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Research Article

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Snapshot of resistance and virulence features in ESCAPE strains frequently isolated from surgical wound infections in a Romanian hospital

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Abstract

***Background**: The aim of this study was to investigate the phenotypic features (adherence, biofilm formation, virulence, antibiotic susceptibility) and the genetic background of antibiotic resistance in nosocomial ESCAPE strains consecutively isolated from surgical wound infections in hospitalized patients. **Methods**: 86 bacterial strains consecutively isolated from various wound infections were analysed by their antibiotic resistance (antibiotic susceptibility testing and PCR for certain antibiotic resistance genes), virulence, biofilm formation and cellular adherence. **Results**: The bacterial isolates were identified as: Enterobacterales (n = 39) including Escherichia coli (n = 9), Klebsiella pneumoniae (n = 14) Proteus mirabilis (n = 7), followed by Staphylococcus aureus (n = 26) and Enterococcus faecalis (n = 20). Compared to other isolates, S. aureus strains exhibited the highest capacity to produce soluble virulence factors and to develop biofilms in vitro, with significant differences between methicillin resistant and methicillin susceptible isolates. Among enterobacterial isolates, K. pneumoniae strains expressed the highest capacity to develop biofilms. The assessment of bacterial adherence to HeLa cells revealed that all bacterial strains adhered to the cellular substrata, showing various adherence patterns. E. faecalis strains exhibited a low soluble virulence factors profile, a lower capacity to adhere to epithelial cells and to develop biofilms. **Conclusions**: The present study could contribute to the understanding of the pathology of infected wounds, depending on the etiological agents, providing data with positive impact on the therapeutic management of surgical wounds infections.*

***Keywords**: biofilm formation, antibiotic resistance, adherence, surgical site infection*

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Introduction

Surgical wound infections, also called surgical site infections (SSI) occur in a wound after a surgical procedure and represent a major source of morbidity and mortality in surgical patients (1). SSI account for 15% to 20% of the healthcare associated infections (2, 3). The most frequent microorganisms associated with SSI are usually represented by *Staphylococcus aureus* strains, followed by coagulase negative staphylococci, enterococci, *Pseudomonas aeruginosa*, *Escherichia coli,* streptococci, *Enterobacter* sp., *Proteus* spp., *Klebsiella pneumoniae/oxytoca* and *Serratia* sp. (4), while other data report a higher prevalence of Gram-negative species (i.e. *K. pneumoniae* or *E. coli*) (1, 5, 6). If these microorganisms acquire antibiotic resistance and/or form biofilms, then the treatment of SSI becomes more complicated and involves prolonged hospitalisation, ultimately leading to an economical burden for the healthcare system (7, 8). Previous data from Romania (2, 6, 9) indicate a higher prevalence of either Gram-positive or Gram-negative species, where Gram-negative non-fermentative species (*P. aeruginosa*) being more resistant to antibiotics compared to other microorganisms involved in SSI, but these studies regarded only antimicrobial resistance. Although biofilm formation in SSI may cause a delay of wound healing, including due to the expression of virulence factors, and increase the risk of infection (8, 10), we found only one previous publication from Romania assessing also biofilm formation capacity of bacterial strains, isolated from chronic skin ulcers (11). Thus, our study aims to extensively investigate the phenotypic features (adherence, biofilm formation, virulence and antibiotic susceptibility) as well as the genetic background of antibiotic resistance in nosocomial strains included in the ESCAPE group (*E. faecium, S. aureus, Clostridioides difficile, Acinetobacter baumannii, P. aeruginosa* and *Enterobacterales*), isolated from surgical wound infections in hospitalized patients.

Material and methods

Microbial strains

This study was performed on 86 microbial strains isolated consecutively from positive samples isolated from various surgical wounds, from inpatients admitted to the Institute for Cardiovascular Diseases "Prof. C.C. Iliescu", Bucharest during March and September 2018.

Microbiological examination

The preliminary identification of isolated strains was performed using standard microbiology procedures (Gram staining, culturing on selective/differential culture media, i.e. Mannitol Salt Agar, Bile Esculin Agar, MacConkey agar), followed by identification using automatic systems (Vitek 2 system, bioMérieux Inc., Durham, NC and Phoenix BD, Beckton–Dickinson). Isolates were stored at –80°C in Trypticase Soy broth supplemented with 20% glycerol, in the Microbial Culture Collection of the Microbiology Laboratory, Faculty of Biology, University of Bucharest.

