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Gianfranco Donelli *Editor*

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The Efficacy of an Electrolysed Water Formulation on Biofilms

Anne-Marie Salisbury and Steven L. Percival

Abstract

Electrolysed water is a basic process whereby an electric current is passed through deionised water containing a low concentration of sodium chloride in an electrolysis chamber, which results in a more complex chemistry resulting in the production of a strong bactericidal and fungicidal solution at the anode. This microbicidal solution contains hypochlorous acid that is fast-acting and environmentally safe, as upon bacterial killing, the equilibrium shifts from hypochlorous acid back to salt and water. Other antimicrobial agents produced in this process include sodium hypochlorite and chlorine. The use of electrolysed water formulations in wound care to control wound bioburden is underway. However, there is limited evidence of the efficacy of electrolysed water on the control of biofilms, which are renowned for their tolerance to a variety of antimicrobials. Therefore this study aimed to assess a new electrolysed water formulation on in vitro *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms. Results showed that the electrolysed water formulation effectively reduced biofilm in all models following a 15 min contact time. Microbial cell counts confirmed the reduction biofilm bacteria. Additional cytotoxicity using L929 fibroblasts confirmed that a 50% and 25% dilution of the electrolysed water formulation was non-cytotoxic to cells. In conclusion, this study has confirmed that the application of a new electrolysed water product effectively removed biofilm after a short exposure time. The use of this technology as a wound cleanser may help to control existing biofilms in complicated, non-healing wounds.

Keywords

Antimicrobial · Biofilms · Electrolysed water · Hypochlorous acid · Wounds

1 Introduction

Electrolysis of deionised water containing a low concentration of sodium chloride (0.1%) in an electrolysis chamber whereby the anode and cathode electrodes are separated by a diaphragm has been shown to impart strong bactericidal, fungicidal and virucidal properties to the water collected from the anode. Much research surrounding the bactericidal, fungicidal and virucidal properties of electrolysed water has been investigated in dentistry and the food industry (Huang et al. 2008; Horiba et al. 1999).

By subjecting the electrodes to direct current voltages, negatively charged ions such as chloride and hydroxide in the diluted salt solution move to the anode to give up electrons and become oxygen

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gas, chlorine gas, hypochlorite ions, hypochlorous acid, hydrochloric acid and a small concentration of sodium hypochlorite (by-product), whilst positively charged ions such as hydrogen and sodium move to the cathode to take up electrons and become hydrogen gas and sodium hydroxide. Therefore, the production of two types of water occurs simultaneously: (1) electrolysed water (EW) (from the anode side) with a low pH (2.3–2.7), high oxidation–reduction potential (ORP) (>1000 mV), high dissolved oxygen concentration and free chlorine (between 10 and 80 ppm) and (2) EW (from the cathode) with a high pH (10.0–11.5), high dissolved hydrogen and low ORP (−800 to −900 mV). EW with a high ORP is often referred to in the literature as acidic electrolysed water or electrolysed oxidising water, whereas the EW with a low ORP is referred to as electrolysed-reduced water (Hsu 2005).

The control of bioburden in open wounds is considered to be of great importance in the promotion of wound healing and is thought to reduce the risk of slow wound closure (Wolcott et al. 2010; Edwards and Harding 2004). The development of microbial biofilms within chronic wounds has been reported (James et al. 2008; Dowd et al. 2008; Kirketerp-Møller et al. 2008). Biofilms are complex societies of microorganisms that attach to a surface and surround themselves with extracellular polymeric substances and have increased tolerance to antimicrobials (Donlan 2002). Therefore as a wound care strategy, the control of these biofilms should be investigated. Electrolysed water has already been reported to have positive effects on wound healing (Sen et al. 2002). In vivo studies have shown that the use of acidic or neutral electrolysed water also increases wound healing in full-thickness cutaneous wounds in rats, potentially via keratinocyte and fibroblast migration (Yahagi et al. 2000).

Therefore the aim of this study was to assess the antimicrobial and anti-biofilm efficacy of a new formulation of electrolysed water against microorganisms associated with complicated chronic wounds.

2 Method

2.1 Microorganisms

The microorganisms *Staphylococcus aureus* ATCC 25923 and *Pseudomonas aeruginosa* NCTC 10662 were employed in all studies.

2.2 Test Solution

The electrolysed water used in this study was produced by passing an electric current through deionised water. Product was collected from the Anode chamber and consisted of Hypochlorous acid, Hypochlorite, Hydroperoxyl, Hydroxide, Hydrogen peroxide, Chlorine and Singlet Oxygen, with the 2 main constituents being Sodium hypochlorite and Hypochlorous acid.

2.3 Planktonic Time-Kill Studies

This test followed the 10 mL sample size procedure outlined in the American Society for Testing and Materials (ASTM) E2783–11. An overnight culture was set up by inoculating 10 mL tryptone soya broth (TSB) (Oxoid, Hampshire, UK) with a single colony and incubating it at 37 °C and 125 rpm overnight. The following day, the overnight culture was adjusted to 0.5 McFarland (~1×10^8 CFU/mL) before adding 0.1–10 mL (~1×10^6 CFU/mL) of the test antimicrobial. Inoculated samples were incubated at 37 °C (shaken at 125 rpm) for 1 min, 5 min and 24 h. For each time point, a 1 mL sample was taken and diluted 1:10 in the dilution blank. The procedure was performed in triplicate. A blank control (no antimicrobial) was included containing a 0.85% sodium chloride solution. Bacterial enumeration was performed by counting bacterial colonies using the spot-plate technique, which was performed in triplicate. The $\log_{10}$ of each

sample was subtracted from the $\log_{10}$ value of the blank control to determine the $\log_{10}$ reduction value. Percentage kill was then calculated using the following formula:

$$\%kill = \left(1 - 10^{-\text{Log } reduction\ value}\right) \times 100$$

2.4 CDC Biofilm Bioreactor Model

The experimental procedure for the CDC biofilm reactor studies followed an adapted version of the ASTM E2871–13, Standard Test Method for Evaluating Disinfectant Efficacy Against *Pseudomonas aeruginosa* Biofilm Grown in the CDC Biofilm Reactor Using Single Tube Method.

TSB was inoculated with bacteria to a concentration of 1×10^8 CFU/ml, which was determined by optical density and total viable counts.

Each CDC reactor contains eight polypropylene rods designed to hold three coupons. In this experiment, polycarbonate coupons were used. The CDC reactor was sterilised before aseptically adding 300 ml of sterile TSB through the inoculation port. Following this, 1 ml of the previously prepared 10^8 CFU/ml inoculum was then added to the reactor. The reactor was placed on a magnetic stir plate. The rotation speed was set to 125 ± 5 rpm. The CDC reactor was operated in batch mode at room temperature (21 ± 2 °C) for 48 h (48 h biofilms).

Following incubation, the rods containing the polycarbonate coupons were removed and rinsed in sterile 0.85% sodium chloride solution to remove planktonic cells. Each coupon was released from the rods into individual sterile 50 ml centrifuge tubes. Each coupon was then treated with 3 mL of the electrolysed water. Treatments of the coupons were performed in duplicate. Antimicrobials used included the electrolysed water at concentrations of 100%, 75%, 50% and 25%. The coupons were then incubated at 37 °C for 5, 15 or 60 min contact times. Following incubation, the antimicrobial was diluted in 27 ml of sterile water. Each 50 ml centrifuge tube containing the coupon and neutraliser was mixed using a vortex for 30 s, followed by sonication for 10 min. The disaggregated biofilm samples were sampled and serially diluted on tryptone soya agar (TSA) (Oxoid, Hampshire, UK) for bacterial enumeration using the spot-plate technique, which was performed in triplicate. Biofilm density was calculated as $\log_{10}$ density for each coupon. The $\log_{10}$ density of each coupon was subtracted from the $\log_{10}$ density of the untreated control coupon to determine the $\log_{10}$ reduction value of each treated biofilm. Percentage kill was then calculated using the following formula:

$$\%kill = \left(1 - 10^{-\text{Log } reduction\ value}\right) \times 100$$

2.5 Cytotoxicity Testing

Indirect cytotoxicity tests performed were in accordance with the international standard ISO 10993–5 Biological Evaluation of Medical Devices – Part 5: Tests for in vitro cytotoxicity. To supplement the guidance in ISO 10993–5, the ASTM 895–11 Standard Test Method for Agar Diffusion Cell Culture Screen for Cytotoxicity was also used. Several negative control samples were used including Dulbecco's Modified Eagle's Medium (DMEM) supplemented with foetal bovine serum (10%) and penicillin-streptomycin (100 units/ml), phosphate-buffered saline and distilled water. For a positive control of cytotoxicity, sodium dodecyl sulphate (SDS) was used at 0.05, 0.10, 0.15 and 0.2 mg/ml.

L929 fibroblast cell cultures were examined microscopically, and any cell monolayers that were not of correct confluency or showed signs of granulation or sloughing were rejected. A 3% solution of agar (suitable for cell culture) was made and autoclaved for 15 min at 121 °C. The autoclaved agar was put into a 45 °C water bath and allowed to cool to 45 °C. DMEM was warmed to 45 °C in a water bath. Equal volumes of the DMEM and 3% Nobel agar were mixed and allowed to cool to approximately 39 °C. The medium from all acceptable cultures was removed and replaced with 2 ml of agar medium. The cultures were placed on a flat surface to solidify at room temperature. Sterile, 10 mm filter discs were saturated in test solution or control

Fig. 1 Image of the indirect cytotoxicity experiment using the agar diffusion assay

solution (0.85% sodium chloride solution) before being placed in each dish in contact with the agar surface (Fig. 1). Triplicate cultures for each test material and both positive and negative controls were performed. All cultures were incubated for 24 ± 1 h. The outline of the specimen was marked on the bottom of the culture dish with a permanent marker, and then the specimen was removed. Two millilitres of 0.01% neutral red solution was added to each dish and incubated for 1 h. Following incubation, the neutral red solution was removed, and each culture was examined microscopically under and around each control and test specimen. The cell culture was deemed to show a cytotoxic effect if microscopic examination revealed malformation, degeneration, sloughing or lysis of the cells within the zone or a moderate-to-severe reduction in cell-layer density. The zone index measures the clear zone in which cells do not stain with neutral red. The lysis index measures the number of cells affected within the zone of toxicity.

2.6 Statistics

Statistical analysis was performed using GraphPad Prism 7 software. Statistical comparisons between microbiological mean log values were performed using the two-way ANOVA.

3 Results

3.1 Efficacy of the Electrolysed Water on Planktonic Microorganisms

Upon bacterial enumeration, no colonies of either *S. aureus* or *P. aeruginosa* were detected following antimicrobial treatment with all dilutions of the electrolysed water at treatment times of 1 min, 5 min and 24 h (Fig. 2a, b) ($p = <0.0001$).

3.2 Efficacy of the Electrolysed Water on Biofilms

The use of the CDC model showed that the electrolysed water at 100, 75, 50 and 25% concentration could significantly reduce *S. aureus* and *P. aeruginosa* biofilms after 5 min contact time (Fig. 3a, b) ($p < 0.0001$).

3.3 Cytotoxicity of the Electrolysed Water

The agar diffusion method allows for the qualitative assessment of cytotoxicity. According to ISO

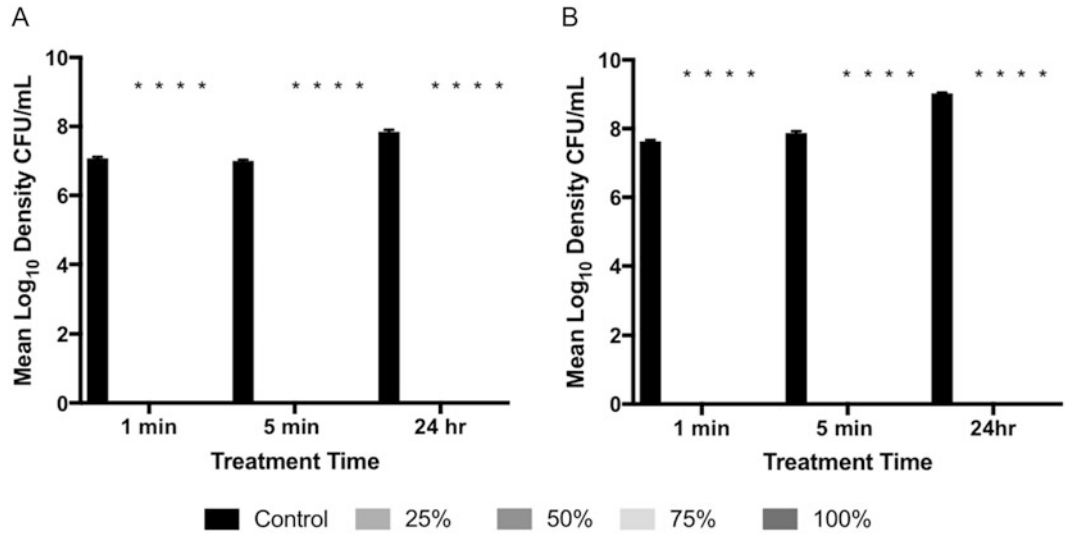

Fig. 2 Log density of *S. aureus* (**a**) and *P. aeruginosa* (**b**) in the electrolysed water time-kill study. Microbial cultures were exposed to the electrolysed water at 100%, 75%, 50% and 25% concentration for 1 min, 5 min and 24 h. Samples were performed in triplicate. The negative control treatment was a 0.85% sodium chloride solution. Mean log density values were obtained and the standard deviation (SD) calculated. Error bars represent SD. All treatment groups showed a significant decrease in bacterial $\log_{10}$ density in comparison with the control group ($p < 0.0001$)

