD, Department of Biochemistry and Microbiology, Plovdiv University "Paisii Hilendarski"

of a dissertation submitted for the award of the educational and scientific degree **"Doctor"**
in: field of higher education 4. Natural Sciences, Mathematics and Informatics,
professional field 4.3 Biological Sciences, doctoral programme Microbiology

Author: Lena Rumenova Ilieva

Topic: Antimicrobial susceptibility and factors of colonisation and invasiveness in nasopharyngeal isolates of *Staphylococcus spp.*

Academic supervisor: Prof. Sonya Kostadinova Trifonova, PhD, Plovdiv University "P. Hilendarski"

1. General description of the submitted materials

By Order No. RD-22-1084 dated 29.0.5.2026 of the Rector of Plovdiv University "Paisii Hilendarski" (PU), I was appointed as a member of the scientific jury responsible for conducting the procedure for the defence of a dissertation entitled "Antimicrobial susceptibility and factors of colonisation and invasiveness in nasopharyngeal isolates of *Staphylococcus spp.*" for the acquisition of the educational and scientific degree "Doctor" in: field of higher education 4. Natural Sciences, Mathematics and Informatics, professional field 4.3 Biological Sciences, doctoral programme Microbiology. The author of the dissertation is Lena Rumenova Ilieva, a full-time doctoral candidate at the Department of Biochemistry and Microbiology, with academic supervisor Prof. Sonya Kostadinova of PU.

The set of materials submitted by Lena Ilieva in electronic and hard-copy form complies with Article 36(1) of the Regulations on the Development of the Academic Staff of PU (PRASPU) and includes the following documents: an application to the Rector of PU for the initiation of a dissertation defence procedure; a curriculum vitae in European format; minutes of the Department Council related to reporting readiness for the initiation of the procedure and to the preliminary discussion of the dissertation; the dissertation; the author's abstract; a list of scientific publications on the topic of the dissertation; copies of the scientific publications; and a declaration of originality and authenticity of the submitted documents.

The submitted documents give me full grounds to determine that the procedure complies with all statutory and internal institutional regulatory requirements.

2. Brief biographical information about the doctoral candidate

Lena Ilieva graduated from the Faculty of Biology of PU and in 2001 obtained the educational qualification degree "Master" in Biology, with a specialisation in Genetics and Microbiology. From 2006 to 2010, she worked as a microbiologist at "Ganchev" EOOD in the field of sanitary microbiology, and from 2010 to 2025 as a microbiologist in the field of clinical microbiology, successively at the Specialised Medical Diagnostic Laboratory "Chronolab" Ltd. and "Synevo - Bulgaria" EOOD. Since August 2025, Lena Ilieva has been an Assistant Professor of Microbiology at the Institute of Food Preservation and Quality at the Agricultural Academy. The doctoral candidate has extensive and indisputable professional experience as a microbiologist.

Lena Ilieva's education and professional competence are entirely within the field of microbiology.

3. Relevance of the topic and appropriateness of the objectives and tasks set

Upper respiratory tract infections are among the leading reasons for consultation and antibiotic treatment in outpatient care, and their high frequency maintains constant selective pressure for the development and spread of antibiotic resistance. Within this clinical context, *Staphylococcus spp.* occupy an important place as microorganisms frequently isolated from nasal and throat samples. Contemporary clinical practice simultaneously faces three problems: increasing antibiotic resistance among staphylococci, the presence of a diverse set of colonisation and invasiveness factors, and the limitations of phenotypic methods for the reliable identification and interpretation of these isolates. The present dissertation focuses on the study of antimicrobial susceptibility and colonisation and invasiveness factors in nasopharyngeal isolates of *Staphylococcus spp.*, with an emphasis on *S. aureus*, the characterisation of their phenotypic and genotypic resistance and virulence profiles, the assessment of their biofilm-forming capacity under conditions mimicking the host environment, and an in vitro investigation of the possibilities for limiting growth and biofilm formation.

I categorically consider the topic of the present dissertation to be particularly relevant from both a practical and a fundamental perspective. The aim of the dissertation is clearly and precisely formulated, and the tasks set are logically connected and arranged in the correct sequence.