Antibiotic susceptibility testing

Antibiotic susceptibility was assessed by Vitek 2, using the AST-P592 cards for Gram-positive bacterial strains and AST-GN for the Gram-negative ones. Prior to testing, frozen specimens were subcultured on Tryptic Soy agar plates containing 5% sheep blood and incubated at 37°C for 18 - 20 h.

Antibiotic resistance genes detection

Antibiotic resistance genes were detected using PCR (polymerase chain reaction) method (simplex or multiplex). Genomic DNA was obtained using a modified alkaline extraction method (12). All reactions were carried in a reaction mix of 20 or 25 µl (PCR Master Mix 2x, Thermo Scientif-

ic), using 1 µl of genomic DNA as template and specific primers for antibiotic resistance genes: bla_{CTX-M}, bla_{SHV}, bla_{TEM} (13), bla_{OXA-48}, bla_{NDM}, bla_{KPC}, bla_{VIM} (14), *aphA1, aadB, aacC1, aadA1, aphA6, aacA4* (15), *qnrA, qnrB, qnrS* (16) for *Enterobacterales*; *blaZ* (17), *tetA*, *ermC*, *ermA* (18), SCC*mec*VJ1, SCC*mec*IVb, SCC*mec*II, SCC*mec*III, SCC*mec*IVa, SCC*mec*IVc, SCC*mec*IVd1 (19), *mecA*, CCrC, CCrB2, SCC*mec*I, *RIF5*, *mecI*, *kdp* , SCC*mec*V, *dcs*, *CIF2* (20) for *S. aureus* and *aacA-aphD1*, *ermA*, *ermC*, *tetM*, *tetK* (18) for *E. faecalis*. The amplification products were visualized by electrophoresis on a 1.5 % agarose gel, stained with ECO Safe Nucleic Acid Staining Solution (Thermo Scientific).

Soluble virulence factors

The phenotypic expression of soluble bacterial virulence factors was assessed by enzymatic tests, using specific culture media: 5% sheep blood agar (haemolysis assessment), 2.5% yolk agar (lecithinase), Tween 80 agar (lipase), 15% casein agar (caseinase), 1% gelatine agar (gelatinase), 10% starch agar (amylase), DNA agar (DN-ase), and 1% esculin iron salts (esculinase).

Biofilm assay

Biofilm forming capacity was tested using 15 µL of 0,5 McFarland bacterial suspensions (1.5×10^8 colony forming units - CFU/mL) prepared in sterile saline water, inoculated in 96 multi-well plates in a final volume of 150 µL nutrient broth. Each strain was tested in triplicate. The plates were subsequently incubated at 37°C for 24, 48 and 72 hrs, then washed gently with phosphate buffered saline (PBS) in order to remove the unattached (planktonic) cells, followed by 5 minute incubation with cold methanol, then dried at room temperature and stained with 0.1% crystal violet solution for 15 minutes. The stain was subsequently re-suspended in 33% acetic acid. Finally, the absorbance of the resulting solution was read spectrophotometrically (492 nm) (21).

Adherence to HeLa cells

Assessment of bacterial adherence was performed using adapted Cravioto's method: HeLa cell monolayers were washed with PBS (there times). Subsequently, 1 mL of fresh medium supplemented with 10% foetal bovine serum was added to each well (22), followed by 1 mL of bacterial cell suspensions adjusted at 10^7 CFU/mL (prepared from mid- logarithmic phase cultures grown in nutrient broth), then incubated for two hours at 37^0C (23). After incubation, the cell monolayer was washed with PBS to remove unattached bacterial cells and then fixed with cold methanol for 5 minutes. Plates were dried at room temperature and cell monolayers were stained with 10% Giemsa solution for 20 minutes. Plates were dried and analysed using the optical microscope (immersion oil, 100x objective).

The adherence patterns were defined as: localized adherence (tight clusters of bacterial cells on the HeLa cell surface), aggregative adherence (displaying a stacked brick pattern both on the eukaryotic cells and the plastic substratum) and diffuse adherence (diffusely adhered bacteria, covering the whole surface of the eukaryotic cell). The adherence index was calculated as the ratio between the number of eukaryotic cells with adhered bacteria and 100 HeLa cells counted on the microscopic field (24).

Statistical analysis

The biofilm formation trend in the selected timescale was analysed using one-way ANOVA repeated measures test and comparison between methicillin-resistant *S. aureus* (MRSA) and methicillin-susceptible *S. aureus* (MSSA) biofilm formation trend was analysed using two-way ANOVA repeated measures. Correlation of biofilm formation and adherence was performed using Pearson correlation. All statistical analyses were performed using GraphPad Prism Software, v. 5.03 (GraphPad Software, La Jolla California USA, www.graphpad.com).