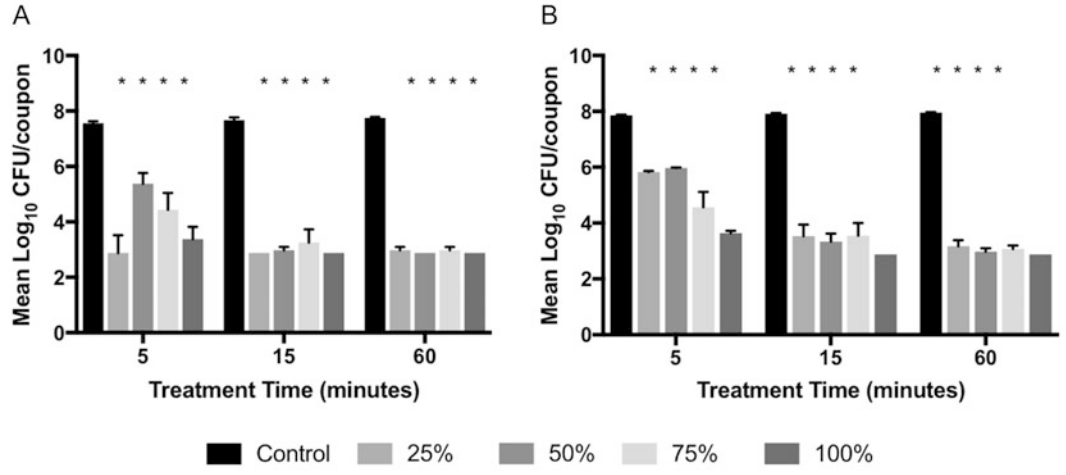

Fig. 3 Mean $\log_{10}$ density of *S. aureus* (**a**) and *P. aeruginosa* (**b**) biofilm in the CDC model. Biofilms were treated with the electrolysed water test solution at 25%, 50%, 75% and 100% concentration. For the control, coupons containing biofilm were treated with a 0.85% sodium chloride solution. The experiment was performed in triplicate. Error bars represent the standard deviation of the mean $\log_{10}$ CFU/coupon. All treatment groups showed a significant decrease in bacterial $\log_{10}$ density in comparison with the control group ($p < 0.0001$)

Table 1 Reactivity grades for zone index

Zone index	Description of zone	Reactivity
0	No detectable zone around or under specimen	None
1	Zone limited to area under specimen	Slight/mild
2	Zone extends less than 0.5 cm beyond specimen	Moderate
3	Zone extends 0.5–0.1 cm beyond specimen	Moderate
4	Zone extends greater than 1.0 cm beyond specimen but does not involve entire dish	Severe

Table 2 Qualitative lysis description

Lysis index	Description of zone	Reactivity
0	Discrete intracytoplasmic granules, no cell lysis, no reduction of cell growth	None
1	Not more than 20% of zone shows rounded cells, loosely attached and without intracytoplasmic granules or show changes in morphology	Slight
2	Not more than 50% of the cells are round, devoid of intracytoplasmic granules, no extensive cell lysis; not more than 50% growth inhibition observable	Mild
3	Not more than 70% of the cell layers contain rounded cells or are lysed; cell layers not completely destroyed, but more than 50% growth inhibition observable	Moderate
4	Nearly complete destruction of the cell layers	Severe

Table 3 Zone index and lysis index for indirect cytotoxicity test

Sample	Zone index	Interpretation	Lysis index	Interpretation
DMEM (negative control)	0	Non-cytotoxic	0	Non-cytotoxic
Water	0	Non-cytotoxic	0	Non-cytotoxic
Phenol	4	Cytotoxic	3	Cytotoxic
Electrolysed water 100%	3	Cytotoxic	3	Cytotoxic
Electrolysed water 75%	3	Cytotoxic	1	Non-cytotoxic
Electrolysed water 50%	2	Non-cytotoxic	1	Non-cytotoxic
Electrolysed water 25%	2	Non-cytotoxic	1	Non-cytotoxic

Zone index measures the clear zone in which cells do not stain with neutral red. The lysis index measures the number of cells affected within the zone of toxicity. All samples were tested in triplicate

10993-5, an achievement of a numerical grade greater than 2 (Tables 1 and 2) is considered a cytotoxic effect. The evaluation of the electrolysed water showed that 100% and 75% of the electrolysed water was cytotoxic to cells. The electrolysed water at 50% and 25% was not cytotoxic to cells (Table 3).

4 Discussion

The use of salt water electrolysis has been used to produce sanitising agents and cleaning agents but has also been utilised to produce hydrogen energy as a potential alternative energy source (Meier 2014). Within this study, the antibacterial and anti-biofilm properties of electrolysed water were assessed in a series of in vitro assays. It was shown that the electrolysed water formulation could kill *S. aureus* and *P. aeruginosa* within 1 min of exposure in planktonic studies. In veterinary medicine, it has been shown that dilute hypochlorous acid solutions are bactericidal against *S. aureus* and *Escherichia coli*, after 1 h exposure (Ramey and Kinde 2015). However, the study failed to evaluate shorter contact times. Another study has demonstrated complete kill of *Vibrio parahaemolyticus*, *Listeria monocytogenes*, *Aeromonas hydrophila* and

Campylobacter jejuni when in contact with acidic electrolysed water for 2 min (Ovissipour et al. 2015). Acidic electrolysed water is a powerful oxidising solution and an incredibly effective antimicrobial. The mechanism of action surrounds the ORP of acidic electrolysed water and the disruption of both the inner and outer membranes of the microorganism (Liao et al. 2007). The use of atomic force microscopy has confirmed the disruption of bacterial membranes, and NMR spectroscopy has shown an influence of electrolysed water on the metabolic state of *E. coli* by reducing nucleotide and amino acid biosynthesis, suppressing energy-associated metabolism, altering osmotic adjustment and promoting fatty acid metabolism (Liu et al. 2017).

Using an ASTM standard procedure, we produced *S. aureus* and *P. aeruginosa* biofilms using the CDC bioreactor model. Using these models, we showed that the electrolysed water could effectively kill microorganisms within a 48 h biofilm after 5 min exposure, demonstrating a fast-acting antimicrobial and anti-biofilm agent. Few other studies have also demonstrated the effectiveness of electrolysed water on in vitro biofilms (Li et al. 2017; Machado et al. 2016; Chen et al. 2013) and viruses (Moorman et al. 2017). The efficacy of strong acid electrolysed water on static and flow *Enterococcus faecalis* biofilms has also been demonstrated with biofilm kill detected after 10 min contact time followed by a 5 min neutralising step with sodium thiosulfate (Cheng et al. 2016). The use of electrolysed water as a combination therapy significantly reduces this contact time. Zhao and colleagues combined a low concentration of neutralised electrolysed water with ultrasound (37 kHz, 80 W), which resulted in a significant reduction of bacterial cells within 5 min of treatment. The combination demonstrated the best sanitising efficacy in comparison with the treatment alone (Zhao et al. 2017).

To determine the potential cytotoxic effects of the electrolysed water if in direct contact with the human skin, we performed in vitro cytotoxicity assays according to ISO 10993–5. Indirect cytotoxicity tests showed that a 25% and 50% solution of the electrolysed water has no cytotoxic effects, whilst 75% and 100% solutions showed mild to moderate cytotoxicity. It is important to note that the contact time of the electrolysed water in the cytotoxicity studies was for 24 h. Given that the eradication of biofilm occurred after 15 min, it is unlikely that this solution of electrolysed water will be left on the wound for 24 h. Further studies into the cytotoxicity of this electrolysed water product at shorter contact times shall be carried out. Nevertheless, clinical studies have demonstrated the safety of using electrolysed water in chronic wounds. A block-randomised, double-blind, parallel-arm, post-marketing study compared the safety and effectiveness of tetrachlorodecaoxide and super-oxidised solution in patients with ulcers, which resulted in significant tissue change (granulation tissue production) and no adverse effects (Parikh et al. 2016). Other studies have determined the cytotoxic effects of electrolysed water in the context of dentistry and the treatment of dental caries. One study compared the bactericidal effects of three preparations of electrolysed water (acid oxidising water, neutral oxidising water and low chlorine acid oxidising water) with their cytotoxicity on epithelial cells. Results showed that all preparations of electrolysed water were bactericidal; however, the low chlorine acid oxidising water demonstrated lower cytotoxicity when compared with acid oxidising water and neutral oxidising water ($P < 0.0001$) (Shimada et al. 2000). In vivo cytotoxicity has shown that the application of acid electrolysed water to guinea pig corneas results in minimal damage when compared to 1% povidone iodine. Furthermore, the eradication of *P. aeruginosa* within 15 s of treatment significantly inhibited the onset of corneal infection (Shimmura et al. 2000).

In conclusion, the assessment of the electrolysed water as an antimicrobial and anti-biofilm agent showed exceptionally fast-acting efficacy. However, various studies have shown difference in the most efficacious contact times, which will undoubtedly be affected by the microbial species involved, the in vitro biofilm model used, whether the biofilm is polymicrobial or not and the maturity of the biofilm. The use of electrolysed water as a method of controlling bioburden and biofilm in complicated chronic wounds could significantly aid wound closure.

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Defining the Relationship Between Phenotypic and Genotypic Resistance Profiles of Multidrug-Resistant Enterobacterial Clinical Isolates

Lamis Galal, Neveen A. Abdel Aziz, and Walaa M. Hassan

Abstract

Fluoroquinolones and aminoglycosides offer effective therapy for extended-spectrum beta-lactamase (ESBL)-producing enterobacterial infections, but their usefulness is threatened by increasing resistant strains. **Objective:** This study was conducted to demonstrate the phenotypic outcomes of the coexistence of genetic determinants mediating resistance to extended-spectrum cephalosporins and quinolones in enterobacterial isolates collected from patients with health-care-associated infections in Egypt. **Methods:** ESBL phenotype was determined using double-disk synergy test (DDST). The PCR technique was used to detect the presence of the genes mediating quinolone resistance (*qnr and aac(6′)-Ib-cr)* and coexistence with ESBL genes. We also examined the association between the genetic makeup of the isolates and their resistance profiles including effect on MIC results. **Results:** Phenotypically ESBLs were detected in 60–82% of the enterobacterial isolates. ESBL, *qnr and aac(6′)-Ib-cr* genes were detected with the following percentages in *Citrobacter* isolates (69%, 69%, and 43%, respectively), *E.coli* isolates (65%, 70%, and 45%, respectively), *Enterobacter* isolates (56%, 67%, and 33%, respectively), and finally *Klebsiella* isolates (42%, 66%, and 25%, respectively). The coexistence of these multiresistant genetic elements significantly increased the MIC values of the tested antibiotics from different classes. **Conclusion:** We suggest using *bla*TEM, *bla*CTX-M-15, *qnr*, and *aac(6′)-Ib-cr* genes for better and faster prediction of suitable antibiotic therapy with effective doses against ESBL-producing isolates harboring plasmid-mediated quinolone resistance (PMQR) determinants. Amikacin, meropenem, gentamicin, and imipenem seem to be better choices of treatment for such life-threatening infections, because of their remaining highest activity.

Keywords

Co-resistance · Extended-spectrum beta-lactamases · MDR · PMQR · Quinolone resistance

All authors meet the ICMJE authorship criteria

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1 Introduction

Extended-spectrum beta-lactams and quinolones are the most important antibiotics used for the treatment of enterobacterial infections. The inappropriate use of these antibiotics has led to the spread of multidrug resistance (MDR) among enterobacterial clinical isolates. Extended-spectrum β-lactamases (ESBLs) are a rapidly evolving group of β-lactamases which hydrolyze the extended-spectrum cephalosporins, the penicillins, as well as aztreonam, but not carbapenems. In addition to the large number of ESBL-TEM and ESBL-SHV variants, other plasmid-encoded ESBLs such as CTX-M-15 enzymes are now frequently reported (Shaikh et al. 2015). The successful spread of ESBLs in a wide range can be attributed to the fact that the genes coding for ESBLs are often located on self-transmissible broad host-range plasmids (Sharma et al. 2010). Many ESBL producers are also resistant to non-β-lactam antibiotics such as quinolones, aminoglycosides, and trimethoprim, further narrowing treatment options (Rawat and Nair 2010). In the last 10 years, the discovery of the three major mechanisms of plasmid-mediated quinolone resistance (PMQR) with the potential for horizontal transfer opened a novel era in resistance to quinolones (Jacoby et al. 2014). Plasmid-mediated quinolone resistance associated with *qnrA*, *qnrB*, and *qnrS* has been reported among enterobacterial species in Asia, the USA, South America, and several countries in Europe (Lavilla et al. 2008). The second mechanism, an aminoglycoside acetyltransferase variant *aac(6′)-Ib-cr*, with two specific amino acid substitutions, enables the acetylation of the piperazinyl substituent of ciprofloxacin and norfloxacin, reducing their activity (Machuca et al. 2016). The existence of multiple resistance genes on the same plasmid and their transfer between ESBL-producing clinical isolates has increased markedly. Some reports have even indicated a strong relationship of quinolone resistance with the production of ESBLs and AmpC beta-lactamase (Jacoby et al. 2014). Rapid diagnostic tests for multiresistance utilizing polymerase chain reaction (PCR)-based methods may decrease the amount of inappropriate prescribing of antibiotics by identifying specific pathogens and their antibiotic resistance characteristics and thus guiding enhanced treatment decisions. Establishing a relation between the results of the PCR-based methods and the classical cultivation protocols for resistance determination is required to conclude optimal empirical antimicrobial therapy and treatment guidelines before obtaining the susceptibility results (Maurer et al. 2017).

This study provides information about understanding prevalence and also dissemination of PMQR determinants among ESBL-producing enterobacterial isolates recovered from patients with health-care-associated infections in Egypt. More importantly, we examined the potential association between the coexistence of these different resistance genes and the MIC values of the tested antibiotics. Finally, we determined the optimal antibiotic of choice for the treatment of infections caused by these species.

2 Materials and Methods

2.1 Bacterial Isolates

This study was conducted over a period of 12 months in which a total of 393 isolates were collected from five hospitals affiliated to the Egyptian health ministry representing various categories of bed size. These hospitals, collectively, admit patients from all socioeconomic strata from Cairo and the surrounding rural areas due to their wide geographic distribution. During the study, all patients admitted to different hospital wards with suspected nosocomial infections were included, i.e., infections occurring within 48 h of hospital admission. Patients with community-acquired infections were excluded. The isolates studied were confined to unrelated first isolates from different patients; duplicate isolates were excluded. The following samples were used for isolation of the clinical specimens: urine, blood, stool, pus, sputum swab, and wound swab. Isolated bacteria were microbiologically identified with standard biochemical methods, and only Gram-negative enterobacterial isolates were chosen for further investigation.

