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Molecular characterization of resistance and biofilm genes of ESKAPE pathogens isolated from clinical samples: examination of the effect of boric acid on biofilm ability by cell culture method

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Abstract

Biofilm formation ranks first among the resistance and virulence factors crucial in forming ESKAPE pathogens. Once biofilm is formed, treating the infection with existing drugs is often futile. Therefore, in this study, resistant ESKAPE pathogens were isolated from intensive care units and sent to Atatürk University Yakutiye Research Hospital Microbiology Laboratory. This study investigated the biofilm formation and molecular characterization of resistant ESKAPE pathogens isolated from intensive care units. The bacteria's biofilm formation abilities, genes responsible for biofilm formation, and resistance characteristics were identified. The effect of boric acid (BA) on resistance and bacterial genes was evaluated by a bacterial infection cell culture model. The highest biofilm formation was observed in *Escherichia coli*, *Enterococcus spp*., and *Pseudomonas aeruginosa Enterococcus spp*. isolates showed the *vanA* gene in 14.6% and the *vanC* gene in 61% of the samples. Among *Staphylococcus spp*. isolates, 48.3% were MSSA, 34.5% were MRCNS, and 17.2% were MRSA. The *KPC* gene was detected in 50%, the *OXA-48* gene in 40%, and the *NDM* gene in 15% of the isolates. In *P. aeruginosa*, the *Las*I and *Las*R quorum sensing system genes were found in 38.5% and 30.8% of the isolates, respectively. In *E. coli* isolates, *OXA-48* was present in 35%, *KPC* in 31.7%, and *TEM* in 12.5%. BA demonstrated significant activity against ESKAPE pathogens. The combined antimicrobial activity of boron compounds showed a decrease in the expression level of the resistance gene. It will be promising for preventing hospital-associated infections.

Keywords Antibacterial Effect, Boric Acid, Biofilm, Biofilm gene, ESKAPE, Resistance Gene

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Introduction

One of the main issues of the modern era is the advent of bacterial infections that have developed resistance to practically all antibacterial agents [1]. These pathogens are also named "superbugs," particularly *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter spp* (ESKAPE) [2, 3]. Various resistance mechanisms, including drug inactivation, alteration of drug binding sites/targets, changes in cell permeability, and mutation, cause antimicrobials' ineffectiveness against certain diseases [4]. The development of enzymes, such as extended-spectrum β-lactamase (ESBL) and carbapenemase like metallo-β-lactamase (MBL), that permanently inactivate β-lactam antibiotics has helped Gram-negative members of ESKAPE pathogens thrive in clinical settings [5]. When treating multidrug-resistant (MDR) Gram-negative bacterial infections, carbapenems are sometimes referred to as resort antibiotics since they also have action against ESBL-producing pathogens. However, ESKAPE that produce MBLs have Gram-negative members resistant to carbapenems, making them a severe concern in clinical settings [5]. A layer of cell clusters formed from microorganisms known as biofilm is encased in an extracellular polysaccharide matrix known as polysaccharide intercellular adhesins. Research has demonstrated that ESKAPE infections can generate biofilms, which provide them resistance to antibiotics by obstructing the entry of the antimicrobial agent [6]. Human plasma boron concentrations have been found to range between 10 and 20 μM, with BA being the most common soluble form of boron in plasma [7]. BA has a wide range of biological impacts, and research into how it affects health is becoming increasingly popular. Research has shown that BA regulates the inflammatory response, reduces oxidative stress in several disorders, and supports bone health and embryonic development [8]. BA is a boron derivative with antibacterial, antifungal, antioxidant, anticancer, and anti-inflammatory properties [9–11]. Antibiotics, which are ineffective in the treatment of biofilm-forming bacterial infections, will increase the effect of antibiotics if combined with antibiofilm agents and thus become available for the treatment of biofilm-associated infections. In line with the data obtained from recent studies, using nanoparticles with antimicrobial and antibiofilm properties against microbial biofilms has shown the necessity of new nanotechnological approaches. Here, we aimed to investigate the resistance genes exhibited by ESKAPE pathogens, biofilm formation, and the effect of BA on these resistance genes in L929 fibroblast cells. In addition, we planned to determine the expression levels of the genes exhibited by the PCR method after BA treatment.

Materials and methods

Ethical consideration and bacterial strains

This study was conducted on 24.02.2022 (Meeting No: 2 Decision No: 37) with the permission of the Atatürk University Non-interventional Clinical Research Ethics Committee. Sample collection was performed following the relevant guidelines and regulations. The patient did not directly participate in this research work and informed Consent was obtained from the patient. Cultures of clinical samples belonging to patients sent from intensive care units to the Microbiology Laboratory of Atatürk University Faculty of Medicine Training and Research Hospital were performed by examining the collected strains. Bacterial identifications were completed using conventional methods (colony morphology, Gram staining characteristics, catalase, and in vitro coagulase tests), and species-level identification was achieved with VITEK 2 in addition to conventional methods. After identifying the bacteria, antibiotic resistance was determined using VITEK 2 and conventional methods. Antibiotic susceptibility testing (ADT) was performed using VITEK 2 or Kirby-Bauer disc diffusion method, using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria. A total of 182 isolates were studied.

Antibiotic susceptibility testing of ESKAPE pathogens and storage of resistant isolates

Antibiotics used for all pathogens were selected, taking EUCAST recommendations into account. It was made using Mueller–Hinton agar, and its values were evaluated according to EUCAST criteria. Resistant ESKAPE isolates were stored at −80 ˚C in a 20% glycerol broth medium.

Biofilm formation

Antibiotics used for all pathogens were selected, taking EUCAST recommendations into account [12]. Biofilm formation was investigated using two different methods.

Detection of biofilm formation by quantitative microdilution plate method

After all strains used in our study were inoculated into Luria Bertani Broth medium (Merck, Germany) following the Mac Farland 0.5 standard, they were incubated at 37 ˚C for 24 h. The next day, 1/100 dilutions were made from the growth media, and 10 μl of bacteria and 190 μl of medium were added to the experimental wells in flat-bottomed microplates. Three wells of each bacterium were used for each experiment. Each sample was transferred to three wells of a 24-well polystyrene plate (Corning, USA). As a negative control, 200 μl of medium alone was added to one well for each sample. After the samples were transferred, the plates were closed and incubated

at 37 ˚C for 48 h. After the incubation period, the wells of the plates were washed gently with phosphate buffer (PBS) (Sigma, USA). After washing, the plates were turned upside down and allowed to dry completely at room temperature. After drying, 200 µl of 1% crystal violet solution (Sigma, USA) was added to the wells and left to stand for 45 min. After waiting for 15 min, the crystal violet solution was washed with phosphate buffer. After washing, the plates were turned upside down and left to dry at room temperature. The biofilm layer stained with crystal violet was dissolved by adding 200 µl of ethanol solution to the completely dried plates. Then, the amount of crystal violet was measured with a UV visible-spectrophotometer device (Thermo Fisher Scientific, USA) at 570 nm. They were compared with controls [13].

Examination of biofilm formation by Congo red agar method

To prepare the Congo red medium, 50 g of sucrose, 37 g of BHI (brain–heart infusion) broth agar (Sigma, USA), and 0.8 g of Congo red (Sigma USA) were thoroughly homogenized into 1 L of distilled water. After the strains were planted on Congo red agar, they were incubated at 37 ˚C for 24 h. As a result of incubation, the strains that formed dry crystallized black colonies were considered biofilm positive, and the strains that formed red or pink colonies were considered biofilm negative. All pathogens were studied in 2 parallels [13].

Molecular analyses

DNA isolation

The boiling method was used to extract genomic DNA. Centrifugation, 3 ml LB bacteria formed after overnight incubation at 37 °C in medium suspension. After dissolving the pellet in 1000 ml, sterile water vortexed once more and centrifuged. These steps were performed three times. After completion, it was vortexed in 300 ml of apyrogenic water, and ten was allowed to boil for minutes. Then, at 13,000 rpm for ten minutes, centrifuged. In PCR experiments, supernatants were used as molds [13].

The primers used for all molecular studies performed are listed in Table 1. All PCR amplification was performed using Bio-Rad (Richmond, USA) for a thermal cycler and Taq DNA polymerase (New England Biolabs (NEB), USA). To prepare the components of the PCR reaction, a total volume of 25 µl of PCR mixture [Genomic DNA: 2 µl, 10X buffer: 2.5 µl, dNTPs: 1 µl, primer forward, 10 pmol: 0.5 µl, primer reverse, 10 pmol: 0.5 µl, Taq polymerase enzyme (Thermo EP0702, 5 U/µl): 0.25 µl, made up to 25 µl with deionized water) was prepared.