4. Knowledge of the problem

The literature review, which forms the basis for assessing the degree of knowledge of the problem, is presented over 32 pages and is based on more than 300 literature sources. The information in the review is comprehensive and structured into 10 sections, and is presented with a high degree of analytical depth. The review begins with data on the clinical and epidemiological significance of *S. aureus* and places emphasis on the molecular mechanisms of nasopharyngeal colonisation, biofilm formation, and antibiotic resistance. The comparative presentation of molecular and genomic approaches for the identification and characterisation of *S. aureus* makes a very good impression. The information regarding the possibilities for applying lactic acid bacteria (LAB) and metabolites arising from their vital activity as alternatives for the control of *S. aureus* is sufficient in scope and comprehensiveness. I highly appreciate the fact that the review concludes with a summary that clearly defines the relevance of the study and the directions of the research, which enables the clear formulation of the aim and tasks of the dissertation.

The scope and manner in which the literature review is organised clearly demonstrate that the doctoral candidate has in-depth knowledge of the scientific problem under investigation, which enables her to formulate the aim clearly and to structure the experimental design of the study correctly.

5. Research methodology

The Materials and Methods section covers 14 pages of the dissertation and describes: (1) classical and contemporary microbiological methods for the isolation and identification of bacteria from clinical samples and fermented food products, reference microbiological methods for determining antimicrobial susceptibility, methods for determining biofilm-inhibiting capacity, and assessment of probiotic potential; (2) molecular biological methods for conventional PCR amplification of the 16S rRNA gene, genome annotation, whole-genome sequencing, genome-based identification, and Multi-Locus Sequence Typing (MLST); (3) chromatographic methods - gas chromatography for determining fatty acid composition (GC) and FPLC; (4) microscopic methods - scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM); (5) electrophoretic methods - SDS-PAGE; and (6) statistical methods for processing the results obtained. The overall experimental design of the study is correctly structured, and the methods applied fully correspond to the objectives of the dissertation research.

The methods applied in the dissertation research have been appropriately selected and enable the generation of reliable and representative results. The range of methods mastered and applied,

as well as the manner in which they are presented, clearly demonstrate that the educational component of the educational and scientific degree "Doctor" has also been successfully completed.

6. Characterisation and evaluation of the dissertation

The dissertation is structured in the generally accepted standard manner and includes the following sections: Introduction (1 page); Literature Review (32 pages); Aim and Tasks (1 page); Materials and Methods (14 pages); Results and Discussion (88 pages); Conclusion (3 pages); Conclusions (2 pages); Contributions (2 pages); References (375 literature sources); and Appendices (40 pages). I consider that optimal proportions have been maintained between the individual sections of the dissertation, with the section presenting and discussing the results being dominant and comprising slightly more than 60% of the substantive part of the work, excluding the references and appendices. The Introduction sets out the seriousness of the problem regarding the frequency of isolation, inaccuracies in identification, and resistance among *Staphylococcus spp.*, clearly defines the relevance of the study, and in this sense fully fulfils its function within the text. As I have already stated in Section 4, the Literature Review is comprehensive, based on current and well-selected literature, and accurately presents the state of scientific knowledge on the problem worldwide. This enables the doctoral candidate to define clearly the aim and tasks of the dissertation research. The experimental design is correctly composed, and the selected methods are of a standard that enables the successful implementation of the research tasks and achievement of the aim of the dissertation. Within the first research task, a large-scale four-year study of the structure of nasopharyngeal infections in the city of Plovdiv was conducted, based on more than 27,000 patient samples. The annual and seasonal dynamics of these infections were monitored, and the age structure of the patient population was assessed. A comparatively low relative proportion of nasopharyngeal isolates among the total number of positive samples was established. The aetiological structure of the positive isolates was investigated, and it was demonstrated that *S. aureus* is detected at the highest percentage in nasal samples. A combined strategy for the correct species-level identification of *S. aureus* was proposed, including a series of phenotypic and biochemical tests combined with sequencing of species-specific genes and genome sequencing. The antimicrobial resistance study conducted under the second research task included 450 species-identified *S. aureus* isolates and used 17 antimicrobial agents from different groups. The results obtained allow the investigated *S. aureus* strains to be defined as having a high resistance burden, characterised by predominant resistance to β-lactam antibiotics, increased methicillin resistance, and practically significant levels of resistance to macrolides, lincosamides, fluoroquinolones, and aminoglycosides. The experimental work within the dissertation continues with an in silico analysis of the

