REVIEW

by Dr. Penka Angelova Moncheva, Professor at the Faculty of Biology, Sofia University "St. Kliment Ohridski" (retired)

on a dissertation for the award of the educational and scientific degree "Doctor" (PhD)

in: Higher Education Area 4. Natural Sciences, Mathematics and Informatics,

Professional Field 4.3. Biological Sciences,

Doctoral Program: Microbiology

Author: Lena Rumenova Ilieva

Topic: "Drug Susceptibility and Factors of Colonization and Invasiveness in Nasopharyngeal *Staphylococcus* spp. Isolates"

Scientific Supervisor: Prof. Dr. Sonya Kostadinova Trifonova, Faculty of Biology, Plovdiv University "Paisii Hilendarski"

1. General Description of the Submitted Materials

By Order No. RD-22-1084 dated May 29, 2026, of the Rector of Plovdiv University "Paisii Hilendarski" (PU), I have been appointed as a member of the Scientific Jury to ensure the procedure for the defense of a dissertation on the topic "Drug Susceptibility and Factors of Colonization and Invasiveness in Nasopharyngeal *Staphylococcus* spp. Isolates" for acquiring the educational and scientific degree "Doctor" in Higher Education Area 4. Natural Sciences, Mathematics and Informatics, Professional Field 4.3. Biological Sciences, Doctoral Program Microbiology. The author of the dissertation is Lena Rumenova Ilieva, a part-time doctoral student at the Department of Biochemistry and Microbiology, with scientific supervisor Prof. Dr. Sonya Trifonova from PU "Paisii Hilendarski".

The package of materials submitted by Lena Ilieva on paper and electronic media complies with Art. 36 (1) of the Regulations for the Development of the Academic Staff of PU and includes the following documents:

- application to the Rector of PU for opening the dissertation defense procedure;
- curriculum vitae in European format;
- dissertation thesis;
- abstract (in Bulgarian and English);
- list of scientific publications on the topic of the dissertation;
- copies of scientific publications;
- declaration of originality and authenticity of the submitted documents;

The doctoral student has attached 2 publications.

The documents are precisely prepared, and the information contained in them is sufficiently comprehensive to prepare an evaluation of the dissertation.

2. Brief Biographical Data of the Doctoral Student

Lena Ilieva completed her higher education with a Master's degree in Biology in 2001 at Plovdiv University "Paisii Hilendarski". Since mid-2025 and currently, she has been an Assistant at the Institute of Food Preservation and Quality – Plovdiv, where she engages in research work in the field of studying food production trends and developing innovative products. Prior to this, she worked as a biologist-lab technician at "Ganchev" Ltd. in the field of baby food production, with main activities related to monitoring food quality and safety, performing microbiological and physicochemical analyses, sampling, etc. (2006–2010). In the period 2010–2016, she worked as a microbiologist at the Independent Medico-Diagnostic Laboratory "Chronolab", Plovdiv, with main activities involving the isolation and identification of pathogenic microorganisms, microbiological analyses of various materials, preparation of antibiograms, etc. She held a similar position with a similar scope of activity at the "Synevo" Medico-Diagnostic Laboratory between 2016 and 2025.

I believe that the professional experience of Asst. Ilieva is a very good foundation, especially in methodological terms, for the development of the dissertation work.

3. Relevance of the Topic and Appropriateness of the Set Goals and Tasks

The scientific problem addressed in the dissertation is exceptionally relevant, given the increasing drug resistance of a number of pathogens, their resistance mechanisms, as well as the excessive and in many cases unjustified use of drugs, including antibiotics, acting as a selective factor for the spread of this resistance. Bacteria of the genus *Staphylococcus* are among the most frequently isolated in upper respiratory tract (URT) infections. Although they are part of the normal microflora, opportunistic and pathogenic species exist among them. The species *Staphylococcus aureus* is of the greatest importance, as it can colonize the nasal cavity and nasopharynx, and under certain conditions can cause local recurrent infections. This ability, combined with its drug resistance, in many cases complicates the treatment of infections caused by it. Methicillin-resistant strains and those with multidrug resistance are of particular concern. The ability of populations of the species to form biofilm on epithelial surfaces enhances their resistance to therapeutic agents. Therefore, the search for alternative agents to traditionally used antibiotics for this purpose is of crucial importance. In this regard, the biological approach chosen by the doctoral student—namely, the study of the anti-staphylococcal potential of lactic acid bacteria (LAB)—fits very well into the search for alternative methods to combat bacteria characterized by drug resistance.