Table 1 Primer sequences used in in house PCR

Gene	*Primers*	*Product (bp)*
icaA	F- 5′- ACACTTGCTGGCGCAGTCAA –3′ R- 5′- -TCTGGAACCAACATCCAACA –3′	188
icaD	F- 5′- ATGGTCAAGCCCAGACAGAG –3′ R- 5′- AGTATTTTCAATGTTTAAAGCAA- 3′	198
van A	F- 5′- GGGAAAACGACAATTGC-3′ R- 5′- GTACAATGCGGCCGTTA-3′	732
van C	F- 5′- GAAAGACAACAGGAAGACCGC-3′ R- 5′- ATCGCATCACAAGCACCAATC-3′	796
gel E	F- 5′- TATGACAATGCTTTTTGGGAT-3′ R- 5′- AGATGCACCCGAAATAATATA –3′	213
esp	F- 5′- AATTGATTCTTTAGCATCTGG-3′ R- 5′- CGGAGGAGTAATTACAAACCTGGCA3′	510
cly-A	F- 5′- ACTCGGGGATTGATAGGC-3′ R- 5′- GCTGCTAAAGCTGCGCTT-3′	688
hyl	F- 5′- ACAGAAGAGCTGCAGGAAATG-3′ R- 5′- GACTGACGTCCAAGTTTCCAA-3′	276
asa1	F- 5′- GCACGCTATTACGAACTATGA-3′ R- 5′- TAAGAAAGAACATCACCACGA-3′	375
acm	F- 5′- GGCCAGAAACGTAACCGATA-3′ R- 5′- AACCAGAAGCTGGCTTTGTC-3′	135
tetA	F- 5′- GGTTCACTCGAACGACGTCA –3′ R- 5′- CTGTCCGACAAGTTGCATGA –3′	576
tet B	F- 5′- TTGGTTAGGGGCAAGTTTTG –3′ R- 5′- GTAATGGGCCAATAACACCG –3′	659
sul1	F- 5′- CGGCGTGGGCTACCTGAACG –3′ R- 5′- GCCGATCGCGTGAAGTTCCG –3′	433
gnr A	F- 5′- CAGCAAGAGGATTTCTCACG –3′ R- 5′- AATCCGGCAGCACTATTACTC –3′	360
gnr B	F- 5′- GGCTGTCAGTTCTATGATCG –3′ R- 5′- GAGCAACGATGCCTGGTAG –3′	488
gnr S	F- 5′- GCAAGTTCATTGAACAGGGT –3′ R- 5′- TCTAAACCGTCGAGTTCGGCG –3′	428
aac(3)-IIa	F- 5′- GCAATAACGGAGGCGCTTCAAAA –3′ R- 5′- TTCCAGGCATCGGCATCTCATACG-3′	563
ant (3)-Ia	F- 5′- TCGACTCAACTATCAGAGG –3′ R- 5′- ACAATCGTGACTTCTACAGCG –3′	542
ant (2-)Ia	F- 5′- CGCCGTGGGTCGATGTTTGATG –3′ R- 5′- TTTTCCGCCCCGAGTGAGGTG –3′	572
bla CTX-M	F- 5′- ATGTGCAGYACCAGTAARGT –3′ R- 5′- TGGGTRAARTARGTSACCAGA –3′	593
bla TEM	F- 5′- CGCCGCATACACTATTCTCAGAATG –3′ R- 5′- ACGCTCACCGGCTCCAGATTTAT –3′	445
bla OXA 48	F- 5′- GCG TGG TTA AGG ATG AAC AC –3′ R- 5′- CAT CAA GTT CAA CCC AAC CG –3′	438
bla SHV	F- 5′- CTTTATCGGCCCTCACTCAA –3′ R- 5′- AGGTGCTCATCATGGGAAAG –3′	237
bla KPC	F- 5′- CGTCTAGTTCTGCTGTCTTG –3′ R- 5′- CTTGTCATCCTTGTTAGGCG-3′	798
bla NDM	F- 5′- GGTTTGGCGATCTGGTTTTC –3′ R- 5′- CGGAATGGCTCATCACGATC –3′	621
bla IMP	F- 5′- GGAATAGAGTGGCTTAAYTCTC –3′ R- 5′- GGTTTAAYAAAACAACCACC –3′	232
bla SPM	F- 5′- AAAATCTGGGTACGCAAACG –3′ R- 5′- ACATTATCCGCTGGAACAGG –3′	271
bla VIM	F- 5′- GATGGTGTTTGGTCGCATA –3′ R- 5′- CGAATGCGCAGCACCAG –3′	390

Table 1 (continued)

Gene	Primers	Product (bp)
LasI	F- 5′- ATGATCGTACAAATTGGTCGGC –3′ R- 5′- GTCATGAAACCGCCAGTCG –3′	605
LasR	F- 5′- ATGGCCTTGGTTGACGGTT –3′ R- 5′- GCAAGATCAGAGAGTAATAAGACCCA –3′	725
mrkA	F- 5′- ACGTCTCTAACTGCCAGGC –3′ R- 5′- TAGCCCTGTTGTTTGCTGGT –3′	115
wbbM	F- 5′- ATGCGGGTGAGAACAAACCA –3′ R- 5′- AGCCGCTAACGACATCTGAC –3′	122
wzm	F- 5′- TGCCAGTTCGGCCACTAAC –3′ R- 5′- GACAACAATAACCGGGATGG –3′	148
luxS	F- 5′- AGTGATGCCGGAACGCGG –3′ R- 5′- CGGTGTACCAATCAGGCTC –3′	148
OXA-23	F- 5′- GAT CGG ATT GGA GAA CCA GA –3′ R- 5′- ATT TCT GAC CGC ATT TCC AT –3′	501
OXA-24	F- 5′- GGT TAG TTG GCC CCC TTA AA –3′ R- 5′- AGT TGA GCG AAA AGG GGA TT –3′	246
OXA-51	F- 5′- TAA TGC TTT GAT CGG CCT TG –3′ R- 5′- TGG ATT GCA CTT CAT CTT GG –3′	353
OXA-51	F- 5′- AAG TAT TGG GGC TTG TGC TG –3′ R- 5′- CCC CTC TGC GCT CTA CAT AC –3′	599

Determination of ESBL genes in E. coli isolates by PCR

Using the PCR method, E. coli isolates were evaluated for molecular screening of *blaKPC* and *blaOXA-48* genes. The amplification program for *KPC* and *OXA-48* genes consisted of initial denaturation at 94 ˚C for 5 min, 36 cycles of denaturation at 94 ˚C for 30 secs, annealing at 62 ˚C for 40 s, extension at 72 ˚C for 50 secs, and final step at 72 ˚C for 5 min [14].

Detection of Intracellular Adhesion (ica) Gene in Staphylococcus spp. Isolates

All *Staphylococcus spp.* isolates were evaluated for molecular screening of *ica*A and *ica*D genes using PCR. The amplification program for *ica*A consisted of an initial denaturation at 94 ˚C for 5 min, 30 cycles of denaturation at 94 ˚C for 60 secs, annealing at 55 ˚C for 60 s, extension at 72 ˚C for 60 secs, and a final step at 72 ˚C for 10 min. The amplification program for *ica*D consisted of initial denaturation at 94 ˚C for 5 min, 30 cycles of denaturation at 94 ˚C for 30 s, annealing at 55 ˚C for 30 s, extension at 72 ˚C for 6 s, and final step at 72 ˚C for 10 min [15].

PCR detection of virulence genes in enterococcal isolates

All enterococcal isolates were evaluated for molecular screening of *esp*, *acm*, *hyl*, *asa*1, *gel*E, and *cyl*A genes and vancomycin resistance genes *van*A and *van*C genes using the PCR method. The amplification program for *esp*, *acm*, *hyl*, *asa*1, *gel*E, and *cyl*A genes consisted of initial denaturation at 95 ˚C for 10 min, 30 cycles of denaturation at 94 ˚C for 30 s, annealing at 56 ˚C for 30 s, extension at 72 ˚C for 30 s and a final step at 72 ˚C for 10 min. *van*A consisted of 10 min initial denaturation at 95 ˚C, 1 min 30 cycle denaturation at 94 ˚C, 1 min annealing at 57 ˚C, 60 s extension at 72 ˚C and 10 min final step at 72 ˚C and *van*C gene consisted of 5 min initial denaturation at 94 ˚C, 1 min 30 cycle denaturation at 94 ˚C, 1 min 30 cycle denaturation at 94 ˚C, 1 min annealing at 50 ˚C, 60 s extension at 72 ˚C and 2 min final step at 72 ˚C [16, 17].

PCR detection of antibiotic resistance genes and virulence genes in Enterobacter spp. isolates

All *Enterobacter* spp. isolates were evaluated for molecular screening of *tet*A, *tet*B, *sul*1, *qnr*ABS, *aac*(3), *ant*(3)-Ia, *ant*(2)-Ia, TEM, SHV, and *CTX-M* genes by PCR. The amplification program for *tet*A, *tet*B, and sul1 genes consisted of initial denaturation at 95 ℃ for 5 min, 34 cycles of denaturation at 94 ˚C for 20 s, annealing at 55 ˚C for 20 s (60 ˚C for sul1), extension at 72 ˚C for 20 s and final step at 72 ˚C for 5 min. The amplification program for AAC(3), *ant*(3)-Ia, *ant*(2)-Ia genes consisted of initial denaturation at 94 ˚C for 5 min, 32 cycles of denaturation at 94 ˚C for 45 s, annealing at 55 ˚C for 1 min (58 ℃ for *ant*(3)-Ia), extension at 72 ˚C for 40 s and final step at 72 ˚C for 10 min [18].

PCR detection of quorum sensing and antibiotic resistance genes in P. aeruginosa isolates

All *P. aeruginosa* isolates were evaluated for molecular screening of OXA-48, KPC, *NDM*, *IMP*, *SPM*, *VIM*, *Las*I, and *Las*R genes using the PCR method. The amplification program for *OXA-48*, *KPC*, *NDM*, *IMP*, *SPM*, and *VIM* genes consisted of initial denaturation at 94 ˚C for 5 min, 36 cycles of denaturation at 94 ˚C for 30 s, annealing at 62 ˚C for 40 s (*IMP*, *SPM* 52 ˚C, *VIM* 55 ˚C for 30 s) and extension at 72 ˚C for 50 s and final step at 72 ˚C for 5 min. The amplification program for *Las*I, *Las*R genes consisted of initial denaturation at 95 ˚C for 5 min, 34 cycles of denaturation at 94 ˚C for 30 s, annealing at 50 ˚C for 30 s, extension at 72 ˚C for 2 min, and final step at 72 ˚C for 10 min [14, 19].