2.2 Antibiotic Susceptibility Testing and Determination of the Minimum Inhibitory Concentrations (MICs)

Antibiotic susceptibility testing was performed with agar diffusion method on Muller-Hinton agar (Difco laboratories, USA). The minimum inhibitory concentrations (MICs) of tested antibiotics were determined by agar dilution and interpreted according to Clinical and Laboratory Standards Institute guidelines (CLSI 2014). Antibiotics used were ampicillin, ampicillin/sulbactam, cefotaxime, ceftazidime, cefepime, imipenem, meropenem, ciprofloxacin, levofloxacin, gatifloxacin, gentamicin, amikacin, and tetracycline.

2.3 Phenotypic Screening and Confirmation of Extended-Spectrum β-Lactamase-Producing Enterobacterial Isolates

The double-disk synergy test was performed by a standard disk diffusion assay on Muller-Hinton agar as recommended by the Clinical and Laboratory Standards Institute guidelines (CLSI 2014). Disks containing aztreonam, ceftazidime, ceftriaxone, and cefotaxime (30 μg each) were placed at distance 30 mm from center to center around the disk containing amoxicillin (20 μg) plus clavulanic acid (10 μg). Enhancement of the inhibition zone toward the amoxicillin-plus-clavulanic acid disk was considered suggestive of ESBL production.

2.4 Polymerase Chain Reaction (PCR)

All phenotypic ESBL producers were screened by PCR to identify their ESBL-carrying genes with specific primers for TEM, SHV, and CTX-M-15 according to a previously described PCR protocol (Monstein et al. 2007). Amplification and identification of plasmid-mediated quinolone resistance (PMQR; *qnrA, qnrB, qnrS* and *aac(6′)-Ib-cr*) genes were performed using previously described primers and PCR protocol (Cattoir et al. 2007; Park et al. 2006). DNA was extracted by a boiling lysis method as described (Olive and Bean 1999), and all the PCR products were visualized using a gel documentation system (Wisd laboratory instruments, DAIHAN Scientific Co.)

2.5 Statistical Analysis

We used the statistical package program (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp). Differences between proportions were analyzed using chi-square test ($\chi 2$). All differences in which the probability of the null hypothesis was $p \leq 0.05$ were considered significant. Descriptive statistical analysis and hierarchical cluster analysis were also performed.

3 Results and Discussion

3.1 Bacterial Identification and Antibiotic Susceptibility Testing

Out of the 393 clinical isolates collected from Egyptian hospitals, 89 enterobacterial isolates were included in this study according to the criteria mentioned in the methods section. The population studied had a sex ratio of 0.82 (40 male, 49 female) and a mean age of 76.2 ± 11.4 years. Patients' age groups and specimens used for isolation are shown in Table 1. Twenty nine isolates were identified as *Escherichia coli* (32.5%), 26 *Citrobacter* species (29%), 23 *Klebsiella pneumoniae* (26%), and 11 *Enterobacter cloacae* (12.5%). Antimicrobial susceptibility testing revealed high resistance frequency to the majority of tested antibiotics as shown in Fig. 1. High percentages of resistance to third- and fourth-generation cephalosporins may be attributed to the production of extended-spectrum beta-lactamases. Phenotypic assessment of extended-spectrum β-lactamases production using double-disk synergy test revealed high

Table 1 Characteristics of multidrug-resistant enterobacterial clinical isolates including patient's age group and type of specimen

	Number	Percent
Age group		
<30 days	17	19.1%
4–12 years	4	4.5%
13–19 years	4	4.5%
20–39 years	25	28.1%
40–59 years	14	15.7%
60–90 years	25	28.1%
Specimen		
Ascetic fluid	1	1.1%
Blood	9	10.1%
Pus	9	10.1%
Spleen	1	1.1%
Sputum	22	24.7%
Stool	2	2.2%
Urine	40	44.9%
Wound	5	5.6%

percentages of ESBL production among our isolates, even higher than reported by several studies from Egypt and other countries (Eldomany and Abdelaziz 2011; Kim et al. 2010; Lee et al. 2010; Paterson et al. 2000). ESBL production was detected in 74% of *E.coli* isolates, 73% of *Citrobacter* species, 82% of *Enterobacter cloacae* isolates, and 60% of *Klebsiella pneumoniae* isolates.

3.2 Bacterial Differentiation Based on the Antibiotic Susceptibility Patterns

The reactions of 89 enterobacterial isolates to 13 antibiotics were analyzed by Ward's hierarchical clustering method. Isolates were differentiated based on the antibiotic susceptibility patterns (Fig. 2). Different profiles of reaction to antibiotics were presented. Groups of isolates with similar profiles were identified. The ten main clusters are presented in a dendrogram (Fig. 2). The analysis of the dendrogram and the characteristics of each cluster are presented in detail in Table 2. Clusters 1, 2, and 3 represent 50% of the isolates with the prevalence of multiple drug resistance species, namely, *Klebsiella pneumoniae* followed by *Escherichia coli* and *Citrobacter species.* In a study to determine resistance profiles of clinical enterobacterial isolates in Egypt, the percentage of resistant isolates exceeded 60% of the total isolates and reached up to 95% according to antibiotic used (Eldomany and Abdelaziz 2011). As noted from the stated features in Table 2, all isolates were sensitive to amikacin, meropenem, and imipenem except isolates in clusters 1 and 2.

To further describe the resistance patterns of these isolates, we examined the relationship between non-susceptibility to extended-spectrum beta-lactams and resistance to non-beta-lactam antibiotics. Resistance to third-generation cephalosporins, namely, cefotaxime and ceftazidime, was highly associated ($p\text{-value} \leq 0.05$) with resistance to all tested non-beta-lactam antibiotics except amikacin and meropenem. On the other hand, we observed a significant association ($p\text{-value} \leq 0.05$) between resistance to cefepime, a fourth-generation cephalosporin, and all tested non-beta-lactam antibiotics (Table 3). Moreover, statistical analysis showed that the incidences of quinolones, tetracycline, aminoglycoside resistance (except amikacin), and carbapenem resistance (except meropenem) were significantly higher among ESBL-producing enterobacterial strains than among ESBL-nonproducing strains ($p\text{-value} \leq 0.05$) (Table 3). This is not unprecedented; Paterson and associates performed a study in seven countries and detected higher resistance patterns among ESBL-producing enterobacterial isolates (Paterson et al. 2000).

3.3 Screening Clinical Isolates for Resistance Genes and the Effect of Their Coexistence on MIC Values

A major goal of this study was to define a good correlation between phenotypic and genotypic resistance profiles. This correlation provides accurate and timely information to conclude optimal antimicrobial therapy guidelines for better

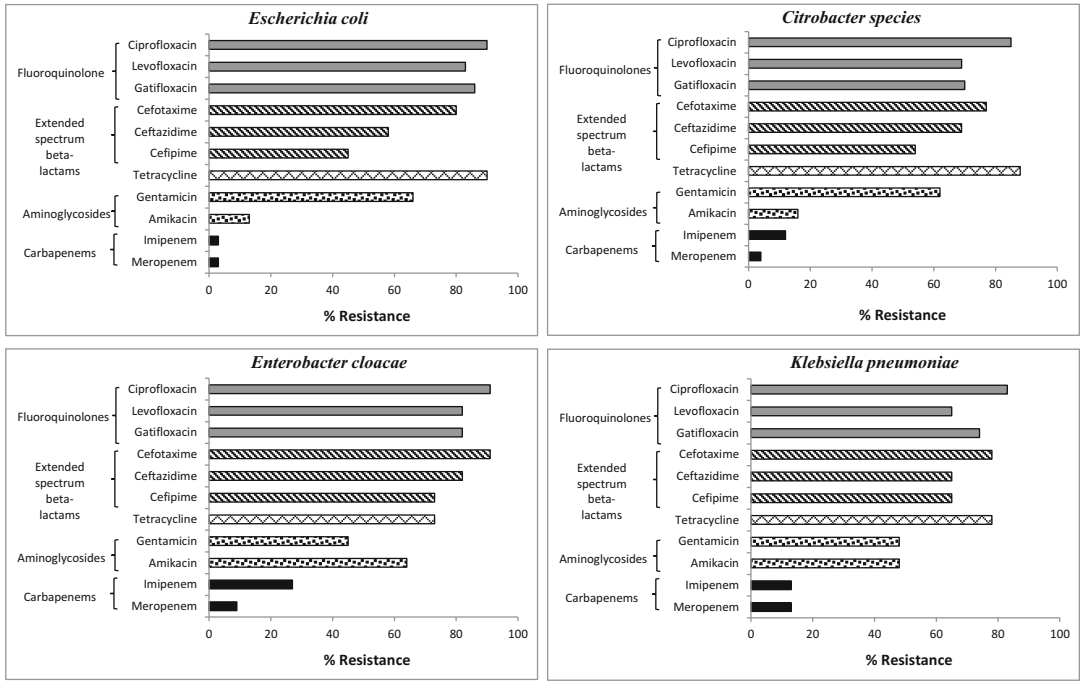

Fig. 1 A clustered bar plot summarizing the antimicrobial resistance patterns of the enterobacterial tested isolates (*Escherichia coli*, *Citrobacter* species, *Enterobacter cloacae*, and *Klebsiella pneumoniae*) to 13 antibiotics (Y axis). Antibiotics are sorted by classes

clinical outcomes. All ESBL-producing clinical isolates were examined by multiplex PCR to determine the presence of TEM, CTX-M-15, and SHV genes. ESBL genes were detected in 69% of the *Citrobacter* isolates, 65% of *E.coli* isolates, 56% of *Enterobacter cloacae* isolates, and finally 42% of *Klebsiella pneumoniae* isolates (Fig. 3). These frequencies were higher than those detected by various investigators (Kim et al. 2010; Lee et al. 2010). The molecular profile of the isolates and their corresponding MIC values of third- and fourth-generation cephalosporins are summarized in Table 4. Results show that the presence of *bla*TEM gene alone or in combination with *bla*CTX-M-15 gene correlated well with the MIC50 values of cefotaxime, ceftazidime, and cefepime, whereas the presence of *bla*CTX-M-15 gene alone or the *bla*TEM and *bla*SHV genes together did not correlate with the MIC50 values of the same antibiotics. However the copresence of *bla*TEM, *bla*CTX-M-15, and *bla*SHV genes predicted higher MIC50 values of cefotaxime, ceftazidime, and cefepime. Notably, no agreement was observed between the molecular profiles of the ESBL genes and the MIC90 values of the tested third- and fourth-generation cephalosporins.

Afterward we studied the prevalence of quinolone resistance among nosocomial Enterobacteriaceae which is increasingly being reported (Mehrad et al. 2015). Plasmid-mediated quinolone resistance (PMQR) genes play an important role in resistance to quinolones due to their horizontal transferability (Goudarzi et al. 2015). The distribution of PMQR genes (*qnrA*, *qnrB*, and *qnrS*) among our ESBL-producing isolates differed among species. They were detected most often in *E. coli* isolates (70%), next most often in *Citrobacter* isolates (69%), then *Enterobacter cloacae* (67%), and least often in *K. pneumoniae* isolates (66%) as shown in Fig. 3.

A novel PMQR gene *aac (6′)-Ib -cr* was first reported in 2003 but is now recognized to be widely disseminated. The results for the prevalence of *aac (6′)-Ib -cr* gene in *E.coli*, *Citrobacter*, *Enterobacter cloacae*, and

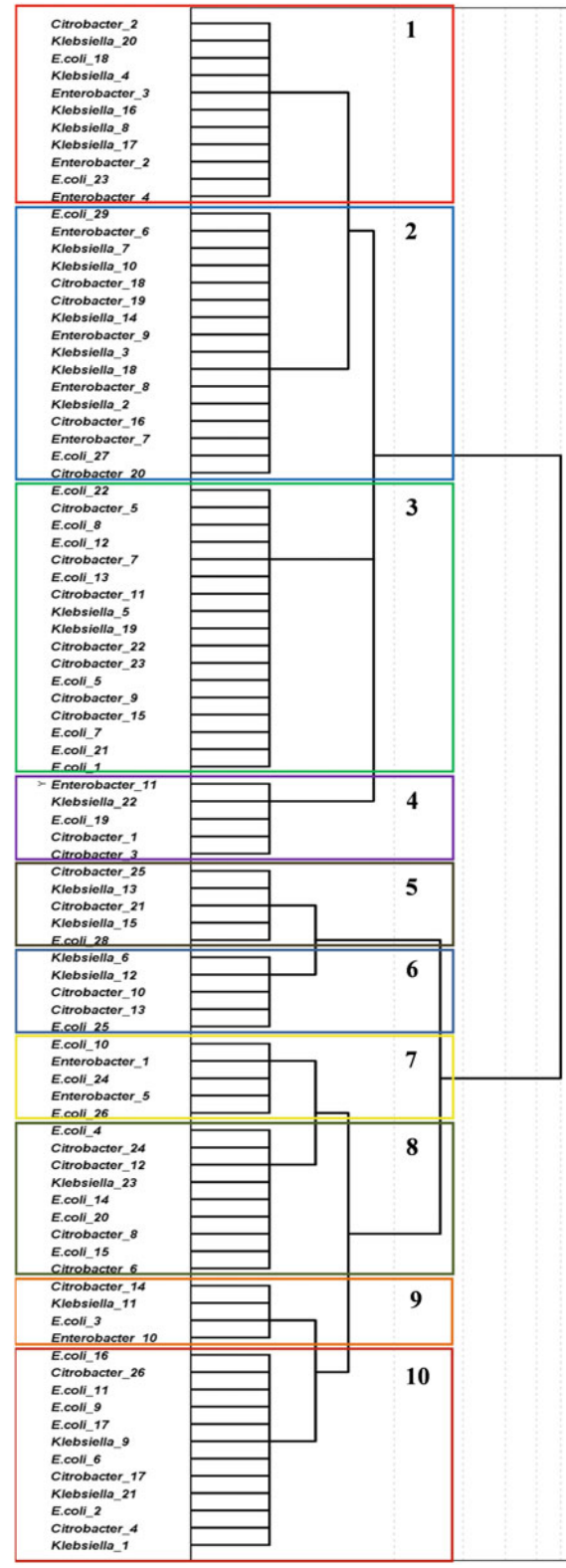

Fig. 2 Dendrogram illustrating 10 clusters of 89 enterobacterial isolates according to Ward's agglomeration method based on their antibiotic susceptibility patterns to 13 antibiotics