I consider the topic of the dissertation to be relevant both scientifically and in terms of potential application, given the widespread antibiotic resistance that poses a risk to public health. The goal of the dissertation is clearly and precisely defined: namely, evaluation and significance of the species *S. aureus* in the etiological structure of symptomatic URT infections in patients from Plovdiv and characterization of its pathobiological potential in relation to opportunities to limit its development.

Five tasks adequate to the goal have been set, the implementation of which leads to its realization.

4. Knowledge of the Problem

In the literature review of the dissertation, Asst. Ilieva provides an overview of the current state of available scientific information on issues related to the topic of the dissertation. The review is structured around several main questions. A brief commentary is made on the etiology, epidemiology, and clinical significance of URT infections and the role of *S. aureus* within them, which underlines and confirms the relevance of the dissertation topic. A review of the taxonomy, biology, and population structure of representatives of the genus *Staphylococcus*, as well as the molecular mechanisms of nasopharyngeal colonization, is presented. Special attention is given to the ability of *S. aureus* to form biofilm, its antibiotic susceptibility, molecular and genetic approaches for identification and characterization, control strategies and novel therapeutic approaches, the application of LAB as a control strategy, the role of postbiotics as a promising approach, and the selection and evaluation of LAB capacity as potential antistaphylococcal agents.

In the literature review, Asst. Ilieva demonstrates very good skills in analyzing and summarizing scientific information related to her dissertation topic, which demonstrates knowledge of the problem, thereby substantiating the purpose of her research. The review is based on scientific information from the last decade, which also confirms the relevance of the subject matter under development.

5. Research Methodology

Given the tasks set for implementation, I consider the methods to be appropriately selected and adequate for executing the tasks and achieving the goal of the dissertation. A very rich and diverse set of methods has been used, including classical microbiological methods for isolating microorganisms; conventional biochemical methods for identifying bacteria of the genus *Staphylococcus*, as well as the standardized biochemical system StaphyTest 24; molecular-genetic methods - 16S rRNA sequencing preceded by DNA isolation and PCR amplification of 16S rRNA; genomic characterization of LAB, aimed at confirming species affiliation, assessing safety and establishing genetic potential for production of antimicrobial peptides and other bioactive compounds (whole genome sequencing, calculation of

average nucleotide identity using the FastANI tool, multilocus sequencing typing-based (MLST) phylogenetic analysis of sequenced strains); whole-genome sequencing and genome annotation (*Staphylococcus* strains were analyzed for antibiotic resistance and virulence genes, and LAB strains for probiotic properties); and the disc diffusion method for phenotypic profiling of antibiotic susceptibility of staphylococci. The following additional methods were used in the experimental work: lyophilization to obtain lyophilized cell-free supernatant (LCFS) from LAB, gas chromatography for determining the lipid fraction of LCFS, in vitro analysis of the probiotic properties of LAB strains comprising 4 tests, diffusion method for determining the antagonistic properties of LAB, a method for evaluating the biofilm-forming capacity of staphylococcal strains, methods for determining the minimum biofilm inhibitory concentration (MBIC), the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC), confocal laser scanning microscopy for evaluating the impact of LCFS on cell viability within the biofilm, scanning electron microscopy, FPLC for purification of low-molecular-weight protein fractions from cell-free LAB supernatant, SDS-PAGE for separation of protein fractions, and statistical processing of the obtained results.

I highly evaluate the choice of methods as well as their impressive number. This unequivocally demonstrates the excellent methodological training of Asst. Ilieva, which was obviously solidly enhanced during her doctoral studies.

6. Characteristics and Evaluation of the Dissertation Thesis

The dissertation thesis is written on 207 standard A4 pages. It is structured in the generally accepted manner for this type of scientific work: List of Abbreviations (2 pages), Introduction (1 page), Literature Review (32 pages), Goal and Tasks (1 page), Materials and Methods (14 pages), Results and Discussion (91 pages), Summary (3 pages), Conclusions (2 pages), Contributions (2 pages), List of Cited Literature (19 pages), and Appendices (41 pages).