PCR detection of antibiotic resistance and biofilm production genes in K. pneumoniae isolates

All *K. pneumoniae* isolates were evaluated for molecular screening of *OXA-48*, *KPC*, *NDM*, *IMP*, *VIM*, *mrk*A, *lux*S, *wbb*M, and wzm genes by PCR. The amplification program for *mrk*A, *lux*S, *wbb*M, and wzm genes consisted of initial denaturation at 94 ˚C for 5 min, 30 cycles of denaturation at 94 ˚C for 60 s, annealing at 55 ˚C for 30 s, extension at 72 ˚C for 1 min and final step at 72 ˚C for 10 min [19, 20].

PCR detection of antibiotic resistance genes in A. baumannii isolates

All *A. baumannii* isolates were evaluated using PCR (Thermo Fisher Scientific, USA) for molecular screening of *OXA*−23, 24, 51, 58, *SPM*, *IMP*, and *VIM* genes. The same protocol was applied for resistance genes as for *P. aeruginosa*. The amplification program for *OXA* genes consisted of initial denaturation at 94 ˚C for 10 min, 36 cycles of denaturation at 94 ˚C for 30 s, annealing at 52 ˚C for 40 s, extension at 72 ˚C for 50 s, and final step at 72 ˚C for 5 min [19, 21].

Effect of BA on biofilm-forming genes by cell culture method

Fibroblast L929 cell line was used in this study. Fibroblast L929 cell line will be produced in 25 cm 2 flasks in 5 mL RPMI 1640 (Sigma, USA) medium containing 10% Fetal Bovine Serum (FBS) (Sigma, USA) in a CO_2 (5%) incubator at 37 ˚C in an antibiotic-free environment. Before the experiment, fibroblast cells were inoculated into a 96-well plate (Corning, USA) with 8×10^4 cells in each well and incubated for 24 h. Bacteria were incubated in MHB (Merck, Germany) medium at 37 ˚C for 18 h.

Infection Model

Bacterial density was measured on a spectrophotometer (Mapada, China) and diluted with 0.9% NaCl to approximately 1×10^6 CFU/ml according to 0.5 McFarland. The wound healing model was done by 100 µl yellow pipet head. Then the bacterial suspension was added for 2 h. 2 h after the infection, the wells were washed twice with PBS, and fresh medium RPMI (containing 10% FBS) was added. BA compound was added to the wells and stayed for 24 h.

Viability measurement analysis of bacterial and eukaryotic cells

After BA was added due to the 4-h- and 18-h incubation periods, CFU and live cell counts were performed in all wells with and without molecule addition. After infection, the wells where boron compounds were/were not applied were washed with Phosphate buffered saline (PBS) (Sigma, USA). Then, Trypsin was treated with Ethylenediaminetetraacetic acid (EDTA) (Sigma, USA) and diluted with 100 µL of medium. Dilutions of 10^3–10^5 were inoculated into MHA medium (Sigma, USA), and colony counting was performed after 18 h. At the same time, live cell count was determined.

Total Oxidant Status (TOS)

In the TOS (Merck, Germany) analysis, the procedure steps were examined using the commercial kit that was readily available, and the calculation was made according to the given formula. Standards and samples were taken in the specified quantity. The specified amount of Reagent 1 was added, and it was read after 30 s. The specified amount of Reagent 2 was added, and after incubation at 37 ˚C, it was read at 530 nm.

TOS Calculation.

➢ A2-A1 = ΔAbs.

➢ Result = ΔAbs sample × 10/ΔAbs Standard.

Total Antioxidant Capacity (TAC)

In the TAC (Merck, Germany) analysis, the procedure steps were examined using the commercial kit that was readily available, and the calculation was made according to the given formula. Standards and samples were taken in the specified quantity. The specified amount of Reagent 1 was added, and it was read after 30 s (A1). The specified amount of Reagent 2 was added, and after incubation at 37 ˚C, it was read at 660 nm.

TAC Calculation.

$$A2 - A1 = \Delta Abs$$

$$\text{Result} = (\Delta Abs\ \text{Water} - \Delta Abs\ \text{sample}) / (\Delta Abs\ \text{Water} - \Delta Abs\ \text{Strandard})$$

Results

Bacterial strains and antibiotic sensitivity of microorganisms results

Of 182 people, 97 female and 85 male patients were included in the study (Fig. 1A). 29 of these patients are between the ages of 0–18, 9 are between the ages of 19–30, 11 are between the ages of 31–45, 33 are between the ages of 46–65, and 100 are over 65 years of age (Fig. 1B). A total of 182 ESKAPE pathogens were included in the study. The most frequently isolated bacteria were *E. coli* ($n=60$, 32.97%), followed by *Enterococcus* spp. ($n=41$, 22.53%), *Staphylococcus* spp. ($n=29$, 15.93%), *K. pneumoniae* ($n=20$, 10.99%), *P. aeruginosa* ($n=13$, 7.14%), *A. baumannii* ($n=11$, 6.04%), and *Enterobacter* spp. ($n=8$, 4.40%) (Fig. 2). There was growth in 115 urine cultures, 20 wound cultures, 11 aspirate cultures, 12 blood cultures, 11 ear cultures, 1 nose culture, 7 sputum cultures, and 5 CSF cultures of 182 patients (Fig. 3A). The majority of ESKAPE pathogens ($n=97$, 53.29%) were outpatients, followed by inpatients ($n=54$, 29.67%) and intensive care unit ($n=31$, 17.03%) (Fig. 3B), and shown antibiotics sensitivity (Fig. 4).

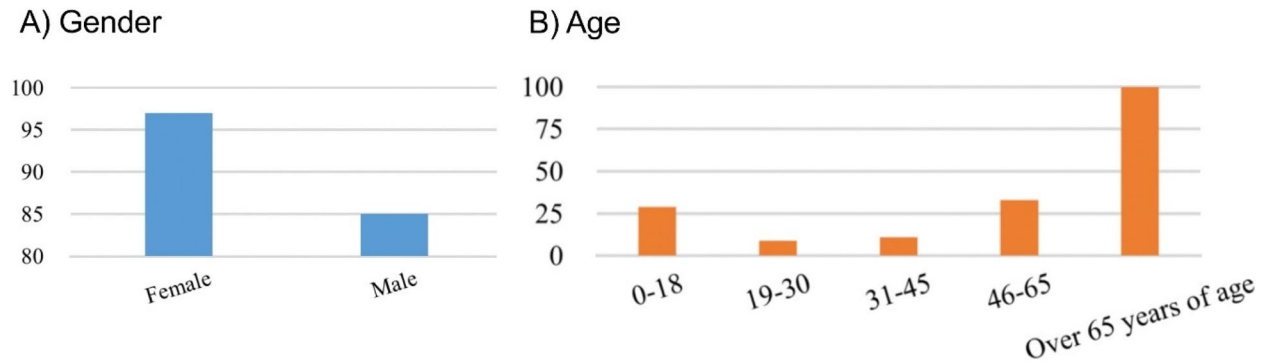


Fig. 1 Distribution of ESKAPE pathogens by (**A**)gender and (**B**) age

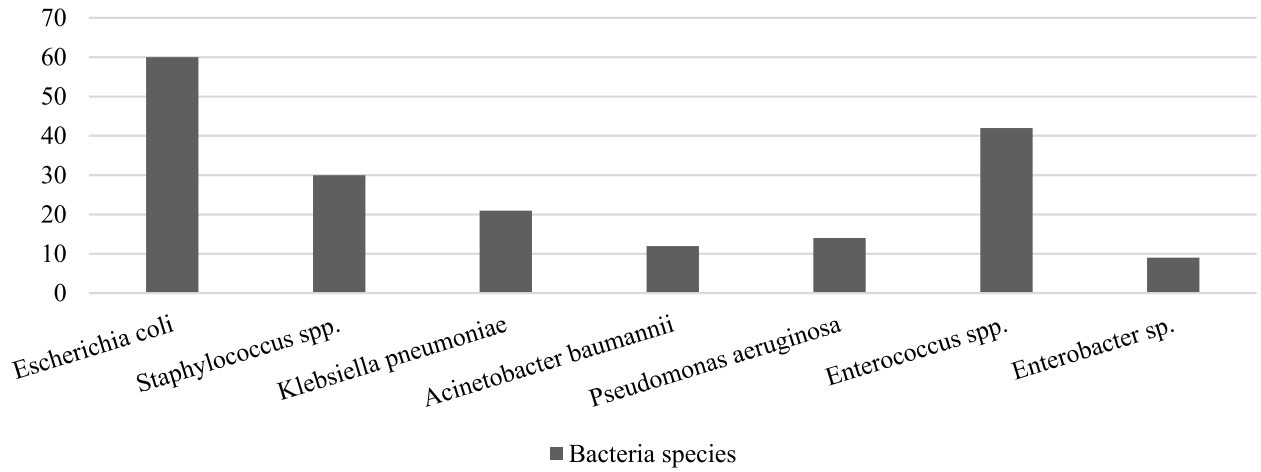


Fig. 2 Distribution of ESKAPE pathogens by bacterial type

Biofilm formation results

Detection of biofilm formation by quantitative microdilution plate method

The presence of biofilms of 182 bacteria was evaluated by the microdilution plate method. The results obtained are given in Fig. 5.

Congo red agar method biofilm formation results

The presence of biofilms belonging to 182 bacteria was evaluated by the Congo red agar method. The results obtained are given in Fig. 6.

In 60 *E. coli* samples, 24 were detected as very strongly positive, 6 as strongly positive, 1 as moderately positive, 4 as slightly positive, and 25 as negative. In 30 *Staphylococcus* spp. samples, 20 were detected as very strong positive, 1 as strong positive, 1 as low positive, and 8 as negative. In 21 K. *pneumoniae* samples, 10 were detected as very strongly positive and 11 as negative. In 12 *A. baumannii* samples, 2 were detected as very strong positive, 1 as low positive, and 9 as negative. Side-negative biofilm was detected in 12 of 14 *P.aeruginosa* samples. 42 *Enterococcus* spp. in the sample, 12 were detected as very strongly positive, 2 as moderately positive, 3 as slightly positive, and 25 as negative (Fig. 7).

