PLOVDIV UNIVRESTITY
"PAISII HILENDARSKI"

FACULTY OF BIOLOGY

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ANTIMICROBIAL SUSCEPTIBILITY AND COLONISATION AND INVASIVENESS FACTORS IN NASOPHARYNGEAL ISOLATES OF *STAPHYLOCOCCUS* SPP.

ABSTRACT

FOR THE ACQUISITION OF THE EDUCATIONAL AND SCIENTIFIC DEGREE "DOCTOR"

Field of Higher Education: 4. Natural Sciences, Mathematics and Informatics, Professional Field: 4.3 Biological Sciences
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Scientific Supervisor:

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The full volume of the dissertation is 207 pages. The PhD thesis includes 33 figures, 10 tables and 8 supplements. The list of references contains 377 titles.

The PhD thesis was discussed and directed for defence at a meeting of the Department of “Biochemistry and Microbiology”, Faculty of Biology, Plovdiv University “Paisii Hilendarski”, which was held on 07.05.2026, with protocol № 217/ 07.05.2026 г.

The public defence of the doctoral dissertation will take place on …… at …… in …… before a scientific jury appointed by Order No. ……/…… of the Rector of Paisii Hilendarski University of Plovdiv.

The materials related to the defence are available at the Department of Biochemistry and Microbiology, Faculty of Biology, Paisii Hilendarski University of Plovdiv, and are published on the university's website.

Scientific jury:

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2. Prof. Mariya Bogomilova Dyankova, DSc
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INTRODUCTION

Upper respiratory tract infections are among the most common reasons for microbiological testing and antibiotic treatment in outpatient practice. Among the isolated microorganisms, members of the genus *Staphylococcus* occupy a special place. They may form part of the normal microbiota, but at the same time include species with marked opportunistic and pathogenic potential. *Staphylococcus aureus* is of the greatest significance, combining the ability for asymptomatic colonisation of the nasal cavity and nasopharynx with the potential to cause local, recurrent and invasive infections.

The clinical significance of the species is determined by the combination of drug resistance, the ability to persist on mucosal surfaces, and the presence of factors that facilitate colonisation and invasive behaviour. Antimicrobial resistance is of particular importance, as the spread of methicillin-resistant and multidrug-resistant strains limits the options for effective therapy and increases the risk of failure to control the infectious process.

Alongside drug resistance, mechanisms that allow *S. aureus* to adhere to epithelial surfaces, evade the local immune response and form biofilms play a significant role. This facilitates the prolonged retention of bacterial cells, reduces access to antimicrobial agents and creates conditions for chronicity and recurrence. Therefore, the evaluation of nasopharyngeal isolates should encompass not only the antibiotic susceptibility profile but also factors related to colonisation, invasiveness and biofilm formation.

The limited options for controlling resistant and biofilm-forming staphylococcal populations have sparked interest in complementary biological approaches. In this context, lactic acid bacteria and their cell-free metabolic products represent a promising source of substances with potential anti-staphylococcal activity. Their evaluation is particularly relevant when aimed not only at suppressing planktonic growth but also at limiting the ability of *S. aureus* to form biofilms. Based on these premises, this doctoral thesis focuses on investigating the drug susceptibility and factors of colonisation and invasiveness in nasopharyngeal isolates of *Staphylococcus* spp., with a focus on *S. aureus*, as well as to assess the potential for influencing growth and biofilm formation through biologically active products of lactic acid bacteria.

AIM AND OBJECTIVES

The aim of this thesis is to assess the role and significance of *Staphylococcus aureus* in the aetiological structure of symptomatic upper respiratory tract infections and to characterise its pathobiological potential in relation to the possibilities for limiting its development.

To achieve this aim, the following objectives have been set:

1. To analyse the involvement of the genus *Staphylococcus* in the aetiological structure of symptomatic upper respiratory tract infections in patients from the city of Plovdiv.
2. To determine the antibiotic susceptibility profile and the main patterns of resistance of clinical isolates of *Staphylococcus aureus*.
3. To perform a genetic characterisation of the virulence potential of clinical isolates of *Staphylococcus aureus.*
4. To assess the biofilm-forming capacity of clinical isolates of *Staphylococcus aureus* in suitable in vitro models.
5. To investigate *in vitro* the potential for limiting the growth of *Staphylococcus aureus* and inhibiting biofilm formation using biologically active substances with potential for use as anti-staphylococcal agents.

MATERIALS AND METHODS

I. Materials

1. **Location and period of the study**. The study was conducted at Independent Medical Diagnostic Laboratory (IMDL) "Synevo-Bulgaria" Ltd, Plovdiv, between 2016 and 2020. The clinical isolates included in the thesis were provided for scientific work on the basis of a cooperation agreement with SMDL "Synevo-Bulgaria" EOOD.

2. **Clinical material.** The study included a total of 8,267 nasal and throat swabs from individuals with upper respiratory tract infections who were admitted for routine microbiological testing. The collection, storage and transport of the clinical material were carried out in accordance with the Guidelines for the Collection, Storage and Transport of Materials for Clinical Microbiological Examinations and Regulation No. 4 of 25 January 2010 on the Approval of the Medical Standard 'Microbiology'. Throat swabs were collected using a sterile dry swab from the tonsils and lateral palatal arches, and nasal swabs were collected using a sterile dry swab with a rotating motion in the nasal passage. Upon arrival at the laboratory, the samples were inoculated onto blood agar, EMB Levine agar and Candida ChromAgar and incubated at 35 ± 2°C for 24 hours.

3. **Bacterial strains**

3.1. **Genus *Staphylococcus.*** The strains identified as representatives of the genus *Staphylococcus* were deposited in a collection at the Department of Biochemistry and Microbiology, Faculty of Biology, Plovdiv University 'Paisii Hilendarski'.

For long-term storage, the strains were suspended in 10% skimmed milk and stored at - 80°C. Prior to experimental work, they were revived on a Tryptic Soy Broth medium.

3.2. **Lactic acid bacteria.** The study included strains of lactic acid bacteria isolated from fermented foods and lactic acid products. Following initial screening for anti- staphylococcal activity, three strains were selected for further analysis: *Lactiplantibacillus plantarum* IZITR_2*4*, *Lactiplantibacillus paraplantarum* IZITR_13 and *Levilactobacillus brevis* DPL5.

II. Methods

1. **Analysis of the aetiological profile.** The aetiological profile of nasal and throat isolates was analysed based on the results of routine microbiological diagnostics for the period 2016–2020. For the purposes of this abstract, summary data on the main bacterial species are presented, with a focus on the prevalence of *S. aureus* among upper respiratory tract isolates.

2. **Determination of antibiotic susceptibility.** Antibiotic susceptibility was determined in 450 nasopharyngeal *S. aureus* isolates using the disc diffusion method on Mueller–Hinton agar. Interpretation was carried out in accordance with EUCAST criteria (2019). Resistance to 17 antimicrobial agents representing major therapeutic classes was analysed. The MAR index was calculated and the distribution of isolates according to the number of antimicrobial classes to which resistance was found was determined.

3. **Whole-genome sequencing and *in silico* analysis**. Ten nasopharyngeal isolates *of S. aureus*, including MRSA and MSSA strains, were selected for whole- genome sequencing. Libraries were prepared using the Ligation Sequencing Kit SQK-RBK114, and sequencing was performed on a MinION platform with an R10 flow cell. Raw data processing was performed using Guppy v6.5.7, adapter sequences were removed using Porechop v0.2.4, and genome assembly was performed de novo using Flye v2.9.2. The sequences were corrected using Racon v1.4.21 and Medaka v1.8.1, and the quality of the assembly was assessed using CheckM v1.1.6. The genome sequences of the studied *S. aureus* strains have been deposited in GenBank under BioProject PRJNA990680. The obtained genomic data were used for *in silico* analysis of the resistome and virulome via ABRicate, utilising the CARD and MEGARes DB databases for antibiotic resistance and the VFDB for virulence-associated genes; additional functional annotation was performed using RAST, KEGG and BlastKOALA.

4. **PCR analysis of virulence-associated genes.** PCR analysis was performed on 40 *S. aureus* strains from the obtained collection to determine the frequency of selected genes associated with the virulence and colonisation potential of the species.The panel included 20 target genes, grouped into major functional categories: adhesion and colonisation (fnbA, fnbB, clfB, cna, fib, ebps), biofilm formation (icaA, icaB, icaD), surface adaptation and persistence (isdA, sdrC, sdrD, tarS), cytotoxicity (hla, hlgA, psmβ1) and toxigenicity (tsst-1, sea, seb, sec). The amplification products were analysed by agarose gel electrophoresis, and the

frequency of occurrence of each target gene is presented as the number and percentage of positive isolates.

5. **Assessment of biofilm-forming capacity.** The assessment was carried out using a microtitre assay with crystal violet. The isolates were cultured in 96-well microtitre plates under various conditions: TSB, TSB with 2% glucose and 2% NaCl (TSBGS), and TSBGS with added human blood plasma. Quantitative assessment was performed by measuring optical density following staining with crystal violet. The classification of isolates as non-biofilm-forming, weak, moderate and strong biofilm-forming was carried out according to Stepanović et al. (2007). The effect of plasma was assessed at concentrations of 1%, 3% and 5%.

6. **Screening for antagonistic activity of lactic acid bacteria.** The antagonistic activity of lactic acid bacteria strains against *S. aureus* was assessed using the agar block diffusion method. Agar blocks containing 24-hour cultures of LAB were placed on a surface culture of *S. aureus*, after which the diameter of the inhibition zones was measured. Based on the results, the three strains used to obtain cell-free supernatants were selected.

7. **Genomic and *in vitro* characterisation of the selected MCPs.** Genomic characterisation was performed on the selected MCP strains, aimed at confirming species identity, assessing safety, and determining genetic potential for the production of antimicrobial peptides and other bioactive compounds. The genomic sequences of the strains have been deposited in GenBank under BioProject PRJNA1113526 and PRJNA1075351. *In vitro* assessment of probiotic potential includes tests for acid tolerance, resistance to bile salts, autoaggregation and coaggregation with test pathogenic microorganisms.

8. **Preparation of lyophilised cell-free supernatant (LCFS)**. The selected MKB strains were cultured in 200 mL MRS broth for 48 h under anaerobic conditions. After cultivation, the cells were separated by centrifugation at 10,000 rpm for 20 min at 4°C. The resulting cell-free supernatant was filtered through a 0.22 μm nitrocellulose membrane, frozen at –80°C and lyophilised in a Labconco FreeZone

4.5 system. After lyophilisation, the samples were stored at –20°C and, prior to use, were rehydrated with deionised water to the required concentrations.

9. **Fatty acid analysis**. The fatty acid composition of the cell-free supernatants was determined by gas chromatography following transesterification with 2% H_2SO_4 in CH_3OH at 50°C according to Christie (2003). The resulting methyl esters were purified by thin-layer chromatography on silica gel plates. Gas chromatographic analysis was performed on a Thermo Fisher Scientific TRACE 1610 GC with an FID detector and a TR-FAME column.

10. **Determination of inhibitory concentrations of LBS.** The effect of LBS on biofilm formation and planktonic growth of *S. aureus* was investigated using concentration gradients. The minimum biofilm-inhibiting concentration (MBIC) was determined using a microtiter plate assay with crystal violet. The minimum

inhibitory concentration (MIC) was determined by monitoring planktonic growth via measurement of optical density (OD_{610}). The minimum bactericidal concentration (MBC) was determined by assessing the absence of viable bacterial growth following exposure. Activity was assessed immediately after lyophilisation and after 12 months' storage at 2–8°C.

11. **Purification of the protein fraction of MKB by FPLC, and agar diffusion test.** To track the protein fraction associated with anti-staphylococcal activity, the cell-free supernatants were subjected to preliminary tangential flow filtration using a hollow-fibre filter at a flow rate of 100 mL min^{-1}, to remove particles and components larger than 0.22 μm. After filtration, the pH of the samples was adjusted from 4.0 to 7.0, and the conductivity was reduced to 6–7 mS cm^{-1}. Cation-exchange FPLC separation was performed on a strong cation exchanger Nuvia S via gradient elution, with fractions collected in 2 mL volumes. The resulting fractions were analysed for protein content and used for subsequent determination of the protein profile by SDS-PAGE and for assessment of antimicrobial activity by agar diffusion test against *S. aureus*.

12. **SDS-PAGE.** The samples were denatured, separated on a polyacrylamide gel, stained with Coomassie Brilliant Blue G-250 and visualised using a ChemiDoc system. The antimicrobial activity of the obtained protein fractions against *S. aureus* was assessed by agar diffusion test at a standardised loading of 10 μg protein per well.

13. **Confocal laser scanning microscopy.** The effect of lyophilised cell-free supernatants on biofilm viability and organisation was investigated using confocal laser scanning microscopy. The *S. aureus* 450G strain was used as a model. The biofilm was formed on sterile borosilicate cover glasses in the presence of 25% LBS MPS. Cell viability was assessed using the Live/Dead BacLight Bacterial Viability Kit, and observations were made using a Nikon Eclipse Ti-U confocal laser scanning microscope.

14. **Scanning electron microscopy.** Scanning electron microscopy was performed on a biofilm of *S. aureus* 450G formed on sterile polystyrene fragments in the presence of 25% LBS MPS. After incubation, the biofilms were washed, fixed with glutaraldehyde and osmium tetroxide, dehydrated in a series of ethanol solutions, mounted on metal holders and coated with gold. Observation was performed using a Lyra Tescan scanning electron microscope.

