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The pygidial gland secretion of *Laemostenus punctatus* (Coleoptera, Carabidae): a source of natural agents with antimicrobial, anti-adhesive, and anti-invasive activities

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In the present study, we investigated *in vitro* the antimicrobial activity of the pygidial gland secretion of the guanophilic ground beetle *Laemostenus* (*Pristonychus*) *punctatus* (Dejean, 1828) and some of its chemicals against resistant and non-resistant bacteria and *Candida* species, the synergistic and additive potential of combinations of selected chemicals and antimicrobial drugs against resistant bacterial and fungal strains, anti-adhesive and anti-invasive potential of the secretion and formic acid alone and in selected combinations with antimicrobial drugs against methicillin-resistant *Staphylococcus aureus* (MRSA) toward spontaneously immortalized human keratinocyte cell line (HaCaT cells). In addition, we examined the antiproliferative activity of the secretion and formic acid *in vitro*. The tested secretion and the standards of formic and oleic acids possessed a significant level of antimicrobial potential against all tested strains ($P < 0.05$). The isolate from guano *Pseudomonas monteilii* showed the highest resistance to the secretion and formic acid, while MRSA achieved a significantly high level of susceptibility to all agents tested, particularly to the combinations of formic acid and antibiotics, but at the same time showed a certain level of resistance to the antibiotics tested individually. *Candida albicans* and *C. tropicalis* were found to be the most sensitive fungal strains to the secretion. Formic acid (MIC 0.0005 mg/mL) and gentamicin (MIC 0.0010 mg/mL) in the mixture achieved synergistic antibacterial activity against MRSA (FICI = 0.5, $P < 0.05$). The combination of formic acid, gentamicin and ampicillin accomplished an additive effect against this resistant bacterial strain (FICI = 1.5, $P < 0.05$). The secretion achieved a better inhibitory effect on the adhesion ability of MRSA toward HaCaT cells compared to formic acid alone, while formic acid showed better results regarding the invasion ($P < 0.001$).

The combinations of gentamicin and ampicillin, as well as of formic acid and gentamicin and ampicillin achieved similar anti-adhesive and anti-invasive effects, with a slight advantage of formic acid and antibiotics in combination ($P < 0.001$). The secretion and formic acid were found to be non-toxic to HaCaT cells *in vitro* ($IC_{50} \geq 401\,\mu g/mL$).

1. Introduction

Natural products and their synthetic analogs contributed significantly to pharmacotherapy in the past, particularly in the treatment of infectious diseases (Atanasov et al., 2021; Zhao et al., 2022). Insects and insect-derived products have been used in traditional medicine for centuries in various regions of the world (Costa-Neto, 2005). Studies published in recent years emphasize the importance of insects and their potential application in biomedicine (Guo et al., 2017; Seabrooks and Hu, 2017; Bairagi, 2019; Nenadić, 2019). Beetles (Coleoptera) nowadays represent the source of biologically active chemicals (Adamski et al., 2019). Many of them, including quinones, glycosides, alkaloids, sugars, polyols, polypeptides, and terpenoids (Costa-Neto, 2005), have been recently identified and shown to have various properties, such as antimicrobial, anti-inflammatory, antiseptic, antioxidant, antitumor, etc. (Cherniack, 2010; Mandal and Mandal, 2011; Seabrooks and Hu, 2017). Combining these compounds with conventional drugs could undoubtedly offer significant advantages for future treatments (Cherniack, 2010). The search for more effective therapies is currently a top priority, particularly because of drug resistance (Murray et al., 2022). Bacterial infections caused by *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Escherichia coli*, and *Pseudomonas aeruginosa* are a major cause of morbidity and mortality in the world (O'Neill, 2014; Asadi et al., 2019). The annual number of deaths from antibiotic resistance may reach approximately 10 million by 2050 (Cars et al., 2021; Laurence et al., 2021; Walsh et al., 2021). Methicillin-resistant *Staphylococcus aureus* (MRSA) strains are thought to be responsible for more than 100,000 fatal cases related to antimicrobial resistance in 2019 (Murray et al., 2022). MRSA strains are specific due to their multi-drug resistance and as such can be the main cause of a number of serious diseases of the skin, soft tissues, bones, and bloodstream, but also could be the cause of serious treatment problems among hospitalized patients (Gordon and Lowy, 2008; Jiang et al., 2021). Although antibiotics are available to treat nosocomial infections, the increasing emergence of antibiotic resistance has rendered many treatments ineffective (Walsh et al., 2021). It has been established that staphylococcal strains are resistant to glycopeptide antibiotics, which are used in the ultimate fight against MRSA strains. In this sense, a new therapeutic approach in recent years refers to the synergistic action of existing antibiotics and natural products (Álvarez-Martínez et al., 2020). In addition, anti-adhesion-based therapies are important to reduce the interaction between pathogens and host tissues by preventing or precluding

bacterial adherence and entry into cells, which may result in invasion, directly leading to the development of infection and disease (Madle et al., 2018; Roberts et al., 2018). A number of fungal infections have also continued to increase over the past 20 years (Wiederhold, 2017; Denning, 2022). Some strains of *Candida glabrata*, *C. krusei*, and the recently emerged species *C. auris* show decreased susceptibility to commonly used antifungal agents (Gow et al., 2022). Increased azole resistance among *Candida* spp. isolates (excluding *C. albicans*) and echinocandin resistance in *C. glabrata* are some of the recent trends in the development of fungal resistance (Wiederhold, 2017). These problems have led scientists to focus on the study of oral, anal, and exocrine gland secretions in insects, as well as related secondary metabolites with potential antimicrobial properties (Giglio et al., 2011). It is generally known that ground beetles (Carabidae), which represent the largest family within the suborder Adephaga (order Coleoptera), possess a pair of abdominal glands known as pygidial (Forsyth, 1972; Eisner et al., 2000; Giglio et al., 2011, 2021; Nenadić et al., 2016a, 2017b; Nenadić, 2019). Recently published papers indicate the importance of the pygidial gland secretions of some *Calosoma* and *Carabus* species, which may contain various organic compounds that possess antimicrobial properties, such as short-chain fatty acids (butyric, isobutyric, formic, acetic, etc.) and aromatic aldehydes (salicylaldehyde) (Giglio et al., 2011; Nenadić et al., 2016a; Vesović et al., 2020). These secretions have demonstrated antimicrobial activity against a wide range of human pathogens, as well as an interesting negligible level of toxicity in several species (Giglio et al., 2011; Nenadić et al., 2016a, 2017a,b, 2018; Nenadić, 2019; Vesović et al., 2020, 2022, 2023). The function of these glands is defense against predators (Giglio et al., 2011), but an additional role is observed in freshwater-dwelling Adephaga (Hydradephaga) (Francke and Dettner, 2005), whose pygidial gland secretions exhibit antimicrobial properties that are very important for their aquatic respiration, allowing inhibition of the growth of microorganisms and preventing contamination and wetting of the cuticular respiratory structures (Kovac and Maschwitz, 1990). The other example is the pupa of ground beetle *Carabus* (*Chaetocarabus*) *intricatus lefebvrei* Dejean, 1826, which produces monoterpenes in its pygidial gland secretion that may presumably have a prophylactic function against environmental pathogens (Giglio et al., 2009).

Laemostenus (*Pristonychus*) *punctatus* (Dejean, 1828) (Coleoptera: Carabidae) represents a predominantly troglophilic and guanophilic ground beetle species, which is widely distributed in the Balkan Peninsula (Casale, 1988). Individuals of this species

TABLE 1 Chemical composition of the pygidial gland secretion of the ground beetle *L.* (*P.*) *punctatus* (after Vesović et al., 2015).

Chemical compounds	Relative percentages of chemical compounds in PGS-LP (%)[a]
Undecane	40.4
Acetic acid	0.6
Formic acid	19.4
Decyl acetate	0.2
Undecyl acetate	0.2
Dodecan-1-ol	1.2
Caproic acid	T
Dodecyl acetate	34.2
7-Hexyldocosane	0.5
9-Methyltetracosane	2.6
Palmitic acid	0.5
Stearic acid	0.1
Oleic acid	0.1

[a]Data obtained by gas chromatography-mass spectrometry on an INNOWax capillary column; PGS-LP, pygidial gland secretion of *L.* (*P.*) *punctatus*; T, compound identified in trace amount (<0.1%).

could be found in burrows of mammals and caves (Casale, 1988), where they usually come into contact with bat guano, which is a significant source of various microorganisms (Nenadić et al., 2017a; Dimkić et al., 2020). Different organic compounds have been detected in the pygidial gland secretion mixture of *L.* (*P.*) *punctatus* (Vesović et al., 2015; Nenadić et al., 2016a). Undecane (with a relative percentage of 40.4%) and dodecyl acetate (with a relative percentage of 34.2%) (Table 1) are the most abundant components of this secretion (Vesović et al., 2015), but there is no clear evidence of their antimicrobial properties. On the contrary, the correlation between some organic constituents, such as various short- and long-chain fatty acids, and their biological activity has been established in the past (Glassman, 1948; Raftari et al., 2009; Desbois and Smith, 2010).