Earlier in this review, I positively evaluated the doctoral student's knowledge of the problem, the set goal of the dissertation, the tasks for its realization, and the applied methodology in the respective sections. I will only add that the literature review is purposeful and based on the analysis and synthesis of a large volume of scientific data contained in 377 literary sources, 44% of which cover the last 5–6 years, showing that the scientific information provided by the doctoral student is modern, up-to-date, and confirms her theoretical training and knowledge of the scientific problem. The methods, besides being appropriately selected, are described in sufficient detail. The "Results and Discussion" chapter accounts for the largest part of the total volume of the dissertation. It presents and discusses the results obtained in the execution of all set tasks. The study was conducted at Laboratory "Synevo-Bulgaria" Ltd., Plovdiv, during the period 2016–2020. The clinical isolates included in this study were provided for scientific work to the doctoral student. A total of **8267 nasal and throat samples** from individuals with upper respiratory tract infections submitted for routine microbiological examination to the laboratory during this period were included in the study. The study included **12 LAB strains**, from which three strains belonging to the species *Lactiplantibacillus plantarum* IZITR_24, *L. paraplantarum* IZITR_13, and *Levilactobacillus brevis* DPL5 were selected through primary screening. Based on the data analysis of a massive number of clinical samples from various materials (**27959**) received at the laboratory during the study period, of which **6657** had confirmed microbiological growth, the annual and seasonal dynamics of positive samples were established. The proportion of nasopharyngeal and throat isolates was determined.

The etiological structure of microorganisms isolated from these samples was investigated to determine the relative proportion of identified species and the place of **S. aureus** among them. In nasal samples, the proportion of this species was the highest (**576 isolates**), but coagulase-negative staphylococci as well as other species were also isolated. In throat samples, **S. aureus** took second place (**184 isolates**), along with other species. Based on phenotypic characterization through classical physiological-biochemical traits and some additional tests, **776 isolates** were identified as belonging to the genus *Staphylococcus*, of which **76%** originated from nasal samples, and those belonging to the species *S. aureus* comprised approximately **97%**, making **574 isolates**. Phenotypic variations were established among the isolates identified as *S. aureus*, defined as intraspecific diversity. The species identity of

isolates phenotypically differing from the main type was confirmed by additional tests. Genotypic identification was performed by 16S rRNA gene sequencing of **20** randomly selected isolates identified to the species level, including both typical and atypical phenotypic traits, as well as other staphylococcal species. The species identity of all isolates (typical and atypical) was confirmed, but the method did not allow their resolution into distinct clusters. For identification purposes and to fulfill subsequent scheduled tasks, whole-genome sequencing of **10** *S. aureus* isolates was carried out, confirming their species identity through this method as well.

Results of *in vitro* phenotypic profiling of antibiotic susceptibility of **450** *S. aureus* isolates against **17** antimicrobial agents are presented, establishing dominant resistance to beta-lactam antibiotics and moderate but clinically significant resistance to macrolides, lincosamides, fluoroquinolones, and aminoglycosides. Both MRSA and MSSA strains were identified, as well as MDR (multidrug-resistant) strains. Based on *in silico* analysis, results regarding the resistome structure of **10** *S. aureus* isolates, including MRSA and MSSA strains, are presented. Confirmations of the preliminary classification of strains into MRSA and MSSA were obtained. Plasmids were identified in **6** of the sequenced genomes. The analysis in this study establishes not only the presence of resistance genes but also provides initial information on their genomic organization, the potential involvement of mobile genetic elements, and the potential for antibiotic resistance expression that is not directly linked to phenotypic manifestation. The *in silico* genomic analysis of the ten whole-genome sequenced *S. aureus* isolates outlines a broad but internally heterogeneous virulence repertoire. A total of **74** genes related to the pathogenesis of the species were identified, with individual strains carrying between 55 and 65 determinants. To verify the *in silico* analysis data, a PCR-based experiment evaluating the virulence potential of **40** nasopharyngeal *S. aureus* isolates (15 of which were identified as MRSA) was conducted for the presence of **20** main functional groups of target genes distributed across key functional categories: adhesion and colonization (*fnb*A, *fnb*B, *clf*B, *cna*, *fib*, *ebps*), biofilm formation capacity (*ica*A, *ica*B, *ica*D), surface adaptation and persistence (*isd*A, *sdr*C, *sdr*D, *tar*S), cytotoxicity (*hla*, *hlg*A, *psm*β1), and toxigenicity (*tss*t-1, *sea*, *seb*, *sec*), which confirmed the in silico analysis data. An evaluation of the biofilm-forming ability of *S. aureus* strains was performed, examining environmental conditions affecting the process.

Based on an initial screening of **12** LAB strains for antagonistic activity against **60** nasopharyngeal *S. aureus* isolates, **3** LAB strains were selected, whose taxonomic identity to *L. plantarum*, *L. paraplantarum*, and *L. brevis* was confirmed by whole-genome sequencing and MLST-based phylogenetic analysis. Results regarding the safety assessment of the strains as well as their genetic potential for synthesizing antimicrobial peptides and other biologically active compounds are presented. Lyophilized cell-free supernatants of the three strains of LAB were obtained, whose activity in terms of limiting the formation of biofilm by *S. aureus* and suppressing its planktonic growth was studied. A quantitative assessment of the established differences in the influence of LCFS on the two processes was made by PROBIT analysis, on the basis of which MBPC, MBC and MPC were calculated, respectively. The chemical analysis of LCFS, including determination of the fatty acid composition and fractionation by FPLC, showed a very low proportion of the lipid component, and that the antistaphylococcal activity of the supernatants is determined mainly by low-molecular protein bacteriocin-like fractions.