15. **Statistical analysis**. The data were analysed using Statistica v.12 and SPSS Statistics v.26. PROBIT analysis was used to determine the MBPC, MPC and MBC. Statistical significance was set at $\alpha = 0.05$.

RESULTS AND DISCUSSION

1. Etiological profile of nasal and throat isolates

To determine the prevalence of *Staphylococcus aureus* among bacterial pathogens from the upper respiratory tract, the aetiological structure of nasal and throat isolates from outpatients was analysed for the period 2016–2020. Over the five-year period, the highest absolute numbers of *S. aureus* were recorded in nasal samples – a total of 574 isolates, followed by *Streptococcus pneumoniae* with 256 isolates (Figure 1). In throat swabs, the most frequently isolated organisms were *Moraxella catarrhalis* – 211 isolates, *S. aureus* – 184 isolates, and *Streptococcus pyogenes* – 89 isolates. The graphical and percentage distribution of the isolated microorganisms shows that *S. aureus*, whilst in the oropharynx a more diverse aetiological spectrum is observed, in which *S. aureus* retains a significant presence alongside other classic pathogens of the upper respiratory tract.

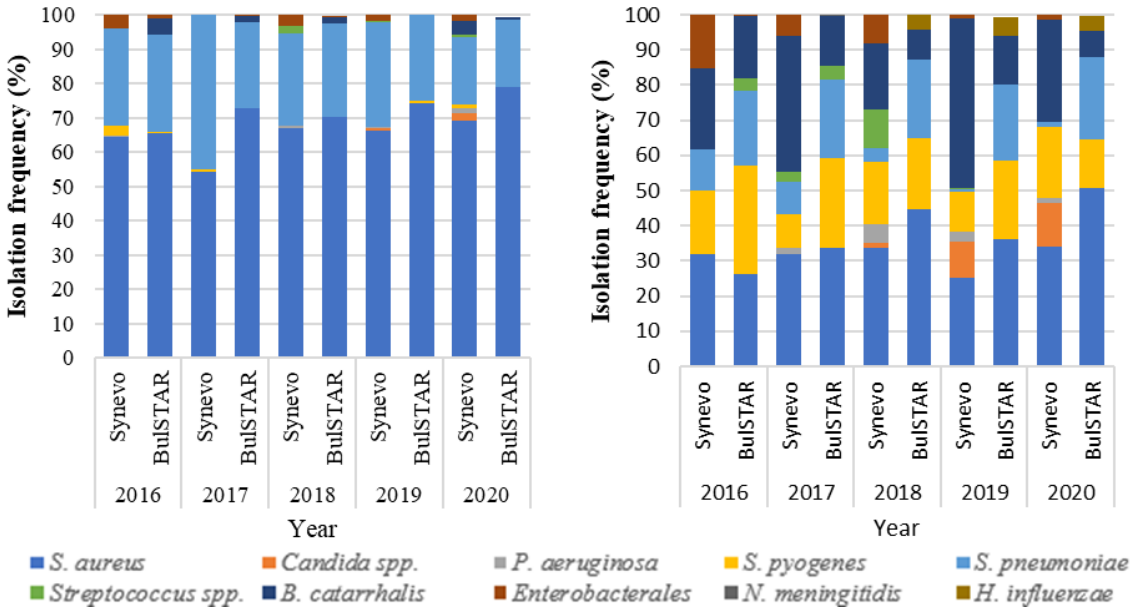

Figure 1: Comparison of the aetiological profile established by the SMLC 'Synevo-Bulgaria', Plovdiv, and data obtained via BulSTAR for the period 2016–2020: a) nasal isolates; b) throat isolates.

The different distribution of *S. aureus* in the two anatomical sites is consistent with the established understanding that the nasal cavity is a primary reservoir for carriage of the species, whilst the oropharynx represents a more dynamic and polymicrobial niche. In this sense, the high prevalence of *S. aureus*, particularly among nasal isolates, justifies the subsequent focus on the species' antimicrobial susceptibility, resistance profile, factors of colonisation and invasiveness, and biofilm-forming capacity.

Antibiotic resistance in *S. aureus* has direct clinical significance, as therapeutic choice in outpatient practice is often based on an empirical approach, and resistance in this species shows significant regional and temporal variations. Therefore, local monitoring of the phenotypic susceptibility profile is necessary both to assess the current resistance situation and to evaluate the reliability of commonly used therapeutic regimens.

2. Antibiotic resistance of nasopharyngeal *Staphylococcus aureus* isolates

Antibiotic resistance in *S. aureus* has direct clinical significance, as therapeutic choice in outpatient practice is often based on an empirical approach, and resistance in this species shows significant regional and temporal variations. Therefore, the investigation of the phenotypic and genotypic resistance profiles of community-acquired nasopharyngeal isolates is necessary to assess the local resistance situation and to interpret therapeutic risk more accurately.

2.1. In vitro analysis of antibiotic resistance

An analysis was conducted of the antimicrobial resistance of a representative sample of 450 nasopharyngeal *S. aureus* isolates from the established collection against 17 antimicrobial agents selected in accordance with EUCAST (2019). The results obtained indicate a predominance of resistance to β-lactam antibiotics, moderate but clinically significant levels of resistance to macrolides, lincosamides, fluoroquinolones and aminoglycosides, as well as preserved activity of antimicrobial agents representing last-resort therapeutic options, including oxazolidinones, amphenicols and glycopeptides (Figure 2).

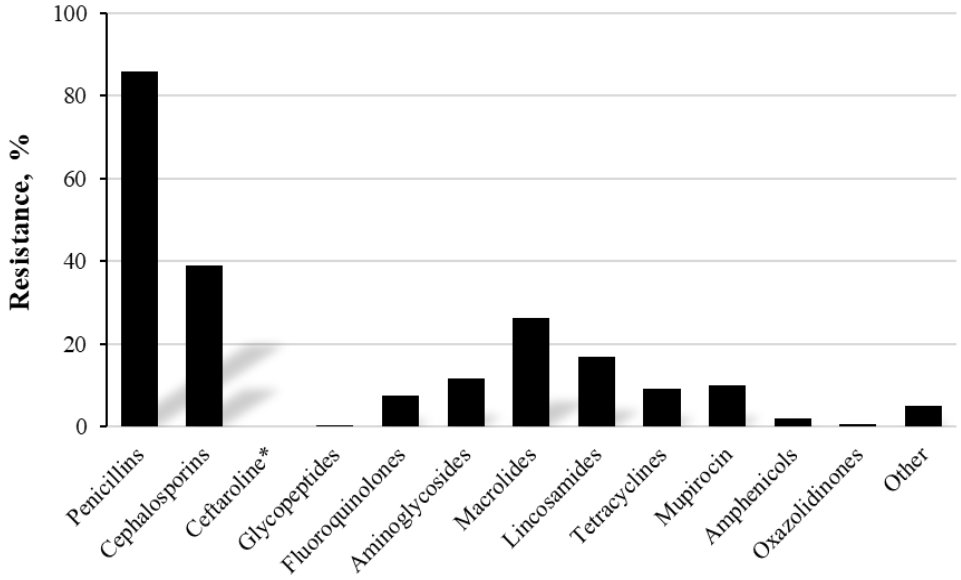

Figure 2. Distribution of resistance in nasal and throat isolates *of Staphylococcus aureus* by antibiotic class.

Of particular significance is the high proportion of methicillin resistance, assessed by cefoxitin, which reaches 38.88%. This figure is significantly higher than the national BulSTAR data for the same period and the European average, indicating a stronger presence of the MRSA phenotype in the nasopharyngeal population studied (BulSTAR, 2021; ECDC, 2024). In addition, moderate levels of resistance to macrolides and lincosamides were observed, at 20.14% and 15.97% respectively, as well as to aminoglycosides – 11.75%. Resistance to fluoroquinolones remains below 10% for individual strains, but retains clinical significance due to the potential for selection of resistant variants in *S. aureus*.

It is particularly important that the phenotypic profile is not limited to single resistance to individual antibiotics, but includes the accumulation of resistance to more than one class of antimicrobials. The distribution of isolates according to the number of classes to which resistance was detected shows a predominance of strains with limited to moderate accumulation of resistance, but at the same time a distinct presence of an MDR component (Figure 3). The mean MAR index value is 0.166 ± 0.151, indicating that the nasopharyngeal population includes not only single-drug-resistant variants but also strains with accumulated class resistance.

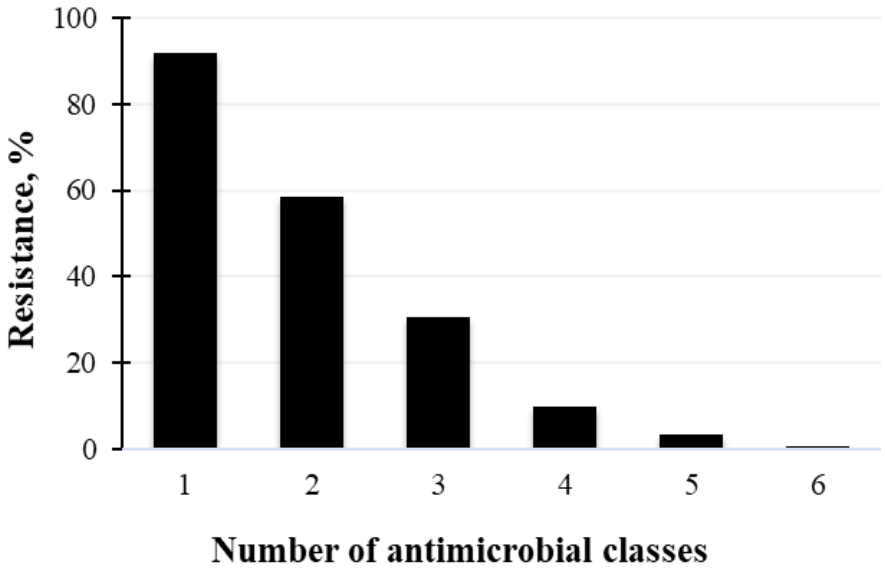

Figure 3. Percentage distribution of nasopharyngeal *Staphylococcus aureus* isolates according to the number of antibiotic classes to which resistance was detected.

Applying the definition by Magiorakos et al. (2012), isolates resistant to three or more antimicrobial classes can be considered MDR phenotypes. In this sense, the observed multidrug resistance increases the clinical significance of the nasopharyngeal isolates studied and identifies them as a potential reservoir of determinants associated with antimicrobial resistance (Magiorakos et al., 2012).

Despite the unfavourable profile of β-lactam and MRSA-associated resistance, some of the therapeutically significant antimicrobials retain good activity. Low levels of resistance were found to chloramphenicol and co-trimoxazole, whilst oxazolidinones and glycopeptides retained their activity against almost all isolates. This indicates that the antibiotic profile of the studied population is unfavourable mainly with regard to β-lactam and multiple resistance, but is not uniformly compromised against all major anti-staphylococcal agents.

2.2. In silico analysis of the resistome in Staphylococcus aureus

The results presented on Figure 4 allows us to track both the overall resistance profile of the sequenced isolates and the differences between MRSA and MSSA strains. The resulting profile shows that the resistance profile comprises several main groups of determinants — genes associated with β-lactam resistance, efflux-related mechanisms, regulatory elements and determinants with a broader

significance for antimicrobial tolerance. This allows both the general resistance background and the differences between MRSA and MSSA isolates to be traced.

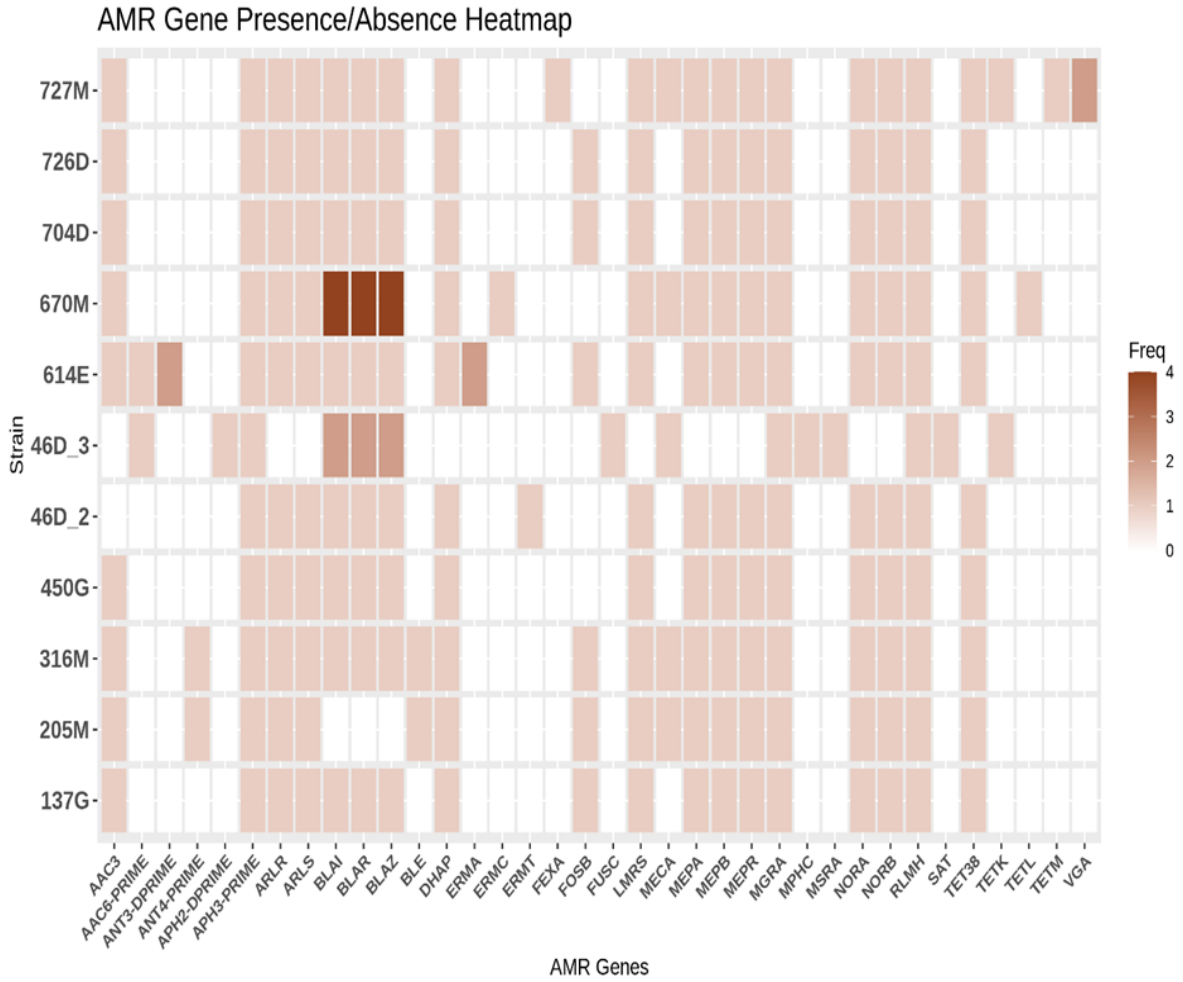

Figure 4: Heatmap of the identified antibiotic resistance genes in the 10 sequenced *Staphylococcus aureus* strains.