Molecular results

Molecular results in E. coli isolates

The presence of *OXA-48* and *KPC* resistance genes in *E. coli* isolates was investigated by single PCR. The 60 isolates, 35% ($n=21$) contained the *OXA-48* gene, and 31.7% ($n=19$) contained the *KPC* gene.

Presence of Intracellular Adhesion (ica) gene in Staphylococcus spp. isolates

The presence of *ica*A and *ica*D adhesion genes in staphylococcal isolates was analyzed. Of the 29 Staphylococcal isolates, 48.3% ($n=14$) were MSSA isolates, 34.5% ($n=10$) were MRCNS isolates, and 17.2% ($n=5$) were MRSA isolates. The presence of *ica*A and *ica*D in the isolates was 55.2% ($n=16$) for both genes. In MSSA isolates, 42.9% ($n=6$) had *ica*A, 57.1% ($n=8$) had icaD, and

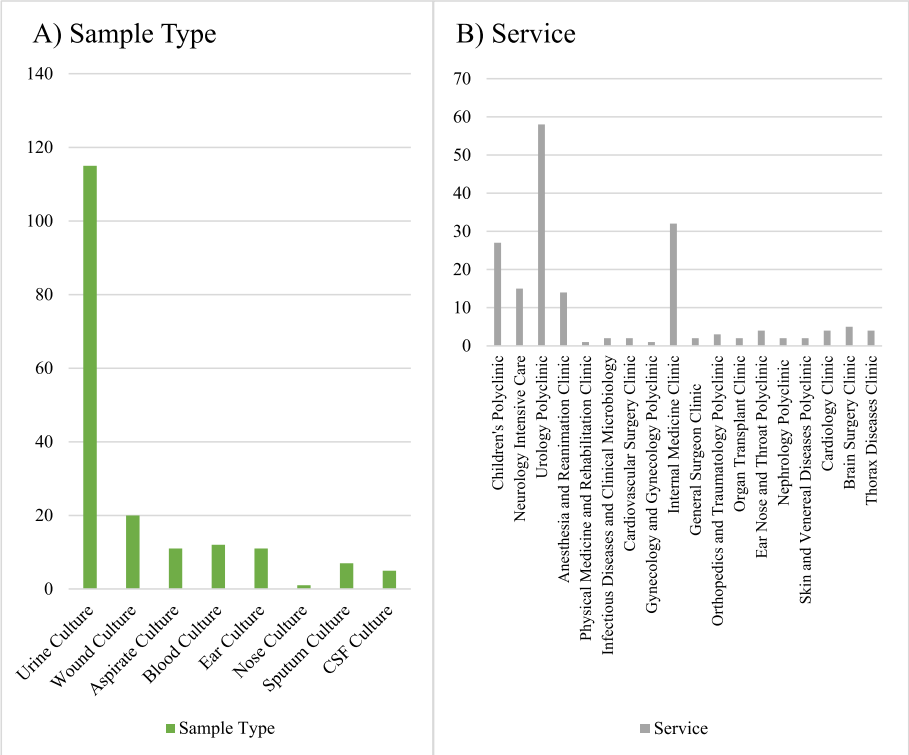


Fig. 3 Distribution of ESKAPE pathogens according to (**A**) sample type and (**B**) service

21.4% ($n=3$) had both genes. In MRCNS isolates, 80% ($n=8$) *ica*A, 40% ($n=4$) *ica*D, and 40% ($n=4$) both genes were detected together. Finally, 80% ($n=4$) *ica*A and 20% ($n=1$) *ica*D genes were detected in MRSA isolates. The presence of both genes together was not observed in MRSA isolates.

Presence of virulence genes in enterococcal isolates

Forty-one enterococcal isolates were analyzed for the presence of resistance genes. 12.2% ($n=5$) vancomycin resistance gene *van*A, 14.6% ($n=6$) vancomycin resistance gene *van*C, 61% ($n=25$) invasion gene (gelatinase) *gel*E and adhesin gene asa1, 48.8% ($n=20$) enterococcal surface protein *esp*, 19.5% ($n=8$) cytotoxin *cyl*A, 9.7% ($n=4$) collagen binding proteins *acm*, and 4.9% ($n=2$) invasion (hyaluronidase) *hyl* gene.

Presence of antibiotic resistance and virulence genes in Enterobacter spp. isolates

A total of 8 *Enterobacter* spp. isolates obtained from clinical samples were analyzed for antibiotic resistance genes and virulence genes. ESBL resistance gene TEM was detected in 12.5% ($n=1$) of the isolates. Other ESBL genes, SHV and *CTX*-M, were not observed. Tetracycline resistance genes *tet*A and *tet*B were detected in 100% ($n=8$) and 12.5% ($n=1$) of the isolates, respectively. *Sul*1, a sulfamethoxazole resistance gene, was observed in 50% ($n=4$) of the isolates. Quinolone resistance gene *qnr*A was detected in 12.5% ($n=1$), *qnr*B in 62.5% ($n=5$), and *qnr*S in 50% ($n=4$). Finally, *aac*(3)Ia gene was detected in 12.5% ($n=1$), *ant*(3)Ia gene in 50% ($n=4$) and *ant*(2) Ia gene in 75% ($n=4$) of the aminoglycoside resistance genes.

Presence of quorum sensing and antibiotic resistance genes in P. aeruginosa isolates

Thirteen *P. aeruginosa* isolates were analyzed for carbapenemase resistance genes and quorum sensing genes. 61.5% ($n=8$) of the isolates produced *OXA-48* and *KPC* genes. Other carbapenemase genes (*NDM-SPM*, *IMP*, and *VIM*) were detected in 7.7% ($n=1$), 15.4% ($n=2$), and 23.1% ($n=3$), respectively. In *P. aeruginosa*, two quorum sensing system genes, *Las*I and *Las*R, were detected in 38.5% ($n=5$) and 30.8% ($n=4$), respectively.

Antibiotic resistance and biofilm production genes in K. pneumoniae isolates

In a total of 20 K. *pneumoniae* isolates obtained from clinical samples, *lux*S, *mrk*A, and *wzm* were detected in 85% ($n=17$), 75% ($n=15$), and 70% ($n=14$) of biofilm-producing genes. Among carbapenemase genes, *IMP* and *VIM* genes were not detected, while the *KPC* gene was

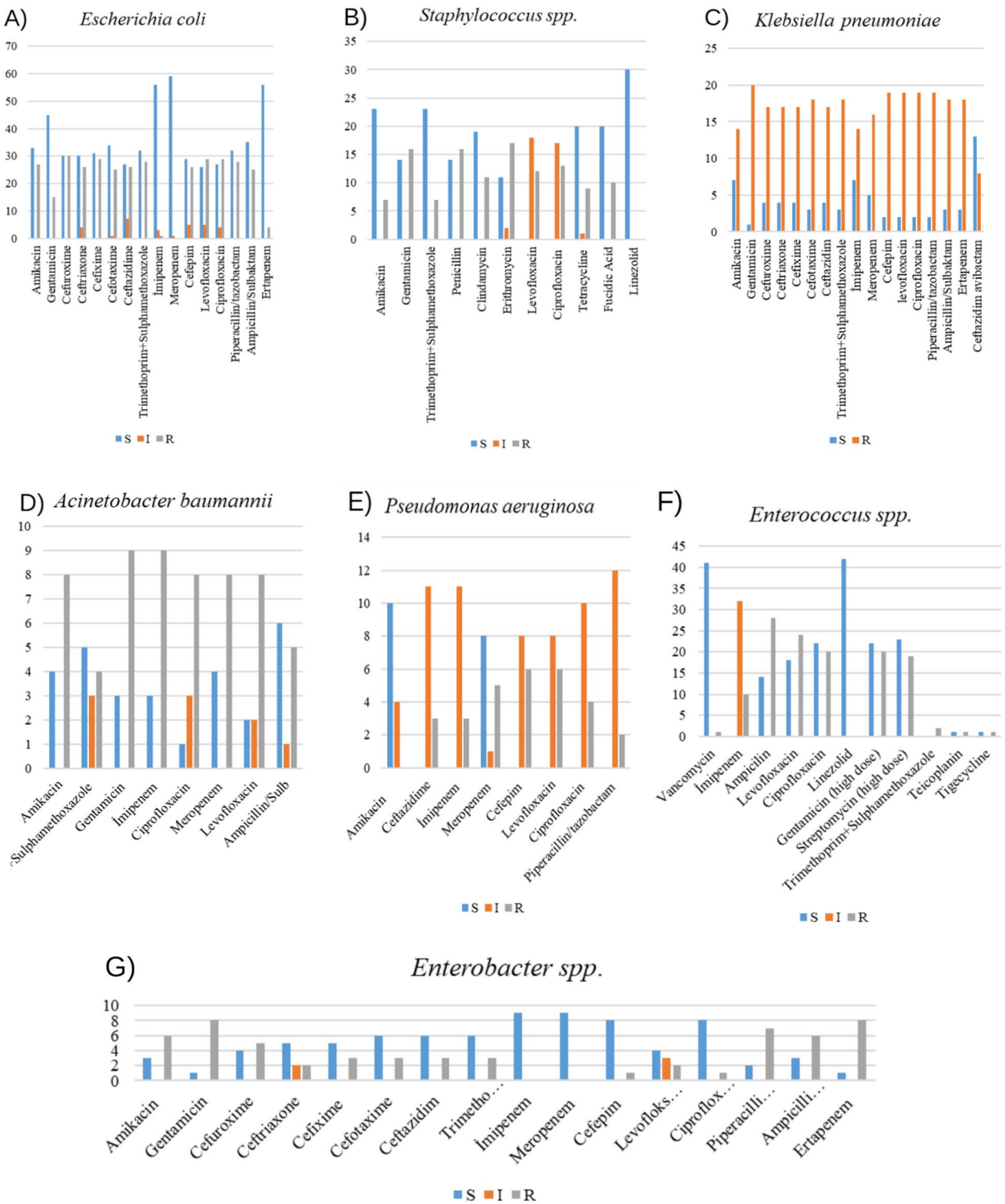


Fig. 4 Antibiotic susceptibility results of ESKAPE pathogens, *E. coli* (**A**), *Staphylococcus* spp. (**B**), *Klebsiella pneumoniae* (**C**), *Acinetobacter baumannii* (**D**), *Pseudomonas aeruginosa* (**E**), *Enterococcus* spp. (**F**), and (**H**) *Enterobacter* spp