Table 2 The characteristics of the dendrogram (Fig. 2) showing detailed features of each cluster of isolates

Cluster	No. of strains	All isolates resistant to antibiotics	All isolates sensitive to antibiotics	ESBL producers (%)	Prevalent strains
1	11	Ampicillin, cefotaxime, cefepime, ciprofloxacin, tetracycline	Gentamicin	54.5%	*Klebsiella pneumoniae*
2	16	Ampicillin, cefotaxime, cefepime, gatifloxacin, tetracycline	None	62.5%	*Klebsiella pneumoniae*
3	17	Ampicillin, cefotaxime, cefepime, ciprofloxacin, levofloxacin, gatifloxacin, tetracycline	Meropenem, amikacin	82.5%	*Escherichia coli*
4	5	Ampicillin, ampicillin/sulbactam, cefotaxime, ceftazidime, cefepime	Meropenem, levofloxacin, gatifloxacin, amikacin	100%	*Citrobacter species*
5	5	None	Ampicillin, ampicillin/sulbactam, ceftazidime, cefepime, imipenem, meropenem, ciprofloxacin, levofloxacin, gentamicin, amikacin	0%	*Klebsiella pneumoniae* and *Citrobacter species*
6	5	Ampicillin	Cefepime, imipenem, meropenem, levofloxacin, gatifloxacin, gentamicin, amikacin	20%	*Klebsiella pneumoniae* and *Citrobacter species*
7	5	Ampicillin, cefotaxime, ciprofloxacin, gatifloxacin	Cefepime, meropenem, gentamicin, amikacin, tetracycline	100%	*Escherichia coli*
8	9	Ampicillin, cefotaxime	Cefepime, imipenem, meropenem, gentamicin, amikacin	44.5%	*Escherichia coli* and *Citrobacter species*
9	4	Ampicillin, cefotaxime, ceftazidime, tetracycline	Cefepime, meropenem gatifloxacin, amikacin	50%	All species were equally prevalent
10	12	Ampicillin, cefotaxime, gatifloxacin, tetracycline	Cefepime, meropenem, amikacin	75%	*Escherichia coli*

Klebsiella pneumoniae clinical isolates were 45%, 43%, 33%, and 25%, respectively (Fig. 3). Similar frequencies of *aac(6′)-Ib-cr* dissemination among ESBL producers have been reported previously (Briales et al. 2012; El-Badawy et al. 2017). Both *qnr* genes and the *aac(6′)-Ib-cr* gene had the same distribution among our isolates which implies that some plasmids can carry both *aac(6′)-Ib-cr* and *qnr* genes and may also suggest that the mechanism for the incidence of *qnr* and/or *aac(6′)-Ib-cr* is related to the particular species (Eftekhar and Seyedpour 2015). Selection pressures from the use of aminoglycosides that are enzyme substrates of *cr* variant of aminoglycoside transferase (kanamycin, tobramycin, and amikacin) and the use of quinolones with a piperazinyl amine that are subject to *N*-acetylation by this variant are predicted to promote *aac(6′)-Ib-cr* gene prevalence. Statistical analysis confirmed a significant association between the *aac(6′)-Ib-cr* and the *qnr* genes ($p\text{-value} \leq 0.05$). Jeong and colleagues (2011) indicated that *qnr* genes were always co-resident with *aac (6′)-Ib* and *aac(6′)-Ib-cr* genes on the same plasmids and that these plasmid-mediated genes may facilitate spread and increase the prevalence of quinolone-resistant strains. We inspected the effect of this association on the MIC values of

Table 3 Results of statistical association between resistance to non-β-lactam antibiotics and non-susceptibility to extended-spectrum beta-lactams, expressed by both the susceptibility results to cephalosporins and ESBL-producing ability

		Cefotaxime			Ceftazidime				Cefepime				Extended-spectrum beta-lactamases		
		R	I	*p-value*	R	I	S	*p-value*	R	S	SDD	*p-value*	Producing	Nonproducing	*p-value*
Ciprofloxacin	R	72	1	0.00004	52	13	8	0.019	46	6	21	0.00000	50	23	0.00094
	I	4	0		2	2	0		1	0	3		3	1	
	S	8	4		4	3	5		2	8	2		4	8	
Levofloxacin	R	62	0	0.00031	46	11	5	0.042	41	2	18	0.00004	41	21	0.0362
	I	5	0		3	1	1		2	2	1		5	0	
	S	17	5		9	6	7		6	10	6		11	11	
Gatifloxacin	R	67	1	0.004	48	14	6	0.031	43	4	21	0.00002	47	21	0.0246
	I	2	0		1	1	0		2	0	0		2	0	
	S	15	4		9	3	7		4	10	5		8	11	
Gentamicin	R	41	0	0.029	33	6	2	0.001	29	1	11	0.004	32	9	0.033
	I	10	0		9	1	0		6	1	3		6	4	
	S	33	5		16	11	11		14	12	12		19	19	
Amikacin	R	21	0	0.335	17	40		0.064	21	0	0	0.00003	10	11	0.065
	I	5	0		5	0	0		4	0	1		5	0	
	S	58	5		36	14	13		24	14	25		42	21	
Tetracycline	R	73	2	0.005	53	13	9	0.041	47	7	21	0.00014	49	26	0.0289
	I														
	S	11	3		5	5	4		2	7	5		8	6	
Imipenem	R	45	0	0.002	42	3	0	0.0000002	41	0	4	0.00000	31	14	0.00016
	I	18	0		9	6	3		7	0	11		17	1	
	S	21	5		7	9	10		1	14	11		9	17	
Meropenem	R	10	0	0.094	10	0	0	0.0002	10	0	0	0.00000	5	5	0.231
	I	32	0		27	5	0		29	0	3		24	8	
	S	42	5		21	13	13		10	14	23		28	19	

R resistant, *S* sensitive, *I* intermediate, *SDD* susceptible dose dependent, interpreted according to Clinical and Laboratory Standards Institute guidelines (CLSI 2014)

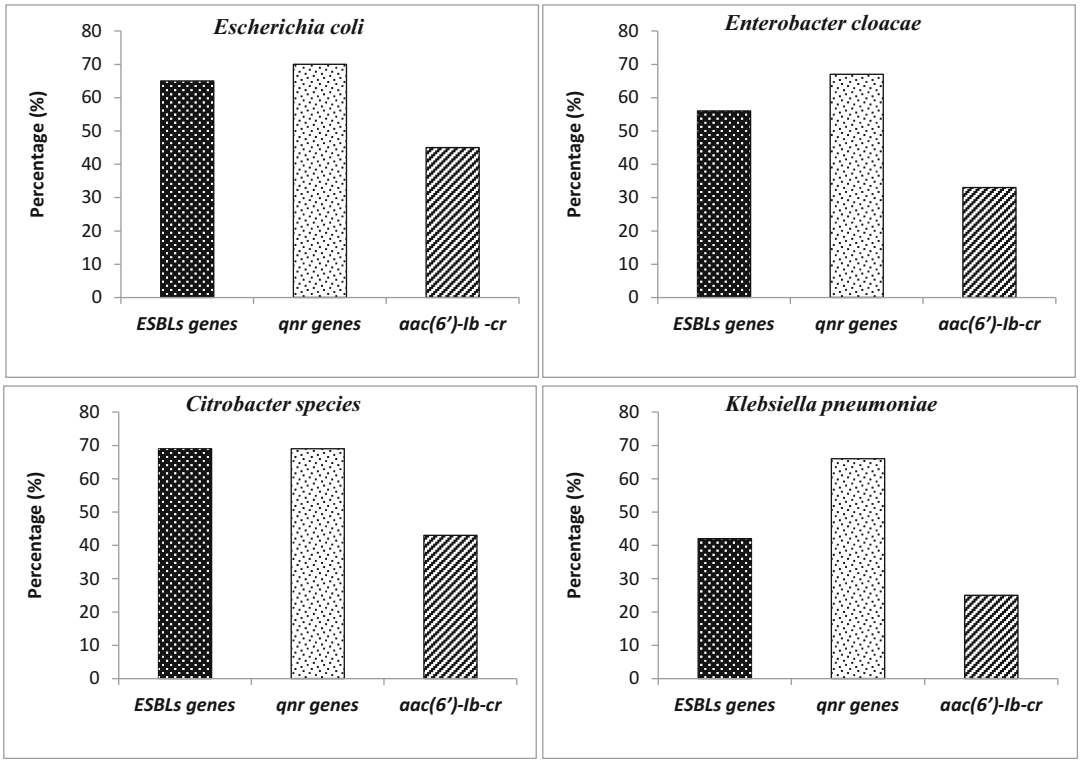

Fig. 3 PCR screening results of ESBL, *qnr* and *aac(6′)-Ib-cr* genes in *E.coli*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, and *Citrobacter* isolates

Table 4 Genotype–phenotype associations of ESBL PCR results and the MIC values of third- and fourth-generation cephalosporins

Molecular profile	No. of isolates	Cefotaxime		Ceftazidime		Cefepime	
		MIC50	MIC90	MIC50	MIC90	MIC50	MIC90
TEM+ M-CTX-15+ SHV	7	512	512	32	64	64	64
TEM+ M-CTX-15	5	256	256	128	256	16	64
TEM+ SHV	2	16	16	4	64	4	64
M-CTX-15	6	64	128	16	128	8	16
TEM	14	256	512	32	256	16	64
None	23	64	512	16	256	8	256

tested quinolones and aminoglycosides. Comparing the MIC values for ciprofloxacin, gatifloxacin, and levofloxacin in the presence of *qnr* genes only and in the presence of both *qnr* and the *aac(6′)-Ib-cr* genes, a significant increase was observed in the latter case specially the MIC50 and MIC90 values of ciprofloxacin (Table 5). This variant of the aminoglycoside acetyltransferase gene, *aac(6′)-Ib-cr*, confers resistance to certain quinolones (norfloxacin and ciprofloxacin) and aminoglycosides (kanamycin, amikacin, and tobramycin). However, the MIC50 and MIC90 values of both gentamicin and amikacin increased by two- to eightfold in isolates harboring both *qnr* and *aac(6′)-Ib-cr* genes, which is quite surprising because *aac(6′)-Ib-cr* was reported to confer resistance to kanamycin but not to gentamicin (Table 5). It is speculated that future shifts in the choice of

Table 5 Genotype–phenotype associations of the molecular profile of *qnr and aac(6′)-Ib* genes and MIC values of tested quinolones and aminoglycosides

Molecular profile	No. of isolates	Ciprofloxacin		Levofloxacin		Gatifloxacin		Gentamicin		Amikacin	
		MIC50	MIC90	MIC50	MIC90	MIC50	MIC90	MIC50	MIC90	MIC50	MIC90
qnrB + qnrS + qnrA+ aac(6′)-Ib-cr	**1**	512	512	128	128	64	64	64	64	64	64
qnrB + qnrS + qnrA	**2**	256	512	128	128	32	64	8	8	4	8
qnrB + qnrS+ aac(6′)-Ib-cr	**11**	128	512	64	128	32	128	32	128	8	64
qnrB + qnrA+ aac(6′)-Ib-cr	**1**	512	512	128	128	32	32	64	64	8	8
qnrB + qnrS	**8**	128	128	64	64	64	128	256	512	8	32
qnrS+ aac(6′)-Ib-cr	**2**	16	64	8	16	16	32	8	64	64	128
qnrB+ aac(6′)-Ib-cr	**2**	64	128	64	128	32	32	4	16	4	32
qnrB	**7**	16	64	8	32	8	32	8	32	2	8
qnrS	**5**	32	256	64	128	32	32	32	64	4	8
qnrA	**5**	8	128	2	128	4	32	16	64	4	128
None	**13**	4	64	2	32	2	32	4	8	1	8

Table 6 The relation between the presence of ESBLs or PMQR genes or both and the MIC values of cephalosporins and aminoglycosides

	Cefotaxime		Ceftazidime		Cefepime		Gentamicin		Amikacin	
Molecular profile	MIC50	MIC90	MIC50	MIC90	MIC50	MIC90	MIC50	MIC90	MIC50	MIC90
ESBL genes	4	256	32	64	2	64	8	64	8	8
PMQR genes	128	512	16	512	16	512	4	64	8	32
ESBL genes + PMQR genes	128	512	32	256	16	64	32	128	8	64

quinolones used to those that are not substrates for *aac(6′)-Ib-cr* might reduce selection pressures for this variant but not for the *qnr* genes.

Several studies suggested that *aac(6′)-Ib-cr* and *qnr* genes are co-associated with genes encoding ESBLs (Jeong et al. 2011; Liu et al. 2016). Our results showed that PMQR genes (both the *qnr* and *aac(6′)-Ib-cr*) are co-expressed with *bla*CTX-M-15, *bla*TEM, and *bla*SHV alleles, and the usage of either a fluoroquinolone or a beta-lactam agent lead to the co-selection of these resistant isolates (Dobiasova et al. 2013). Further analysis of data revealed that the coexistence of PMQR genes with ESBL genes increased the MIC values of all tested third- and fourth-generation cephalosporins by two- to 32-folds. On the other hand, the MIC values of tested quinolones were not affected by the coexistence of PMQR and ESBL genes. Lastly, we noticed a significant increase in the MIC50 and MIC90 values of both gentamicin and amikacin in the presence of both PMQR and ESBL genes (Table 6). This association is clinically significant since it limits the therapeutic options for these multiresistant enterobacterial isolates.

Finally, our results suggest that amikacin followed by meropenem, gentamicin, and then imipenem are the best choices for treatment of ESBL-related infections harboring PMQR determinants based on the isolates' susceptibility rates to these agents (74%, 49%, 34%, and 16%, respectively).