The resulting profile shows that the resistance profile of the isolates studied comprises several main groups of determinants – genes associated with β-lactam resistance, efflux-related mechanisms, regulatory elements, and determinants with broader significance for tolerance to antimicrobial agents. This organisation corresponds to current genomic data on *S. aureus*, according to which some of the resistance determinants belong to the core chromosomal background, whilst others are acquired via mobile genetic elements and reflect more dynamic processes of selection and horizontal transfer (Contarin et al., 2024).

Among the most significant findings is the detection of mecA in five of the ten analysed strains, which corresponds to their preliminary distribution into the MRSA and MSSA groups. The biological significance of mecA is linked to the encoding of PBP2a, which has low affinity for β-lactam antibiotics; consequently, the gene remains a key diagnostic marker for methicillin resistance in *S. aureus* (Lade et al., 2023). Concurrently, the β-lactamase module blaI/blaR/blaZ was detected in nine of the ten strains, indicating an almost universal prevalence of the

genetic potential for penicillinase-mediated resistance.

Comparison with phenotypic results shows the highest concordance precisely for the main β-lactam markers. All mecA-positive isolates are phenotypically resistant to cefoxitin, whilst mecA-negative isolates remain susceptible to this antibiotic. Similarly, the presence of blaZ corresponds to the widely established resistance to penicillin and ampicillin. This indicates that, for the main acquired mechanisms of β-lactam resistance, genomic data provide a reliable basis for interpreting the phenotypic profile.

The interpretation of efflux-related and regulatory determinants is more complex. The presence of genes such as norA, norB, mepA, lmrS, mgrA, arlRS and tet38 indicates a genetic potential for involvement in reduced susceptibility to various antimicrobial agents, but this alone is not sufficient to confer a clinically significant resistant phenotype. In these mechanisms, phenotypic expression depends on the level of expression, the genetic background, the presence of concomitant mutations, and environmental conditions. Therefore, they should be interpreted not as standalone markers of resistance, but as part of the strain's broader adaptive potential (Gagetti et al., 2023; Hu et al., 2024).

The overall picture shows that WGS analysis expands the information on resistance by enabling the simultaneous detection of key acquired genes, regulatory elements and potential efflux-related mechanisms. At the same time, the data obtained confirm the need to interpret resistance in conjunction with phenotypic testing for antibiotic susceptibility. In the nasopharyngeal isolates studied, the relationship between genotype and phenotype is most direct for mecA and blaZ, whilst for efflux and regulatory determinants it is graded and functionally dependent.

3. Genetic characterisation of the virulence potential of nasopharyngeal *Staphylococcus aureus* isolates

3.1. In silico analysis of the overall virulence potential in Staphylococcus aureus

Based on genomic data from the ten sequenced nasopharyngeal *S. aureus* isolates, an analysis was performed of the genes associated with the pathogenesis, colonisation and invasive behaviour of the species. A total of 74 genes were identified, with individual strains carrying between 55 and 65 determinants. The richest gene set was found in strains 670M and 704D, and the most limited in 727M and 450G. The summary profile of virulence-associated genes is presented in Figure 5.

The sequenced isolates do not form a completely homogeneous group, but rather a population with a common conservative background and a variable superstructure of varying extent. The most consistently represented determinants are those associated with nasopharyngeal adaptation, primary attachment, stabilisation of the biofilm structure, iron uptake and interaction with the immune system. This group includes genes for surface adhesins, components of the ica

operon, the Isd system, factors for interaction with complement and immunoglobulins, as well as major cytolytic proteins. The distribution of toxic, secretory and motility-related determinants is more variable, which corresponds to the organisation of the virulome characteristic of *S. aureus* – a conserved core of genes for colonisation and basic invasiveness, combined with more variable additional factors (Hait et al., 2021; Zhou et al., 2021).

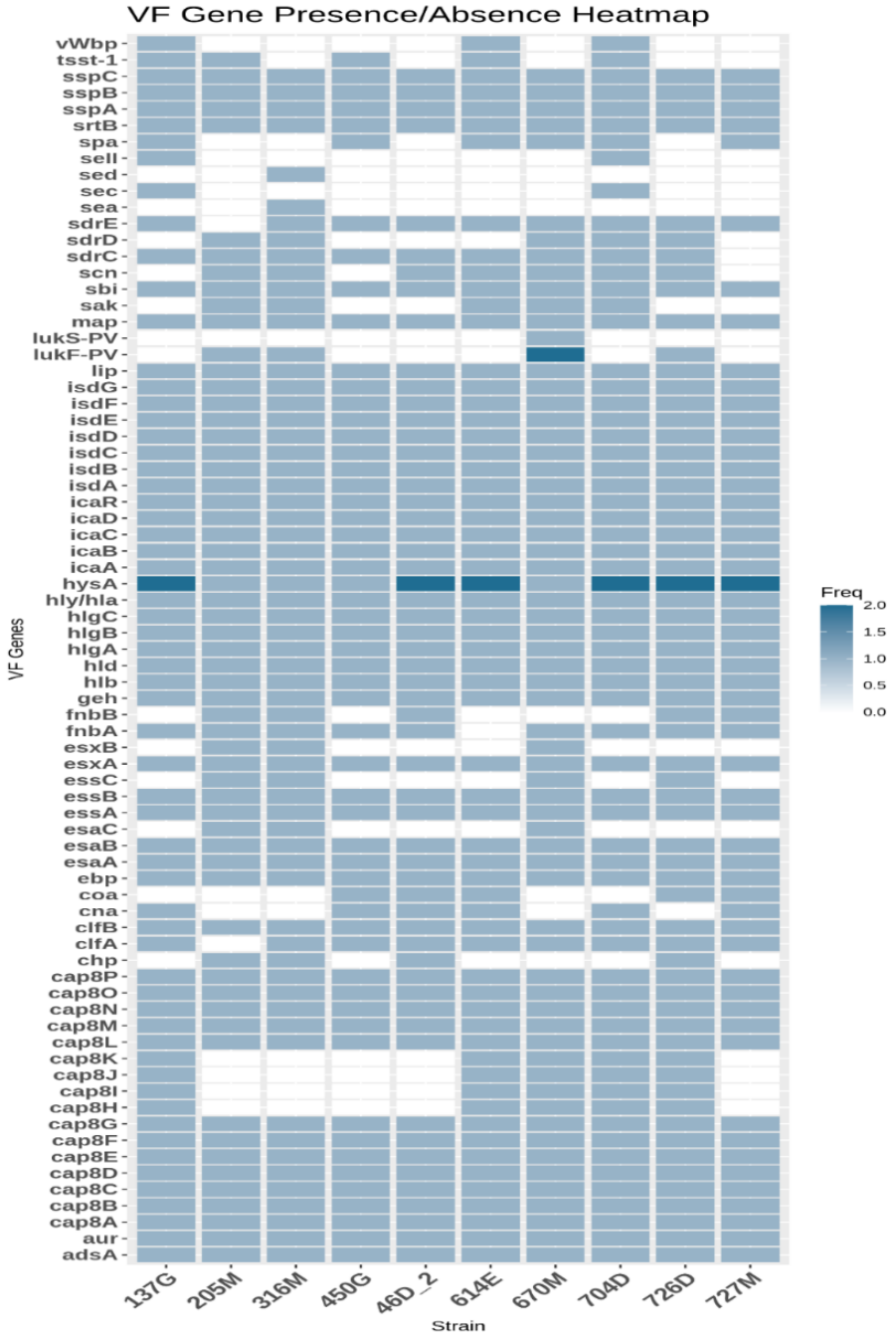

Figure 5. Profile of virulence-associated genes in 10 sequenced nasopharyngeal *Staphylococcus aureus* isolates.

The adhesion profile observed is similar for MRSA and MSSA strains. This suggests that the differences between the two types are more clearly distinguished by resistance genes than by the overall set of core adhesion and colonisation

factors. This finding is significant for the interpretation of nasopharyngeal MRSA isolates, as methicillin resistance is not accompanied by a reduction in basic colonisation potential. A similar relationship has been described in other genomic studies, in which MRSA and MSSA often share a significant portion of the adhesion and immunomodulatory repertoire, whilst the main differences are concentrated in the resistance determinants and mobile genetic elements (Zhou et al., 2021).

The most conserved in the studied series is the block of genes associated with the establishment and maintenance of colonisation in the nasopharyngeal niche. The universal presence of clfB, icaABCDR, isdA–isdG, adsA, sbi and the major haemolysins identifies strains genetically adapted not only for transient presence but also for prolonged persistence in the nasopharyngeal and oropharyngeal systems. This is consistent with the understanding that *S. aureus* occupies an intermediate position between a commensal and an opportunistic pathogen, with nasopharyngeal colonisation potentially serving as a starting point for subsequent localised or invasive disease (Sakr et al., 2018).

The genomic profile indicates that nasopharyngeal isolates harbour both determinants necessary for mucosal persistence and a set of genes which, under suitable conditions, may facilitate tissue damage and more invasive behaviour. In this sense, the virulome of the studied strains should not be interpreted merely as a catalogue of available genes, but as a functionally layered system in which the conservative colonisation core is combined with variable toxic and secretory elements. These data reflect genetic potential rather than directly proven expression or phenotypic activity of the relevant determinants; therefore, the distribution of selected genes was also investigated in a broader group of clinical isolates.

3.2. In vitro analysis of virulence potential in Staphylococcus aureus

To experimentally validate the data from the *in silico* analysis, a PCR panel has been developed, comprising genes involved in various stages of the infectious process: initial attachment and colonisation (fnbA, fnbB, clfB, cna, fib, ebps), biofilm formation (icaA, icaB, icaD), adaptation to the oral environment and interaction with the host (isdA, sdrC, sdrD, tarS), cytotoxic potential and tissue damage (hla, hlgA, psmβ1), as well as toxigenicity (tsst-1, sea, seb, sec). This screening allows not only the presence of individual virulence-associated genes to be assessed, but also their distribution across a broader collection of strains, so as to determine the extent to which the profile outlined by WGS is confirmed in an expanded set of isolates (O'Brien et al., 2002; Brown et al., 2012; Mulcahy et al., 2012; Tam and Torres, 2019).

PCR analysis was performed on 40 *S. aureus* strains from the collection. Initially, multiplex PCR was used to simultaneously detect the species-specific marker Staur, the methicillin resistance gene mecA, and the tsst-1 gene. A 1250 bp fragment was detected in all strains, resulting from the amplification of a region of the 23S rRNA gene using the *S. aureus*-specific primer pair Staur4/Staur6

(Straub et al., 1999). This confirms the species affiliation of the isolates studied and provides a reliable basis for the subsequent analysis of the target genes.

The frequency of occurrence of individual genes in the study group is summarised in Table 1, and the profiles obtained for the 20 target genes are presented in Figure 6. The results show a widespread distribution of genes associated with adhesion, biofilm formation and surface adaptation. In the group of adhesion and colonisation determinants, clfB was detected in all isolates, fnbA in 77.5%, cna and ebps in 62.5%, and fnbB and fib in 42.5%. This profile indicates that the studied strains possess a set of determinants directed towards interaction with epithelial structures, components of the extracellular matrix and plasma proteins.

Genes associated with biofilm formation are also present at a high frequency. icaA was detected in all strains, whilst icaB and icaD were found in 92.5%. This indicates a widespread genetic potential for the synthesis of polysaccharide intercellular adhesins and for stabilising the biofilm structure. In the context of nasopharyngeal isolates, this result is of particular significance, as biofilm formation may facilitate persistence on mucosal surfaces and hinder the elimination of the bacterial population.