Results

Microbiological examination

The microbial strains isolated from SSI were identified as *Enterobacterales* (n=39), of which: *Klebsiella pneumoniae* (n=14), *Escherichia coli* (n=9), *Proteus mirabilis* (n=7), *Citrobacter* spp. (n=2), *Morganella morganii* (n=2), *Providencia stuartii* (n=1), *P. rettgeri* (n=1), *Enterobacter cloacae* (n=1), *E. aerogenes* (n=1), *Serratia marcescens* (n=1), followed by *S. aureus* (n=26), out of which MRSA (n=17) and MSSA (n=9) and *E. faecalis* (n=20) .

Antibiotic susceptibility profiles

Antibiotic susceptibility profiles, assessed by both classical and automatic approaches, revealed that *S. aureus* strains exhibited high resistance rates to penicillin (100%), methicillin (71%), tetracycline (71%), erythromycin (63%), clindamycin (59%), and lower rates of resistance to other classes of antibiotics (fig. 2). Moreover, two strains exhibited resistance to teicoplanin, one of them being concomitantly resistant to linezolid. All *S. aureus* strains were susceptible to vancomycin and tigecycline. Ten strains exhibited inducible MLS_B (macrolide-lincosamide-streptogramin B) phenotype. According to the criteria for multiple-drug resistance (25), 16 (59%) of the strains were multidrug-resistant (MDR), out of which 14 were MRSA, the correlation between the MDR and MRSA phenotypes being statistically significant (p = 0.0009). PCR analyses revealed that the majority of the MRSA tested strains harboured the staphylococcal cassette chromosome *mec* (SCC*mec*) type IV (n=11), followed by SCC*mec* type III (n=3), SCC*mec* type I (n=1). The SCC*mec* was non-typeable in one strain. The *S. aureus* strains harboured various antibiotic resistance genes (table 1), which in a decreasing frequency order were: *mecA* (all MRSA strains), *blaZ* (85%), *ermC* (63%), *tetK* (52%), *ermA* (15%) and *tetM* (7%). The *blaZ* gene was encountered in strains belonging to different SCC*mec* cassettes, *ermC + tetK* genes were encountered in strains belonging to SCC*mec*IV, while the association *ermA +tetM* in strains of SSC*mec*III type.

The antibiotic susceptibility profiles for *E. faecalis* revealed that all tested strains were resistant to erythromycin (100%), followed by high level resistance rates to tetracycline (75%), aminoglycosides (streptomycin high level-60%, gentamicin high level-50%), and fluoroquinolones (ciprofloxacin 55%). Moreover, 50% of the strains were MDR. All strains harboured *ermC* gene, while *tetM* was encountered in 75% and *aacA-aphD1* in 50% of the tested strains, only in combination with the *ermC+tetM* genes.

Antibiotic susceptibility profiles for *Enterobacterales* revealed high level of resistance to penicillins (83%), amoxicillin plus clavulanic acid (43%), fluoroquinolones (ciprofloxacin-40%, ofloxacin-43%, moxifloxacin-43%), tetracycline (43%), and trimethoprim sulfamethoxazole (43%). Moreover, 64% of the strains were MDR. The genetic background of antibiotic resistance was variable according to the species: in *E. coli* there were encountered bla_{TEM} (56%), $bla_{CTX\text{-}M}$ (33%), $bla_{OXA\text{-}48}$ (11%), *aphA1* (11%), *aadA1* (11%), *qnrB* (11%) and *qnrS* (11%). *K. pneumoniae* strains harboured bla_{SHV} (100%), $bla_{OXA\text{-}48}$ (86%), $bla_{CTX\text{-}M}$ (79%), bla_{TEM} (79%), *aphA1* (57%), *aadA1* (14%) and *P. mirabilis* tested positive for *aphA1* (86%), bla_{TEM} (71%), $bla_{OXA\text{-}48}$ (71%) and *aadB* (14%).