In this study, we assume that, apart from the dominant components, only minor chemical constituents that have at least a hint of antimicrobial properties can contribute to the antimicrobial potential of the tested secretion mixture. The effects of minor constituents, such as dodecan-1-ol, decyl acetate, undecyl acetate, caproic acid, 7-hexylcocosane, and 9-methyltetracosane (Table 1), were not investigated as there is no evidence in the literature of their possible antimicrobial properties. However, this is not the case with acetic acid (Table 1), which antimicrobial potential has been demonstrated in the past (Ryssel et al., 2009; Fraise et al., 2013), or oleic acid, which antimicrobial properties have been proved several times in recent decades, particularly when it comes to inhibiting the growth of gram-positive bacteria, such as *S. aureus* (Speert et al., 1979; Dilika et al., 2000; Stenz et al., 2008; Desbois and Smith, 2010). Palmitic and stearic acids also exhibit antibacterial activity against both gram-positive and gram-negative bacteria (Casillas-Vargas et al., 2021). The production of formic acid as a defensive chemical is widespread in different ground beetles (Francke and Dettner, 2005; Lečić et al., 2014; Nenadić et al., 2016b). It is well-known that formic acid has been added to poultry diets to limit

Salmonella spp. and other foodborne pathogens in the feed and potentially in the gastrointestinal tract (Gómez-García et al., 2019; Ricke et al., 2020).

Nenadić et al. (2016b) gave the very first data on the antimicrobial potential of the pygidial gland secretion of *L.* (*P.*) *punctatus* against various human pathogens (clinical and laboratory gram-negative and gram-positive bacterial strains and fungal species), while Dimkić et al. (2020) analyzed the antimicrobial activity of this secretion against guano-dwelling bacterial and fungal strains. Prior to these studies, taxa within the ground beetle subfamily Platyninae were generally poorly known in this regard.

In the current study, we presented the first data on the antimicrobial activity of the pygidial gland secretion of *L.* (*P.*) *punctatus* against resistant bacterial strains and *Candida* species, as well as on its cytotoxicity and inhibitory effect on the adhesion and invasion ability of MRSA toward spontaneously immortalized human keratinocyte cell line (HaCaT cells). Therefore, the objectives of this study were to investigate: (i) the antimicrobial activity of the pygidial gland secretion of *L.* (*P.*) *punctatus* and selected secretion's chemicals against resistant and non-resistant strains of various bacterial (laboratory, clinical, and guano-dwelling) and *Candida* species; (ii) the type of interaction between selected secretion's chemicals and antimicrobial drugs in combinations; (iii) the antimicrobial potential of these combinations against all resistant strains tested using a two-dimensional (2D) and a three-dimensional (3D) checkerboard method; (iv) *in vitro* inhibitory effect of the secretion and formic acid alone and in combination with antimicrobial drugs on the adhesion and invasion ability of MRSA toward HaCaT cells; and (v) the antiproliferative activity of the secretion and formic acid toward HaCaT cells to test the cytotoxicity *in vitro*.

2. Materials and methods

2.1. Collection and handling of specimens of *L.* (*P.*) *punctatus* under laboratory conditions

A total of 79 adult specimens of *L.* (*P.*) *punctatus* were manually collected by S. Ćurčić, N. Vesović, V. Žikić, and S. Stanković on 27th September 2020 in the Ogorelička Pećina Cave, village of Sićevo, Svrljiške Planine Mts., southeastern Serbia (average environmental conditions recorded: temperature 13°C, aerial humidity 70.5%). After collection, the live beetles were transported and preserved in cold and portable plastic chamber for a few days before sampling the contents of their pygidial glands according to a previously established protocol (Nenadić et al., 2016a). Preservation of beetles was performed with the aim of reducing the stressful state of insects to the minimum under controlled laboratory conditions (the moist soil obtained from the collection sites, complete darkness, a constant temperature of 10°C, and feeding on earthworms and springtails collected from the Ogorelička Pećina Cave). After isolation and quantitative analysis of the pygidial gland secretion, the dried beetle specimens were deposited in the collection of the Institute of Zoology, University of Belgrade - Faculty of Biology, Belgrade, Serbia.

2.2. *In vitro* antimicrobial assay

2.2.1. Isolation procedure and quantitative assessment of biological material

Pygidial gland secretion was isolated and quantitatively analyzed under sterile conditions at room temperature according to a previously established protocol (Nenadić et al., 2016a). Analysis was performed immediately after sampling to reduce the impact of composition-altering oxidation, possible evaporation and degradation of the chemical constituents of the secretion. The total amount of the pygidial gland secretion from 79 specimens of *L.* (*P.*) *punctatus* was obtained by stimulating their legs and pressing the tip of their abdomen to discharge pygidial gland secretion into a 2 mL glass vial. The secretion was measured on a Sartorius Model 2405 analytical balance (Sartorius Group, Göttingen, Germany) for weighing up to 30 g maximum, with 0.001 mg resolution in the laboratory of the Department of Organic Chemistry, University of Belgrade - Faculty of Chemistry, Belgrade, Serbia. The secretion mass was measured as described in Nenadić et al. (2016a).

To check the validity of the results, all measurements were performed three times.

2.2.2. Preparation of biological material for *in vitro* antimicrobial susceptibility testing

The extracted biological sample was prepared by dissolving it in 0.01 M phosphate buffer solution (PBS) (Sigma-Aldrich, Munich, Germany) under sterile conditions at room temperature and then stored in a freezer at $-20°C$ in the laboratory of the Institute of Zoology, University of Belgrade - Faculty of Biology, Belgrade, Serbia until the antimicrobial effect is tested.

2.2.3. Antimicrobial drugs

The following synthetic commercial antibiotics were used as positive controls in this study: streptomycin (1 mg of active substance in 1 mL of physiological solution) (Sigma-Aldrich, Munich, Germany), ampicillin (1 mg of active substance in 1 mL of physiological solution) (Panfarma, Belgrade, Serbia), gentamicin (500 mg of amoxicillin trihydrate in 500 mL of saline) (Panfarma, Belgrade, Serbia), and cephalexin (1 mg of active substance in 1 mL of physiological solution) (Panfarma, Belgrade, Serbia).

The synthetic commercial antifungal drugs used as positive controls in this study were amphotericin B (1 mg of active substance in 1 mL of physiological solution) (Sigma-Aldrich, Munich, Germany) and nystatin (1 mg of active substance in 1 mL of physiological solution) (Sigma-Aldrich, Munich, Germany).

2.2.4. Commercial standards tested

The following commercial standards were tested in this study: acetic acid (ethanoic acid, IUPAC) 99.8% (Sigma-Aldrich, St. Louis, MO, USA) (1 mg of active substance in 1 mL of 0.01 M PBS), dodecyl acetate (lauryl acetate, IUPAC) 97% (Sigma-Aldrich, St. Louis, MO, USA) (1 mg of active substance in 1 mL of 0.01 M PBS), formic acid (methanoic acid, IUPAC) 95% (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) (1 mg of active substance in 1 mL of 0.01 M PBS), oleic acid [(9Z)-octadec-9-enoic acid, IUPAC] 99% (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) (1 mg of active substance in 1 mL of 0.01 M PBS), palmitic acid (hexadecanoic acid, IUPAC) 95% (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan)

(1 mg of active substance in 1 mL of 0.01 M PBS), stearic acid (octadecanoic acid, IUPAC) 98% (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) (1 mg of active substance in 1 mL of 0.01 M PBS), and undecane (hendecane, IUPAC) 99.5% (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) (1 mg of active substance in 1 mL of 0.01 M PBS).

2.2.5. Bacterial and fungal strains tested

In total 16 microbial pathogens (12 bacterial and four fungal strains), including resistant and non-resistant strains, have been tested in this study. The human pathogens used in this research were: *Escherichia coli* ATCC 25922, *E. coli* resistant strain IBRS E003, *Pseudomonas aeruginosa* ATCC 27853, *P. aeruginosa* resistant strain IBRS P001 (gram-negative bacteria), *Staphylococcus aureus* ATCC 11632, methicillin-resistant *S. aureus* (MRSA) IBRS 011 (clinical isolate) (gram-positive bacteria), *Candida albicans* ATCC 10231, *C. krusei* H1/16 (clinical isolate), *C. tropicalis* ATCC 750, and *C. auris* CDC B11903 (fungal strains). They are deposited in the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research "Siniša Stanković" - National Institute of the Republic of Serbia, University of Belgrade, Belgrade, Serbia. In addition, we tested the following bacterial strains obtained from bat guano: *Bacillus subtilis* GT1, *B. clausii* GT2, *B. toyonensis* GT3, *Peribacillus simplex* GT4, *Pseudomonas monteilii* GT5, and *Staphylococcus arlettae* GT6. These were previously identified and deposited in the laboratory of the Department of Biochemistry and Molecular Biology, University of Belgrade - Faculty of Biology, Belgrade, Serbia.

2.2.6. Preparation of the overnight cultures of bacterial and *Candida* species

All tested strains of bacterial and *Candida* species, previously stocked in 80% glycerol at $-80°C$, were seeded on 2 mL Tryptic Soy Broth (TSB) growth medium (15 g of casein, 5 g of peptone, 5 g of NaCl, 15 g of agar, 1 L of K_2HPO_4, and 1,000 mL of distilled water) (Biolife Italiana, Milan, Italy). Overnight cultures with 1.0×10^9 colony-forming units (CFU)/mL were established after incubation for 24 h at $37°C$ in a thermostat. The final solution of culture and medium (1.0×10^8 CFU/mL) was prepared by dissolving 100 mL of primary culture in 900 mL of TSB and stored in a freezer at $-20°C$ until use in the antimicrobial assay. The final concentration tested per well of a 96-well microtiter plate was 1.0×10^6 CFU/mL.