S. aureus resistome using the innovative Oxford Nanopore Technologies platform. An in silico analysis of the virulence potential of the isolates was conducted and was also confirmed by data from in vitro analyses. The in silico analysis established that the clinical *S. aureus* isolates possess a stable set of genes associated with adhesion, persistence, and interaction with the host immune system. A very extensive amount of experimental work was carried out within the fourth research task, relating to the assessment of the biofilm-forming capacity of the isolates. It was established that 82% of them form biofilm, and 60 strains were identified as having high biofilm-forming potential. The experiments relating to the optimisation of biofilm formation conditions, particularly with regard to the composition of the medium, also merit attention, as they enabled the ability of the isolates to form biofilm to be assessed in the best possible manner. In view of the search for alternative possibilities for controlling nasopharyngeal *S. aureus* isolates and their ability to form biofilm, the doctoral candidate proceeded to isolate lactic acid bacteria (LAB) from fermented lactic acid products. A biochemical and genetic characterisation of the probiotic potential of LAB with antistaphylococcal activity was performed. Through a set of chromatographic and genetic methods, the bacteriocin nature of the antimicrobial agents produced by the promising isolates identified to species level and assigned to the species *Lactoplantibacillus plantarum*, *L. paraplantarum*, and *Levilactobacillus brevis* was demonstrated. In the concluding part of the experimental work, the ability of a lyophilised cell-free supernatant from the selected LAB to inhibit the growth of nasopharyngeal *S. aureus* isolates and their ability to form biofilm was determined. The section concludes with a summary of the results obtained, demonstrating their significance and potential possibilities for application.

All results have been correctly interpreted and compared with the literature data. On the basis of the results obtained, 10 conclusions have been formulated, reflecting the most significant achievements of the dissertation research. Scientific and scientific-applied contributions have been identified, which I fully accept and define as being original and advancing in nature.

I consider the dissertation to represent a comprehensive and completed study based on a large-scale experimental design and a broad range of correctly combined methods. The results obtained are highly representative and significant in both scientific and applied terms. The conclusions and contributions are correctly formulated.

7. Assessment of the publications related to the dissertation

Two scientific publications related to the dissertation have been submitted, in *Int. J. Mol. Sci.* and *Microorganisms*. Both journals are in quartile Q1, and the publication in *Int. J. Mol. Sci.* has

already received 2 citations, while the publication in *Microorganisms* has received 13, which is indisputable evidence of their quality.

8. Personal contribution of the doctoral candidate

I consider the doctoral candidate's personal contribution to the implementation of the experimental work, the writing of the dissertation, and the publications to be indisputable and commensurate with the level of her professional competence.

9. Author's abstract

The author's abstract meets the requirements and reflects all of the more significant results of the dissertation.

10. Critical remarks, recommendations, and questions

I have no critical remarks or recommendations regarding either the study conducted or the set of materials.

I have the following questions for the doctoral candidate:

1. To what extent could the proposed comprehensive scheme for the identification of *S. aureus* find practical application in clinical laboratory practice?
2. Are other means of controlling the biofilm-forming capacity of *S. aureus* known, and how effective are they in comparison with bacteriocins from LAB?

11. Personal impressions

My personal impressions of the doctoral candidate Lena Ilieva are entirely positive. She is an accomplished specialist in the field of clinical microbiology with extensive practical experience. I highly appreciate her positive attitude and ease of communication within a team.

CONCLUSION

The dissertation *contains scientific, scientific-applied, and applied results that represent an original contribution to science* and meet all the requirements of the Development of the Academic Staff in the Republic of Bulgaria Act (ZRASRB), the Regulations for the Implementation of ZRASRB, and the corresponding Regulations of Plovdiv University "Paisii Hilendarski".

The dissertation demonstrates that the doctoral candidate Lena Rumenova Ilieva possesses in-depth theoretical knowledge and professional skills in the scientific speciality of Microbiology, while demonstrating the qualities and skills required to conduct independent scientific research.

In view of the above, I confidently give my *positive assessment* of the study conducted, as presented in the dissertation, author's abstract, results, and contributions reviewed above, and I *propose that the esteemed scientific jury award the educational and scientific degree "Doctor"* to Lena Rumenova Ilieva in field of higher education 4. Natural Sciences, Mathematics and Informatics, professional field 4.3 Biological Sciences, doctoral programme Microbiology.

14.07.2026 Reviewer: .....

Prof. Velizar Gochev, PhD