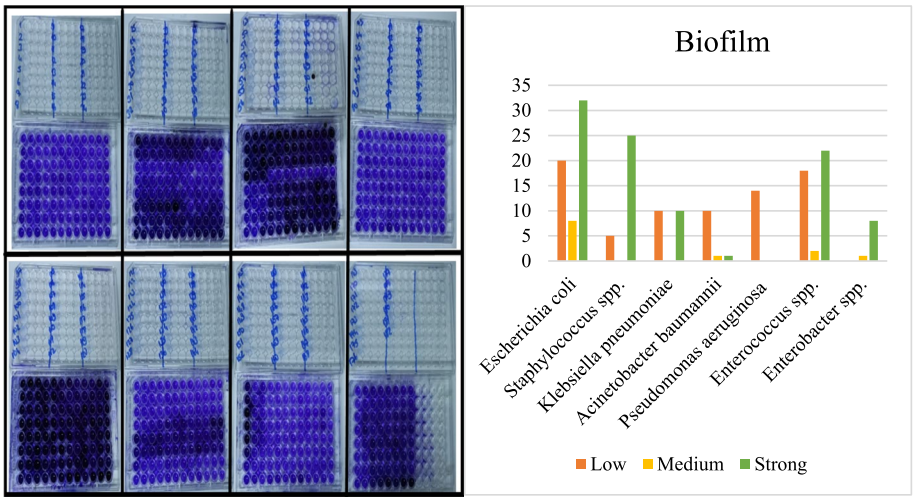


Fig. 5 Determination of biofilm formation of 182 bacteria by microdilution plate method

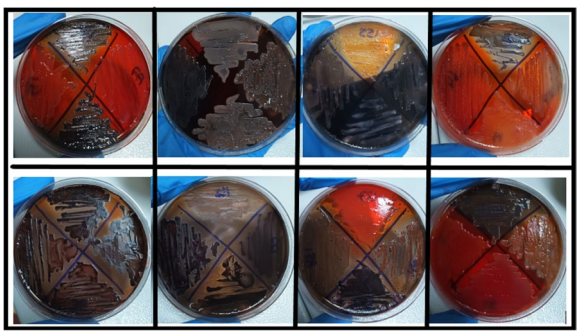


Fig. 6 Demonstration of the presence of biofilm by the Congo red agar method. Black: Strong positive biofilm, Red/Pink: Low positive, White: Negative

detected in 50% ($n=10$), the *OXA-48* gene in 40% ($n=8$), and the *NDM* gene in 15% ($n=3$).

Presence of antibiotic-resistance genes in A. baumannii isolates

Carbapenem resistance genes *OXA-23*, *OXA-51*, and *OXA*-58 were not observed in 11 *A. baumannii* isolates obtained from clinical samples. *IMP* was observed in 81.8% ($n=9$), *OXA-24* and *VIM* in 63.6% ($n=7$), and *SPM* in 27.3% ($n=3$) of the isolates.

Cell culture results

The efficacy of BA against the infection caused by ESKAPE pathogens on the L929 cell line was evaluated. The results obtained are given in Fig. 8. BA in Fig. 8-A shows toxicity in 8 and 16 mM/mL doses ($P<0.05$ and $P<0.001$ respectively). BA 1, 2 and 4 mM/mL did not show any difference in comparison with the control group. Figure 8 shows that boric acid decreased the *E. coli* population in the 2 and 4 mM/mL concentrations compared to the control group. BA decreased bacterial population in *Staphylococcus* spp., *P. aeruginosa, Enterococcus,* and *Enterobacter* 2 mM/mL ($P<0.05$). *K. pneumoniae* population decreased after 4 mM/mL administration ($P<0.05$). *A. baumannii* did not affect meaningfully ($P>0.05$).

TAC results

The efficacy of BA against the infection caused by ESKAPE pathogens on the L929 cell line was evaluated by TAC level. The results obtained are given in Fig. 9. BA in Fig. 9-A shows TAC capacity decreased in 8 and 16 mM/mL groups ($P<0.05$ and $P<0.001$ respectively). BA 1, 2 and 4 mM/mL did not show any difference in comparison with the control group. Figure 9 shows that BA 1, 2, and 4 mM/mL groups did not show a significant decrease in *E. coli, P. aeruginosa, Enterococcus,* and *Enterobacter* ($P>0.05$). Trolox level shows an increase in BA 2 mM/mL in *Staphylococcus, A. baumanni* ($P<0.05$), and 4 mM/mL in *K. pneumoniae* ($P<0.05$).

TOS results

The efficacy of BA against the infection caused by ESKAPE pathogens on the L929 cell line was evaluated by TOS level. The results obtained are given in Fig. 10. BA in Fig. 10-A shows TOS capacity decreased in 8 and 16 mM/mL groups ($P<0.05$ and $P<0.001$ respectively). BA 1, 2 and 4 mM/mL did not show any difference in

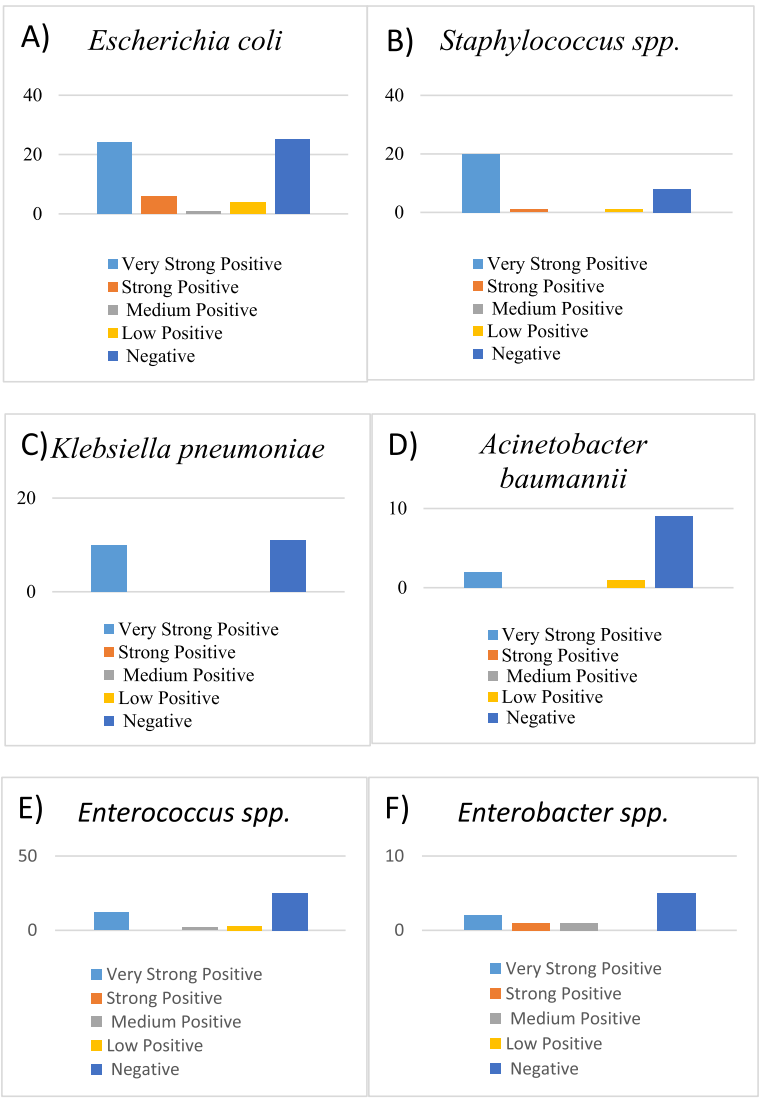


Fig. 7 Biofilm results of ESKAPE pathogens, *E. coli* (**A**), *Staphylococcus* spp. **B**, *Klebsiella pneumoniae* (**C**), *Acinetobacter baumannii* (**D**), *Enterococcus* spp. (**E**) and (**F**) *Enterobacter* spp

comparison with the control group. Figure 10 shows that BA 1, 2, and 4 mM/mL groups did not show a significant decrease in *E. coli, P. aeruginosa, A. baumanni*, *Enterococcus,* and *Enterobacter* ($P > 0.05$).

H_2O_2 level shows a decrease in BA 2 mM/mL in the *Staphylococcus* group ($P < 0.05$). BA 1, 2, and 4 mM/mL groups in *K. pneumoniae* show significantly decreased H_2O_2 levels ($P < 0.001$).

Discussion

The World Health Organization (WHO) prepared a global "priority pathogen list" for MDR bacteria in 2017 to promote research and create new effective antibiotics [22]. Antimicrobial-resistant ESKAPE pathogens pose a global threat to human health. The acquisition of antimicrobial resistance genes by ESKAPE pathogens has reduced treatment options for serious infections,

(See figure on next page.)