In conclusion, our findings are of concern since different selective pressures might contribute to the persistence and broad dissemination of these multiresistant genetic elements. The co-carriage of PMQR determinants and ESBL enzymes provides an evolutionary benefit to these strains in an antibiotic-rich environment and leads to their selection under both beta-lactam and quinolone pressure. In this respect, *aac(6′)-Ib-cr* provides an even greater advantage by also conferring resistance to certain aminoglycosides. Therefore, it is important to control such strains closely with early routine screening of these determinants in order to prevent different selective pressures and reduce their spread. This study demonstrates the need to combine phenotypic and molecular methods to understand the aspects of co-resistance to fluoroquinolones, aminoglycosides, and β-lactam antibiotics in developing countries. We demonstrated the association between several genetic determinants, such as *bla*TEM, *bla*CTX-M-15, *qnr*, and *aac(6′)-Ib-cr* genes, and the significant increase in the MIC values of tested cephalosporins and aminoglycosides. This genotype–phenotype association has a direct influence on choosing the appropriate antimicrobial agent and concluding the effective therapeutic doses. Out of the detected ESBL genetic determinants, *bla*TEM and/or *bla*CTX-M-15 exhibited the highest predictive value of an increased cephalosporin MIC result. Concerning plasmid-mediated quinolone resistance, *qnr* and *aac(6′)-Ib-cr* genes showed better estimation of resistance level to quinolones and aminoglycosides when used together for screening purposes. Optimal antibiotic therapy not only treats a patient's specific infection but also minimizes collateral damage to society more generally by reducing the risk of resistance due to unnecessary or nontargeted use of antibiotics.

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Conflict of Interest The authors declare that they have no conflict of interest.

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Lesions Consistent with Tuberculous Spondylitis in Domestic and Wild Swine and Their Potential Use as a Model for Pott Disease in Humans

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Abstract

During a routine abattoir inspection of pig carcasses and control activities of hunted wildlife in 2013, 118 large white pigs *Sus scrofa domesticus*, 474 Nebrodi black pigs and 135 wild boars *Sus scrofa scrofa* were submitted to anatomopathological examination to evaluate the presence of tuberculosis-like lesions. Localized and generalized granulomatous lesions were detected with a prevalence of about 25% in large white pigs, 13% in Nebrodi black pigs and 8.15% in wild boars. Localized lesions involved mainly the submandibular lymph nodes, but when the disease was spread throughout the body, the inner organs and, also, in some cases, udders and/or bones were injured. The highest prevalence of generalized lesions (15/30) was observed in large white pigs, the only ones in which tuberculous granuloma affected also the spine. The bovine tuberculous spondylitis cases observed showed some similarities with Pott disease in humans regarding aspect and localization of lesions and age of the affected animals.

Tissue samples of the positive animals were collected and submitted to bacteriological analysis, and the bone samples were also subjected to histological and immunohistochemistry analysis. *M. bovis* was isolated in all the analysed samples, and the granuloma encapsulation was found often incomplete indicating that the disease was in an active phase. The presence of lesions associated to tuberculous spondylitis in pigs suggests the possibility to use this animal species as model for the study of Pott disease in humans.

Keywords

Anatomopathology survey · Bovine tuberculosis in swine · Pott disease · Tuberculous spondylitis

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1 Introduction

Bovine tuberculosis (TB) is a chronic bacterial disease in animals and humans characterized by the progressive development of specific granulomatous lesions (tubercles) in the involved tissues. Although the infection is under control in many European countries, it is still present in farm animals and wildlife in some regions such as Sicily in Italy, probably related to the rural farm management with pastures shared among different livestock species and wildlife. Tuberculosis in pigs is predominantly caused by *Mycobacterium avium*. The lesions are mainly confined to the lymph nodes of the head (mandibular, parotid and retropharyngeal) (Martìn-Hernando et al. 2007; Bailey et al. 2013), and the most common route of infection is by ingestion. Susceptibility to *Mycobacterium bovis* and the presence of both generalized and localized lesions have been reported in Sicily, Italy (Di Marco et al. 2012; Amato et al. 2016). Bone tuberculosis in humans (Pott disease) can constitute up to 35% of extrapulmonary forms of tuberculosis and about 2% of total reports (Mohideen and Rasool 2013). Forms of tuberculous spondylitis have been documented in wildlife such as red-tailed hawk of Jamaica (*Buteo jamaicensis*) (Sadar et al. 2015), kangaroos (Kennedy et al. 1978), rhesus macaques (Fox et al. 1974; Martin et al. 1968) and lions (Kirberger et al. 2006) and in domestic animals such as horses (Hewes et al. 2005; Perdue et al. 1991), cattle, goats and poultry. Bovine tuberculosis affecting the bones in pigs dates back to the 1930s (Ottonsen. 1931), and recently it has been extensively described (Guarda et al. 2013; Amato et al. 2017). The aim of this study is to describe the main pathological findings associated with bovine tuberculosis in pigs underlining the differences inside the same species and focusing on bone lesions to evaluate the possible use of pigs as model to study clinical and latent tuberculosis in humans.

2 Material and Methods

Sample collection: During a routine abattoir inspection of pig carcasses in 2013, 118 large white pigs and 474 Nebrodi black pigs were examined together with 135 wild boars coming from the hunting seasons. The age of the subjects was estimated between 8 and 18 months.

2.1 Gross Pathology

Thoracic and abdominal organs and lymph nodes were carefully dissected following a standardized protocol of gross examination. Tuberculous-like lesions were evaluated considering aspect, dimension, colour and distribution. The axial and appendicular skeleton of each animal was also submitted to anatomopathological inspection to assess the spreading of the disease throughout the body.

2.2 Histology

Tissue biopsies were fixed in formalin (10% neutral buffered formalin) and processed and embedded in paraffin wax (FFPE blocks). The FFPE tissue blocks were serially sectioned (5 μm thickness) and stained with haematoxylin and eosin (H&E) and Ziehl-Neelsen.

2.3 Immunohistochemistry

Immunohistochemistry was performed using the following protocol: sections were deparaffinised in xylene and hydrated in graded ethanol, and antigen sites were unblocked by heating for 20 min in citrate buffer (pH 6·0). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 30 min. Non-specific staining was minimized by incubation with Background Sniper (Biocare Medical,

CA, USA) for 15 min. Sections were incubated in each of the four primary antibodies (anti-*Mycobacterium bovis*, Cd3, Cd79 and HLA-DR) for 30 min as the manufacturer's recommendations. The Mach1 Mouse Probe (Biocare Medical, CA, USA) was used as a linker, followed by visualization with the DAB chromogenic (Dako) and nuclear visualization using Mayer's haematoxylin. T cells were detected using anti-human CD3 antibody (polyclonal rabbit, Dako) and B cells using CD79 (Clone HM57, monoclonal mouse, Dako). The major histocompatibility complex II (MHC II) antigen was identified by HLA-DR (human leucocyte antigen). The HLA-DR (Clone TAL.1B5, monoclonal mouse, Dako) was used for macrophages, monocytes and dendritic cells. Mycobacterial antigen was stained by anti-*Mycobacterium bovis* antibody (polyclonal rabbit, Biorbyt).

Negative control slides (no primary antibody used but treated with an appropriate dilution of animal serum) were included with each immunolabelling procedure. Sections of no pathological lymph node were used as positive controls for Cd3, Cd79 and HLA-DR immune staining. All sections were analysed using Leica DMLB microscope equipped with a Nikon DS-Fi1 digital camera.

2.4 Bacteriological Analysis

All tissue samples showing tuberculous lesions were cultured in liquid and solid media (Middlebrook 7H9 broth and Löwenstein-Jensen medium) according to the official culture protocol for bovine tuberculosis (OIE Manual 2009).

Molecular identification and genotyping of strains were performed by spoligotyping and mycobacterial interspersed repetitive unit variable number tandem repeat (MIRU-VNTR) analysis.

Spoligotyping was performed as described (Kamerbeek et al. 1997). The primers used were DRa (GGTTTTGGGTCTGACGAC, 59 biotinylated) and DRb (CCGAGAGGGGA CGGAAAC).

MIRU-VNTR analysis was carried out by amplifying 12 individual VNTR loci: ETRA, ETRB, ETRC, ETRD, ETRE, VNTR 2163a, VNTR 2163b, 4052, 3155, 1895, 3232 and MIRU 26 (Boniotti et al. 2009).

3 Results

3.1 Clinical-Anatomopathological Examination

Tuberculosis-like lesions were detected in 30/118 of commercial white pigs (25%), 63/474 of black Nebrodi pigs (13%) and 11/135 of wild boars (8.15%).

Both localized and generalized lesions were observed in both white and black Nebrodi pigs and wild boars as showed in Table 1. Among the large white pigs, one subject showed bone lesions similar to the human Pott disease, a form of bone tuberculosis due mainly to *M. tuberculosis* and sometimes to *M. bovis*. The farm from which the pig belonged was detected, and epidemiological investigations and subsequent follow-up were performed on the remnant pigs. The farm was officially free for brucellosis, enzootic bovine leucosis and also bovine tuberculosis; after that a tuberculosis breakdown in cattle was opened and closed a couple of years before, according to the Italian official eradication plan. The remnant apparently healthy pigs present inside were submitted to skin test and IFN-γ assay resulting all positive. These pigs were reared in promiscuity with sheep, goats and cattle and fed with bovine whey, vegetables and food waste. During the clinical exam, animals showed a good general state of nutrition and health with the exception of a sow that showed a form of moderate lameness at rear left limb.

After slaughtering, the anatomopathological examination of large white pig carcasses revealed severe forms of protracted generalization that interested the lymph nodes of all districts (head, thorax and abdomen) and organs such as the pleura, lungs, liver, spleen, and omentum in 15 animals. The involvement of kidneys was evident in one pig, while in the mammary glands of

Table 1 Prevalence and diffusion of tuberculosis-like lesions in the positive animals

Breeds	Generalized	Udder lesions	Bone lesions
White pig	15/30 (50%)	2/15 (13%)	7/15 (47%)
Black Nebrodi pig	30/63 (48%)	2/30(7%)**	Not detected
Wild boar	2/11 (18%)*	Not detected	Not detected

Note: The subjects with bone lesions were three females and four males
*Two of the three females had, also generalized udder lesions
**In one case only udder lymph nodes were involved

two other subjects, necrotic confluenting lesions and colliquation phenomena that are indicative of a post-primary infection period were detected. The ribs showed varying size of nodular oval and/or elongated lesions that appeared isolated and/or confluent and smooth or rough with a hard texture. After cutting, the degree of bone deformation associated with the gradual replacement of the mineralized tissue with caseous material was evident. The spine was affected by erosions of varying length at the vertebral bodies and spinous processes especially in the thoracic, lumbar and sacral-coccygeal tract. The erosions were associated with yellowish, caseous necrosis (Fig. 1) surrounded by pearly colour tissue of fibro-connective nature probably due to reparative/replacement function. The transverse processes were difficult to assess due to the routinely cut of the carcasses into two halves. Sometimes the caseous necrotic material protruded into the spinal canal compressing the spinal cord (Fig. 2). In addition granulomatous inflammatory phenomena were evident into the spinal canal (walls and floor) affecting also in a form of granulomatous meningitis. Signs of ankylosis or degenerative phenomena of the intervertebral discs were not detected. Similar findings were observed in human cases of bone tuberculosis (Mousa 2007).

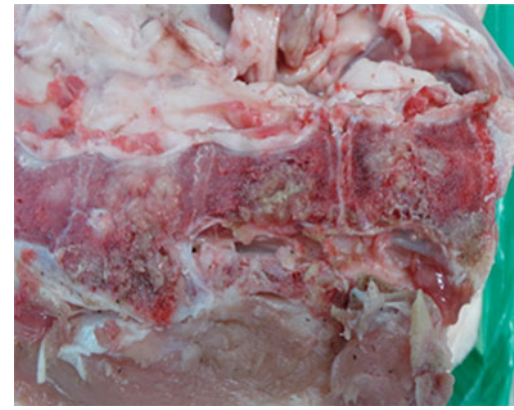

Fig. 1 Gross pathology, vertebral column, pig. Necrotic lesions

Fig. 2 Gross pathology, vertebral column, pig. Compression of the spinal cord

3.2 Histology

Histologically, tuberculous granulomas were observed in ribs and vertebrae. In particular, the tubercle (typical granuloma) contained a central core of necrotic tissue, sometimes characterized by dystrophic calcification phenomena, surrounded by epithelioid cells and more

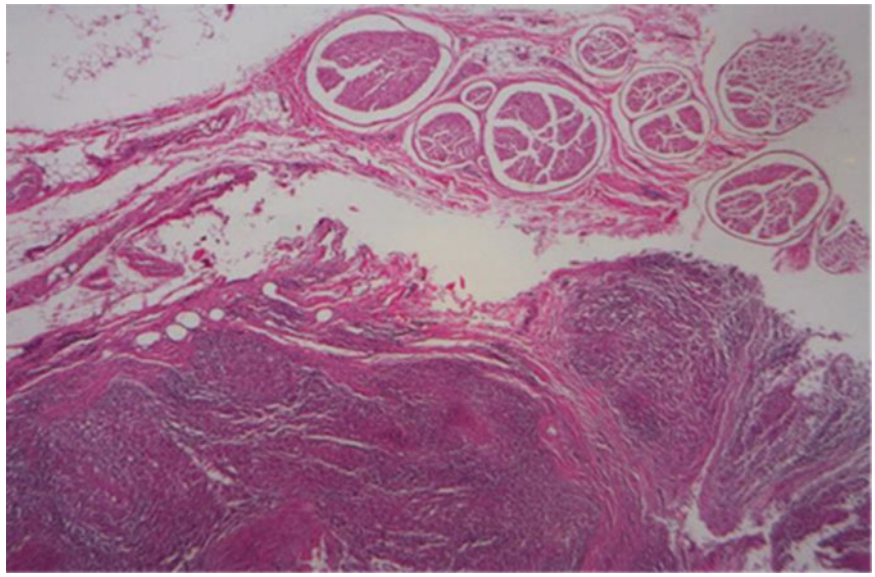

Fig. 3 Histopathology, vertebrae, pig. Granuloma formation close to several nerve bundles and surrounded by connective tissue

peripherally by macrophages and lymphocytes with varying degrees of fibroplasia and bone resorption phenomena. Encapsulation of the granulomas was often incomplete (see Fig. 3).

The active bone resorption associated to moderate osteogenesis is highly indicative of a chronic inflammatory process.