2.2.7. *In vitro* broth microdilution

The *in vitro* antimicrobial assay was performed using the broth microdilution method (Hänel and Raether, 1988; Daouk et al., 1995; CLSI, 2009; Soković et al., 2010; Nenadić et al., 2016a, 2017b) to determine the minimum inhibitory concentrations (MICs), minimum bactericidal concentrations (MBCs), and minimum fungicidal concentrations (MFCs) of the tested agents. The method was carried out using a 96-well system, i.e., 96-well sterile microtiter plates with a flat bottom (F-microtiter plate with a cover) (Spektar, Čačak, Serbia), and through 2-fold serial dilutions of the tested samples dissolved in 0.01 M PBS (pygidial gland secretion and the commercial standards of selected chemicals) or physiological solution (antimicrobial drugs). Each well of the first row of microtiter plates had a total volume of 200 μL (180 μL of added tested agents and 20 μL of TSB), whereas the

sterility control was inoculated only with 100 μL of TSB. Finally, each well was inoculated with 10 μL of bacterial/fungal culture $(1.0 \times 10^8$ CFU/mL). After performing 2-fold serial dilutions, the suspensions were adjusted to a final volume of 100 μL. The microtiter plates were incubated at 37°C for 24 h.

The antibiotics streptomycin, ampicillin, cephalexin, and gentamicin, as well as the antimycotics nystatin and amphotericin B were used as positive controls in final tested concentration ranges of 0.001–0.900 mg/mL (antibiotics) and 0.028–0.450 mg/mL (antimycotics), respectively, while 0.01 M PBS was used as a negative control. The final tested concentration of the secretion of *L. (P.) punctatus* was in a range of 0.004–0.112 mg/mL for 12 bacterial strains, and 0.024–0.096 mg/mL for four strains of *Candida* species, respectively. The final tested concentration range of the selected chemicals tested was 0.001–0.600 mg/mL for 12 bacterial strains, and 0.008–0.600 mg/mL for four strains of *Candida* species, respectively.

2.2.8. Determination of MICs, MBCs, and MFCs

Minimum inhibitory concentrations (MICs) are defined as the lowest concentrations that cause inhibition of bacterial or fungal growth, which can be observed under a binocular microscope (Rodríguez-Tudela et al., 2003), while MBCs and MFCs represent the lowest concentrations with no bacterial or fungal growth observed, indicating that 99.5% of the original inoculum has been killed (Soković et al., 2010).

Minimum inhibitory concentration values were determined by using a colorimetric microbial viability assay based on the reduction in color of *p*-iodonitrotetrazolium violet, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT) (Sigma-Aldrich, Munich, Germany) (0.2 mg of the substance in 1 mL of double distilled water). Colonies were stained by adding 40 μL of INT and then incubated at 37°C for 2 h.

Pale yellow color pointed to inhibition and unstained microtiter plate chambers indicated that observed tested agent achieved bactericidal effect (CLSI, 2009). A microplate reader (Tecan Austria GmbH, Grödig, Austria; Eppendorf AG, Hamburg, Germany) was used to assess the intensity of yellow color and MICs were determined by counting cell numbers using spectrophotometric methods (Nenadić et al., 2016a, 2017b). The optical density of each well was measured at a wavelength of 655 nm and compared to a blank (broth medium and diluted agent) and a positive control. In addition, MBCs and MFCs were confirmed by reinoculating of 2 μL of the tested dilutions with bacterial or fungal inoculum from each well without color change into 100 μL of TSB and incubating at 37°C for 24 h, after which no growth was detected. MIC, MBC, and MFC values were obtained as mg/100 μL and finally expressed in mg/mL.

2.2.9. *In vitro* 2D and 3D checkerboard assay

To determine the interactions (synergism, additive effect, indifference or antagonism) between selected agents (formic and oleic acids, the antibiotics streptomycin, ampicillin, and gentamicin, and the antifungal agents amphotericin B and nystatin) in combinations and the antimicrobial potential of the tested combinations *in vitro*, selected resistant bacterial strains (*E. coli*, *P. aeruginosa*, and MRSA) and the resistant *C. auris* strain were studied using a 2D and a 3D checkerboard method. For this

assay, we used 96-well microtiter plates containing TSB growth medium with the tested acids and antimicrobial agents combined in a checkerboard style at concentrations ranging from 1/8 MIC to $2 \times$ MIC (0.0005–1 mg/mL). The microtiter plates were incubated at 37°C for 24 h (Jacqueline et al., 2005). To evaluate the effect of three selected components (formic acid, ampicillin, and gentamicin), the 3D checkerboard method according to Stein et al. (2015) was performed using 96-well microtiter plates containing TSB medium with the tested compounds. The bacterial and fungal inoculum per well was 1.0×10^6 CFU/mL.

Fractional Inhibitory Concentration Index (FICI) was determined according to the following formula:

$$\mathrm{FICI} = \mathrm{FIC1}^0/\mathrm{MIC1}^0 + \mathrm{FIC2}^0/\mathrm{MIC2}^0$$

where $\mathrm{FIC1}^0$ and $\mathrm{FIC2}^0$ are the MIC values of the tested compounds and antibiotics in combination, while $\mathrm{MIC1}^0$ and $\mathrm{MIC2}^0$ represent the MIC values of the individual activity of selected agents. For the interpretation of the obtained results, the following threshold values according to Lorian (2005) were used: FICI $\leq$ 0.5 = synergistic effect, $2 \geq$ FICI $>$ 0.5 = additive effect, $4 \geq$ FICI $>$ 2 = indifferent effect, and FICI $>$ 4 = antagonistic effect.

2.2.10. *In vitro* assay for evaluation of the adhesion and invasion ability of MRSA toward HaCaT cells

Percentages of adhesion and invasion were determined as described by Ahmed et al. (2014), with some modifications. HaCaT cells were prepared and grown in 24-well plates with an adhesive bottom. Afterward, the medium was removed and fresh Dulbecco's Modified Eagle Medium (DMEM) without fetal bovine serum (FBS) (Invitrogen, Waltham, MA, USA) containing the tested agents in concentrations of the determined MIC and MBC values was added to the cells. The first incubation was then carried out for 15 min at 37°C, after which 100 μL of MRSA culture $(1.0 \times 10^8$ CFU/mL) was added to the cells and incubated again with the cells for 1 h at 37°C for testing adhesion capacity, and 2 h at 37°C for testing invasion capacity, respectively. To kill the adhering and free-floating bacterial cells, gentamicin at a concentration of 300 μg/mL was added to the cells for 1 h. Finally, the cells were washed three times with DMEM without FBS and lyzed for 30 min at 37°C with 1 mL of 1% (v/v) Tween 20 (Sigma-Aldrich, Munich, Germany).

For the adhesion assay, the previously prepared sample was diluted 100 times. For the invasion test, the prepared sample was not diluted. A total of 10 μL of both samples were inoculated and spread on tryptone soy agar (TSA) growth medium (Torlak, Belgrade, Serbia) using a sterile stick. After 18 h of incubation at 37°C, the number of CFU was determined. The final results of this assay were expressed as percentages (%). Gentamicin and ampicillin in combination were used as positive controls, while untreated cultures were used as negative controls (100% of adhesion/invasion).

This assay was performed in triplicate and three independent experiments were repeated.

2.2.11. *In vitro* crystal violet cytotoxicity assay

In vitro analysis of cytotoxicity of the tested secretion of *L. (P.) punctatus*, commercial standards and their combinations with

antibiotics was performed on HaCaT cells using a modified crystal violet assay as described by Stojković et al. (2020).

HaCaT cells were grown in high-glucose DMEM supplemented with 10% FBS, 2 mM L-glutamine and 1% antibiotic/antimycotic (Invitrogen, Waltham, MA, USA) in 5% CO_2 at 37°C. Cells were prepared and incubated in each well of 96-well microtiter plates with an adhesive bottom 48 h prior to treatment. After the initial incubation, the clear growth medium was removed, and a new medium containing dissolved tested agents at various concentrations (up to a final concentration of 8 mg/mL) was added to the cells, which were treated for 24 h. Afterward, the medium was removed and the cells were washed twice with PBS. In addition, the cells were stained with 0.4% crystal violet staining solution at room temperature for 20 min. The crystal violet staining solution was then removed, the cells were washed in a stream of tap water and air-dried at room temperature. The absorbance of dye dissolved in methanol was measured at 570 nm (OD 570) using a microplate reader (Tecan Austria GmbH, Grödig, Austria; Eppendorf AG, Hamburg, Germany).

The results were expressed as IC_{50} values, indicating 50% inhibition of proliferation of HaCaT cells compared to the untreated positive control. The criterion for determining and classifying the antiproliferative activity of the tested agents and combinations toward HaCaT cells was as follows: $IC_{50} \leq 20$ µg/mL = highly cytotoxic effect, IC_{50} 21–200 µg/mL = moderately cytotoxic effect, IC_{50} 201–400 µg/mL = weakly cytotoxic effect, and $IC_{50} \geq 401$ µg/mL = no cytotoxicity observed (Stojković et al., 2020).