Fig. 8 Cell viability was assessed via the MTT test ($n = 12$). The consequences of *Escherichia coli, Staphylococcus spp., Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterococcus spp.,* and *Enterobacter spp* combined on the viability of L929 fibroblast cell line. BA 1, 2, 4, 8, and 16 mM/mL were used as a treatment. L929 Fibroblast (**A**), *E. Coli* (**B**), *Staphylococcus spp* (**C**), *Klebsiella pneumoniae* (**D**), *Acinetobacter baumannii* (**E**), *Pseudomonas aeruginosa* (**F**), Enterococcus *spp.* (**G**), and *Enterobacter spp.* (**H**) were applied to cell cultures in 96-well plates for 24 h. The average of three different experiments is used to represent the results. All results were compared to the control group. Meaningful statistically: * $P < 0.05$; ** $P < 0.001$

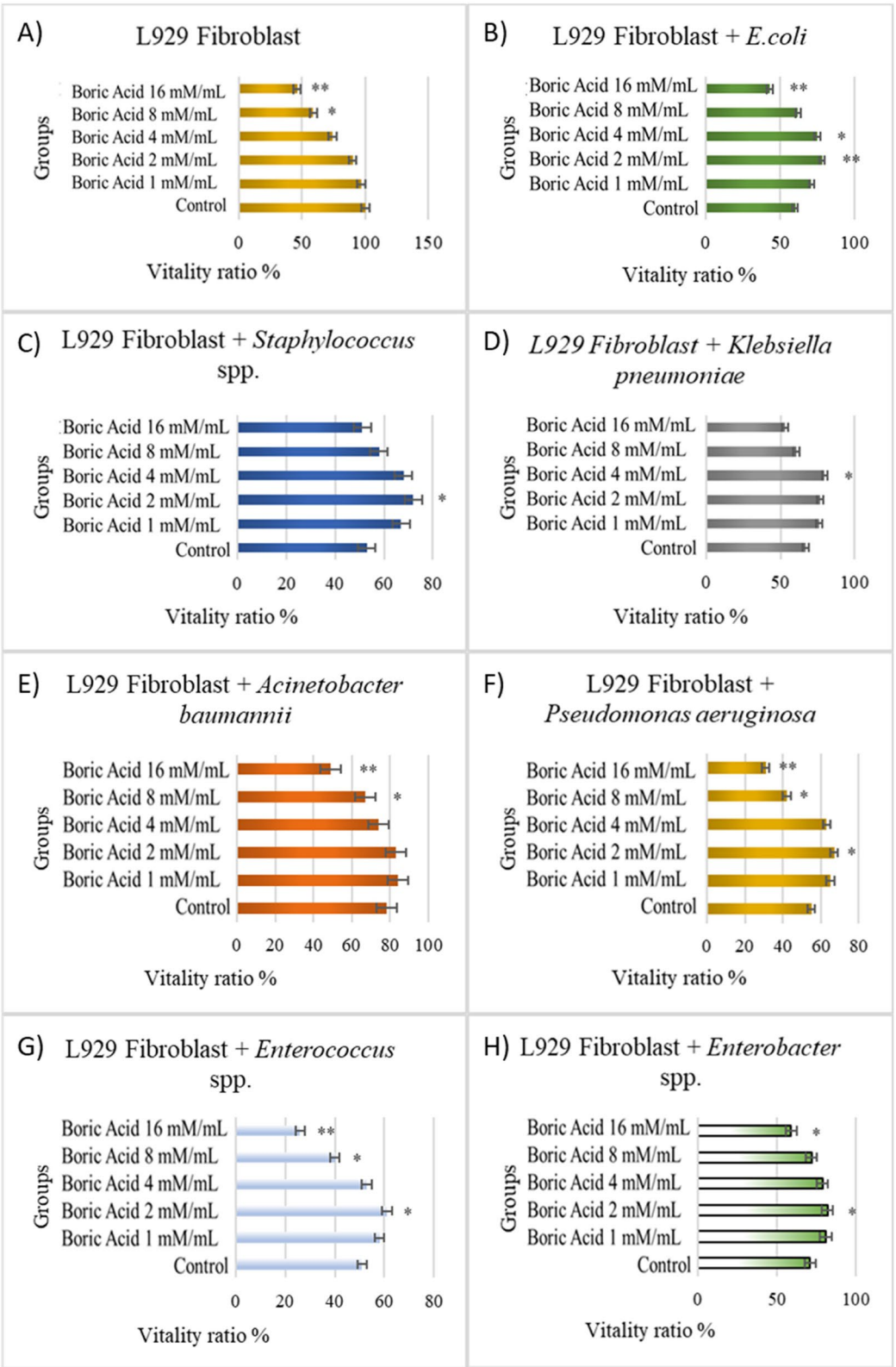


Fig. 8 (See legend on previous page.)

increased disease burden, and increased mortality rates due to treatment failure, thus requiring a coordinated global response for antimicrobial resistance surveillance [2, 23–25].

Among the various clinical samples processed, the incidence of infection with ESKAPE pathogens was highest in urine (63.19%), followed by wound (10.99%) and blood (6.59%) samples. This result is similar to that of Pandey et al. who isolated 49.9% from urine and 22.3% from wound samples in a similar study on 452 ESKAPE pathogens isolated from clinical samples in a hospital in Nepal [26]. The most frequently isolated bacteria were *E. coli* 60 (32.97%), followed by *Enterococcus* spp. 41 (22.53%), *Staphylococcus* spp. 29 (15.93%). This finding is consistent with the rates of Gram-positive and Gram-negative bacteria isolated from diabetic foot wounds by Orfali et al. Orfali et al. reported that *E. coli* with a rate of 9.5% and *Staphylococcus* spp. with a rate of 15.6% were among the most frequently detected microorganisms [27]. In fact, in studies conducted with gram-positive and gram-negative bacteria in different countries, it was found in Egypt (27.7%; 56%) and India (79%; 21%), respectively [28, 29].

The increase in antimicrobial resistance threatens the use of many antimicrobial agents currently available to treat infection. *E. coli* and *P. aeruginosa* (50%; 46.15%) isolates were resistant to cephalosporins (cefuroxime, cefotaxime, ceftazidime and cefepime), Staphylococcus spp. (58.62%) to macrolide (erythromycin), *K. pneumoniae* and *A. baumannii* (100%; 81.81%) to aminoglycosides (gentamicin), and *Enterococcus* spp. (68.29%) to penicillin-derived antibiotics (ampicillin). These findings are similarly dramatic to the antimicrobial resistance rates of ESKAPE pathogens in Arab countries between 2000 and 2020 [30]. At the same time, the antimicrobial resistance rates of the study were higher than European countries [31, 32].

The high level of antimicrobial resistance and biofilm formation among ESKAPE isolates indicate that biofilm formation is one of the important factors in the spread of antimicrobial resistance [26]. We evaluated the possible relationship between antimicrobial resistance and the ability to form biofilms among the collected isolates and found that 53.40% of the isolates were biofilm producers; and this result coincides with similar studies of Pandey et al. [26], Cepas et al. [33] Sanchez et al. [34].

In our study, when biofilm formation was examined by the quantitative microdilution plate method, the highest biofilm formation was determined in *E. coli, Enterococcus*, and *Pseudomonas* spp. samples, while the highest rate of biofilm formation by the Congo red agar method was observed in samples of *Staphylococcus, Klebsiella,* and *Enterobacter* species. In the study conducted by Chen et al. (2020) using the colorimetric microtiter plate method of *S. aureus* strains isolated from food samples and determining their ability to form biofilms [35]. They found that 72% of 97 *S. aureus* samples formed biofilms, and their results are high, consistent with our study. A doctoral thesis study in 2019, it was investigated biofilm formation using the quantitative microdilution plate method using 79 uropathogenic *E. coli* isolates and determined that all strains were biofilm producers [35]. In another thesis study, Tursun (2018) examined biofilm formation with 150 non-fermentative Gram-negative bacterial isolates using three different methods and determined that over 50% of the bacteria produced biofilm in each method [36]. When we look at the studies, we see results parallel to our research on biofilm formation, and the ability to form biofilms appears to be positively related to antimicrobial resistance.

Antibiotics are powerful weapons used in the treatment of bacterial infections. The development of antibiotic resistance increases with the inappropriate use of drugs and emerges as a global public health problem [37]. ESKAPE pathogens have developed resistance mechanisms to oxazolidinones, lipopeptides, macrolides, fluoroquinolones, tetracyclines, beta-lactams, beta-lactam-beta-lactamase inhibitor combinations, and last-line antibiotics, including carbapenems, glycopeptides, and clinically inappropriate polymyxins [23].

E. faecium is a leading cause of healthcare-associated infections, and hospital isolates are becoming increasingly resistant to vancomycin [38]. Glycopeptide resistance in enterococci involves the acquisition of van gene clusters that coordinate them. Nine different van gene clusters have been classified to date, with most human VRE infections being attributed to *E. faecium* and *E. faecalis* isolates carrying the *van*A and *van*B gene clusters. *van*A-mediated resistance occurs most frequently and is characterized by high levels of resistance to both vancomycin and teicoplanin [39]. In our study, *Enterococcus* spp. in the PCR scans of the isolates, the *van*A gene was

(See figure on next page.)