Soluble virulence factors production

The analysed strains frequently expressed lecithinases, caseinases and esculin hydrolase and only moderately haemolysins and gelatinase. All *E. faecalis* strains produced caseinase (100%), and with less frequency, gelatinase (35%) and lecithinase (30%) and were negative for DNase, amylase and lipase production. The tested *S. aureus* strains, both MRSA and MSSA, ex-

Table 1. Soluble virulence factors, antibiotic resistance profiles and antibiotic resistance genes identified in the strains isolated from SSI

		Virulence [a]	Antibiotic resistance profiles [b]	Resistance phenotypes	Antibiotic resistance genes
***S. aureus* (27)**	**MRSA SCC*mec*I (1)**	HEM - α, CAS, LEC	Pen, Oxa		*mecA, blaZ*
	MRSA SCC*mec*III (3)	HEM - α (1), CAS, LEC (2), ESC (1)	Pen, Oxa, Gen, Cip, Mox, Ery, Cli, Tet (2), Rif, Sxt (1)	**MDR** MLSB-I (2)	*mecA, ermA* (2), *tetM* (2), *blaZ*
	MRSA SCC*mec*IV (11)	HEM-α (5), HEM-β (5), CAS, LEC (10), ESC (7), GEL (1)	Pen, Oxa, Gen (3), Cip (3), Mox (3), Ery (9), Cli (8), Lzd (1), Tec (2), Tet, Fsa (3), Rif (4), Sxt (3)	**MDR (10)** MLSB-I (4)	*mecA, ermC* (10), *tetK* (9), *blaZ* (10)
	MRSA SCC*mec* NT* (1)	CAS, LEC, ESC	Pen, Oxa, Gen, Tet, Ery, Cli	**MDR** MLSB-I	*mecA, ermC, tetK, blaZ*
	MSSA (11)	HEM-α (4), HEM-β (3), CAS (10), LEC (3), ESC (8), GEL (2)	Pen (9), Ery (4), Cli (3), Tet (4), Rif (1)	**MDR (1)** MLSB-I (3)	*ermA* (2), *ermC* (5), *tetK* (4), *blaZ* (8)
***E. faecalis* (20)**		LEC (6), CAS, ESC, GEL (7)	Amp (1), Gen (10), Str (12), Cip (11), Ery, Tet (15)	**MDR (10)**	*aacA-aphD1* (10), *ermC*, *tetM* (15)
***Enterobacterales* (39)**	***K. pneumoniae* (14)**	CAS (8), ESC (14), GEL (8)	Amc, Tzp (8), Fox (9), Caz (10), Cro (11), Ctx (11), Cpd (11), Fep (11), Atm (11), Etp (5), Imp (5), Mem (5), Amk (3), Gen (11), Tob (11), Cip (11), Lev (8), Tet (8), Tig (2), Sxt (9), Col (10), Fos (2)	ESBL+**MDR (11)**	bla_{TEM} (11), bla_{SHV}, bla_{CTX-M} (11), bla_{OXA-48} (12), *aphA1* (8), *aadA1* (2),
	***E. coli* (9)**	LEC (2), CAS (4), AMY (2), DNA (2), ESC (4), GEL, LIP (1)	Amp (8), Amc (4), Tzp (2), Fox (1), Caz (5), Cro (4), Ctx (5), Cpd (5), Fep (4), Atm (4), Gen (1), Tob (2), Cip (4), Lev (4), Tet (6), Tig (1), Sxt (3), Col (1), Fos (1)	ESBL + **MDR (4), MDR (1)**	bla_{TEM} (5), bla_{CTX-M} (3), bla_{OXA-48} (1), *aadA1* (1), *qnrB* (1), *qnrS* (1)
	***P. mirabilis* (7)**	LEC (5), AMY (6), DNA (6), GEL	Amp (4), Gen (3), Tob (4), Cip (4), Lev (3), Sxt (4), Fos (2)	**MDR (6)**	bla_{TEM} (4), bla_{OXA-48} (5), *aphA1* (6), *aadA1 (5),*
	***Citrobacter* sp. (2)**	CAS, GEL	Tzp (1), Caz (1), Cro (1), Ctx (1), Cpd (1), Fep (1), Atm (1), Etp (1), Imp (1), Mem (1), Amk (1), Gen (1), Tob (1), Cip (1), Lev (1), Tet, Tig (1), Sxt (1), Col (1), Fos (1)		bla_{OXA-48}, *aphA1* (1),
	***M. morganii* (2)**	CAS (1), GEL	Caz (1), Ctx (1), Cpd (1), Gen (1), Cip, Lev, Tet, Sxt, Fos	**MDR** ESBL (1)	bla_{OXA-48} (1), *aphA1, aadA1* (1),
	***P. stuartii* (1)**	-	Cpd, Gen, Tob, Cip, Lev, Tet, Sxt, Fos	**MDR**	-
	***E. cloacae* (1)**	ESC, GEL	Cpd,		bla_{OXA-48}
	***E. aerogenes* (1)**	ESC	-		
	***S. marcescens* (1)**	CAS, ESC, GEL, LIP	-		bla_{OXA-48}
	***P. rettgeri* (1)**	ESC	Caz, Atm, Cip, Lev, Fos	**MDR**	