For each sample, concentration procedures were carried out in triplicate and three independent experiments were performed.

2.2.12. Statistical analysis of the results

One-way analysis of variance (ANOVA) was used to interpret the results, which were presented as mean values (MEAN) and standard errors (SE), followed by Tukey's HSD test with a significance level of 0.05. Statgraphics Centurion software XVI, version 16.2 was used, and graphs were generated using GraphPad Prism, version 8.0.

3. Results

3.1. Total amount of discharged pygidial gland secretion

The total amount of the discharged pygidial gland secretion obtained from 79 adult individuals of *L.* (*P.*) *punctatus* was 0.395 ± 0.001 mg, which was used for further investigations in this study. The average amount of the secretion per one adult specimen of the analyzed species was 0.005 ± 0.001 mg.

3.2. *In vitro* screening of antibacterial activity

The initial analysis indicated that all tested bacterial strains showed a high susceptibility to undecane, dodecyl acetate, and acetic acid (MIC values were not obtained due to the bactericidal

effect of undecane, dodecyl acetate, and acetic acid even at the lowest concentration tested) and simultaneously a high resistance to palmitic and stearic acids (MIC values were not detected due to the observed growth of bacterial cultures even at the highest concentration tested), so these chemicals were excluded from further examinations.

The pygidial gland secretion of *L.* (*P.*) *punctatus* and the standards of two secretion's compounds (formic and oleic acids), tested in comparison with commercial antibiotics, were found to have a certain antibacterial potential that differed between all resistant and non-resistant strains ($P < 0.05$). Resistant bacterial strains generally showed lower susceptibility to all tested agents compared to non-resistant bacteria. The beta-lactam antibiotics tested (ampicillin and cephalexin), the aminoglycoside streptomycin, and the standard of oleic acid showed a lower antibacterial potential compared to the secretion of *L.* (*P.*) *punctatus* and formic acid (Table 2).

With respect to the tested standards of organic constituents contained in the secretion mixture of *L.* (*P.*) *punctatus*, formic acid achieved much higher antibacterial potential against all tested bacteria (MIC 0.001–0.112 mg/mL) compared to oleic acid (MIC 0.150–0.600 mg/mL) (Table 2).

Formic acid was found to have antimicrobial activity equivalent to the aminoglycoside gentamicin, with MIC values 2–15 times lower than the MICs of the secretion of *L.* (*P.*) *punctatus*, except for the strain from guano *P. monteilii*, which showed the highest resistance to formic acid (MIC 0.112 ± 0.000 mg/mL, MBC 0.240 ± 0.000 mg/mL) and the secretion of *L.* (*P.*) *punctatus* (MIC 0.060 ± 0.000 mg/mL, MBC 0.112 ± 0.000 mg/mL).

Regarding resistance to gentamicin and formic acid, *S. aureus* and MRSA were found to be more resistant to gentamicin, whereas the resistant *E. coli* and *P. aeruginosa* showed a higher resistance to formic acid in relation to the non-resistant *E. coli* and *P. aeruginosa* that displayed equal susceptibility. Gentamicin and formic acid accomplished the highest bactericidal potential against resistant strains, thus both were tested in combination in further steps of this study.

Escherichia coli and MRSA were found to be the most sensitive to the secretion of *L.* (*P.*) *punctatus* among the resistant strains (MIC 0.015 ± 0.000 mg/mL, MBC 0.030 ± 0.000 mg/mL), while the non-resistant *E. coli* showed the highest susceptibility to the secretion (MIC 0.004 ± 0.000 mg/mL, MBC 0.008 ± 0.000 mg/mL) compared to all tested bacteria (Table 2).

The resistant *P. aeruginosa* strain was found to be less sensitive to the secretion of *L.* (*P.*) *punctatus* (MIC 0.030 ± 0.000 mg/mL, MBC 0.060 ± 0.000 mg/mL) compared to the other strains tested.

All guano-dwelling bacterial strains showed a similar susceptibility to the secretion of *L.* (*P.*) *punctatus*, with a MIC range of 0.015–0.060 mg/mL (Table 2). With respect to the tested secretion, *B. subtilis*, *B. clausii*, and *S. arlettae* proved to be the most sensitive strains (with MIC 0.015 ± 0.000 mg/mL and MBC 0.030 ± 0.000 mg/mL, respectively), while the remaining three guano-dwelling strains tested (*B. toyonensis*, *P. monteilii*, and *P. simplex*) showed a higher level of resistance.

The resistant *E. coli* and MRSA showed a similar susceptibility to the secretion of *L.* (*P.*) *punctatus*, approximately the same as the guano-dwelling *B. subtilis*, *B. clausii*, and *S. arlettae* strains (with MIC 0.015 ± 0.000 mg/mL and MBC 0.030 ± 0.000 mg/mL, respectively) that were found to be less resistant to the secretion compared to other isolates from guano tested.

TABLE 2 *In vitro* screening of antibacterial activity displayed as mean values (MEAN) of the minimum bactericidal concentrations (MBCs) and minimum inhibitory concentrations (MICs) of the pygidial gland secretion of *L.* (*P.*) *punctatus* and the standards of formic and oleic acids alone compared to positive controls (antibiotics streptomycin, ampicillin, cephalexin, and gentamicin).

No.	Bacterial strains	Agents MBC (MEAN ± SE) (mg/mL) MIC (MEAN ± SE) (mg/mL)						
		PGS-LP	Formic acid	Oleic acid	Streptomycin	Ampicillin	Cephalexin	Gentamicin
1.	*E. coli* ATCC 25922[NR]	0.008 ± 0.000[e] 0.004 ± 0.000[e]	0.002 ± 0.000[g] 0.001 ± 0.000[g]	0.600 ± 0.000[a] 0.300 ± 0.000[b]	0.113 ± 0.000[d] 0.056 ± 0.000[d]	0.038 ± 0.000[c] 0.019 ± 0.000[c]	0.019 ± 0.000[f] 0.009 ± 0.000[g]	0.002 ± 0.000[c] 0.001 ± 0.000[d]
2.	*E. coli* IBRS E003[R]	0.030 ± 0.000[c] 0.015 ± 0.000[c]	0.004 ± 0.000[f] 0.002 ± 0.000[f]	0.600 ± 0.000[a] 0.300 ± 0.000[b]	0.450 ± 0.000[b] 0.225 ± 0.000[b]	0.075 ± 0.000[b] 0.038 ± 0.000[b]	0.075 ± 0.000[d] 0.038 ± 0.000[e]	0.002 ± 0.000[c] 0.001 ± 0.000[d]
3.	*P. aeruginosa* ATCC 27853[NR]	0.015 ± 0.000[d] 0.008 ± 0.000[d]	0.002 ± 0.000[g] 0.001 ± 0.000[g]	0.300 ± 0.000[b] 0.150 ± 0.000[c]	0.225 ± 0.000[c] 0.113 ± 0.000[c]	0.038 ± 0.000[c] 0.019 ± 0.000[c]	0.038 ± 0.000[e] 0.019 ± 0.000[f]	0.002 ± 0.000[c] 0.001 ± 0.000[d]
4.	*P. aeruginosa* IBRS P001[R]	0.060 ± 0.000[a] 0.030 ± 0.000[b]	0.008 ± 0.000[e] 0.004 ± 0.000[e]	0.600 ± 0.000[a] 0.300 ± 0.000[b]	0.450 ± 0.000[b] 0.225 ± 0.000[b]	0.150 ± 0.000[a] 0.075 ± 0.000[a]	0.300 ± 0.000[b] 0.150 ± 0.000[c]	0.002 ± 0.000[c] 0.001 ± 0.000[d]
5.	*S. aureus* ATCC 11632[NR]	0.030 ± 0.000[c] 0.015 ± 0.000[c]	0.002 ± 0.000[g] 0.001 ± 0.000[g]	0.600 ± 0.000[a] 0.300 ± 0.000[b]	0.225 ± 0.000[c] 0.113 ± 0.000[c]	0.075 ± 0.000[b] 0.038 ± 0.000[b]	0.075 ± 0.000[d] 0.038 ± 0.000[e]	0.008 ± 0.000[b] 0.004 ± 0.000[c]
6.	MRSA IBRS 011[R]	0.030 ± 0.000[c] 0.015 ± 0.000[c]	0.004 ± 0.000[f] 0.002 ± 0.000[f]	0.600 ± 0.000[a] 0.300 ± 0.000[b]	0.900 ± 0.000[a] 0.450 ± 0.000[a]	0.150 ± 0.000[a] 0.075 ± 0.000[a]	>0.600 ± 0.000[a] 0.300 ± 0.000[b]	0.008 ± 0.000[b] 0.004 ± 0.000[c]
7.	*B. subtilis* GT1[G]	0.030 ± 0.000[c] 0.015 ± 0.000[c]	0.030 ± 0.000[d] 0.015 ± 0.000[d]	0.600 ± 0.000[a] 0.300 ± 0.000[b]	0.450 ± 0.000[b] 0.225 ± 0.000[b]	0.075 ± 0.000[b] 0.038 ± 0.000[b]	0.300 ± 0.000[b] 0.150 ± 0.000[c]	0.060 ± 0.000[a] 0.030 ± 0.000[b]
8.	*B. clausii* GT2[G]	0.030 ± 0.000[c] 0.015 ± 0.000[c]	0.030 ± 0.000[d] 0.015 ± 0.000[d]	0.600 ± 0.000[a] 0.300 ± 0.000[b]	0.450 ± 0.000[b] 0.225 ± 0.000[b]	0.075 ± 0.000[b] 0.038 ± 0.000[b]	0.300 ± 0.000[b] 0.150 ± 0.000[c]	0.060 ± 0.000[a] 0.030 ± 0.000[b]
9.	*B. toyonensis* GT3[G]	0.060 ± 0.000[b] 0.030 ± 0.000[b]	0.060 ± 0.000[c] 0.030 ± 0.000[c]	>0.600 ± 0.000[a] 0.600 ± 0.000[a]	>0.900 ± 0.000[a] >0.450 ± 0.000[a]	0.150 ± 0.000[a] 0.075 ± 0.000[a]	0.600 ± 0.000[a] 0.300 ± 0.000[b]	>0.060 ± 0.000[a] 0.060 ± 0.000[a]
10.	*P. monteilii* GT4[G]	0.112 ± 0.000[a] 0.060 ± 0.000[a]	0.240 ± 0.000[a] 0.112 ± 0.000[a]	>0.600 ± 0.000[a] 0.600 ± 0.000[a]	>0.900 ± 0.000[a] >0.450 ± 0.000[a]	0.150 ± 0.000[a] 0.075 ± 0.000[a]	>0.600 ± 0001[a] >0.600 ± 0.001[a]	>0.060 ± 0.000[a] 0.060 ± 0.000[a]
11.	*P. simplex* GT5[G]	0.060 ± 0.000[b] 0.030 ± 0.000[b]	0.112 ± 0.000[b] 0.060 ± 0.000[b]	>0.600 ± 0.000[a] 0.600 ± 0.000[a]	>0.900 ± 0.000[a] >0.450 ± 0.000[a]	0.150 ± 0.000[a] 0.075 ± 0.000[a]	>0.600 ± 0.000[a] 0.300 ± 0.000[b]	>0.060 ± 0.000[a] 0.060 ± 0.000[a]
12.	*S. arlettae* GT6 28.2[G]	0.030 ± 0.000[c] 0.015 ± 0.000[c]	0.030 ± 0.000[d] 0.015 ± 0.000[d]	>0.600 ± 0.000[a] 0.600 ± 0.000[a]	0.900 ± 0.000[a] 0.450 ± 0.000[a]	0.075 ± 0.000[b] 0.038 ± 0.000[b]	0.150 ± 0.000[c] 0.075 ± 0.000[d]	>0.060 ± 0.000[a] 0.060 ± 0.000[a]