Fig. 9 Total Antioxidant Capacity was assessed via the TAC test ($n=6$) and Trolox Equiv/L.$^{-1}$. The consequences of *Escherichia coli, Staphylococcus spp., Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterococcus spp.,* and *Enterobacter spp* combined on the viability of L929 fibroblast cell line. BA 1, 2, 4, 8, and 16 mM/mL were used as a treatment. L929 Fibroblast (**A**), *E. Coli* (**B**), *Staphylococcus spp* (**C**), *Klebsiella pneumoniae* (**D**), *Acinetobacter baumannii* (**E**), *Pseudomonas aeruginosa* (**F**), Enterococcus *spp.* (**G**), and *Enterobacter spp.* (**H**) were applied to cell cultures in 96-well plates for 24 h. The average of three different experiments is used to represent the results. All results were compared to the control group. Meaningful statistically: * $P<0.05$; ** $P<0.001$

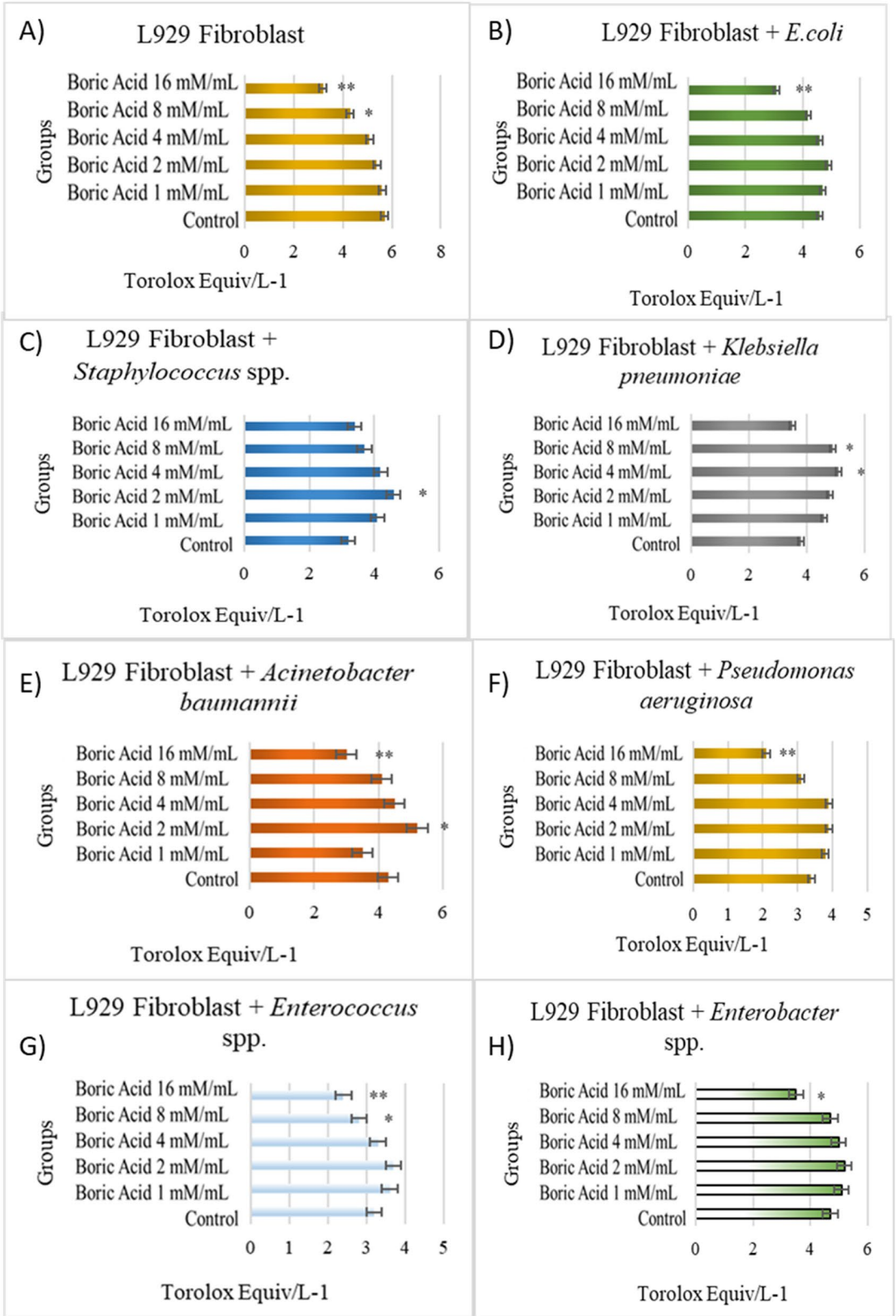


Fig. 9 (See legend on previous page.)

identified as 14.6%, and the *van*C gene was identified as 61%. Additionally, invasion gene (gelatinase) *gel*E and adhesin gene asa1, 48.8% ($n=20$) enterococcal surface protein esp, 19.5% ($n=8$) cytotoxin *cyl*A, 9.7% ($n=4$) collagen binding proteins *acm* and 4.9% ($n=2$.) The presence of virulence genes such as invasion (hyaluronidase) *hyl* was assessed by PCR. In a study conducted by Sattari Maraji et al. in 2019, the *van*A gene was high in the samples, and they also found the *acm* gene to be high among the virulence genes and stated that the *esp* and *gel*E genes followed it [40].

S. aureus is an essential human pathogen that causes both hospital- and community-acquired infections and can be an opportunistic pathogen in some cases [41]. Biofilm formation is the leading cause of biomaterial-borne infections, where these organisms adhere to medical devices [42]. The Ica operon in *Staphylococcus aureus* contains *ica*ADBC genes, which play a vital role in biofilm formation. Several studies have shown that biofilm-associated infections in *Staphylococcus aureus* are caused by the presence of both *ica*A and *ica*D genes [43]. In our study, 48.3% ($n=14$) of the 29 staphylococcal isolates consisted of MSSA samples, 34.5% ($n=10$) MRKNS samples, and 17.2% ($n=5$) MRSA samples, and the presence of *ica*A and *ica*D was detected for both genes. It was found to be 55.2% ($n=16$). In a study conducted by Saba et al. 2018 with 81 *S. aureus*, which was determined to be the majority (MRSA), they determined that 75% of the isolates formed biofilm and 42.2% contained *ica*A and *ica*D [44].

Cephalosporin and carbapenem classes of antibiotics have been the mainstay in treating serious infections caused by enterobacteria such as *K. pneumoniae*. Still, this effectiveness has been compromised by the widespread acquisition of genes encoding enzymes such as extended-spectrum beta-lactamases (ESBLs) [45]. High mortality rates, often exceeding 40%, have been associated with serious infections caused by carbapenem-resistant Enterobacteriaceae (CRE) [46]. Carbapenem-resistant *K. pneumoniae* (CRKP) strains are the most clinically significant CRE [47]. In our study, while *IMP* and *VIM* genes were not among the carbapenemase genes, the *KPC* gene was in 50% ($n=10$), the *OXA-48* gene was 40% ($n=8$), and the *NDM* gene was 15% ($n=3$). Additionally, the presence of biofilm genes was investigated. Among these genes, *lux*S was found to be positive in 85% ($n=17$), *mrk*A and *wzm* in 75% ($n=15$), and *wbb*M in 70% ($n=14$). In a study by Chen et al. in 2020, they searched the relationship between biofilm formation and biofilm genes in *K. pneumoniae* samples and found that *lux*S and biofilm genes play an important role in biofilm architecture [48].

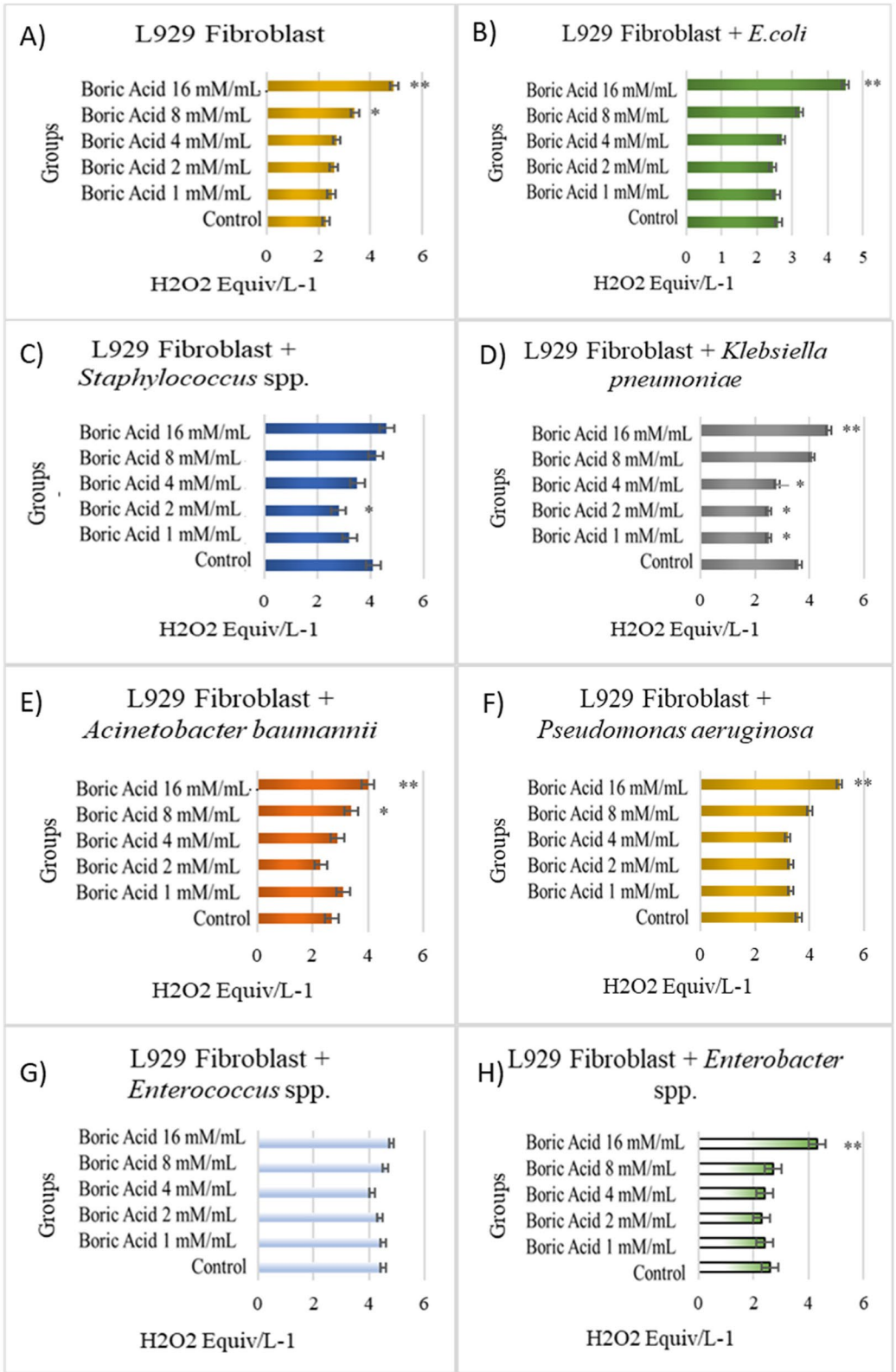
P. aeruginosa is inherently resistant to a wide range of antimicrobial agents, currently resisting multiple classes of antibiotics [49]. Additionally, the widespread distribution of *P.aeruginosa* nosocomial isolates resistant to last-resort polymyxin and carbapenem-class antibiotics has been documented [50]. In addition, *P. aeruginosa* produces biofilm with the quorum sensing (QS) system. Two main interrelated QS systems exist las and rhl [51]. In our study, 61.5% ($n=8$) of the isolates produced *OXA-48* and *KPC* genes. Other carbapenemase genes (*NDM-SPM*, *IMP*, and *VIM*) were detected in 7.7% ($n=1$), 15.4% ($n=2$), and 23.1% ($n=3$), respectively. In *Pseudomonas aeruginosa*, two quorum sensing system genes, *Las*I and *Las*R, were detected in 38.5% ($n=5$) and 30.8% ($n=4$), respectively.