Among the factors for surface adaptation and persistence, isdA and tarS are found in all isolates, whilst sdrC and sdrD are detected in 72.5% and 57.5% of isolates, respectively. This group of genes complements the profile of strains adapted to a mucosal environment, in which successful persistence depends on the combination of attachment, surface modification, utilisation of limited nutritional resources, and interaction with local defence mechanisms.

Table 1: Frequency of occurrence of target genes in 40 *Staphylococcus aureus* isolates

Functional group	Gene	n (%)	Gene	n (%)
Adhesion and colonization	*fnbA*	31 (77.5%)	*fnbB*	17 (42.5%)
	clfB	40 (100%)	*cna*	25(62.5%)
	fib	17 (42.5%)	*ebps*	25(62.5%)
Biofilm	*icaA*	40 (100%)	*icaB*	37 (92.5%)
	icaD	37 (92.5%)		
Surface Adaptation and Persistence	*isdA*	40 (100%)	*sdrC*	29 (72.5%)
	sdrD	23 (57.5%)	*tarS*	40 (100%)
Regulation and Cytotoxicity	*hla*	40 (100%)	*hlgA*	40 (100%)
	psmβ1	40 (100%)		
Superantigens and Enterotoxins	*tsst-1*	12 (30%)	*sea*	9 (22.5%)
	seb	6(15%)	*sec*	40 (100%)

The *hla*, *hlgA* and *psmβ1* genes were detected in all isolates, indicating almost complete preservation of determinants associated with cytotoxic potential. This result requires cautious interpretation. PCR analysis of genomic DNA confirms the presence of the relevant genes, but does not provide information on the extent of their expression under conditions of mucosal colonisation. This is of particular significance in *S. aureus*, as the production of haemolysins and other secreted effector molecules is determined by a complex regulatory network and by the specific conditions in the host (Cheung et al., 2011; Oogai et al., 2011; Jenul and Horswill, 2019). Nevertheless, the universal presence of these genes indicates that even isolates adapted to mucosal colonisation retain the potential for tissue damage under suitable conditions.

The distribution of superantigens and enterotoxins is more variable. The *tsst-1* gene was detected in 30% of the strains studied, sea in 22.5%, seb in 15%, whilst sec was detected in all isolates. This indicates that some of the nasopharyngeal strains carry genetic determinants associated with the production of toxins of potential systemic significance. The presence of tsst-1 in some of the isolates suggests a genetic predisposition for the synthesis of the toxin causing toxic shock syndrome, but cannot be interpreted as evidence of actual toxin production under specific conditions.

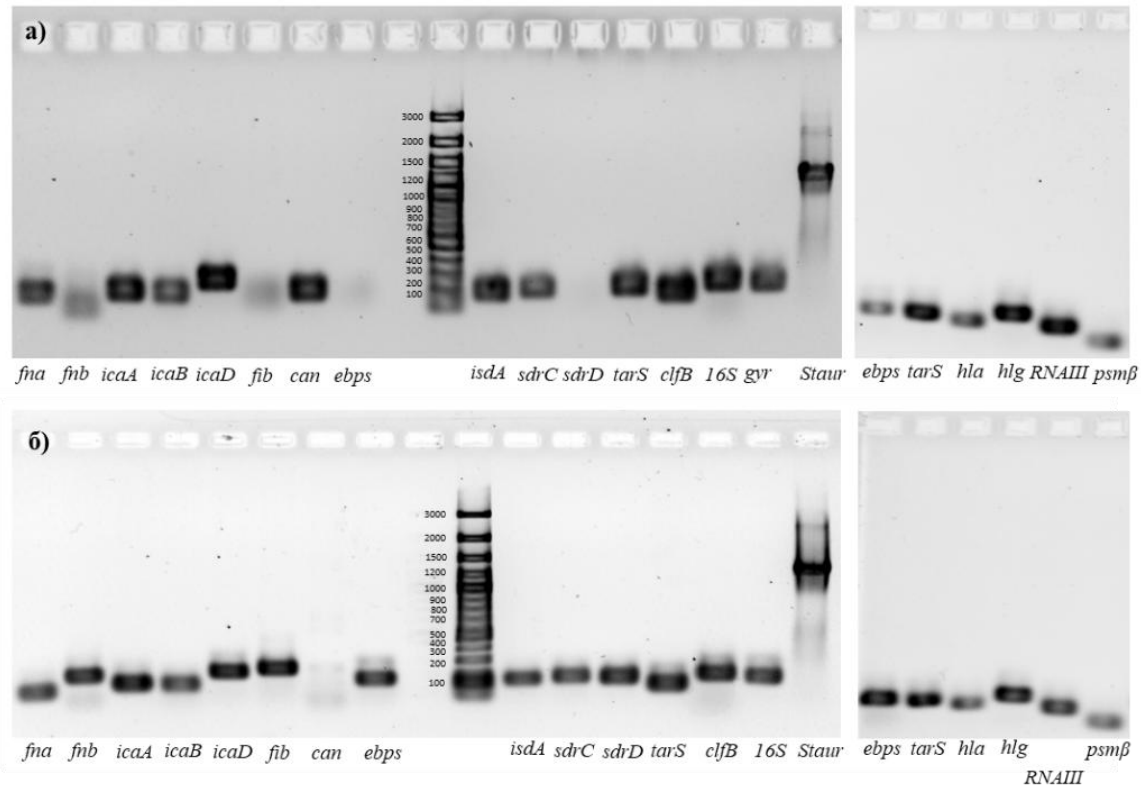

Figure 6: PCR profiles of 20 target genes associated with virulence and colonisation potential in *Staphylococcus aureus* isolates

Comparison of the individual functional groups shows that, in the isolates studied, the block of genes associated with adhesion, biofilm formation and surface adaptation is the most highly conserved. The frequency of *clfB, icaA,*

icaB, icaD, isdA and *tarS* delineates a stably conserved functional core, whilst the frequency of *fnbA, sdrC, sdrD*, *cna* and *ebps* indicates that a variable periphery of additional adhesion determinants is added to it. This organisation is particularly significant for nasal and oropharyngeal isolates, as in these cases the ability to attach, adhere and persist in the mucosa is likely to be of greater importance than direct tissue invasiveness.

PCR analysis in the expanded group confirms that the main trends identified by WGS are replicated in a broader clinical sample. Outpatient nasal and oropharyngeal *S. aureus* isolates combine a conserved set of genes supporting adhesion, retention and biofilm formation with a more variable distribution of superantigenic and enterotoxin determinants. The results thus obtained indicate that the studied population is genetically adapted for mucosal persistence, with some strains also exhibiting toxin-associated potential.

4. Biofilm-forming capacity of clinical *Staphylococcus aureus* isolates

The studied collection of nasopharyngeal *S. aureus* isolates exhibits distinct phenotypic heterogeneity with regard to biofilm formation. According to the classification by Stepanović et al. (2007), the strains are divided into four groups: non-biofilm-forming, weak, moderate and strong biofilm-forming isolates. The results indicate that biofilm formation in the studied strains is not a binary trait, but a quantitatively variable phenotype, the expression of which depends both on the characteristics of the individual strain and on environmental conditions (Otto, 2018). To clarify the influence of the culture medium on biofilm-forming potential, a comparative analysis was conducted during cultivation in standard soybean-casein broth (TSB), in TSB medium with 2% glucose and 2% NaCl (TSBGS), and in TSBGS with 1% human blood plasma added (TSBGSP). The results, presented in Figure 7, show minimal biofilm formation in standard TSB medium, whilst the addition of glucose and sodium chloride significantly stimulates the process ($p = 0.0007$). The most pronounced biofilm formation was observed during cultivation in TSBGSP, indicating that the presence of plasma components further enhances the phenotypic expression of this trait.

Under standard conditions, some of the isolates are categorised as weak biofilm formers or non-biofilm formers, but following enrichment of the medium with glucose, NaCl and plasma, a shift towards higher categories of biofilm formation is observed. This indicates that the assessment of biofilm-forming capacity depends significantly on the model used and that the standard culture medium may underestimate the potential of some clinical isolates. This result is consistent with the genetic profile of the isolates, in which widespread ica-related genes and surface adhesins were identified.

To assess the influence of plasma components more precisely, biofilm formation was analysed upon the addition of human blood plasma at concentrations of 1%, 3% and 5%. The results presented in Figure 8 show that all tested concentrations statistically significantly stimulate biofilm formation ($p < 0.01$), with the effect observed even in strains initially categorised as non-biofilm-

forming. The highest values were observed with 3% plasma, whilst biofilm formation was also recorded at 1% and 5%, but with lower intensity.

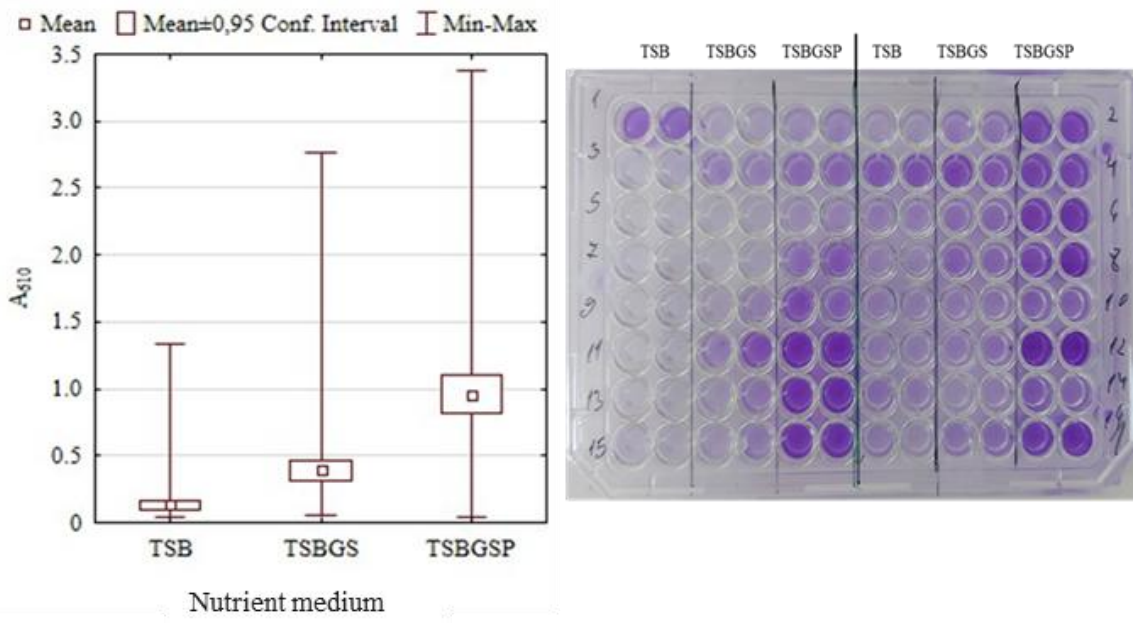

Figure 7. Biofilm formed after 24 hours of cultivation of *Staphylococcus* strains in 96-well microtitre plates. TSB – soybean-casein broth; TSBGS – TSB with 2% glucose and 2% NaCl added; TSBGSP – TSBGS with 1% human blood plasma added.

These results indicate that human plasma acts as a potent stimulator of biofilm formation in nasopharyngeal *S. aureus* isolates, with the most pronounced effect observed at 3%. The presence of an optimal range for plasma-induced stimulation indicates that this effect is not simply the result of general enrichment of the medium, but of a specific interaction between bacterial surface structures and plasma components. In this sense, the model used allows for a more accurate assessment of biofilm-forming potential under conditions close to those of interaction with the host.

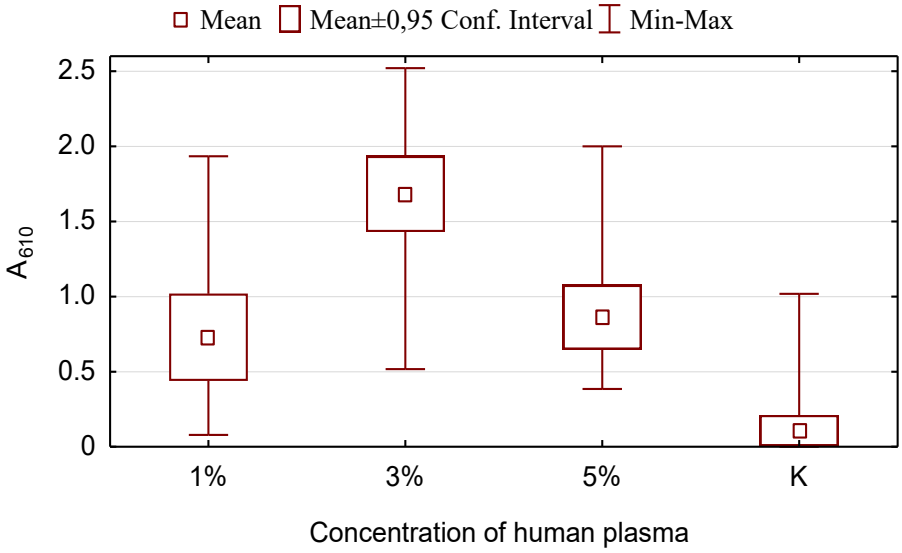

Figure 8. Biofilm formation by different *S. aureus* strains in the presence of varying concentrations of human blood plasma.