All concentration values show significant differences ($P < 0.05$). Small letters in superscript indicate the levels of statistical significance. [G], guano-dwelling strains; [NR], non-resistant strains; PGS-LP, pygidial gland secretion of *L.* (*P.*) *punctatus*; [R], resistant strains; SE, standard error; >, not determined at the maximum tested concentration.

Formic acid was found to achieve a stronger antibacterial activity against most clinical and laboratory strains compared to the secretion of *L.* (*P.*) *punctatus*, but the secretion was more effective against two guano-dwelling strains — *P. monteilii* (MIC 0.060 ± 0.000 mg/mL) and *P. simplex* (MIC 0.030 ± 0.000 mg/mL). The other guano-dwelling strains tested showed the same susceptibility to formic acid.

In contrast to all resistant strains, MRSA showed a higher susceptibility to the secretion of *L.* (*P.*) *punctatus* (MIC 0.015 ± 0.000 mg/mL) and formic acid (MIC 0.002 ± 0.000 mg/mL), but exhibited a higher resistance to all positive controls. Among all positive controls tested, gentamicin and ampicillin achieved the highest antimicrobial potential against MRSA. Based on these facts, this bacterial strain was tested in further steps of this research.

3.3. *In vitro* screening of antifungal activity

The initial analysis indicated that all tested strains of *Candida* species showed a high susceptibility to undecane, dodecyl acetate, and acetic acid (MIC values were not obtained due to the fungicidal effect of undecane, dodecyl acetate, and acetic acid even at the lowest concentration tested) and simultaneously a high resistance to palmitic and stearic acids (MIC values were not detected due to the observed growth of fungal cultures even at the highest concentration tested), thus these chemicals were excluded from further examinations.

The tested pygidial gland secretion of *L.* (*P.*) *punctatus* and the standards of formic and oleic acids achieved a certain antifungal potential against all tested *Candida* species, including the resistant *C. auris* and non-resistant strains, compared to the commercial antimycotics nystatin and amphotericin B ($P < 0.05$) (Table 3).

Candida albicans and *C. tropicalis* were found to be more sensitive to the secretion of *L.* (*P.*) *punctatus* (with MIC 0.024 ± 0.000 mg/mL and MFC 0.048 ± 0.000 mg/mL, respectively) compared to the clinical isolate *C. krusei* and the resistant *C. auris* strain (with MIC 0.048 ± 0.000 mg/mL and MFC 0.096 ± 0.000 mg/mL, respectively). *Candida krusei* and the resistant *C. auris* showed a higher susceptibility to formic acid (with MIC 0.008 ± 0.000 mg/mL and MFC 0.015 ± 0.000 mg/mL, respectively) (Table 3).

Oleic acid achieved a significantly weaker antifungal activity compared to all tested agents, with MIC value in a range of 0.150–0.300 mg/mL.

TABLE 3 *In vitro* screening of antifungal activity displayed as mean values (MEAN) of the minimum fungicidal concentrations (MFCs) and minimum inhibitory concentrations (MICs) of the pygidial gland secretion of *L.* (*P.*) *punctatus* and the standards of formic and oleic acids alone compared to positive controls (antimycotics nystatin and amphotericin B).

No.	Fungal strains	Agents MFC (MEAN ± SE) (mg/mL) MIC (MEAN ± SE) (mg/mL)				
		PGS-LP	Formic acid	Oleic acid	Nystatin	Amphotericin B
1.	*C. albicans* ATCC 10231[NR]	0.048 ± 0.000[b] 0.024 ± 0.000[b]	0.030 ± 0.000[a] 0.015 ± 0.000[a]	0.600 ± 0.000[a] 0.300 ± 0.000[a]	0.056 ± 0.000[c] 0.028 ± 0.000[d]	0.056 ± 0.000[b] 0.028 ± 0.000[a]
2.	*C. krusei* H1/16[NR]	0.096 ± 0.000[a] 0.048 ± 0.000[a]	0.015 ± 0.000[b] 0.008 ± 0.000[b]	>0.600 ± 0.000[a] 0.300 ± 0.000[a]	0.225 ± 0.000[a] 0.113 ± 0.000[d]	0.113 ± 0.000[a] 0.056 ± 0.000[b]
3.	*C. tropicalis* ATCC 750[NR]	0.048 ± 0.000[b] 0.024 ± 0.000[b]	0.030 ± 0.000[a] 0.015 ± 0.000[a]	0.300 ± 0.000[b] 0.150 ± 0.000[b]	0.056 ± 0.000[c] 0.028 ± 0.000[d]	0.113 ± 0.000[a] 0.056 ± 0.000[b]
4.	*C. auris* CDC B11903[R]	0.096 ± 0.000[a] 0.048 ± 0.000[a]	0.015 ± 0.000[b] 0.008 ± 0.000[b]	>0.600 ± 0.000[a] 0.300 ± 0.000[a]	0.450 ± 0.000[a] 0.225 ± 0.000[d]	0.056 ± 0.000[b] 0.028 ± 0.000[a]

All concentration values show significant differences ($P < 0.05$). Small letters in superscript indicate the levels of statistical significance. [NR], non-resistant strains; PGS-LP, pygidial gland secretion of *L.* (*P.*) *punctatus*; [R], resistant strain; SE, standard error; >, not determined at the maximum tested concentration.

When comparing the positive controls, amphotericin B proved to have a better antifungal activity (MIC 0.028–0.056 mg/mL) than nystatin (MIC 0.028–0.225 mg/mL).

With respect to all *Candida* strains tested, the secretion of *L.* (*P.*) *punctatus* and formic acid showed higher antifungal potential compared to positive controls.

3.4. *In vitro* screening of antimicrobial activity of selected agents tested in combinations using a 2D and a 3D checkerboard assay

An *in vitro* checkerboard method showed that among the selected combinations against resistant laboratory and clinical strains tested only formic acid (MIC 0.0005 ± 0.0000 mg/mL) in combination with gentamicin (MIC 0.0010 ± 0.0000 mg/mL) achieved a synergistic antibacterial effect against MRSA (FICI = 0.5, $P < 0.05$) (Figure 1 and Table 4), whereas the other combinations tested achieved an additive effect ($P < 0.05$), with the exception of the combination of formic and oleic acids that showed indifference to all tested bacteria and *C. auris*, as well as the combination of formic acid and gentamicin that demonstrated indifference to the resistant *P. aeruginosa* strain (Figure 1). Based on this fact, only formic acid in combination with selected antimicrobial drugs was further tested using a 2D and a 3D checkerboard method.