3.3 Immunohistochemical Examination

The immunohistochemistry characterization of bone granulomas showed the presence of lymphocytes with the labelled antibody for the CD3 receptor which seems to be predominantly close to the fibrous capsule in the proximity of the richest areas of macrophage cells (Fig. 4). Epithelioid cells that showed a high expression of the major histocompatibility complex (MHC) type II were also identified. B cells (marked with CD79) and giant cells were considered as minor populations in the composition of the granuloma. Abundant presence of free forms of tuberculous bacilli with higher percentage of positivity in the peripheral areas rather than in the central areas of the tubercle was observed. The immunohistochemistry characterization of bone granulomas is poorly used in human medicine for diagnostic purposes, but it is widely applied in the veterinary field as an auxiliary examination in the presence of tuberculosis-like lesions.

3.4 Bacteriology and Genotyping of the Bone Isolates

Mycobacterium bovis was isolated from all the granulomatous lesions sampled in the three different breeds (large white, black Nebrodi and wild boars).

Mycobacterium bovis, spoligotype SB0120 MIRU-VNTR profile 5,3,5,3,3,10,4,4,3,3,6,5, was isolated from all positive bone samples, indicating that a single clone was involved inside the farm. Spoligotype SB0120 is widespread in Sicily among different animal species at prevalence of almost 50% in swine and 45% in cattle (Amato et al. 2017).

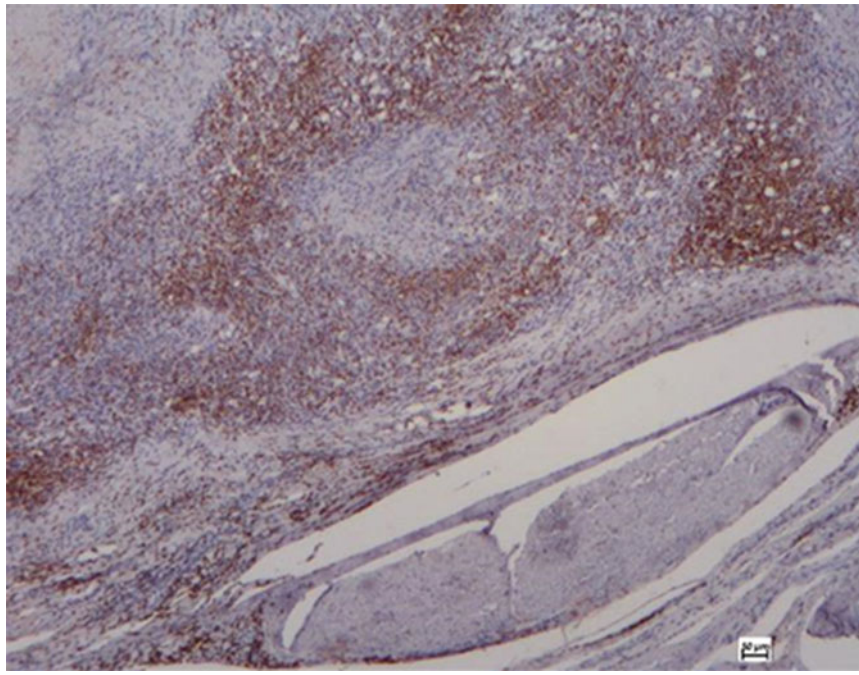

Fig. 4 Immunohistochemistry, vertebrae, pig. Peripheral rim of a large tubercle showing a majority of lymphocytes labelled with rabbit anti-human CD3 antiserum. Bar, 50 μm

4 Discussion

The results of pig carcasses inspection for tuberculosis-like lesions during routinely activity in 2013 showed how high the prevalence of this disease is especially in large white pigs (*Sus scrofa domesticus*) (Table 1). Tuberculosis infection due to *M. bovis* in pigs is present in Sicily at almost 7% of prevalence representing a risk for human infection (Di Marco et al. 2012). Sharing pastures can facilitate contacts between infected and uninfected animals of both the same and different species. The case of bone tuberculosis described in this paper occurred in a farm where pigs were reared together with cattle, sheep and goats and fed with cow milk whey. The farm had a cattle bTB outbreak previously, but isolation and genetic analysis were not performed. The *M. bovis* isolate from the bone lesions was characterized as spoligotype SB0120 which is very common in cattle and pigs in Sicily (Amato et al. 2017). The tropism of mycobacteria for the bones is closely related to the high degree of their vascularization.

In humans bone tuberculosis is also reported (Pott disease), predominantly affecting the vertebrae, although potentially the infection can affect indiscriminately any bone of the axial and appendicular skeleton (Mousa 2007). From the clinical point of view, the involvement of spine is manifested by localized pain, progressive deformation of the spine (kyphosis) due to the collapse of one or more vertebrae, paresis, paralysis up to paraplegia related to the involvement of the spinal canal and, consequently, of the meninges (meningitis) (Aydın et al. 2016). These symptoms could be associated to non-specific and systemic symptoms such as fatigue, fever and weight loss. In the preliminary stage of infection, the epiphysis (long bones) and the physis (the growth plate) are interested. Later the mycobacteria spread progressively to the articular cavities with the consequent involvement of the capsule. Inflammatory processes lead to the formation of granulation tissue and progressive erosion of the underlying cartilage.

The presence of osteomyelitis with bone destruction, minimum regeneration and lack of sequestra is highly indicative of an articular spondylitis in active phase (Kahn and Pritzker 1973). When the joint is affected, it appears swollen and painful to touch with a consequent limitation of the movements due also to the presence of cold infiltrating abscesses that surround muscles. Young adults, African people and women appear to be more susceptible. Frequent childbirths and lactation periods seem to be risk factors in women (Mousa 2007). Although the most frequently isolated agent is *Mycobacterium tuberculosis*, some cases due to *Mycobacterium bovis* and MOTT (mycobacteria other than tuberculosis) have also been reported (Mousa 2007; Pigrau-Serrallach and Rodríguez-Pardo 2013). From the pathogenic point of view, the spread of tubercle bacilli in the osteoarticular district is mainly haematogenous from foci of pulmonary origin. In case of MOTT infection, generally, lesions follow surgical or traumatic events, whereas the vertebral infection caused by *Mycobacterium bovis* is due to the ascent of the pathogen from the bladder through the venous plexus of Batson (epidural venous plexus) (Pigrau-Serrallach and Rodríguez-Pardo 2013).

The use of animal models for the study of tuberculosis infection is widely documented in the literature (Ordonez et al. 2016; Rahyussalim et al. 2016; Van Leeuwen et al. 2014). The pig is considered a more suitable model for comparative studies compared to mouse (Schook et al. 2015). Some genetic, anatomical and physiological aspects as well as innate and adaptive components of the swine immune system are similar in large part to humans, so this species should be considered a good candidate for the study of infectious diseases such as tuberculosis (Meurens et al. 2012). Bovine tuberculosis osteomyelitis cases in pigs described in this study showed remarkable similarities to the cases described in humans regarding the type of lesions (erosive, osteopenic forms with formation of bone cysts) (Aydın et al. 2016). The age of the affected individuals (young subjects) and the preferential involvement of the spine and less of the appendicular skeleton are also similar to Pott disease. However further studies need to clarify the exact pathogenetic dynamics involved in the development of bone lesions in pigs as well as the composition of the granuloma; the cell populations involved in the immune response and their subsequent activation and interaction in this species are not thoroughly characterized.

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Usnic Acid: Potential Role in Management of Wound Infections

Iolanda Francolini, Antonella Piozzi, and Gianfranco Donelli

Abstract

Usnic acid (UA) is a secondary lichen metabolite extensively studied for the broad variety of biological features. The most interesting property of UA is its antimicrobial activity against Gram-positive bacteria growing either in planktonic or in biofilm mode. In this chapter, the most relevant studies assessing usnic acid activity against microbial biofilms have been summarized and the potential role of UA in the management of biofilm-based wound infections has been critically discussed. Additionally, an overview of the main strategies adopted so far to reduce drug toxicity and increase bioavailability is given in the perspective of a safe use of UA in the clinical management of infected wounds.

Keywords

Drug delivery · Microbial biofilm · Topical applications · Usnic acid · Wound infections

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1 Introduction

Over the last two decades, there has been a renewed interest toward lichens since these consortia of fungal and photosynthetic partners are considered among the most important sources for pharmaceutically active compounds (Huneck 1999). Indeed, lichens produce a significant amount of diversified exclusive metabolites that are thought to have an adaptive value, including protection toward a large spectrum of virus and bacteria as well as toward environmental stress factors such as ultraviolet rays (Huneck and Yoshimura 1996).

The di-benzofuran compound usnic acid (UA) is undoubtedly the most extensively studied lichen metabolite due to its broad variety of biological activities (Cocchietto et al. 2002; Araújo et al. 2015; Alahmadi 2017). It was first isolated by W. Knop in 1844 (Knop 1844) and further identified in several lichen genera including *Usnea*, *Cladonia*, *Lecanora*, *Ramalina*, *Evernia*, *Parmelia*, and others.

As recently reported, UA has been demonstrated to possess antimicrobial, anti-inflammatory, antioxidant, antiviral, and antitumoral properties (Alahmadi 2017). Focusing on its antimicrobial activity, this compound is a potent antimicrobial agent against Gram-positive bacteria, including *Staphylococcus* (Lauterwein et al. 1995), *Enterococcus* (Lauterwein et al. 1995) and *Streptococcus*

(Ghione et al. 1988) species, *Mycobacterium tuberculosis* (Ingólfsdottir et al. 1997), and some pathogenic fungi (Pires et al. 2012). UA antimicrobial activity has been confirmed also on sessile-growing bacteria (Francolini et al. 2004), opening interesting perspectives for the use of this compound for prevention or treatment of biofilm-based infections.

Serious issues concerning the low UA solubility in water and the reported high toxicity (Guo et al. 2008) have so far denied the systemic use of this compound in humans. For this reason, in the last decade, investigations have been focused on topical applications of UA mostly targeting microorganisms involved in skin lesions, scurf, and dermatitis.

The aim of the present review is to summarize the most relevant studies on usnic acid activity against microbial biofilms and to highlight its potential role in the management of wound infections. An overview of the main strategies pursued to reduce drug toxicity and to increase drug bioavailability is also given in the perspective of a safe use in clinics of UA to counteract wound infections and promote wound healing.

2 The Chemistry of Usnic Acid

Usnic acid exists in two enantiomeric forms, which differ in the orientation of the methyl group located in position 9b (Fig. 1). The dextrorotatory enantiomer of UA has the R-configuration at C(9b) (Huneck et al. 1981; Bazin et al. 2008). Both enantiomers exhibit biological activity.

Three hydroxyl groups are present in the molecule: (i) the enolic -OH (position 3) having the strongest acidic feature, with pKa = 4.4, due to the inductive effect of the keto-group; (ii) the two phenol groups in position 7 and 9, with pKa values of 8.8 and 10.7 (Sharma and Jannke 1966; Sokolov et al. 2012). The lower acidity of these groups is presumably related to the inductive effect of the acetyl group in position 6 and involvement in hydrogen bond interactions.

Intramolecular hydrogen bonds are considered responsible for the low UA water solubility (less than 0.1 mg/mL), which limits UA bioavailability. UA lipophilia, instead, contributes to drug toxicity since it promotes UA membrane-permeability.

3 Usnic Acid and Microbial Biofilms

Microbial biofilm refers to a complex community of microbes growing attached to a surface showing a mushroom-like three-dimensional structure in which microbes are encased and kept together by a self-produced extracellular matrix (Costerton et al. 1995). Microbial cells growing in biofilm are physiologically distinct from their planktonic counterparts, and, of most relevance, they show high antibiotic tolerance. Proposed mechanisms behind this phenomenon are many, the most accredited being (i) the biofilm matrix, mostly anionic in nature, restricting drug diffusion; (ii) the presence of an altered microenvironment (pH, oxygen) leading to heterogeneity in growth rate and metabolic activity of cells in different biofilm layers; and (iii) a high frequency of mutation and horizontal gene transfer occurring in multiclonal communities.

Biofilms have been found to be involved in a wide variety of human infections such as urinary tract infections, pulmonary infections in cystic fibrosis, medical device-related infections, and chronic wound infections (Donelli and Vuotto 2014). Several promising experimental approaches have been investigated in the last two decades to prevent biofilm formation onto medical device surfaces (Donelli et al. 2006; Taresco et al. 2015a) or on the wound bed (Percival et al. 2015). Besides such new technological approaches, it is of crucial importance to find out novel antimicrobial compounds possessing activity not only toward bacteria in a planktonic state of growth but also against biofilm-growing bacteria.

The first investigation focused on the assessment of usnic acid ability to affect biofilm formation by clinically relevant pathogens was carried on by our group in 2004 (Francolini et al. 2004). Specifically, usnic acid was adsorbed on properly

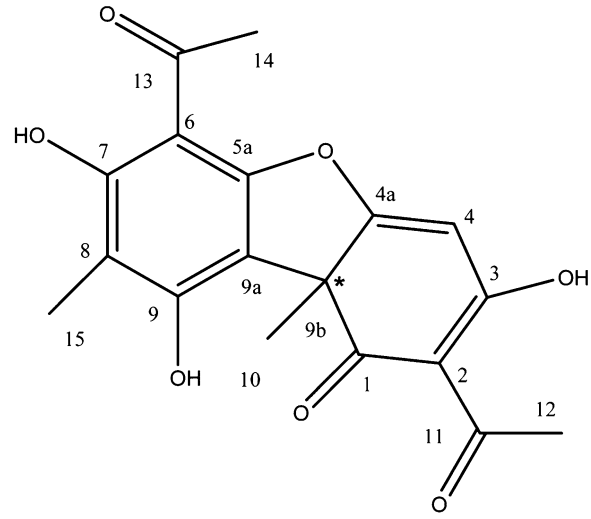


Fig. 1 Usnic acid structural formula (R = (+)-usnic acid; S = (−)-usnic acid)

functionalized-polyurethane thin films and tested in terms of capacity to control biofilm formation by either *Staphylococcus aureus* or *Pseudomonas aeruginosa*, under laminar flow conditions. UA-loaded polyurethane surfaces successfully inhibited biofilm formation by *S. aureus* and altered the morphology of *P. aeruginosa* biofilm, possibly indicating an interference of UA with bacterial signaling pathways. Later, Kim et al. (2011) incorporated usnic acid in polymethylmetacrylate (PMMA) bone cements for orthopedic applications. UA-impregnated bone cement coupons significantly diminished biofilm formation by methicillin-resistant *S. aureus* (MRSA). This result was confirmed more recently by Pompilio et al. (2013) who assessed UA antibacterial and antibiofilm activity against three strains each of methicillin-susceptible and methicillin-resistant *S. aureus* isolated from cystic fibrosis patients. Usnic acid resulted to be more active than Atranorin against MRSA both in planktonic and in sessile modes of growth. The same research group demonstrated by real-time PCR analysis that antibiofilm activity of UA is multi-target and mainly related to impaired adhesion to the host matrix binding proteins and decrease of lipase and thermonuclease expression (Pompilio et al. 2016).