Numbers in braces indicates the number of strains for which a certain feature was encountered. No number in braces indicates that the respective feature is presented in all strains of one certain group. * NT – non-typeable. [a] Virulence: AMY – amylase; CAS – caseinase; DNA – DNase, LIP - lipase. ESC – esculin hydrolysis; GEL – gelatinase, HEM-α – α-haemolysis; HEM-β – β-haemolysis; LEC – lecithinase; [b] Antibiotics: AMC – amoxicillin-clavulanic acid; AMK – amikacin; AMP – ampicillin; ATM – aztreonam; CAZ – ceftazidime; CIP – ciprofloxacin; CLI – clindamycin; COL – colistin; CPD – cefpodoxime; CRO – ceftriaxone; CTX – cefotaxime; ERY – erythromycin; ETP – ertapenem; FEP – cefepime, FOS – fosfomycin; FOX – cefoxitin; FSA – fusidic acid; GEN – gentamycin; IMP – imipenem; MEM – meropenem; MOX – moxifloxacin; OXA – oxacillin; PEN – penicillin; RIF – rifampicin; SXT – trimethoprim-sulfamethoxazole; TEC – teicoplanin; TET – tetracycline; TOB – tobramycin; TZP – piperacillin-tazobactam.

pressed the majority of soluble enzymes associated with bacterial pathogenesis: hemolysins (75%/64%), caseinases (100%/91%), lecithinases (88%/73%), esculin hydrolases (56%/27%) and gelatinases (6%/18%) and were negative for amylase and lipase production and surprisingly, were negative also for DN-ase production.
In *Enterobacterales*, the most frequent soluble virulence factors were: gelatinase (100%), caseinase (44%), esculin hydrolase (44%), DN-ase (33%), lecithinase (22%), amylase (22%) and lipase (11%) in *E. coli*, while *P. mirabilis* strains produced gelatinase (100%), DN-ase (86%), amylase (86%), lipase (71%) and lecithinase (71%). Although exhibiting the highest rates of antibiotic resistance among *Enterobacterales* strains, *K. pneumoniae* expressed fewer virulence factors. i.e.: esculin hydrolases (100%), gelatinases (57%) and caseinases (57%).

Cellular adherence

The assessment of adherence to the cellular substratum represented by HeLa cells highlighted that all bacterial strains isolated from surgical wounds possess the ability to attach to the HeLa cells, revealing all main adherence patterns (diffuse, localized, aggregative) or a mixed pattern (e.g., aggregative-diffuse) with adherence rates ranging from 5% to 100%. 50% of *S. aureus* strains demonstrated an aggregative adherence pattern, while the adherence rates in *Enterobacterales* isolates were about 30%, and less than 10% for *Enterococcus* sp. The localized pattern was recorded in 80% of the *Enterococcus* sp., 40% of the *S. aureus* strains, while for *Enterobacterales* in 45% of the strains. A lower frequency, 15-25%, was observed for the diffuse adherence, while 14% of *S. aureus* strains expressed a mixed diffuse-aggregative adherence pattern (Fig. 1, Table 2).

Biofilm development assessment

The results of biofilm development on the inert substratum assay highlighted that all the evaluated microbial strains present different abilities to develop monospecific biofilm, therefore they were clustered in 3 groups, depending on the absorbance value reported to the positive control (0.5), being expressed as: low (+) (absorbance value of 0.5-1.0), moderate (++) (1.0-2.0), and strong/high (+++) (2.0-3.0) (26). Biofilm development results followed a similar temporal dynamic for the strains belonging to a certain group (fig. 2) with a maximum of development at 46 hrs for *S. aureus* and of 72 hrs for *Enterobacterales*.
Biofilm assessment revealed that most of the *S. aureus* strains presented a moderate and high capacity to develop biofilms, with an increased