E. coli causes urinary tract infections, neonatal meningitis, sepsis, and intestinal infections more frequently than other members of the Enterobacteriaceae family and is responsible for 80% of community-acquired urinary tract infections [52]. The increasing use of carbapenem, the first treatment method, has revealed the problem of carbapenem resistance in ESBL-producing *E. coli* infections [53]. Our study investigated the presence of *OXA-48* and *KPC* resistance genes in *Escherichia coli* isolates by single PCR. Of the 60 isolates, 35% ($n=21$) were determined to be positive for the *OXA-48* gene, and 31.7% ($n=19$) were positive for the *KPC* gene. In the study conducted by Candan and Aksöz in 2017, 22% of the isolates carried *OXA-48*, and this plasmid-borne resistance was reported in Turkey and the world and was more common than other types of resistance [54].

Over the last 35 years, *E. aerogenes* and *E. cloacae* species have posed significant threats to neonatal and intensive care unit patients, especially those dependent on mechanical ventilation. These two *Enterobacter*

(See figure on next page.)

Fig. 10 Total Oxidant Status was assessed via the TOS test ($n=6$) and H_2O_2 Equiv/L.$^{-1}$. The consequences of *Escherichia coli, Staphylococcus spp., Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterococcus spp.*, and *Enterobacter spp* combined on the viability of L929 fibroblast cell line. BA 1, 2, 4, 8, and 16 mM/mL were used as a treatment. L929 Fibroblast (**A**), *E. Coli* (**B**), *Staphylococcus spp* (**C**), *Klebsiella pneumoniae* (**D**), *Acinetobacter baumannii* (**E**), *Pseudomonas aeruginosa* (**F**), Enterococcus *spp.* (**G**), and *Enterobacter spp.* (**H**) were applied to cell cultures in 96-well plates for 24 h. The average of three different experiments is used to represent the results. All results were compared to the control group. Meaningful statistically: * $P<0.05$; ** $P<0.001$



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spp. emerged as clinically significant MDR pathogens in concurrent epidemic waves [55]. In this regard, the *Enterobacter* spp. included in our study were examined for the presence of antibiotic-resistance genes and virulence genes. *TEM*, an *ESBL* resistance gene, was detected in 12.5% ($n=1$), while the presence of *SHV* and *CTX-M* genes was not detected. Gelmez et al.'s (2021) study on *Enterobacter* spp. in the beta-lactamase genes research they conducted with their species, the ratio of *TEM*, *SHV*, and *CTX-M* enzymes was found to be relatively high in ESBL-positive strains [56]. In our study, other resistance genes *tet*A and *tet*B were detected in 100% ($n=8$) 12.5% ($n=1$), respectively, and the sulfamethoxazole resistance gene sul1 was seen in 50% ($n=4$) of the isolates. Among the quinolone resistance genes, *qnr*A was detected in 12.5% ($n=1$), *qnr*B 62.5% ($n=5$), and *qnr*S 50% ($n=4$). Finally, among the aminoglycoside resistance genes, the *aac*(3)-Ia gene was detected in 12.5% ($n=1$), the *ant*-(3)Ia gene was detected in 50% ($n=4$), and the *ant*(2)-Ia gene was detected in 75% ($n=4$). This bacterial species is a member of the ESKAPE group, as it has recently contributed to the infection problem in human health [57, 58]. Consequently, it is crucial to examine the various pathways used by *E. aerogenes* and *E. cloacae* to detect and respond to modifying environmental conditions and the presence of drugs in the environment.

Additionally, effective antimicrobial agents are not designed to target biofilms. Therefore, new strategies are needed to combat ESKAPE biofilms effectively. Research is developing methods to combat resistant biofilms by focusing on quorum sensing (QS). Different novel targets, such as electrochemical signaling, have been identified in Acinetobacter baumannii, which can be used as targets for anti-biofilm molecules [59].

Limayem et al. showed the bactericidal effects of silver hydrosol nanotherapeutics against biofilms of antibiotic-resistant *E. faecium* [60]. It has been demonstrated that Ag-hydrosol nanoparticles exhibit relatively high antibiofilm activity and low cytotoxicity. Therefore, bioactive Ag-hydrosol NPs may be a promising nanotherapeutic agent against drug-resistant pathogens. In a similar study conducted by Tiwari et al., polyvinylpyrrolidone-capped silver nanoparticles inhibit infection of the carbapenem-resistant *A. baumannii* strain in human lung epithelial cells. The study concluded that PVP-AgNPs could be developed instead of carbapenem to control infection caused by carbapenem-resistant *A. baumannii* [61].

Kasparova et al. demonstrated that non-thermal plasma is effective against resistant biofilms of *P. aeruginosa* by inhibiting the production of *Las*-B elastase, protease, and pyocyanin, which release biofilm cells [62]. Similarly, in another study, Deng et al. demonstrated that DNase I significantly inhibited early biofilm formation in a dose-dependent manner in models of empyema caused by *P. aeruginosa* and *S. aureus* [63].

These strategies, such as DNase treatments, non-thermal plasma, and silver hydrosol, appear to be crucial step in overcoming drug resistance in ESKAPE pathogens. However, when designing any compound to combat biofilm, observing its appropriate delivery in any in-vivo system at a particular site is always important. The dosage of the anti-biofilm compound is another essential factor that requires extensive research.

Boron compounds are used in many areas, from medicine to industry. Using boron is very important in overcoming increasing antibiotic resistance [64]. Biofilm-forming and MDR microorganisms pose a worldwide health problem. Increasing interest in alternative treatments in recent years has increased the search for potential antibacterial agents. This increases the use of boron compounds in antifungal, antibacterial, and anti-biofilm studies [65].

Boron nitride (BN) and molybdenum disulfide (MoS2) are two dichalcogenide nanomaterials that are being investigated as antibacterial and anti-biofouling agents to prevent biofilm formation. They are more often used to disrupt mature biofilm [66]. BN may exhibit biofilm inhibition and bactericidal activity due to its unique properties. BN directly interacts with bacterial cells, causing cell damage and death. In one study, a low-density polyethylene (LDPE) polymer embedded in BN nanoflakes was formulated to eliminate the biofilm of *P. aeruginosa* and *S. aureus* [67]. In another study, boric corrosive and borax were determined to successfully reduce the colony number of *Brucella* spp., *E.coli,* and *Staphylococcus* spp. [68]. In a study by Çelebi et al. (2023), the synergistic effects of boron compounds were examined, as resistance to antimicrobials applied in pathogen-focused treatments led to the search for alternatives [69]. It has been reported that boron compounds inhibit this enzyme by binding to the serine residue, especially with the overexpression of beta-lactamase classes. Boron compounds have also taken their place in the literature in studies to discover new compounds that can be applied with beta-lactam antibiotics.

Conclusions

In light of this information, in our study, the effect of BA against the infection on the L929 cell line was investigated, and by showing the survival rate of BA at different concentrations, it was determined that BA had significant

effectiveness against ESKAPE pathogens at the TACTOS level. It was thought that the combined antimicrobial activity of boron compounds could be promising for preventing hospital-associated infections. BA has a great effect in 2 and 4 mM/mL doses. Bacterial resistance has been a major problem of our decade so BA is a good candidate. It is suggested animal study can show us the immune response, symptoms, and prognosis of the infection more realistic.

Abbreviations

ESKAPE	*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter spp.*
ESBL	Extended-spectrum β-lactamase
MBL	Metallo-β-lactamase
MDR	Multidrug-resistant
BA	Boric acid
ADT	Antibiotic susceptibility testing
EUCAST	European Committee on Antimicrobial Susceptibility Testing
PBS	Phosphate buffer
BHI	Brain–heart infusion
TOC	Total oxidant capacity
TAC	Total antioxidant capacity
CRAB	Carbapenem-resistant *Acinetobacter*
CRE	Carbapenem-resistant *Enterobacteriaceae*

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None.

Authors' contributions
All the authors contributed to the study conception and design.

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Data availability
Data and materials described in this article will be made available on request.

Declarations

Ethics approval and consent to participate
This study was conducted on 24.02.2022 (Meeting No: 2 Decision No: 37) with the permission of Atatürk University Non-interventional Clinical Research Ethics Committee. Sample collection was performed in accordance with the relevant guidelines and regulations. The patient did not directly participate in this research work, and informed Consent was obtained from the patient.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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