5. Lactic acid bacteria as a strategy for controlling *Staphylococcus aureus*

5.1. Screening of lactic acid bacteria for antagonistic activity against *Staphylococcus aureus*

In order to select strains of lactic acid bacteria with potential for anti-staphylococcal application, a preliminary screening of antagonistic activity against clinical isolates *of S. aureus* was carried out. A diffusion method using agar blocks was employed, which allows the assessment of pathogen growth inhibition upon direct interaction with 24-hour cultures of the lactic acid bacteria under investigation.

The results show a clearly expressed strain-specific nature of the antagonistic activity. A wide range in the degree of inhibition was observed between the individual Lactobacillus strains, as well as variability in the sensitivity of the different *S. aureus* isolates. This indicates that the anti-staphylococcal effect cannot be regarded as a general property of all lactic acid bacteria, but depends on the specific strain and the composition of the antimicrobial substances it produces.

The antagonistic activity of the studied strains is presented in Figure 9. The most pronounced and reproducible zones of inhibition were observed for the strains *Lactiplantibacillus plantarum* IZITR_24, *Lactiplantibacillus paraplantarum* IZITR_13 and *Levilactobacillus brevis* DPL5, which justifies their inclusion in subsequent genomic and functional analyses.

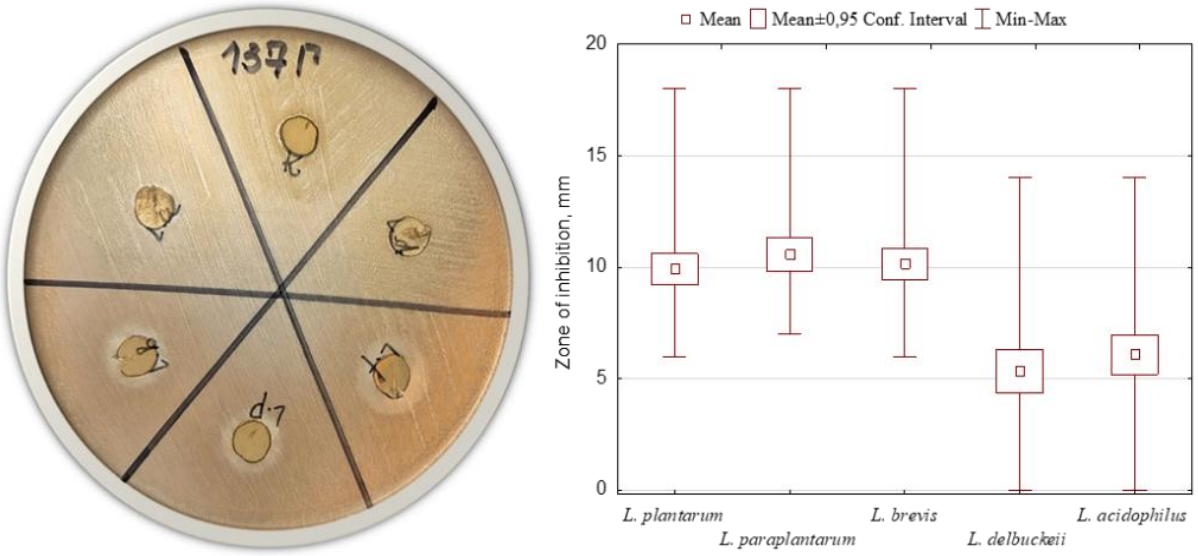

Figure 9. Antagonistic activity of lactic acid bacteria strains against *Staphylococcus aureus*, determined by the plug diffusion test.

The observed variability in the diameter of the inhibition zones should be regarded not only as a characteristic of the MKB antagonists, but also as a reflection of the heterogeneity of the *S. aureus* group under investigation. Such strain specificity is to be expected, as *S. aureus* exhibits significant phenotypic plasticity, including differences in susceptibility to acid stress, in the activity of

defence systems, and in the expression of surface and regulatory mechanisms (Otto, 2014; Piewngam and Otto, 2024).

5.2. Genomic characterisation of lactic acid bacteria

Genomic characterization was performed on the three selected strains, focusing on species identification, safety assessment, and analysis of their genetic potential for the production of antimicrobial peptides and other bioactive compounds. Genomic analysis confirmed that the strains belong to Lactiplantibacillus plantarum IZITR_24, Lactiplantibacillus paraplantarum IZITR_13, and Levilactobacillus brevis DPL5, and revealed the presence of genetic determinants associated with anti-staphylococcal activity.

In the strains from the L. plantarum complex—IZITR_13 and IZITR_24—class II bacteriocin systems typical of this taxon were identified. L. paraplantarum IZITR_13 exhibited the most complex profile, including plnEF and plnJK, genes encoding ABC transporters (Figure 10), a pediocin-encoding cluster, and a gene for enterolysin A (Figure 11).

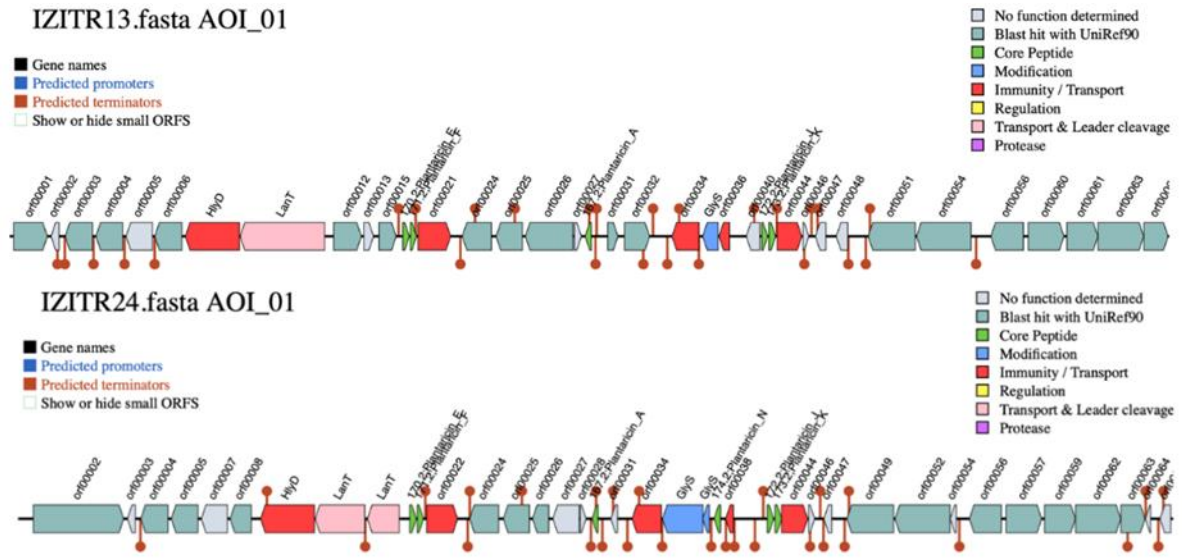

Figure 10. Genomic organisation of the clusters encoding plantarin/class II bacteriocins in *Lactiplantibacillus paraplantarum* IZITR_13 and *Lactiplantibacillus plantarum* IZITR_24.

This indicates antimicrobial potential with various mechanisms of action, including membrane-targeting mechanisms and mechanisms targeting the cell wall. In *L. plantarum* IZITR_24, a similar but more conservative organisation is observed, which corresponds to the classical model of plantaricin-associated bacteriocin systems characteristic of members of the *L. plantarum* group (Perez et al., 2013).

Of particular interest is *L. brevis* DPL5, in which typical plantaricin-like clusters are absent, but genes associated with the synthesis of lantiotic peptides, RiPP-like compounds and a region encoding a type III polyketide synthase are identified (Figure 12). This profile points to alternative antimicrobial potential based on more complex modified peptides and secondary metabolites, which may

be characterised by higher structural stability and a broader spectrum of activity (Alvarez-Sieiro et al., 2016).

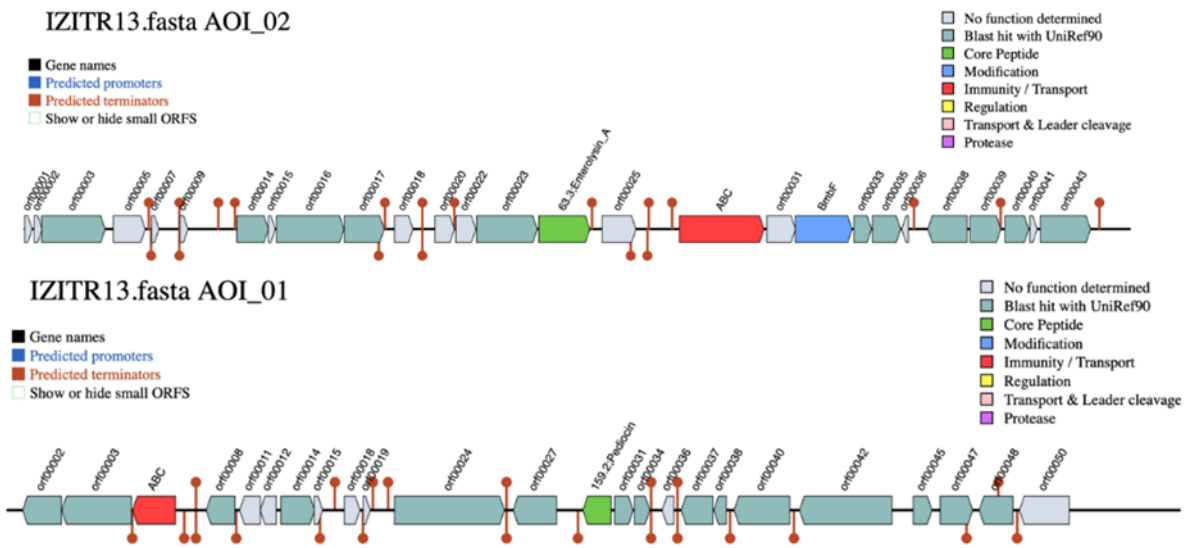

Figure 11. Genomic organisation of the clusters in *Lactiplantibacillus paraplantarum* IZITR_13 for regions encoding enterolysin A (bacteriolysin) and pediocin (class IIa bacteriocin).

The results obtained indicate that the selected strains do not rely on a single type of bacteriocin system, but possess structurally and functionally distinct complexes of antimicrobial determinants. This difference is significant for the subsequent study of cell-free supernatants, as it suggests that the observed anti-staphylococcal activity may be due to different molecular mechanisms in the individual strains.

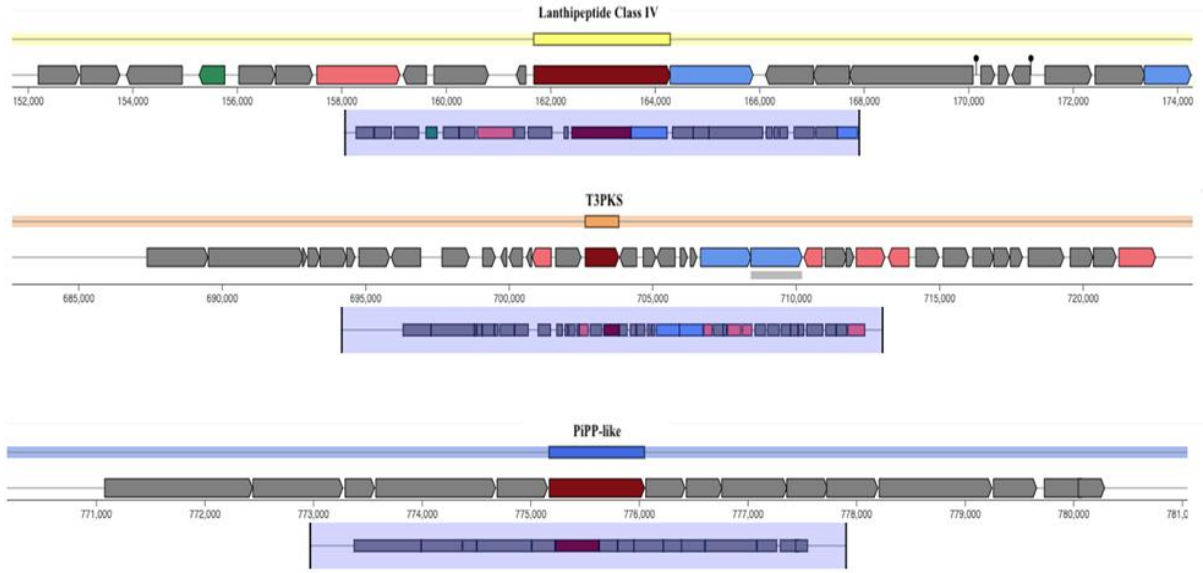

Figure 12. Genomic organisation of the clusters encoding class IV lantipeptides, the T3PKS region and RiPP-like complexes in *Lactobacillus brevis* DPL5.

5.3. In vitro assessment of the probiotic potential of the selected lactic acid bacteria strains

In vitro evaluation of *L. plantarum* IZITR_24, *L. paraplantarum* IZITR_13 and *L. brevis* DPL5 shows that the three strains demonstrate varying degrees of resistance to conditions mimicking gastrointestinal stress, as well as strain-specific differences in aggregation behaviour and interaction with test pathogenic microorganisms. These results highlight functional heterogeneity among the selected strains, whilst confirming the preservation of key properties related to survival, autoaggregation and coaggregation.

Tests for acid tolerance and resistance to bile salts revealed differences between the individual strains, indicating that their functional profile is not homogeneous. At the same time, all selected strains retained viability under some of the tested stress conditions, characterising them as resistant to varying degrees to factors associated with the gastrointestinal environment. Aggregation tests demonstrate the ability for autoaggregation and coaggregation, with values varying between the individual strains and the test microorganisms used.