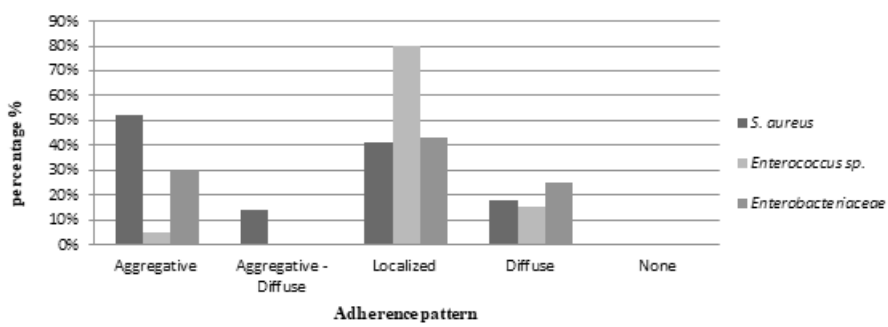

Fig. 1. Adherence pattern of the isolated strains to the cellular substrata represented by HeLa cells.
Table 2. Adherence patterns and indexes observed for strains isolated from SSI

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		Adherence patterns and indexes [a]	Adherence index	No. of strains exhibiting specific index
***S. aureus* (27)**	**MRSA SCC*mec*I (1)**	DIF	90%	1
	MRSA SCC*mec*III (3)	AGG	85%	1
			100%	1
		LOC	100%	1
	MRSA SCC*mec*IV (11)	LOC	25%	1
			30%	1
			100%	1
		AGG	80%	1
			100%	5
		DIF-AGG	100%	2
	MRSA SCC*mec* NT* (1) **MSSA (11)**	DIF-AGG	40%	1
		AGG	100%	6
		DIF	80/	1
			85/	1
			95/	1
			100%	1
		DIF-LOC	100%	1
***Enterobacterales* (39)**	***E. faecalis* (20)**	LOC	10/	2/
			15/	1/
			30/	1/
			40/	3/
			50/	5/
			60/	3/
			70%	1
		DIF	70%	2
			80%	1
		AGG	90%	1
	***K. pneumoniae* (14)**	AGG	100%	12
		DIF	100%	2
	***E. coli* (9)**	LOC	90%	2
			100%	1
	***P. mirabilis* (7)**	LOC	90%	2
			100%	3
		DIF	90%	1
			100%	1
	***Citrobacter* sp. (2)**	LOC	40%	1
			90%	1
	***M. morganii* (2)**	LOC	40%	1
			60%	1
	***P. stuartii* (1)**	LOC	40%	1
	***E. cloacae* (1)**	LOC	10%	1
	***E. aerogenes* (1)**	LOC	70%	1
	***S. marcescens* (1)**	LOC	40%	1
	***P. rettgeri* (1)**	LOC	5%	1

[a] Adherence patterns: DIF – diffuse; AGG – aggregative; LOC – localized;