The effect of LBS on the viability and organization of the *S. aureus* biofilm was visualized by confocal laser scanning microscopy, and the biofilm of one of the strains was examined by scanning microscopy. The experimental results obtained were statistically processed using appropriate analyses, enabling the determination of their reliability and the establishment of various correlations. The results are presented in **32** very well-designed figures and **10** tables. Additionally, **8** appendices display highly detailed data from some of the analyses.

An enormous volume of experimental research work has been carried out, yielding an exceptionally large number of primary data, which have been analyzed, summarized, and presented in tabular or graphical form in excellent order. The results are analyzed and discussed in detail with great skill based on available scientific literature on the topic.

Based on the experimental results obtained, **10 conclusions** have been drawn, summarized below:

1. The role of the species *Staphylococcus aureus* in the etiological structure of symptomatic upper respiratory tract infections remained significant and stable during the study period (2016–2020) in the city of Plovdiv.

2. Taxonomic identification of *S. aureus* in outpatient practice using phenotypic methods is not sufficiently reliable and should be confirmed by routine molecular-biological methods, thereby reducing the risk of misinterpretation of clinical isolates, which is important for subsequent therapy.

3. *S. aureus* populations formed during the study period exhibit a high resistance burden with dominant resistance to β-lactam antibiotics, elevated methicillin resistance, and significant levels of resistance to macrolides, lincosamides, fluoroquinolones, and aminoglycosides, with established genetic determinants.

4. *In silico* analysis shows that clinical isolates of *S. aureus* possess a stable set of genes associated with adhesion, persistence, and interaction with the host immune system. Superantigenic and certain enterotoxin determinants have a more limited and uneven distribution.

5. The majority of the examined *S. aureus* isolates possess the ability to form biofilm, which varies among strains and depends on environmental conditions.

6. The antagonistic activity of screened LAB against *S. aureus* is strain-specific and ranges from growth inhibition to inhibition of biofilm formation.

7. Based on chemical analyses combined with FPLC fractionation, it was proven that the primary antistaphylococcal effects of cell-free supernatants of LAB are associated with low-molecular-weight protein bacteriocin-like fractions, while the lipid component has no independent inhibitory activity against *S. aureus* growth and biofilm formation.

8. The presence of gene clusters associated with the potential synthesis of antimicrobial peptides was proven in the genomes of selected LAB, with no genetic determinants identified that would compromise their probiotic potential.

9. The lyophilized cell-free supernatants from *Levilactobacillus brevis* DPL5, *Lactiplantibacillus paraplantarum* IZITR_13, and *Lactiplantibacillus plantarum* IZITR_24 exhibit pronounced dose-dependent antistaphylococcal activity.

10. The cell-free supernatants of the three MKB strains quantitatively reduce biofilm formation by *S. aureus*, alter the architecture of the mature biofilm and lead to well-pronounced changes in its structure, cell surface and morphology of bacterial cells, thus modifying the behavior of *S. aureus* related to biofilm formation.

7. Contributions and Significance of the Work for Science and Practice

The results obtained from the conducted research are of a contributing nature with scientific and applied scientific significance.

Contributions of a Scientific Nature

- Based on a comprehensive study involving epidemiological, phenotypic, and genetic characterization of outpatient isolates, the clinical significance of the species *S. aureus* as an etiological agent in nasopharyngeal infections has been proven
- Scientific information was obtained regarding the resistome structure of a local Plovdiv population of *S. aureus*, identifying major genetic determinants conferring resistance to beta-lactams, macrolides, and lincosamides.
- It was established that the virulence profile of *S. aureus* isolates is complex, with a predominance of adhesion and persistence determinants and a relatively limited distribution of superantigens and enterotoxins.
- It was proven that the presence of genetic resistance determinants does not always have phenotypic expression, which depends on environmental conditions.
- The characteristics of biofilm formation in clinical isolates of *S. aureus* were characterized under conditions mimicking the host environment, showing that medium enrichment with glucose, sodium chloride, and human blood plasma significantly enhances biofilm formation.