Besides biofilms of Gram-positive bacteria, those formed by fungal pathogens, such as *Candida orthopsilosis* (Pires et al. 2012), *Candida parapsilosis* (Pires et al. 2012), and *Candida albicans* (Peralta et al. 2017), were shown to be susceptible to UA. However, Kvasnickova et al. (2015) failed in demonstrating UA antibiofilm activity versus *Candida parapsilosis* and *Candida krusei*.

4 Usnic Acid Application in Management of Wounds

The major causes of skin loss are surgical interventions, burn injuries, and long-term chronic wounds such as venous, diabetic, and pressure ulcers. Wound healing is a complex and dynamic process consisting of different phases, namely hemostasis, inflammation, proliferation, and tissue resolution, normally occurring in a determined set of time. However, the wound healing process can be seriously delayed or even hindered by several different factors, among which wound contamination by bacteria and consequent biofilm development have been identified as the most crucial factors impairing wound healing (Davis et al. 2008; Percival et al. 2015a). In Fig. 2, field

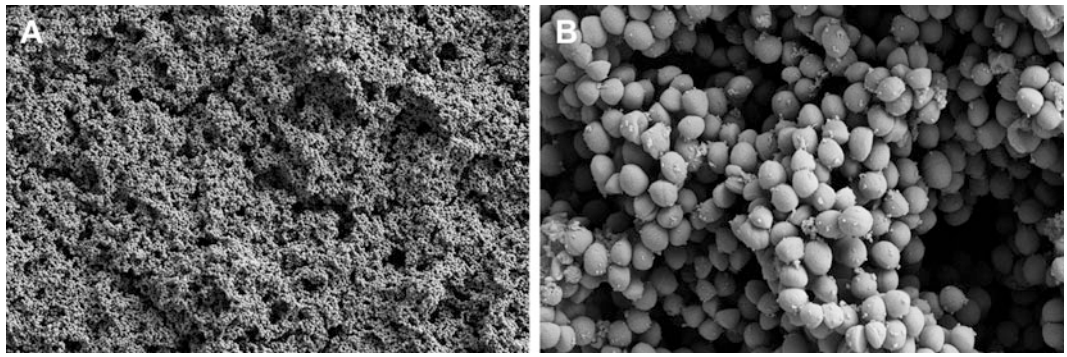


Fig. 2 FESEM micrographs at low (A, 2,000X) and high magnification (B, 20,000X) of a single species biofilm formed by a *Staphylococcus aureus MRSA* strain, isolated from an infected pressure ulcer

emission scanning electron microscopy (FESEM) micrographs showing a single species biofilm formed by a *S. aureus* MRSA strain, isolated from infected pressure ulcer of a patient admitted to the Fondazione Santa Lucia Hospital, are reported. The role of microbial biofilms in chronic wounds has been recently reviewed in detail (Percival 2018).

A large variety of antiseptics (Percival et al. 2016) and wound dressings (Valle et al. 2014) have been investigated for local wound treatment, some of them being on the market. In this regard, usnic acid may represent an interesting compound for topical treatment of wounds, given its ability to affect biofilm formation by the Gram positives *Staphylococcus epidermis* and *S. aureus*, which are the main pathogens commonly isolated from infected wounds (Han et al. 2011). For that reason, recently, a number of investigations have been focused on the assessment of UA potential role in promoting wound healing.

Zhang et al. (2018) topically applied usnic acid sodium salt on dermal injuries and studied wound healing rates by contraction experiments, histological analysis, and immuno-histochemistry analysis. Wound healing rates were higher and re-epithelialized times were shorter with topical application of sodium usnic acid, as compared to the control group. A reduction in inflammatory cells and an increase in fibroblast proliferation, granulation tissue, and vascular regeneration were also observed by usnic acid sodium salt application. The authors related the UA activity in promoting wound healing to anti-inflammatory effects at the wound bed. The anti-inflammatory activity of UA had been already demonstrated by experiments in rats in both acute and chronic wound models (Vijayakumar et al. 2000).

Promotion of HaCaT keratinocytes regeneration and strong wound closure effects of UA were also demonstrated by Burlando et al. (2009). Interestingly, similar remarkable cicatrizing properties were also displayed by UA enamine derivatives obtained through nucleophilic attack of amino acids or decarboxyamino acids at the acyl carbonyl of the enolized 1,3 diketone (Bruno et al. 2013). The wound repair properties of these derivatives were evaluated using in vitro and in vivo assays. Two of the synthesized compounds were able to combine low cytotoxicity with high wound healing performance.

On the basis of this proven UA ability to promote tissue regeneration and wound healing, several UA-loaded wound dressings were developed in the last few years (Nunes et al. 2011; Grumezescu et al. 2013; Grumezescu et al. 2014a, b; Araújo et al. 2016; Nunes et al. 2016; dos Santos et al. 2018). Nunes et al. (2011) reported the effect of collagen-based films containing usnic acid as a wound dressing for dermal burn healing. The efficacy of this dressing was evaluated in an in vivo rat model of burn

wound. The use of usnic acid improved burn healing process in rats, providing more rapid substitution of collagen type-III with type-I on the 14th day and increasing collagen density on the 21st day. More recently, the same research group investigated the activity of a gelatin-based membrane containing UA/liposomes in promoting healing of burns in pigs (Nunes et al. 2016). The activity of such a membrane was compared with silver sulfadiazine ointment and duoDerme® polyurethane dressing. The group treated with the UA/liposomes-containing membrane displayed a degree of maturation of granulation tissue and scar repair that was comparable to that obtained by duoDerme® dressing, and better than that produced by treatment with sulfadiazine silver ointment. Any clinical sign of secondary infections was not observed.

Improved wound dressings based on collagen, dextran, silica network, and usnic acid were developed by Grumezescu et al. (2013). In in vitro experiments, the fabricated wound dressing resulted to be not cytotoxic, did not influence interaction with the mesenchymal stem cells, and exhibited good antibiofilm properties. The year after, Grumezescu and colleagues (2014a, b) investigated a resorbable and bioactive wound dressing prototype, based on sodium alginate or carboxymethylcellulose plus UA-loaded magnetic nanoparticles (Fe_3O_4@AU). The obtained dressings exhibited good structural properties, low cytotoxicity on human progenitor cells, and antibiofilm activity against *S. aureus*.

Usnic acid-loaded electrospun fibers of Eudragit L-100 and polyvinylpyrrolidone for potential use in wound dressing fabrication showed good activity in vitro against *S. aureus* (Araújo et al. 2016). Finally, dos Santos et al. (2018) have recently reported the antimicrobial activity against *Escherichia coli* and *S. aureus* of UA-loaded polyaniline/polyurethane foam wound dressings. The conducting polymer polyaniline was deposited onto polyurethane foams, and interestingly, UA acted both as antimicrobial compound and dopant agent.

5 Looking for Solution to Issues Concerning UA Low Solubility and Toxicity

Safe and successful application of UA in wound management requires the finding of solutions to improve UA water solubility (bioavailability) and reduce toxicity. Indeed, if we consider the Biopharmaceutics Classification System (BCS) of drugs, UA may be classified as a class II drug, that is a drug with low solubility and high membrane permeability. It is known that UA lipophilia greatly contributes to both features, hindering drug solvatation and providing the drug with the ability to interact with the cell membrane promoting membrane uncoupling (Abo Khatwa et al. 1996). Usnic acid hepatotoxicity, proved both in in vitro (Han et al. 2004) and animal studies (Ribeiro-Costa et al. 2004), was shown to be related to the inhibition of the mitochondrial function and oxidative stress.

Over the past decades, significant efforts have been made to reduce UA side effects mainly by following two strategies: (i) UA derivatization and (ii) UA encapsulation in micro- or nanoparticles for the development of pharmaceutically accessible formulations.

5.1 UA Derivatives

UA possesses three carbonyl groups and three hydroxyl groups (Fig. 1) that, at least in principle, can undergo derivatization. However, the intramolecular hydrogen bonds occurring in UA scaffold strongly reduce reactivity of these groups making UA functionalization a complex issue.

Various UA derivatives have been synthesized through modification of either the carbonyl group of the "triketone" moiety or the group in the phenolic fragment. Sokolov et al. (2012) reported in detail the most important approaches investigated for UA derivatization, classifying the different reactions in two groups: (i) reactions with retention of the C skeleton and

(ii) reactions accompanied by destruction of C skeleton. The tri-ketone system of UA has been demonstrated to be of fundamental importance for its antibacterial action (Luzina and Salakhutdinov 2016). Therefore, the disruption of such system drastically drops UA activity. In contrast, modification of the tri-ketone system to obtain di-keto-enamine derivatives often brings about compounds with high antibacterial activity (Luzina and Salakhutdinov 2016; Victor et al. 2018). UA enamine derivatives can be obtained by reaction of UA carbonyl groups and primary amines (Fig. 3). Different kinds of amines have been investigated to obtain UA-enamine derivatives, including amino acids, diamines, triamines at different lengths, and the natural polyamine Spermine (Fig. 4).

Tomasi et al. (2006) conjugated UA with several diamines ($3 < n < 8$, Fig. 4) and triamines ($3 < n < 5$, $3 < m < 6$, Fig. 4). Interestingly, UA conjugation with short amines ($n = 3$ and $n = 4$) permitted to obtain compounds with antimicrobial activity against *S. aureus* and *Listeria monocytogenes* greater than UA. Unfortunately, data on toxicity of such derivatives were not provided. However, 2 years after, Bazin et al. (2008) obtained similar enamine-derivatives by reacting UA with two diamines ($n = 4$ and $n = 8$, Fig. 4) and two polyamines, specifically spermidine ($n = 3$, $m = 4$, Fig. 4) and spermine (Fig. 4). All of the synthesized UA derivatives exhibited cytotoxicity toward eight cancer cell lines ($2.7 < IC_{50} < 15.3$ μM) higher than UA ($19.5 < IC_{50} < 105.4$ μM). Particularly, the derivative obtained with short amine ($n = 4$) was less cytotoxicity ($IC_{50} = 12$ μM) than the one obtained with longer amine ($n = 8$, $IC50 = 3$ μM). Therefore, derivatization of UA with short amines seems to be a good strategy to have a suitable compromise between antimicrobial activity and cytotoxicity. That was later confirmed by Luzina et al. (2015) who conjugated UA with ethylenediamine ($n = 2$, Fig. 4) and quaternized the nitrogen in the diamine substituent, obtaining a derivative active against Gram-positive pathogens as much as UA but, of great concern, with an acute toxicity (LD_{50}) significantly lower (975 mg/kg) than that of UA (75 mg/Kg UA).

5.2 UA Formulations

The development of UA formulations based on UA combination with suitable delivery systems is of great research and applicative interest because it may allow improving UA therapeutic and toxicological properties by a controlled and targeted release (Dikmen et al. 2011). Drug delivery strategies may improve patient compliance and reduce dose and dose-related toxicity (Kalepu and Nekkanti 2015). Indeed, encapsulation of drug inside the nano- or microparticle core may permit to mask the drug thereby reducing systemic toxicity associated with drugs. In addition, targeting strategies of such delivery systems can improve the accumulation of drug at the disease site (Kumari et al. 2014).

In this regard, UA was either complexed with hydrophilic compounds (Nikolić et al. 2013; Francolini et al. 2013; Tang et al. 2014) or loaded onto nanocarriers, including liposomes (Lira et al. 2009; Nadvorny et al. 2014; Ferraz-Carvalho et al. 2016; Si et al. 2016), polymeric microparticles (Ribeiro-Costa et al. 2004; Martinelli et al. 2014; Grumezescu et al. 2014a, b), and hybrid core/shell nanoparticles (Grumezescu et al. 2011; Taresco et al. 2015a, b).

Molecular inclusion complexes of UA with β-cyclodextrin (β-CD) and 2-hydroxypropyl β-cyclodextrin (HP β-CD) improved solubility of UA in water, drug antimicrobial activity remaining unchanged (Nikolić et al. 2013). Our group complexed UA with a basic polyacrylamide obtaining a molecular dispersion of the drug in the polymer and a complete drug/polymer miscibility for all the tested compositions. The polymer/drug complex also possessed antimicrobial activity against a standard strain of *S. epidermidis* greater than free drug (Francolini et al. 2013). More recently, Tang et al. (2014) filed a patent concerning the preparation of a fatty alcohol polyoxyethylene ether/UA complex with improved water solubility.