The results of the *in vitro* 3D checkerboard method showed that the combination of formic acid, gentamicin, and ampicillin expresses an additive effect (FICI = 1.5, $P < 0.05$) (Figure 1) against the MRSA strain, with the following MIC values in the tested combination, respectively: 0.0010 ± 0.0000 mg/mL (formic acid), 0.0020 ± 0.0000 mg/mL (gentamicin), and 0.0380 ± 0.0000 mg/mL (ampicillin) (Table 4).
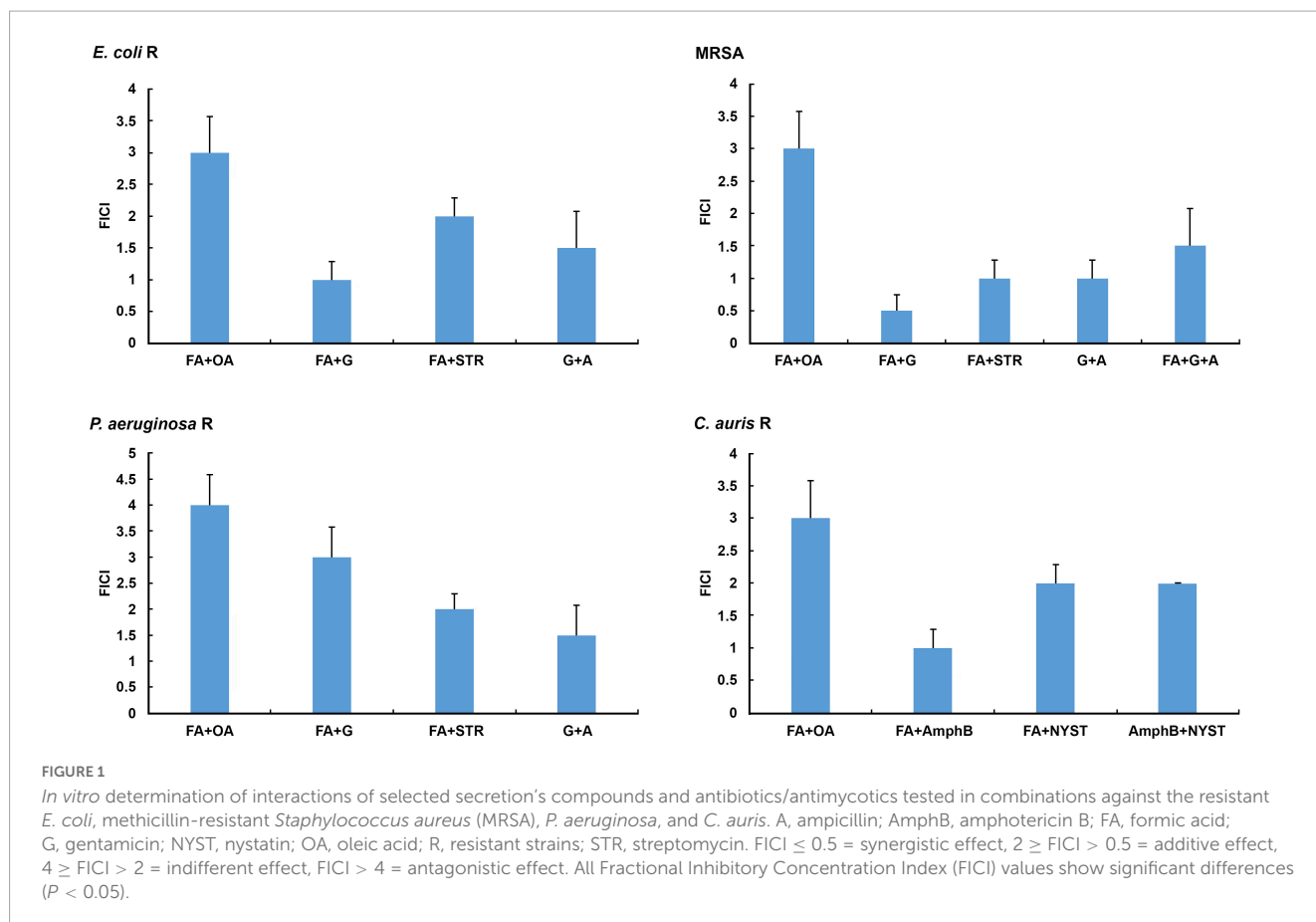
All tested bacteria showed higher resistance to oleic acid in combinations, while formic acid and gentamicin in combination showed stronger antimicrobial activity against the tested strains (Table 4).

Candida auris showed the highest susceptibility to the combination of formic acid and amphotericin B (Figure 1 and Table 4).

3.5. *In vitro* screening of anti-adhesive, anti-invasive, and cytotoxic effect toward HaCaT cells

In vitro antimicrobial screening indicated that MRSA showed a significantly high level of susceptibility to all tested agents compared to other resistant strains, especially to the secretion of *L.* (*P.*) *punctatus* (MIC 0.015 ± 0.000 mg/mL, MBC 0.030 ± 0.000 mg/mL, $P < 0.05$), formic acid (MIC 0.002 ± 0.000 mg/mL, MBC 0.004 ± 0.000 mg/mL, $P < 0.05$) and, in particular, the combinations of formic acid and antibiotics acting additively and synergistically, but at the same time showed a certain level of resistance to the antibiotics tested individually. In accordance with the preceding facts, this strain was used to analyze the effects of the selected agents tested (secretion, formic acid, formic acid in combination with gentamicin, and ampicillin) on the adhesion and invasion ability of MRSA toward HaCaT cells. Likewise, the relative proliferation rate of HaCaT cells after treatment with different concentrations of the secretion of *L.* (*P.*) *punctatus* and formic acid (MIC 0.025-0.400 mg/mL) was observed to exhibit the level of cytotoxicity. All tested agents alone and in combination achieved a significant level of anti-adhesive and anti-invasive effect compared to the negative control (100% of adhesion and invasion) ($P < 0.001$) (Figures 2A, B). The secretion of *L.* (*P.*) *punctatus* with a MIC of 0.015 ± 0.001 mg/mL caused a significant decrease in the adhesion rate of MRSA toward HaCaT cells to 44.075 ± 6.585%, which was higher than the one of formic acid (adhesion rate 59.471 ± 0.290%) ($P < 0.001$) (Figure 2A). The combination of formic acid, gentamicin, and ampicillin proved to have the highest level of anti-adhesive effect compared to the negative control and the other tested agents by reducing the adhesion rate of MRSA toward HaCaT cells to 1.623 ± 0.491% ($P < 0.001$), including gentamicin and ampicillin in combination (adhesion rate 2.366 ± 0.409%), but with no significant difference

FIGURE 1

In vitro determination of interactions of selected secretion's compounds and antibiotics/antimycotics tested in combinations against the resistant *E. coli*, methicillin-resistant *Staphylococcus aureus* (MRSA), *P. aeruginosa*, and *C. auris*. A, ampicillin; AmphB, amphotericin B; FA, formic acid; G, gentamicin; NYST, nystatin; OA, oleic acid; R, resistant strains; STR, streptomycin. FICI ≤ 0.5 = synergistic effect, 2 ≥ FICI > 0.5 = additive effect, 4 ≥ FICI > 2 = indifferent effect, FICI > 4 = antagonistic effect. All Fractional Inhibitory Concentration Index (FICI) values show significant differences ($P < 0.05$).
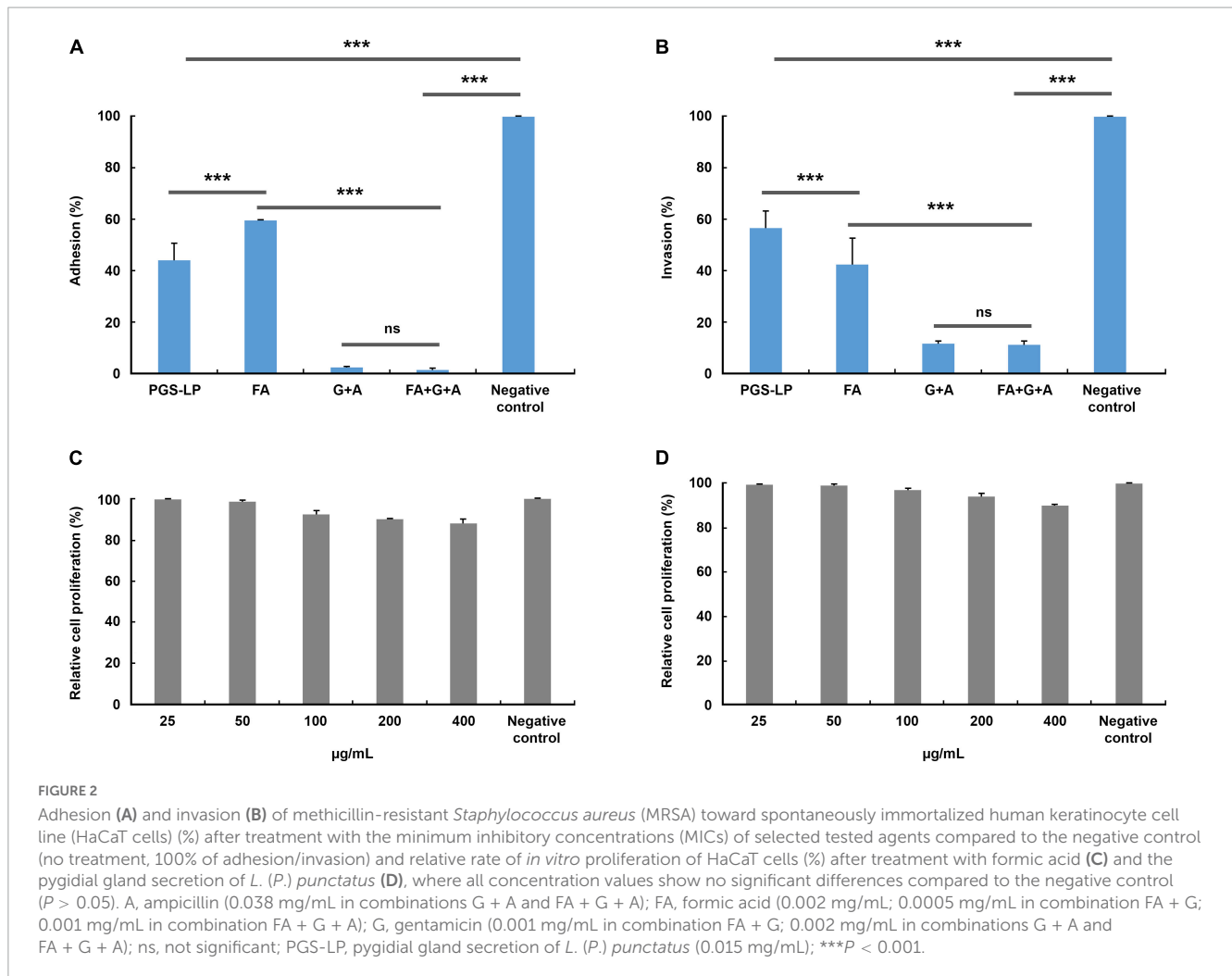
TABLE 4 *In vitro* screening of the antimicrobial activity of the tested combinations against resistant strains displayed as mean values (MEAN) of the minimum inhibitory concentrations (MICs) of the mixtures.