Taken together, the *in vitro* results indicate that the three selected MKB strains possess a distinct yet functionally significant profile regarding resistance to stress factors, aggregation behaviour, and interaction with pathogenic bacteria. These characteristics complement the data from the antagonistic screening and the genomic characterisation of the strains.

6. Inhibitory activity of lyophilised cell-free supernatant (LSFC)

To assess the antimicrobial activity of *L. brevis* DPL5, *L. plantarum* IZITR_24 and *L. paraplantarum* IZITR_13 against *S. aureus* strains, lyophilised cell-free supernatants were used as postbiotic preparations. This approach focuses the analysis on the extracellular bioactive molecules secreted into the culture medium and allows for the assessment of their effect on two main phenotypes of *S. aureus* – biofilm formation and planktonic growth.

6.1. Fatty acid composition of cell-free supernatants

The fatty acid composition of the cell-free supernatant was analysed with a view to determining the possible contribution of the lipid fraction to the observed anti-staphylococcal activity. The results show that in the three strains studied, fatty acids are present in a very low total proportion, with the lipid fraction remaining below 0.1% of the sample. In all three supernatants, the relative composition is dominated by oleic acid, whilst palmitic, linoleic and stearic acids are present in significantly lower quantities. In addition, small amounts of short-chain fatty acids are detected, with *L. paraplantarum* showing a relatively higher proportion of butyric and caproic acids, as well as a more pronounced presence of linoleic acid, whilst caprylic acid is not detected in *L. brevis*. Regardless of these inter-strain differences, the overall picture remains the same – the fatty acid profile is quantitatively underrepresented and does not indicate a lipid fraction with a dominant chemical presence in the cell-free supernatant (Table 2).

6.2. Inhibition of the ability of Staphylococcus aureus to form biofilms

In the present study, a clear dose-dependent inhibition of *S. aureus* biofilm formation was observed under the influence of LSFS from the three strains, but with varying degrees of potency and a different transition profile between complete, partial and minimal inhibition. The most intense inhibition of biofilm formation was observed with LSFS from *L. brevis* DPL5, followed in terms of activity by *L. plantarum* IZITR_24 and *L. paraplantarum* IZITR_13. The observed dependence is determined not only by the final concentration at which complete inhibition is achieved, but also by the nature of the response in the intermediate concentration range, reflecting the width and smoothness of the dose-response curve.

Table 2. Fatty acid composition of lyophilised cell-free supernatants from the studied lactic acid bacteria strains.

Fatty acid	***L. plantarum***	***L. paraplantarum***	***L. brevis***
Butyric acid $C_{4:0}$	0.31	1.87	0.35
Caprolic acid $C_{6:0}$	0.5	2.04	0.43
Caprilic acid $C_{8:0}$	2.69	1.02	n. d
Myristic acid $C_{14:0}$	0.48	n. d	0.58
Palmitic acid $C_{16:0}$	5.17	7.51	6.47
Stearic acid $C_{18:0}$	2.13	3.36	2.58
Oleic acid $C_{18:1}$	68.65	57.28	74.49
Linoleic acid $C_{18:2}$	3.23	8.53	3.11
Arachidic acid $C_{20:0}$	0.24	n. d	0.20

In the case of LBS from *L. brevis* DPL5, a significant reduction in biofilm formation was observed at 5–10 mg mL^{-1}, whilst complete inhibition was achieved at 20 mg mL^{-1} across the entire test panel (Figure 13a). For *L. plantarum* IZITR_24, a distinct dose-dependent effect was also observed, but complete inhibition was achieved at a higher concentration – 40 mg mL^{-1} (Figure 13b). Below this threshold, the inhibition of biofilm formation persists over a wide range of lower concentrations, indicating a more gradual transition to complete inhibition. LSFS from *L. paraplantarum* IZITR_13 exhibits weaker activity, with the inhibitory effect becoming more pronounced at higher concentrations (Figure 13c).

The results of the LSFS tests following long-term refrigerated storage indicate that the ability to inhibit *S. aureus* biofilm formation is retained, but with a less pronounced effect and a shift in activity towards higher concentrations (Figure 13d– e).

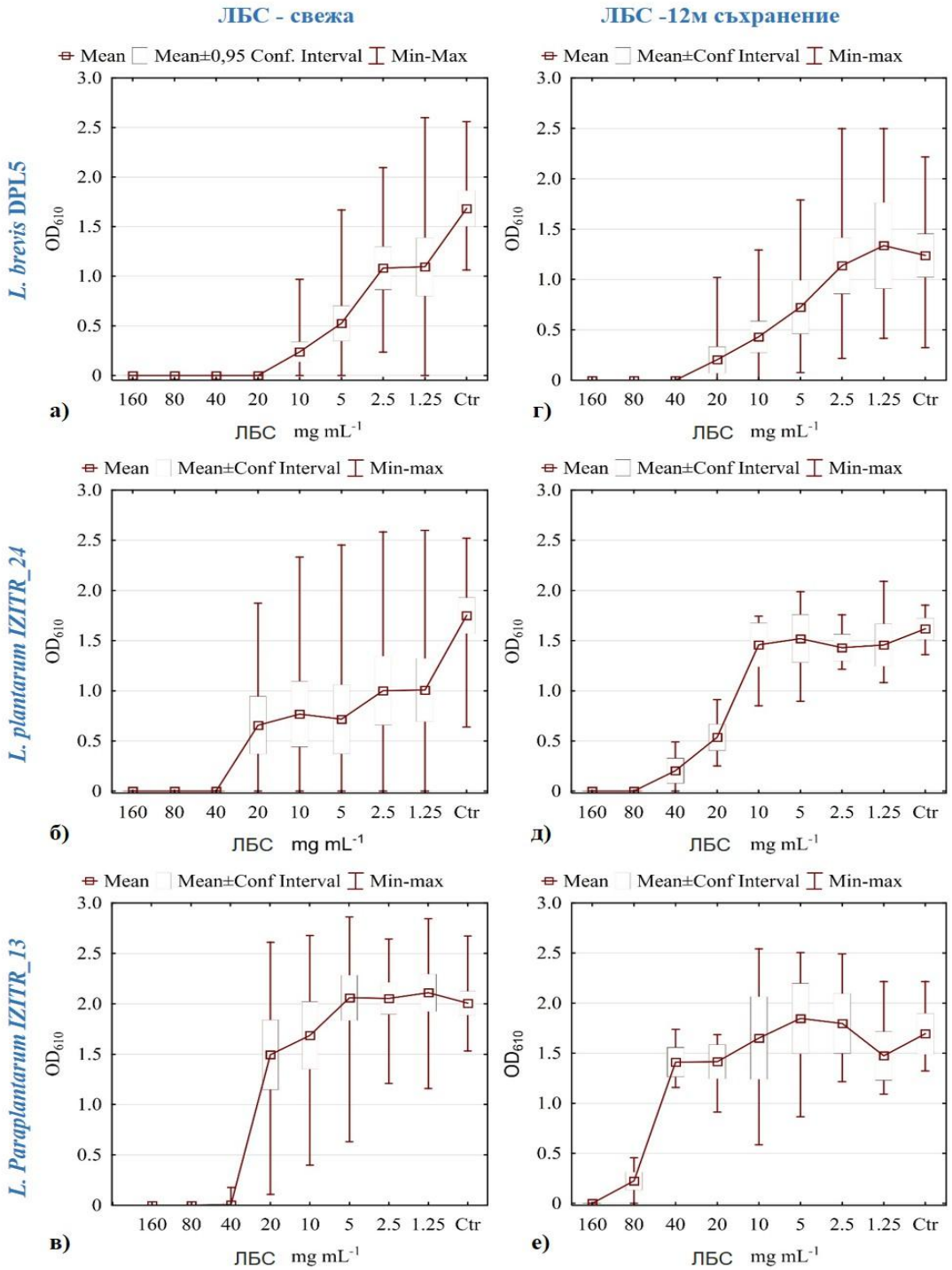

Figure 13. Inhibitory effect of lyophilised cell-free supernatants from lactic acid bacteria on biofilm formation in 50 clinical isolates of *Staphylococcus aureus*, assessed immediately after lyophilisation and after 12 months' storage of the lyophilizate at 2-8°C.

A comparison between biofilm inhibition and the identified genetic determinants for bacteriocins shows that the strength of the phenotypic response is not a direct function of the number of bacteriocin loci detected. Both strains from the *Lactiplantibacillus* complex carry conservative class IIb plantarinicin systems plnE/F and plnJ/K, whilst IZITR_13 also possesses additional determinants associated with pediocin- and enterolysin-like molecules.

Nevertheless, a more pronounced inhibition of biofilm formation is observed in IZITR_24. This indicates that the genetic presence of bacteriocin systems alone is not sufficient to explain the intensity of the observed effect.

L. brevis DPL5 lacks typical plantarin-like systems, but whole-genome analysis identifies clusters associated with RiPP/lantipeptide and other secondary metabolites. It is precisely this strain that exhibits the strongest phenotypic effect on *S. aureus* biofilm formation, suggesting the involvement of a different type of extracellular bioactive component.

6.3. Inhibition of planktonic growth

When growth was monitored by measuring optical density (OD), it was found that all three LBSs act in a dose-dependent manner on the planktonic cells of *S. aureus*. However, for a marked inhibition of growth, higher concentrations are required than those at which biofilm formation is already restricted. This indicates an earlier influence on the processes associated with biofilm formation, whilst the effect on the planktonic population manifests at higher exposure levels. The LSFS from *L. brevis* DPL5 stands out as having the highest activity, with an inhibitory effect observed as low as 10 mg mL^{-1}. Complete growth inhibition is achieved at 20 mg/mL. LBS from *L. plantarum* IZITR_24 occupies an intermediate position, with the full effect being achieved at higher concentrations – 40 and 80 mg mL^{-1}. At 5-20 mg mL^{-1}, a partial but distinct growth inhibition is observed. The effect of the LBS from *L. paraplantarum* IZITR_13 is the least pronounced; a convincing inhibitory effect is observed at > 40 mg mL^{-1}, whilst at 5 and 2.5 mg mL^{-1} growth is significantly similar to the control values.

The quantitative assessment of the differences between the effects on biofilm formation and planktonic growth was performed using PROBIT analysis. Based on this, the minimum biofilm-inhibiting concentration (MBIC), the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) were calculated. For all variants studied, the MBCV values were significantly lower than the corresponding MICs ($p < 0.001$), indicating that processes associated with biofilm formation are affected at a lower exposure level than that required for a clearly pronounced inhibition of planktonic growth. A proven bactericidal effect was observed only for LBS from *L. brevis* DPL5 and *L. plantarum* IZITR_24, whereas for *L. paraplantarum* IZITR_13, the MIC was not determined within the concentration range studied.

A comparison of the three LBS strains reveals a consistent hierarchy of activity across both *S. aureus* phenotypes studied. The strongest effect was observed for *L. brevis* DPL5, followed by *L. plantarum* IZITR_24, whilst the weakest activity was recorded for *L. paraplantarum* IZITR_13. The difference between MBPC and MPC indicates that the inhibition of biofilm formation is not merely a consequence of general growth inhibition, but rather an independent and earlier-manifesting effect of the LBS on the processes associated with attachment, accumulation and stabilisation of the biofilm structure.

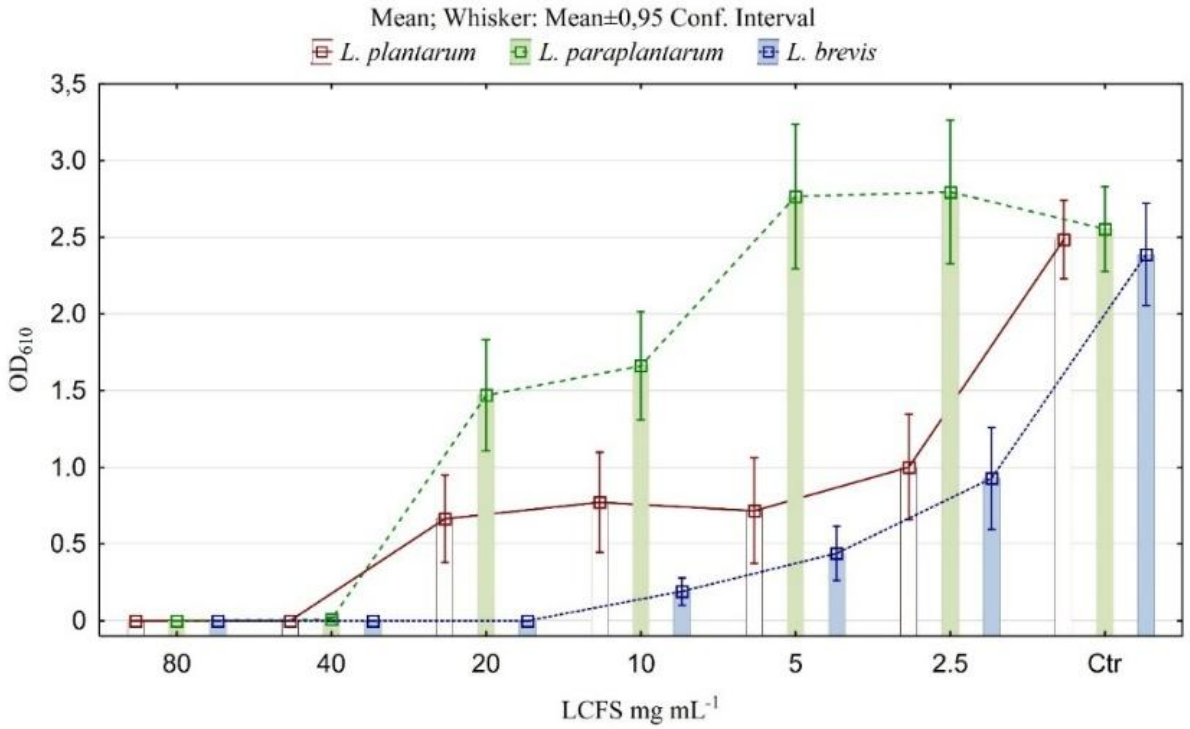

Figure 14. Dose-dependent inhibition of planktonic growth of *S. aureus* under the action of lyophilised cell-free supernatants from *L. brevis* DPL5, *L. plantarum* IZITR_24 and *L. paraplantarum* IZITR_13.