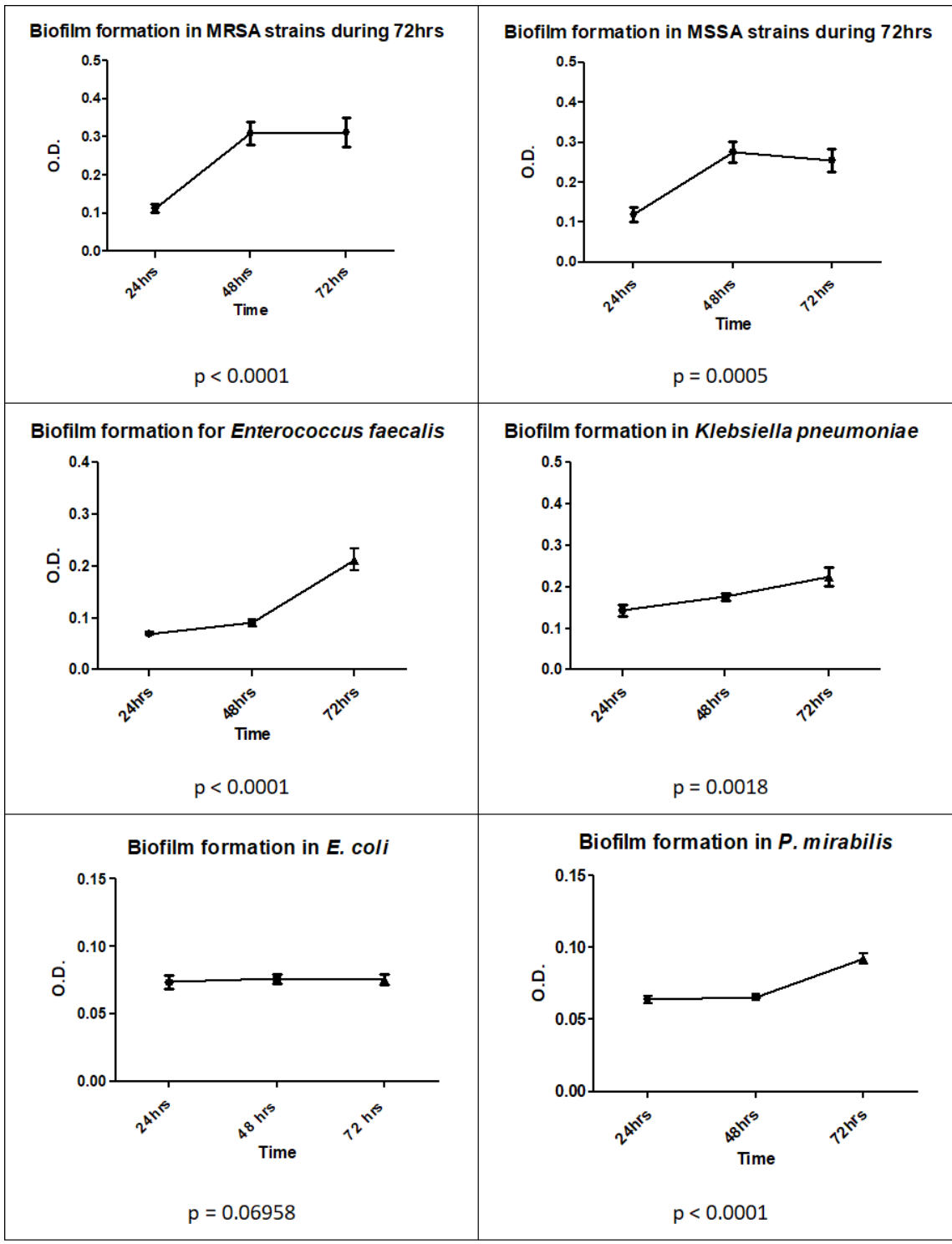

Fig 2. Biofilm development assessed at 24, 48 and 72h of incubation

capacity in MRSA compared to MSSA (two-way ANOVA, $p < 0.0001$). *E. faecalis* strains exhibited a low to moderate biofilm formation activity.
The evaluated *Enterobacterales* expressed a lower potential of biofilm formation on inert substrata, but significant differences (two-way ANOVA, $p<0.0001$) were noticed between *K. pneumoniae* (with moderate-high capacity) and the other *Enterobacterales* strains (mostly low-moderate capacity) analysed in this study. (Fig. 3, 4)
The correlation between bacterial adherence to cellular substrate and biofilm formation was significant in Gram-negative species ($p = 0.0179$), while in *E. faecalis* and *S. aureus* this correlation was not significant ($p = 0.1153$ and 0.3160, respectively). These results could suggest that the Gram-negative strains are more capable to form biofilm both on cellular and inert substrata, as compared to Gram-positive bacteria, which are more probably to colonize the inert substrata and therefore to produce medical device-associated biofilm infections. This conclusion is supported by different studies showing that the Gram-positive bacteria, and especially *S. aureus* and coagulase-negative staphylococci, are mostly involved in the aetiology of these infections (27-29).

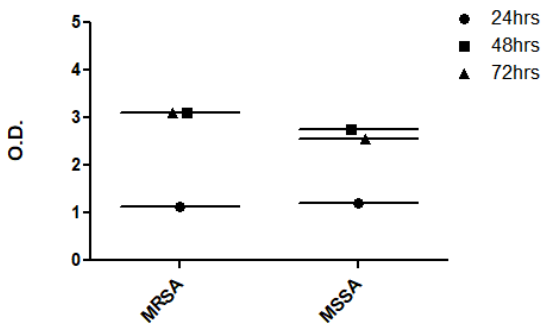

Fig. 3. The MRSA strains had a higher capacity of biofilm formation compared to MSSA strains ($p < 0.0001$)

Discussion

Our study provides an analysis of a subset of bacterial strains consecutively isolated from surgical wound infections from hospitalised patients in 2018. We observed that the most frequent strains were Gram-negative *Enterobacterales*, followed by Gram-positive *S. aureus* and *E. faecalis* strains. All these strains are included in the short list of the most dangerous microorganisms in terms of resistance, known under the acronym ESCAPE (*E. faecium, S. aureus, Clostridioides difficile, A. baumannii, P. aeruginosa*, and *Enterobacteriaceae*).
The strains included in this study exhibited high rates of antibiotic resistance, 56% being MDR, and produced a spectrum of soluble virulence factors involved in the colonization of the wounded tissue with the subsequent occurrence of the wound infection, coupled with the relatively high rates of biofilm production on the inert substratum and adherence to eukaryotic cells (30). Our findings are consistent with previous studies, including *S. aureus* strains exhibiting a MDR phenotype (31) or harbouring the Panton-Valentine leukocidin (PVL) (32) and MDR *Enterobacterales* (33). These results raise concerns regarding the prophylactic antibiotic