No.	Resistant strains	Agents MIC^A (MEAN ± SE) (mg/mL) MIC^B (MEAN ± SE) (mg/mL) MIC^C (MEAN ± SE) (mg/mL)							
		Formic acid^A + Oleic acid^B	Formic acid^A + Gentamicin^B	Formic acid^A + Streptomycin^B	Gentamicin^A + Ampicillin^B	Formic acid^A + Gentamicin^B + Ampicillin^C	Formic acid^A + Amph. B^B	Formic acid^A + Nystatin^B	Amph. B^A + Nystatin^B
1.	*E. coli* IBRS E003	0.0020 ± 0.0000^{Ad} 0.6000 ± 0.0000^{Ba}	0.0010 ± 0.0000^{Ae} 0.0005 ± 0.0000^{Bf}	0.0020 ± 0.0000^{Ad} 0.2250 ± 0.0000^{Bb}	0.0005 ± 0.0000^{Af} 0.0190 ± 0.0000^{Bc}	nt	nt	nt	nt
2.	*P. aeruginosa* IBRS P001	0.0080 ± 0.0000^{Ad} 0.6000 ± 0.0000^{Ba}	0.0080 ± 0.0000^{Ad} 0.0010 ± 0.0000^{Bf}	0.0040 ± 0.0000^{Ae} 0.2250 ± 0.0000^{Bb}	0.0010 ± 0.0000^{Af} 0.0380 ± 0.0000^{Bc}	nt	nt	nt	nt
3.	MRSA IBRS 011	0.0040 ± 0.0000^{Ad} 0.3000 ± 0.0000^{Ba}	0.0005 ± 0.0000^{Ag} 0.0010 ± 0.0000^{Bf}	0.0010 ± 0.0000^{Af} 0.2250 ± 0.0000^{Bb}	0.0020 ± 0.0000^{Ae} 0.0380 ± 0.0000^{Bc}	0.0010 ± 0.0000^{Af} 0.0020 ± 0.0000^{Be} 0.0380 ± 0.0000^{Cc}	nt	nt	nt
4.	*C. auris* CDC B11903	0.0160 ± 0.0000^{Ad} 0.3000 ± 0.0000^{Ba}	nt	nt	nt	nt	0.0040 ± 0.0000^{Af} 0.0140 ± 0.0000^{Be}	0.0040 ± 0.0000^{Af} 0.2250 ± 0.0000^{Bb}	0.0280 ± 0.0000^{Ac} 0.2250 ± 0.0000^{Bb}

All concentration values show significant differences ($P < 0.05$). According to the checkerboard method, all presented agents showed different effects in combinations. Small letters in superscript indicate the levels of statistical significance, while capital letters in superscript indicate the order of compounds in the combination. Amph. B, amphotericin B; nt, not tested; SE, standard error.

(**Figure 2A**). In contrast, the secretion of *L.* (*P.*) *punctatus* showed a significantly lower inhibitory effect against MRSA, with the invasion rate of 56.189 ± 6.676%, compared to the rate of formic acid of 42.105 ± 10.255% ($P < 0.001$) (**Figure 2B**). Likewise, the combination of formic acid, gentamicin, and ampicillin achieved a similar effect in an invasion assay (invasion rate 11.011 ± 1.266%) as did gentamicin and ampicillin in combination

(11.444 ± 0.987%), with no significant difference. The combination of formic acid, gentamicin, and ampicillin significantly reduced adhesion and invasion compared to the secretion of *L.* (*P.*) *punctatus* and formic acid alone ($P < 0.001$). An *in vitro* crystal violet assay showed that the secretion and formic acid had no antiproliferative activity against HaCaT cells (IC$_{50}$ values were ≥ 0.401 mg/mL for all samples tested) (**Figures 2C, D**).

FIGURE 2

Adhesion **(A)** and invasion **(B)** of methicillin-resistant *Staphylococcus aureus* (MRSA) toward spontaneously immortalized human keratinocyte cell line (HaCaT cells) (%) after treatment with the minimum inhibitory concentrations (MICs) of selected tested agents compared to the negative control (no treatment, 100% of adhesion/invasion) and relative rate of *in vitro* proliferation of HaCaT cells (%) after treatment with formic acid **(C)** and the pygidial gland secretion of *L.* (*P.*) *punctatus* **(D)**, where all concentration values show no significant differences compared to the negative control ($P > 0.05$). A, ampicillin (0.038 mg/mL in combinations G + A and FA + G + A); FA, formic acid (0.002 mg/mL; 0.0005 mg/mL in combination FA + G; 0.001 mg/mL in combination FA + G + A); G, gentamicin (0.001 mg/mL in combination FA + G; 0.002 mg/mL in combinations G + A and FA + G + A); ns, not significant; PGS-LP, pygidial gland secretion of *L.* (*P.*) *punctatus* (0.015 mg/mL); ***$P < 0.001$.

4. Discussion

In recent years, the number of publications focused on the importance of insect products in biomedicine is increasing (Ratcliffe et al., 2014; Seabrooks and Hu, 2017; Nenadić, 2019). These studies have revealed that among organic compounds, salicylaldehyde, short-chain fatty acids, such as formic, butyric, and isobutyric, and long-chain fatty acids could be associated with the antimicrobial activity of insect products (Glassman, 1948; Raftari et al., 2009; Desbois and Smith, 2010; Nenadić et al., 2016a, 2017b; Nenadić, 2019). One of the latest *in vitro* studies indicates that the defensive gland secretion of *Luprops tristis* Fabricius, 1801 (Coleoptera: Tenebrionidae) could be a very effective antimicrobial agent against *S. aureus* and *E. coli* as it contains oleic acid and specific compounds, such as 2,3-dimethyl-1,4-benzoquinone, 1,3-dihydroxy-2-methylbenzene, 2,5-dimethylhydroquinone, tetracosane, hexacosane, pentacosane, 7-hexadecenal, and tert-hexadecanethiol (Sabira et al., 2022).

The antimicrobial activity of the secretions of representatives of the ground beetle subfamily Platyninae was initially investigated by Nenadić et al. (2016b), who presented the first *in vitro* antimicrobial screening of the pygidial gland secretion of *L.* (*P.*) *punctatus* against various human microbial pathogens, including clinical and laboratory strains. Although undecane and dodecyl acetate were found in its secretion mixture in the highest percentage (Vesović et al., 2015), their possible antimicrobial properties have not been reported in the literature. Likewise, all tested bacterial and fungal strains showed high susceptibility to undecane and dodecyl acetate in the current study. Hydrocarbons, such as undecane, are thought to act as penetration facilitators of active compounds through the lipophilic cuticle of predators (Blum, 1981). Despite the fact that formic acid was present in a smaller percentage (19.4%) in this secretion than undecane and dodecyl acetate (Vesović et al., 2015), there is evidence of the antimicrobial properties of this compound (Gómez-García et al., 2019; Luise et al., 2020; Peh et al., 2020). Oleic acid, which was represented in this secretion mixture with 0.1% (Vesović et al., 2015), proved to have certain antimicrobial properties in this study, so it could probably give an additional contribution to the antimicrobial activity of this secretion (Vesović et al., 2015; Nenadić, 2019).