- Based on whole-genome sequencing of three newly isolated LAB strains with antistaphylococcal activity, their capacity to synthesize antimicrobial peptides and the absence of genetic determinants compromising their safety were proven.

Contributions of an Applied Nature:

- An updated local database on antimicrobial susceptibility and genetic resistance determinants of a local upper respiratory tract *S. aureus* population was created, applicable for optimizing empirical therapy and decolonization strategies.
- A comprehensive model combining phenotypic, biochemical, molecular, and whole-genome approaches for routine and extended characterization of clinical *S. aureus* isolates in a microbiological laboratory was developed.
- For the first time, the applicability of whole-genome sequencing using the Oxford Nanopore Technologies platform for diagnostic and epidemiological purposes was proven.
- The established modification of *S. aureus* growth and biofilm formation upon treatment with cell-free LAB supernatant provides a prerequisite for their practical application in therapeutic treatment.

8. Assessment of Publications on the Dissertation

Doctoral student Lena Ilieva has presented 2 scientific publications in prestigious journals, referred and indexed in world-renowned scientific databases (WoS and Scopus), one of which is in a Q1 journal (*International Journal of Molecular Sciences*) and the other in Q2 (*Microorganisms*), with a total IF = 10.3 and SJR = 2.393, which fulfills and exceeds the minimum national requirements for the educational and scientific degree "Doctor". The publications present results of research included in the dissertation work. The doctoral student is the lead author in one of the publications, demonstrating her primary role in conducting the experimental part. The publications have received 12 citations for one and 2 citations for the other to date, which is a positive assessment of their scientific significance.

9. Personal Contribution of the Doctoral Student

Given the position the doctoral student occupies in the authorship team of the presented articles related to the dissertation, as well as the indication of each co-author's contribution in them, I consider that Lena Ilieva has played a leading role in conducting the experimental part of the dissertation work and in preparing the publications themselves. Given her professional qualifications in both methodological and theoretical terms, I consider the formulated contributions and obtained results to be her personal credit, without diminishing the role of her scientific supervisor and the members of the team working on the topic.

10. Author's Abstract

The abstract has been prepared according to the requirements of the Regulations for the Application of the Act on the Development of Academic Staff in the Republic of Bulgaria and those of Plovdiv University, reflecting the main results contained in the dissertation thesis and their contributing nature.

11. Critical Remarks and Recommendations

I have no critical remarks and recommendations regarding the doctoral student or the dissertation.

12. Questions

- Are there any other scientific reports, besides yours, about the presence of genetic determinants from other lactobacilli simultaneously for plantaricin, pediocin and enterolysin A?
- Since you investigated the probiotic properties of LAB showing antistaphylococcal activity, do you have results from *in vitro* analyses of their antibiotic resistance?
- How is the presence of genetic determinants for antibiotic resistance, but the absence of phenotypic expression, and vice versa, explained?

- Do you consider it realistic for the identification of *S. aureus* in prehospital practice by phenotypic methods to be confirmed by routine molecular biological methods and which ones do you think are most appropriate?
- Do you know whether there are postbiotics based on lactic acid bacteria implemented in practice for the treatment of nasopharyngeal infections or upper respiratory tract infections, in particular those caused by *S. aureus*, and if so, in what form are they administered?

13. Personal Impressions

I do not know the doctoral student personally, but my overall impression of her, although obtained indirectly, is very positive and based on the high quality of the presented dissertation thesis, the immense experimental work, the presentation of an impressive volume of results in appropriately selected ways, their discussion, and the very good scientific style in which this work is written.

CONCLUSION

The dissertation thesis contains scientific and applied scientific results that constitute an original contribution to the respective scientific field and meet all the requirements of the Act on the Development of Academic Staff in the Republic of Bulgaria, the Regulations for its Application, and the relevant Regulations of Plovdiv University "Paisii Hilendarski".

The dissertation demonstrates that doctoral student Lena Ilieva possesses deep theoretical knowledge and scientific-methodological skills in the scientific specialty of Microbiology, demonstrating qualities for independently conducting scientific research and formatting it into a scientific work, along with very good presentation and discussion of the obtained results.

In light of the above, I convincingly give my positive evaluation of the conducted research presented by the above-reviewed dissertation, abstract, achieved results, and contributions, and propose to the honorable Scientific Jury to award the educational and scientific degree "Doctor" to Asst. Lena Rumenova Ilieva in Higher Education Area 4. Natural Sciences, Mathematics and Informatics, Professional Field 4.3. Biological Sciences, Doctoral Program Microbiology.

August 10, 2026

Reviewer: .....
(Prof. Dr. Penka Moncheva)