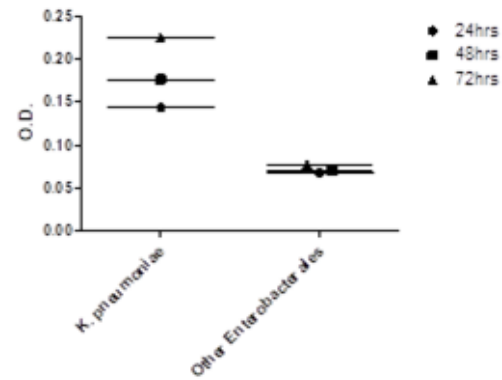

Fig. 4. ***K. pneumoniae*** **had a higher capacity to biofilm formation, compared to other** ***Enterobacterales*** **strains ($p < 0.0001$) analysed in this study.**

treatment of patients in order to prevent these infections (34), the potential of the nosocomial spreading and also the potential of a severe outcome in cases like bacteraemia and sepsis (11). Moreover, the capacity of biofilm formation may lead to antimicrobial tolerance and ultimately to therapeutic failure (11, 35).

The virulence and antibiotic resistance of bacterial strains isolated from SSI was scarcely investigated in the neighbouring countries. The prevalence of enterobacterial species, especially *K. pneumoniae*, in the aetiology of SSI was observed in a Bulgarian teaching hospital, emphasizing the high rates of ESBL (36), consistent with the present study. On the other hand, a study from Ukraine (37) reports high prevalence of *S. aureus*, followed by *E. coli*, *P. aeruginosa* and *E. faecalis*, highlighting the high rates of resistance to oxacillin and teicoplanin amongst Gram-positive isolates.

The high percentage of resistance to first line antibiotics (i.e. erythromycin, amoxicillin plus clavulanic acid, ciprofloxacin, etc.) described in the analysed strains points to (at least initial) therapeutic failure, since these antibiotics are usually chosen as empirical therapy until the antibiotic susceptibility testing results are available. Furthermore, our results pointed out the bacterial adherence to eukaryotic cells, with variable patterns and intensity, which is a key step in biofilm development and tissue invasion.

This study was designed to provide a snapshot of the virulence and antibiotic resistance of bacterial strains isolated from positive samples of SSI, hence its limitations, by not taking into account the type of surgical wound, the bacterial strain clonality, as well as not considering information regarding patient outcome.

Conclusions

This study represents the first comprehensive analysis of bacterial strains isolated from SSI of hospitalized patients in Romania. The analysed strains expressed a large spectrum of virulence features, coupled with high rates of antibiotic resistance (especially for *S. aureus* and enterobacteria). These features explain the survival and resistance of the bacterial strains in the hospital environment (i.e. biofilm formation), the ability to initiate an infectious process and to produce tissue lesions (through the production of pore-forming toxins, proteolytic enzymes and DN-ases).

These data suggest that microbial resistance and virulence phenotypes of the wound-related pathogens may be diverse, and they could differently impact on the outcome of the disease.

Abbreviations

ESCAPE - *Enterococcus faecium, Staphylococcus aureus, Clostridioides difficile, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacterales*
PCR – polymerase chain reaction
CFU – colony forming units
PBS - phosphate buffered saline
MRSA - methicillin-resistant *S. aureus*
MSSA - methicillin-susceptible *S. aureus*
MDR – multidrug resistance
MLS_B – Macrolide-lincosamide-streptogramin B
SCC*mec* – staphylococcal cassette chromosome *mec*
SSI – surgical site infection
PVL - Panton-Valentine leukocidin

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Authors' contribution

SMR – conceptualization, methodology, validation, formal analysis, investigation, data curation, writing – original draft preparation; WNA – draft preparation, review and editing; HBM – methodology, validation; ICB – methodology, formal analysis, software, investigation, data curation, writing – original draft preparation; AMH – methodology, formal analysis, resources; IG – methodology, validation, resources; OB – resources; OSS – investigation, resources; MCC – writing – review and editing, funding acquisition, supervision; GM – supervision, project administration

Conflict-of-Interest Statement

The authors declare no conflict of interests.

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