In previous studies, it was assumed that the antimicrobial activity of the secretion of *L.* (*P.*) *punctatus* is probably caused by the interaction of several chemical components acting synergistically (Nenadić et al., 2016b; Nenadić, 2019), but in this study we found that the selected fatty acids from the secretion were indifferent in combination as formic acid achieved much stronger antimicrobial activity compared to oleic acid.

In this study, the beta-lactam antibiotics ampicillin and cephalexin and the aminoglycoside streptomycin were found to have weaker antibacterial activity than the tested secretion and formic acid. In contrast, Nenadić et al. (2016b) noted that the commercial antibiotics streptomycin and ampicillin and the antifungal agents ketoconazole and bifonazole generally exhibited higher antimicrobial potential against all tested bacterial and fungal strains, with the exception of *Penicillium ochrochloron*, which was found to be more resistant to ketoconazole and more sensitive to the pygidial gland secretion of *L.* (*P.*) *punctatus* (MIC 1.1 mg/mL, MBC 2.2 mg/mL). Interestingly, the secretion displayed a higher antibacterial potential against the non-resistant *E. coli*, *P. aeruginosa*, and *S. aureus* strains in the current study, with MIC values about 100 times lower.

Nenadić et al. (2016b) showed that the non-resistant *E. coli* ATCC 35210 proved to be the most sensitive bacterium to the secretion of *L.* (*P.*) *punctatus*, while in the current study the non-resistant *E. coli* ATCC 25922 strain showed the highest sensitivity to this secretion compared to all tested bacteria. On the other hand, in this study, the resistant *E. coli* strain and MRSA showed higher sensitivity compared to all resistant strains tested. Regarding antifungal activity, the secretion and formic acid showed higher antifungal potential in the present study compared to the positive controls amphotericin B and nystatin.

Nenadić et al. (2017a) reported the first evidence of the antimicrobial properties of the same secretion against the cave-dwelling micromycetes *Talaromyces duclauxii*, *Aspergillus brunneouniseriatus*, *Penicillium* sp., *Rhizopus stolonifer*, and *Trichoderma viride*. The most sensitive fungal isolate proved to be *Penicillium* sp. (MIC 0.500 ± 0.029 mg/mL), while the other isolates displayed a high level of resistance to the secretion. According to Dimkić et al. (2020), the most sensitive guano-dwelling fungal strains to this secretion were *Meyerozyma guilliermondii*, *Penicillium expansum*, and *Trichoderma harzianum* (each with MIC 0.150–0.300 mg/mL). These studies indicate that the defensive strategy of *L.* (*P.*) *punctatus*, primarily aiming to protect the beetle from predators, can also be associated with defense against microorganisms from the same habitat (Nenadić et al., 2017a; Nenadić, 2019; Dimkić et al., 2020).

According to Dimkić et al. (2020), the pygidial gland secretion of *L.* (*P.*) *punctatus* proved to have certain antibacterial properties against 12 tested guano-dwelling bacterial isolates. Likewise, Dimkić et al. (2020) revealed that gram-positive bacterial isolates generally showed a higher level of sensitivity to this secretion compared to gram-negative strains. This is confirmed in the current study as the gram-negative bacterium *P. monteilii* proved to be the most resistant guano-dwelling strain to the secretion compared to other tested gram-positive guano-dwelling isolates. *Escherichia fergusonii* showed a pronounced sensitivity to the secretion, almost twice as high compared to the tested antibiotic streptomycin. The most sensitive isolate proved to be *Enterococcus eurekensis*, with the lowest recorded MIC value of 0.007 mg/mL (Dimkić et al., 2020), while the guano-dwelling strains with the highest susceptibility in the current study were *B. subtilis*, *B. clausii*, and *S. arlettae*, each with a MIC value of 0.015 mg/mL. Interestingly, in this study, *B. subtilis*, *B. clausii*, *B. toyonensis*, and *S. arlettae* showed the same level of susceptibility to the secretion and the standard of formic acid, with simultaneous resistance to the antibiotics streptomycin, cephalexin, ampicillin, and gentamicin, while oleic acid achieved

inhibitory effect at the highest concentration tested, with 20 times higher MIC compared to the secretion and formic acid alone. The resistant *P. aeruginosa* strain was the least susceptible bacterium to the secretion, together with *P. monteilii*. As guano-dwelling bacteria showed a significant level of sensitivity to the secretion of *L.* (*P.*) *punctatus* and formic acid as an important antibacterial constituent of this mixture (Nenadić, 2019) ($P < 0.05$), the cause of proved antimicrobial, anti-adhesive, and anti-invasive potential of this secretion and formic acid against clinical and laboratory strains probably lies in a possible defensive strategy against potential microbial pathogens from the same habitat, with which these insects frequently come into contact (Nenadić, 2019; Dimkić et al., 2020). It is worth mentioning that the bacterial community of guano may contain pathogens responsible for various diseases important in human medicine (representatives of the genera *Bacillus*, *Pseudomonas*, *Staphylococcus*, *Escherichia*, *Enterococcus*, etc.), which further confirms the significance of the relevant studies (Banskar et al., 2016; Dimkić et al., 2020). This means that the antimicrobial properties of the tested secretion, apart from its ecological importance, could also have medical significance in the future.

Methicillin-resistant *Staphylococcus aureus* undeniably represents a significant strain due to its resistance to many antibiotics, such as methicillin, penicillin, oxacillin, cloxacillin, cefazolin, cefoxitin, etc. (Larsen et al., 2022; Nandhini et al., 2022). For this reason, but also due to its demonstrated increased susceptibility to the natural product (secretion) and formic acid compared to other resistant bacteria, this strain was the focus of this study. Regarding the antimicrobial properties of insect products against MRSA, literature data mainly refer to the effect of antimicrobial proteins. Li et al. (2017) showed that insect defensins DLP2 and DLP4 have higher antimicrobial potential, better efficiency, faster action, and longer post-antibiotic effect compared to the antibiotic vancomycin. A novel strategy against resistant microorganisms is a synergistic or combination therapy, particularly when it involves the combinations of existing commercial medications and natural products (Pancu et al., 2021). Regarding the antimicrobial activity of commercial antimicrobials in combination with natural products against MRSA, there is evidence of higher efficacy of gentamicin in combination with plant extracts. According to Blesson et al. (2015), the synergistic antibacterial activity of gentamicin and an aqueous extract of *Colocasia esculenta* (L.) Schott, a widely used traditional medicinal plant, may be a promising future therapy in the fight against MRSA infections. Similarly, the combination of formic acid and monolaurin may reduce intestinal inflammation caused by enterotoxigenic *E. coli* in piglets by inhibiting their NF-κB/MAPK pathways (Ren et al., 2020). In this study, we found that the combination of formic acid, gentamicin, and ampicillin showed an additive effect against MRSA strain. Simultaneously, the same combination significantly reduced the adhesion rate of MRSA toward HaCaT cells. Nenadić et al. (2018) investigated the antitumor and antiproliferative potential of the pygidial gland secretion of *L.* (*P.*) *punctatus*. Although this secretion showed certain level of inhibition of porcine liver primary cell proliferation (14.72 ± 0.86%) (Nenadić et al., 2018), it wasn't found to be antiproliferative toward HaCaT cells in this study, which was also the case with formic acid (IC$_{50}$ ≥ 401 μg/mL). Bioactive natural products in combination with commercial antibiotics

and antifungal drugs could represent an alternative source of antimicrobial agents. In recent years, a new therapeutic approach based on the synergistic effect of existing antibiotics and natural products has been mainly explored. On the other hand, anti-adhesion-based therapies are promising in reducing the interaction between pathogens and host tissues, which could lead to the prevention of any possibility of infection and disease development (Ofek et al., 2003; Jiang et al., 2012; Asadi et al., 2019). The current study suggests that the secretions of ground beetles and other insects, presumably with the aim of protecting themselves against pathogens from the same habitat (Giglio et al., 2011; Nenadić, 2019; Dimkić et al., 2020), have unpredictably become a source of potential therapeutics, which is very important for the fight against the current global antimicrobial resistance. Likewise, this study indicates that the future of treating bacterial and fungal infections lies in the use of anti-adhesive therapeutics. Since current scientific research on the potential therapeutic functions of insects suggests that these organisms may become an important source of novel drugs (Feng et al., 2009; Nenadić, 2019), the present study may inspire the development of new synthetic analogs with potential medical application in combined therapies in the future. Further research will aim to better understand the biological activities of antimicrobial agents from the pygidial gland secretions of ground beetles *in vitro* and *in vivo*.

Data availability statement

The original contributions presented in this study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

MN: study design, conceptualization, software - statistical analysis, processing of figures, writing—original draft preparation, and visualization. MN, MS, AĆ, DS, ID, and SĆ: methodology - adaptation. SĆ, NV, and SSS: fieldwork investigation and collection of insect samples. LV and MT: quantitative analysis. MN and AĆ: methodology - *in vitro* screening of antimicrobial activity. MN and DS: methodology - *in vitro* assay for evaluation of anti-adhesive and anti-invasive potential of tested agents and *in vitro* cytotoxicity assay. ID and TJ: preparation of cultures of guano-dwelling bacterial strains. NV: software - figures editing. SĆ, AĆ,

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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