6.4. Antimicrobial activity of the low-molecular-weight protein fraction from cell-free supernatants

To demonstrate the protein nature of the anti-staphylococcal activity, the cell-free supernatants were subjected to cation-exchange FPLC. The purification was aimed at enriching and concentrating the low-molecular-weight protein fraction, without seeking complete analytical separation of individual molecules. During sample preparation, the supernatants were first subjected to tangential flow filtration to remove particles larger than 0.22 μm, after which the pH was adjusted from 4 to 7 and the conductivity to 6–7 mS cm^{-1}. The separation was carried out on a strong cation exchanger, Nuvia S, using gradient elution and collection of 2 mL fractions. Using this scheme, a single elution peak with a volume of 32 mL was obtained, starting at a conductivity of 7.5 mS cm^{-1}, with a maximum at 38.7 mS cm^{-1} and an end at 55 mS cm^{-1}, with the elution profile matching the distribution of protein concentration in the fractions. This indicates that the method enables the concentration of a limited set of charged protein components within a relatively narrow elution window, without the need for additional steps such as precipitation, drying, rehydration and subsequent purification. In *L. plantarum* IZITR_24 and L. *paraplantarum* IZITR_13, the proteins are concentrated in a limited set of adjacent fractions (Table 3).

SDS-PAGE analysis confirms the presence of protein in these samples for both strains, showing a predominance of components in the low-molecular-weight range (Figure 15). In *L. plantarum* IZITR_24, a more compact low-molecular-

weight zone is observed, whereas in *L. paraplantarum* IZITR_13, the proteins are evenly distributed among the individual eluted fractions.

Table 3. Inhibitory activity of protein fractions obtained after FPLC purification of cell-free supernatant.

L. paraplantarum IZTR_13			*L. plantarum* IZTR_24		
Фракция №	C (mg mL^{-1})	ИЗ* (мм)	Фракция №	C (mg mL^{-1})	ИЗ* (мм)
E6	0.169	0	E6	0	
E7	0.238	0	E7	0	
E8	0.308	0	E8	1.096	21
E9	0.37	0	E9	1.112	20
E10	0.466	7	E12	0	
E11	0.501	16	E13	0	
E12	0.476	16	E14	0	
E13	0.456	19	E15	0.058	0
E14	0.418	17	E16	0.067	0
E15	0.349	16	E17	0.094	0
E16	0.299	0			

An agar diffusion test was performed to determine the potential antimicrobial activity of the obtained protein fractions against *S. aureus*. The test was performed at a standardised loading of 10 μg protein per well, allowing the comparison to be based on the actual activity of the protein content rather than on differences in total protein yield (Figure 16). For *L. plantarum* IZITR_24, inhibition zones were observed in fractions E8 and E9 – 21 and 20 mm respectively, whilst no activity was detected in E14 to E16 despite the presence of measurable protein. For *L. paraplantarum* IZITR_13, no activity was detected in E6–E10, but appeared only from E11 onwards, with the inhibition zone being 7 mm in E10, 16 mm in E11 and E12, and reaching 19 mm in E13. These results indicate that antimicrobial activity does not correlate directly with total protein content, but is localised in specific low- molecular-weight protein fractions.

7. Visualisation of the biofilm

7.1. Confocal laser scanning microscopy

Confocal laser scanning microscopy (CLSM) with fluorescent staining was used to visualise the effect of lyophilised cell-free supernatants on cell viability and the spatial organisation of the biofilm. Viable cells are visualised in green, and non- viable cells in red. A concentration equal to 25% of the MBPC was used to track the structural impact on the biofilm in the presence of residual biofilm mass.

In the control, untreated sample, a well-developed biofilm is observed, dominated by viable, metabolically active cells and with a limited presence of non- viable cells. The biomass is dense, with clear layering of cell layers and pronounced vertical growth, characteristic of a mature staphylococcal biofilm. Compared to the control, the treated samples appear predominantly monolayered, with a sparser distribution of cells and disrupted layering of cell layers. In the LBS from *L. brevis* DPL5 and *L. plantarum* IZITR_24, the cells are more sparsely distributed and form small aggregates, composed mainly of non-viable cells. Enlarged cells are observed in all treated samples (Figure 17).

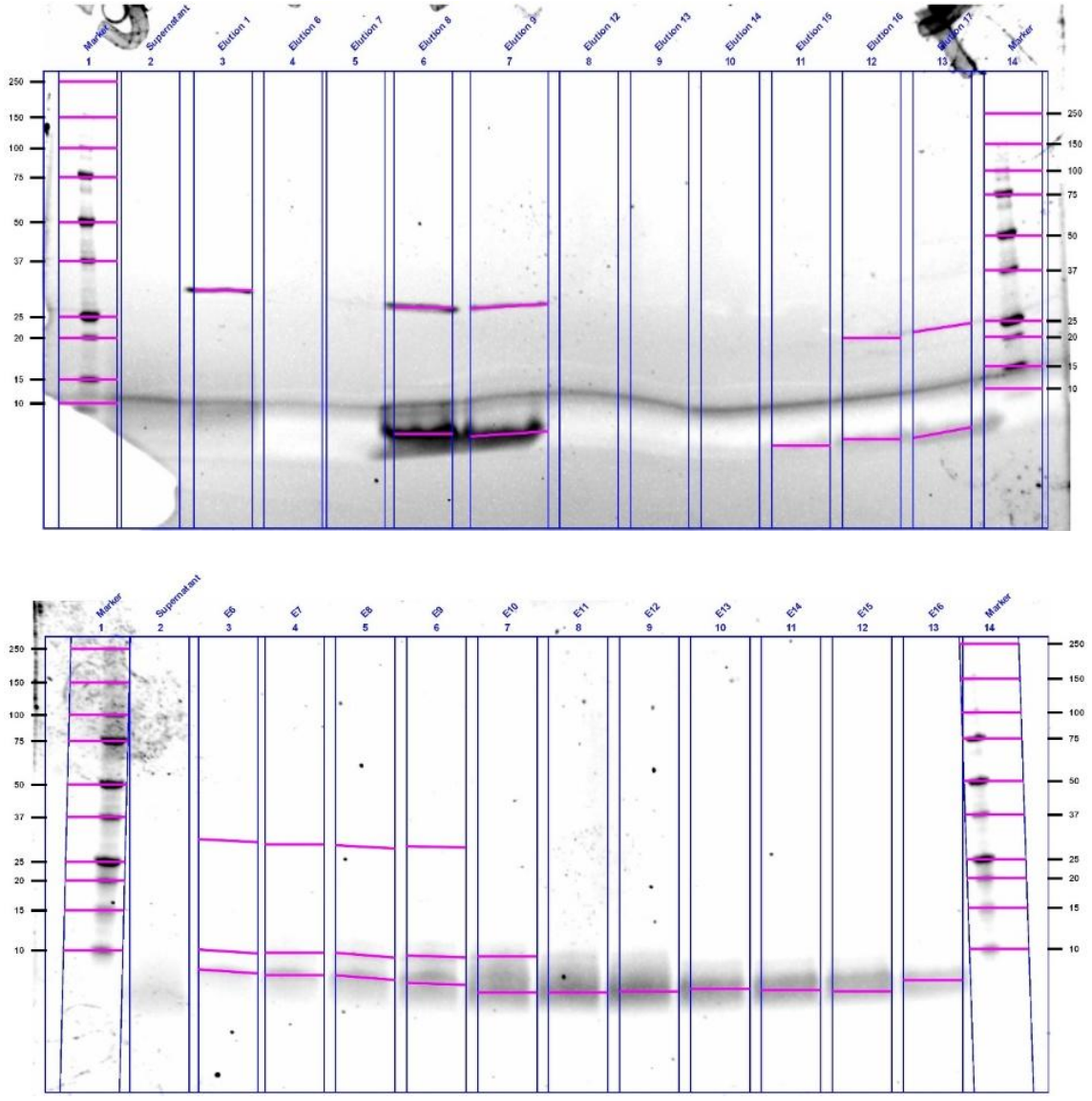

Figure 15. SDS-PAGE analysis of the protein fractions obtained following FPLC purification of the cell-free supernatant from *L. plantarum* IZITR_24 (a) and *L. paraplantarum* IZITR_13 (b).

CLSM findings indicate that the LBSs studied not only reduce the amount of biofilm formed but are also associated with a decrease in cell viability within the residual biofilm structure. The reduction in vertical growth and the transition to monolayer structures suggest a disruption of the processes responsible for biofilm mass accumulation and the stabilisation of the three-dimensional organisation.

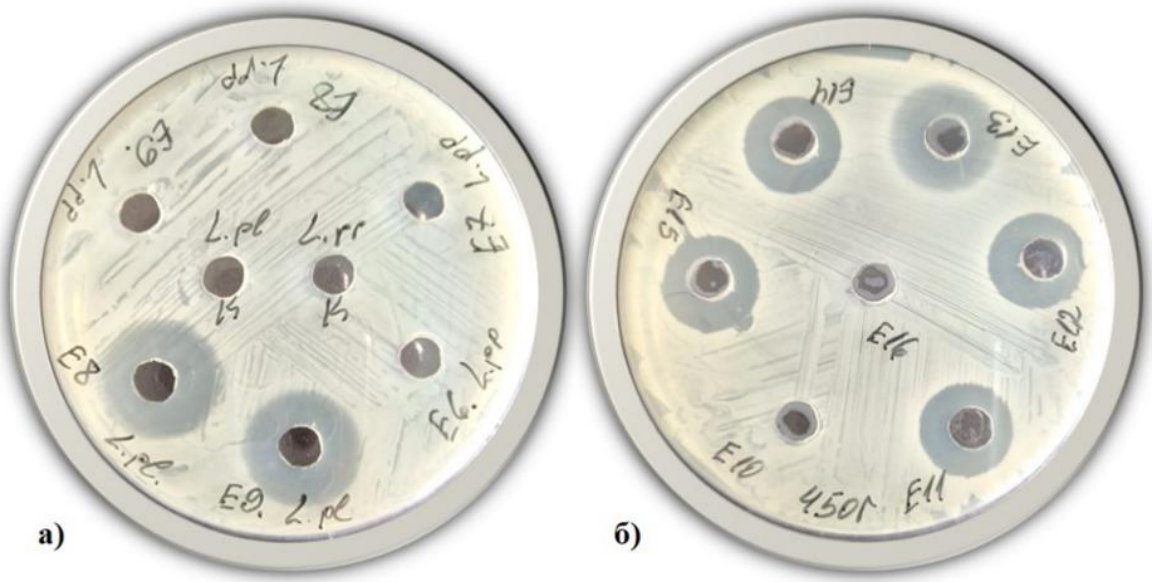

Figure 16. Agar diffusion test for antimicrobial activity against *S. aureus* of the protein fractions obtained after FPLC purification of cell-free supernatants: a) fractions E8 and E9 from *L. plantarum* IZITR_24 and fractions E6–E9 from *L. paraplantarum* IZITR_13. In the centre – controls for both strains; b) fractions E10–E16 from *L. paraplantarum* IZITR_13.
**Control – a proportional mixture of all active fractions, treated with pepsin*

7.2.Scanning electron microscopy

Scanning electron microscopy (SEM) was used to investigate the three-dimensional organisation of the biofilm and possible morphological abnormalities in the bacterial cells. The *S. aureus* strain was cultured in TSBGSP for 48 h in the presence of a sub-inhibitory dose (25% of MIC) of LBS from *L. brevis* DPL5, *L. plantarum* IZITR_24 and *L. paraplantarum* IZITR_13. In the control sample, the biofilm was well-developed and structured, with multilayered cell consortia and a clearly defined three-dimensional organisation. Visible openings are observed in the interstitial cavities, and at the cellular level, spherical cells are identified, with dimensions typical of the species – approximately 0.5–1.0 μm, a smooth surface, and clearly defined boundaries (Figure 18A and 18A1).

Distinct morphological changes were observed in the treated samples. In biofilms cultured in the presence of LBS from *L. plantarum* IZITR_24, damaged cells with significant invaginations were observed, including radial indentations along the length of the cells, with a pattern consistent with the leakage of cellular contents (Figure 18B). Upon treatment with LBS from *L. paraplantarum* IZITR_13, atypical cells were identified which, instead of the characteristic spherical shape, exhibited flattened and polygonal outlines (Figure 18C). In the

samples cultured in the presence of LBS from *L. brevis* DPL5, cells with unusual indentations were again observed, as well as cells with dimensions exceeding the standard for the species (Figure 18D).

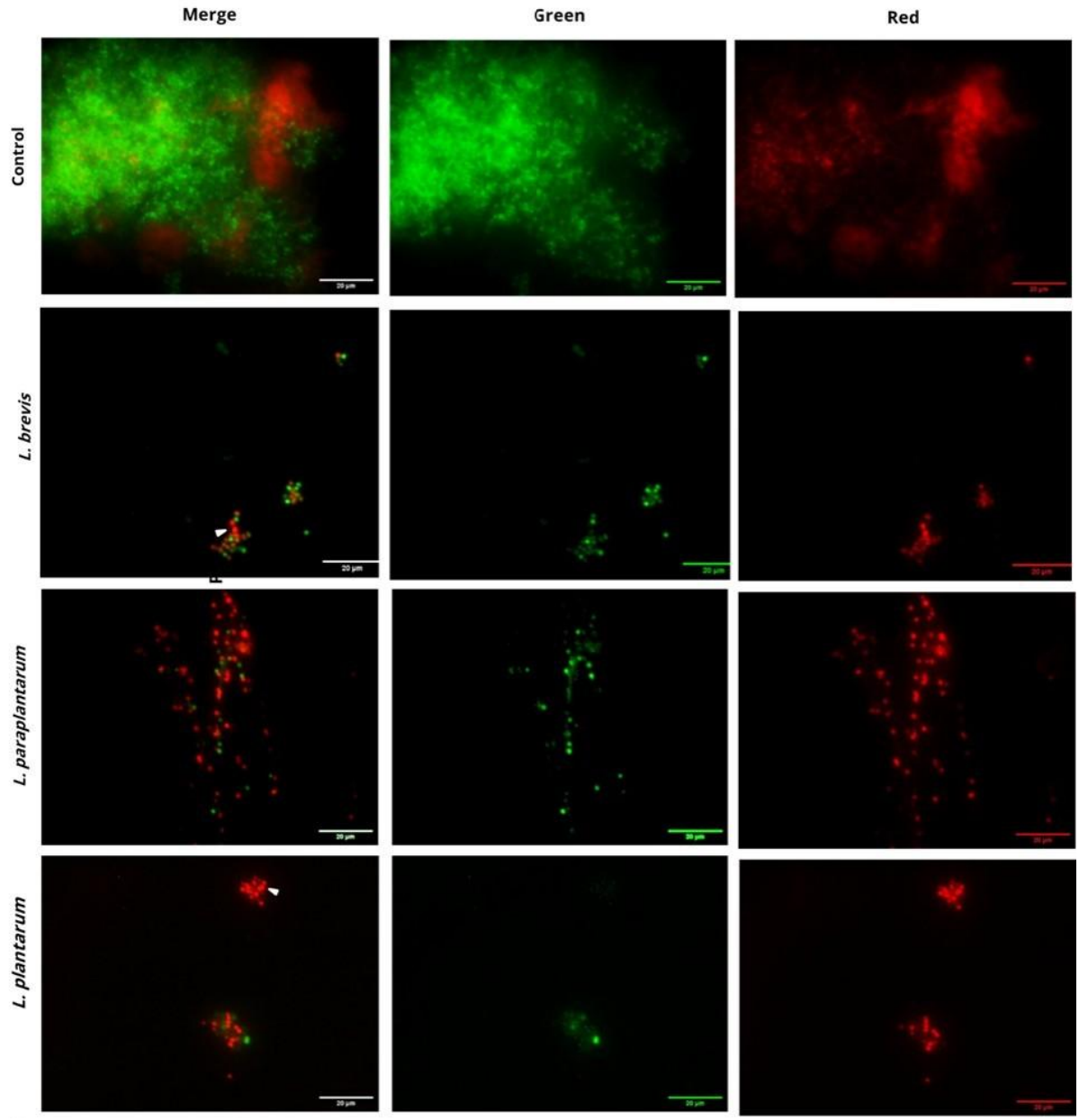

Figure 17. Determination of the viability of bacterial cells in the *S. aureus* biofilm, cultured in the presence of various lyophilised cell-free supernatants, using confocal laser scanning microscopy. Viable cells are stained green and non-viable cells red. Scale bar = 20 µm.

In parallel with the cellular changes, the biofilm architecture is significantly reduced. Compared to the control, the treated samples show thinner, single- or double-layered structures, lacking the characteristic multi-layered complexity. The SEM results confirm the CLSM findings and demonstrate that the effect of LBS is not limited to a quantitative reduction in the biofilm, but also involves changes in its three-dimensional organisation and the morphological integrity of the bacterial cells.

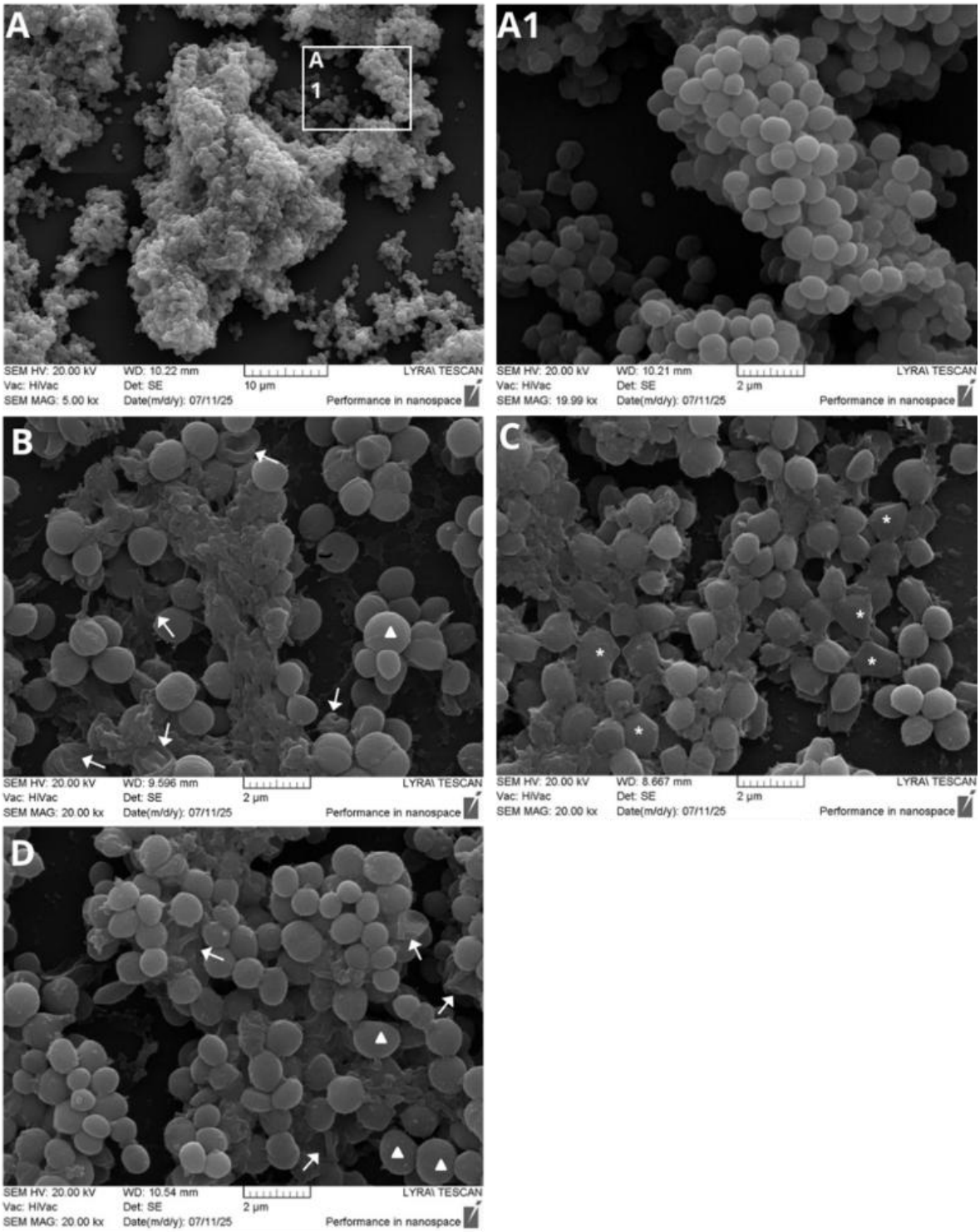

Figure 18. Scanning electron microscopy of S. aureus biofilms cultured in the presence of: A) control – untreated sample; A1) magnified view of a selected field from the control sample; B) LBS from *L. plantarum* IZITR_24; C) LBS from *L. paraplantarum* IZITR_13; D) LBS from *L. brevis* DPL5; White arrow – cellular invaginations; white star – abnormally shaped cells; white triangle – enlarged cells. Scale bars = 2 µm.

CONCLUSIONS

1. *Staphylococcus aureus* occupies a stable and significant place in the aetiological structure of symptomatic upper respiratory tract infections in the population established at SMDL 'Sinevo-Bulgaria' EOOD.
2. Phenotypic identification of *Staphylococcus aureus* in outpatient practice is associated with a potential for errors and deviations from the typical profile; therefore, its use alone does not provide sufficient reliability, whereas support from molecular biological methods significantly increases accuracy and reduces the risk of misinterpretation of clinical isolates
3. The *Staphylococcus aureus* strains isolated during the study form a population with a high resistance burden, characterised by dominant resistance to β-lactam antibiotics, increased methicillin resistance and clinically significant levels of resistance to macrolides, lincosamides, fluoroquinolones and aminoglycosides, with identified genetic determinants that largely explain the observed phenotype.
4. *In silico* analysis shows that clinical isolates of *Staphylococcus aureus* possess a stable set of genes associated with adhesion, persistence and interaction with the host immune system. Superantigenic and some enterotoxinic determinants have a more limited and uneven distribution.
5. Most *Staphylococcus aureus* isolates demonstrate the ability to form biofilms, with the intensity of biofilm formation varying significantly between strains and increasing under conditions mimicking the host environment.
6. Screening of lactic acid bacteria revealed a clear strain-specificity of antagonistic activity against *Staphylococcus aureus*, with a wide range of degrees of inhibition of growth and biofilm formation among the individual isolates.
7. Chemical analysis, combined with fractionation by FPLC, shows that the main anti-staphylococcal effects of the cell-free supernatants of lactic acid bacteria are associated with low-molecular-weight protein bacteriocin-like fractions, whilst the lipid component does not exhibit independent inhibitory activity against the growth and biofilm formation of *Staphylococcus aureus*.
8. Genomic analysis of the selected lactic acid bacteria confirms the presence of gene clusters associated with the potential for the synthesis of antimicrobial peptides, in the absence of genetic determinants that would call into question their probiotic potential and safety.
9. The lyophilised cell-free supernatants from *Lactobacillus brevis* DPL5, *Lactiplantibacillus paraplantarum* IZITR_13 and *Lactiplantibacillus plantarum* IZITR_24 exhibit pronounced dose-dependent anti-staphylococcal activity, with minimum inhibitory concentrations against biofilm formation and planktonic growth of *Staphylococcus aureus* determined for all strains.
10. Data from scanning electron microscopy indicate that the cell-free supernatants of the three strains modify the biofilm-associated behaviour of *Staphylococcus aureus* – they quantitatively reduce biofilm formation, alter the architecture of the mature biofilm and lead to distinct changes in the biofilm structure, cell surface and morphology of the bacterial cells.

CONTRIBUTIONS

Scientific contributions

1. The clinical significance of *Staphylococcus aureus* as an aetiological agent in the nasopharyngeal niche has been demonstrated, based on an integrated epidemiological, phenotypic and genetic characterisation of community-acquired isolates.

2. A detailed assessment of the resistance profile of a local population of *Staphylococcus aureus* has been carried out, identifying the main genetic determinants associated with resistance to β-lactam antibiotics, macrolides and lincosamides.

3. A comprehensive virulence profile of *Staphylococcus aureus* strains was established, demonstrating a predominance of adhesion and persistence determinants with a relatively limited distribution of superantigens and enterotoxins.

4. It has been demonstrated that genomic analysis reflects functional potential, but not necessarily its phenotypic expression; it has been established that the genotype-phenotype relationship in the strains studied is graded in nature and depends on environmental conditions.

5. The characteristics of biofilm formation in clinical isolates of *Staphylococcus aureus* under conditions mimicking the host environment have been characterised, and it has been shown that enrichment of the medium with glucose, sodium chloride and human blood plasma leads to a significant enhancement of biofilm formation.

6. The whole genome of newly isolated strains of lactic acid bacteria with anti-staphylococcal activity has been investigated, and the production of antimicrobial peptides and the absence of determinants compromising their safety have been demonstrated.

Applied contributions

1. An up-to-date local database has been created on the antimicrobial susceptibility and genetic determinants of resistance in *Staphylococcus aureus* from the upper respiratory tract, with immediate applicability in optimising empirical therapy and decolonisation strategies.

2. An original comprehensive model has been developed for the combined application of phenotypic, biochemical, molecular and whole-genome approaches in the routine and extended characterisation of clinical isolates of *Staphylococcus aureus* in a microbiology laboratory

3. The applicability of whole-genome sequencing using the Oxford Nanopore

Technologies platform for diagnostic and epidemiological purposes has been demonstrated for the first time.

4. The ability of products from the cell-free supernatant of lactic acid bacteria to modify the growth and biofilm formation of *Staphylococcus aureus* has been experimentally demonstrated, paving the way for future development.

Publications related to the dissertation

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