Liposomes have been investigated as carriers for UA, since they permit the loading of hydrophobic drugs at high content. In addition, liposomes are versatile nanomaterials being

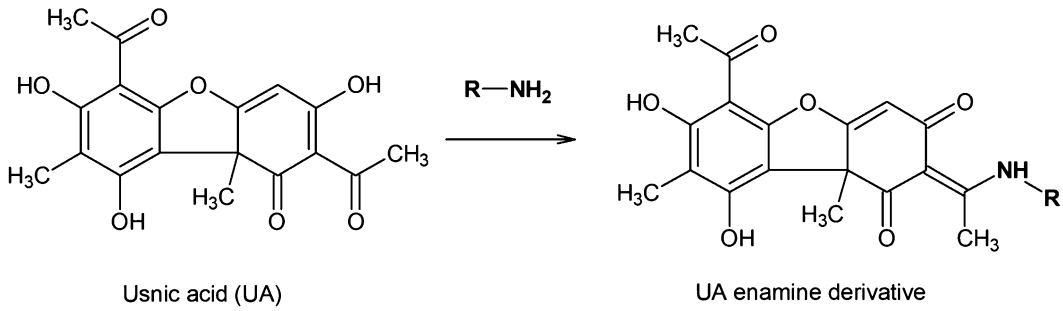
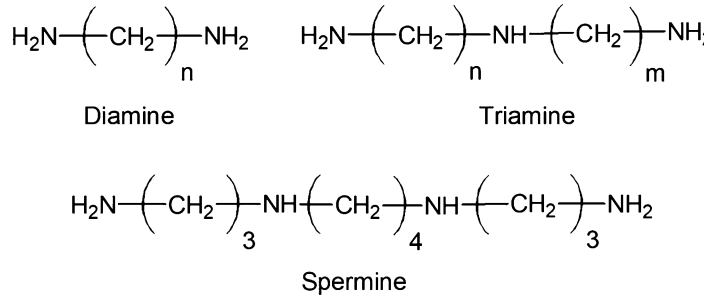


Fig. 3 Reaction of UA with amines to obtain enamine derivatives

Fig. 4 Examples of amines used to obtain UA-enamine derivatives



susceptible to modifications in terms of physico-chemical properties, bilayer composition, membrane fluidity, size, and surface charge. Lira et al. (2009) first loaded UA in positively charged liposomes obtaining a bimodal in vitro release kinetics profile, with an initial burst release of ca. 20% in the first 8 h followed by a first-order kinetics with a constant release rate in the interval from 8 to 72 h. UA-loaded liposomes exhibited a minimum bactericidal concentration (MBC) against *Mycobacterium tuberculosis* twice as low as that of free UA (16 μg/mL vs. 32 μg/mL). Interestingly, an improvement in the intracellular uptake of UA-loaded liposomes in J774 macrophages was found in comparison to free UA, presumably related to the passive targeting of UA-loaded liposomes to J774 macrophages. More recently, the same research group tested UA-loaded liposomes, alone or in combination with rifampicin or isoniazid, against multidrug-resistant tuberculosis clinical isolates (Ferraz-Carvalho et al. 2016). A synergistic interaction between RIF and UA-loaded liposomes was found suggesting this combination as a valid treatment for infections caused by *Mycobacterium tuberculosis*. Nadvorny et al. (2014) demonstrated by dynamic simulations that UA causes a lamellar to non-lamellar transition in di-palmitoyl-phosphatidyl-choline (DPPC) and di-oleoyl-phosphatidyl-choline (DOPC) membranes, thus suggesting the confinement of UA within liposomes as a possible strategy to reduce drug toxicity. Finally, the effect of free UA and UA-loaded liposomes on *Toxoplasma gondii* was recently studied in vitro and in vivo (Si et al. 2016). The authors reported that UA and UA-loaded liposomes extensively prolonged the survival time of mice about 90.9% and 117%, respectively.

Polymeric micro- and nanoparticles were also investigated as potential carriers for usnic acid to increase drug bioavailability and decrease toxicity. Biodegradable poly(lactic-co-glycolic acid) (PLGA)-microspheres were loaded with UA by

Ribeiro-Costa et al. (2004) and tested in terms of both cytotoxicity against HEp-2 cells and anticancer activity in mice against Sarcoma-180 tumor. UA encapsulation resulted in enhancement of tumor inhibition by 21%, although there was no significant difference between the cytotoxicity of free and encapsulated UA. Differently, da Silva Santos et al. (2006) showed that the injection of UA-loaded poly(lactic acid-co-glycolic acid) nanoparticles in Swiss mice permitted to achieve a 70% tumor inhibition, improving the activity of UA by 26.4% compared to free UA, and a significant reduction in drug hepatotoxicity presumably due to drug targeting. Indeed, histological observation of the tumor and liver of the animals revealed extensive necrotic areas on the tumor and liver tissues when treated with free UA, while such abnormality was not significant on the liver tissue of the animals treated with UA-loaded nanoparticles.

An increase of UA activity was also obtained by loading UA onto carboxylated poly(L-lactide) (cPLLA) microparticles (Martinelli et al. 2014). Specifically, UA-loaded cPLLA microparticles were able to promote the killing of a 24 h-old *S. epidermidis* biofilm more efficaciously than free UA. Moreover, poly-lactic-co-glycolic acid/polyvinyl alcohol (PLGA-PVA) microspheres were loaded with UA and deposited by matrix-assisted pulsed laser evaporation onto surfaces obtaining coatings able to inhibit the initial attachment of *S. aureus* as well as development of mature biofilm (Grumezescu et al. 2014a, b).

Finally, hybrid core/shell iron oxide magnetic nanoparticles loaded with UA were developed by Grumezescu et al. (2011) and applied to surfaces to obtain antibiofilm coatings. Results showed ability of the coating to decrease *S. aureus* biofilm formation, as revealed by viable cell counts and confocal laser scanning microscopy observations. Surface-engineered manganese/iron oxide magnetic nanoparticles displaying positively charged (cationic) groups were also developed by Taresco et al. (2015b) for UA loading. The developed nano-system displayed superior capacity of UA loading and activity versus *S. epidermidis*. Presumably, the cationic groups present on the nano-system surface contributed to enhance affinity of the nano-system for the anionic bacterial cell membrane, thus promoting UA uptake.

6 Future Perspectives

Usnic acid has many interesting biological activities, the antimicrobial one being the most promising, and also the most studied, for therapeutic applications. There is now a well-established knowledge about the antimicrobial activity of UA toward drug-resistant Gram-positive bacteria as well as toward biofilm-growing bacteria. Additionally, UA was shown to have multicell targets and to exert antimicrobial activity by different mechanisms (Abo Khatwa et al. 1996; Pompilio et al. 2016). This feature is definitely pivotal for overcoming the resistance of bacterial infections. Alongside this, remarkable cicatrizing effects have been reported for UA mainly thanks to the phenolic fragment providing UA with anti-inflammatory activity.

All these features point toward a promising application of UA for treatment of biofilm-based wound infections. So far, positive results have been obtained both by direct application of UA on the wound site and by UA incorporation into wound dressings. However, the relatively low number of studies available make necessary to perform further investigations for strengthening these findings. In the design of UA-based topical ointments or wound dressings, the use of UA/liposomal or UA/polymeric formulations is definitely recommended given their potential ability to reduce drug toxicity and improve bioavailability. Finally, the combination of UA with other therapeutic compounds, such as antibiotics, antimicrobial peptides, and other quorum sensing inhibitors, for the treatment of wound infections is still a poorly investigated area that undoubtedly deserves future research efforts.

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Biological Profiling of Coleoptericins and Coleoptericin-Like Antimicrobial Peptides from the Invasive Harlequin Ladybird *Harmonia axyridis*

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Abstract

The spread of antibiotic-resistant human pathogens and the declining number of novel antibiotics in the development pipeline is a global challenge that has fueled the demand for alternative options. The search for novel drug candidates has expanded to include not only antibiotics but also adjuvants capable of restoring antibiotic susceptibility in multidrug-resistant (MDR) pathogens. Insect-derived antimicrobial peptides (AMPs) can potentially fulfil both of these functions. We tested two coleoptericins and one coleoptericin-like peptides from the invasive harlequin ladybird *Harmonia axyridis* against a panel of human pathogens. The AMPs displayed little or no activity when tested alone but were active even against clinical MDR isolates of the Gram-negative ESKAPE strains when tested in combination with polymyxin derivatives, such as the reserve antibiotic colistin, at levels below the minimal inhibitory concentration. Assuming intracellular targets of the AMPs, our data indicate that colistin potentiates the activity of the AMPs. All three AMPs achieved good in vitro therapeutic indices and high intrahepatic stability but low plasma stability, suggesting they could be developed as adjuvants for topical delivery or administration by inhalation for anti-infective therapy to reduce the necessary dose of colistin (and thus its side effects) or to prevent development of colistin resistance in MDR pathogens.

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Anti-infectives · Antimicrobial peptides · Biological profiling · Coleoptericin · *Harmonia axyridis*

1 Introduction

The increasing prevalence of multidrug-resistant (MDR) bacteria and the lack of novel antibiotics in the development pipeline are a challenge to healthcare systems worldwide and have prompted the search for new antibiotic candidates, especially those active against Gram-negative bacteria (access to Medicine Foundation 2018; Delaney and Butter 2018; O'Neill 2016; Stern et al. 2017; WHO 2017). One promising class of candidates are the antimicrobial peptides (AMPs), which are produced by most if not all eukaryotic organisms but are particularly diverse and extensive among insects (Tonk and Vilcinskas 2017). Many families of insect AMPs demonstrate promising activity against human pathogens, e.g., certain insect cecropins (cationic, α-helical linear peptides) show potent in vivo activity against MDR *Acinetobacter baumannii* (Jayamani et al. 2015) and certain insect defensins (globular peptides with β-sheets stabilized by intramolecular disulfide bridges) are active against MDR *Staphylococcus aureus* (Li et al. 2017).

In insects with the most extensive AMP repertoires, there is evidence that multiple AMPs are co-expressed in response to infection and they interact to maximize their combined activity in vitro and in vivo (Pöppel et al. 2015). Beneficial combinatorial AMP interactions include potentiation (one AMP enabling or enhancing the activity of others) or synergy (the combined antimicrobial effects are greater than the sum of the individual activities). This enhances the efficacy of antimicrobial immune responses and reduces the resources reallocated to the innate immune system by increasing the antimicrobial activity of AMPs at lower concentrations (Rahnamaeian et al. 2016). The achievement of robust antimicrobial responses by the co-expression of AMPs with distinct modes of action explains why some insect-derived AMPs show little or no detectable antimicrobial activity when tested alone (Bolouri Moghaddam et al. 2016). This natural principle can be translated to medical applications, i.e., several insect-derived AMPs have been shown to interact synergistically with conventional antibiotics, suggesting they could be used to restore antibiotic sensitivity in MDR pathogens. For example, a cecropin produced by the mosquito *Aedes aegypti* was recently shown to act synergistically with tetracycline against *Pseudomonas aeruginosa*, which is responsible for most hospital-acquired diseases (Zheng et al. 2017). Similarly, a defensin from the beetle *Tribolium castaneum* was shown to act synergistically with telavancin and daptomycin against MDR *S. aureus* (Rajamuthiah et al. 2015).

Here we present the first biological profile of coleoptericins and coleoptericin-like peptides, which are specific for beetles (Coleoptera), from the harlequin ladybird *Harmonia axyridis* against a panel of human pathogens (Mylonakis et al. 2016). We selected several candidates from this species, which is native to Central and Eastern Asia but which has been introduced as a biological control agent in Northern America and Europe (Koch and Costamagna 2017; Roy et al. 2016). In the past two decades, it has become an invasive species that successfully outcompetes native ladybird species in the newly colonized areas (Roy et al. 2016), partly due to its superior immune system (Verheggen et al. 2017). *H. axyridis* constitutively produces an antibacterial and antiparasitic alkaloid called harmonine (Rohrich et al. 2012; Schmidtberg et al. 2013) but also carries inducible genes for up to 49 AMPs (Vilcinskas et al. 2013), which is much more extensive than the 15 genes found in the native seven-spotted ladybird *Coccinella septempunctata* and the 10 genes of the two-spotted ladybird *Adalia bipunctata* (Vogel et al. 2017). During the evolution of *H. axyridis*, the defensin and coleoptericin gene families have undergone unprecedented expansion, with 14 coleoptericins in *H. axyridis* but only 2 in *C. septempunctata* and 3 in *A. bipunctata* (Vogel et al. 2017). Another feature of the immune system which differs remarkably

among these three species is the maximum induction levels of some AMPs following a bacterial challenge, with the response in *H. axyridis* several orders of magnitude higher than in the two native ladybird species (Vogel et al. 2017). Remarkably, we discovered recently that coleoptericin1 (Col1) also shows population-specific expression patterns in *H. axyridis*, with invasive populations expressing higher maximum levels of Col1 than noninvasive populations. When the *col1* gene is silenced by RNA interference, *H. axyridis* becomes more susceptible to its natural pathogen *Pseudomonas entomophila*, but this susceptibility can be reversed by the injection of a synthetic Col1 peptide (Gegner et al. 2018). Taken together, these results inspired us to determine the activity of synthetic analogs of coleoptericins and coleoptericin-like peptides from *H. axyridis* against a panel of human pathogens. In addition, we investigated whether these beetle-derived AMPs displayed combinatorial activity with the peptide-based reserve antibiotic colistin, which was abandoned in the 1970s because of its severe side effects but is now being reintroduced due to the lack of alternative treatment options (Kelesidis and Falagas 2015; Tangden and Giske 2015).

2 Materials and Methods

2.1 Coleoptericins and Coleoptericin-Like Peptides

The amino acid sequences of the peptides Col1, Col6, and ColLC as well as their natural occurring derivatives Col4 (Col1 derivative), Col15 (Col6 derivative), and ColLA (ColLC derivative) are listed in Table 1. While Col1, Col4, Col6, and Col15 belong to the coleoptericin-type peptides, ColLA and ColLC are coleoptericin-like peptides. A sequence alignment, performed with the COBALT algorithm (Papadopoulos and Agarwala 2007), is depicted in Fig. S1. The peptides were produced by solid-phase synthesis on a polymeric carrier resin (GenScript, Piscataway, NJ, USA). They were analyzed by reversed-phase chromatography on a 4.6 × 250 mm Alltech Alltima C18 column (Thermo Fischer Scientific, Waltham, MI, USA) with an ascending acetonitrile gradient in water in the presence of a small amount of trifluoroacetic acid (0.05–0.065%). The peptides were detected by measuring the UV absorption at 220 nm as well as by electrospray ionization mass spectrometry (ESI-MS). The peptide purity was at least 90%.

2.2 Biological Isolates and Culture Conditions

The microbial isolates that were tested against the *H. axyridis* peptides are listed in Table S1. They were obtained from the American Type Culture Collection (Manassas, VA, USA) or the German Collection of Microorganisms and Cell Cultures GmbH (Braunschweig, Germany). Furthermore, meropenem-resistant and colistin-resistant clinical isolates derived from hospitalized patients in Germany were provided by Dr. Yvonne Pfeifer (Robert Koch Institute (RKI), Wernigerode, Germany). These isolates were identified by a RKI strain number. All isolates were cultivated in cation-adjusted Mueller-Hinton broth (CAMB) or (*Mycobacterium smegmatis* only) in brain heart infusion (BHI) medium supplemented with 1% Tween-80. All isolates were cultivated at 37 °C and 85% relative humidity, shaking at 180 rpm, and merely *Candida albicans* was cultivated at 28 °C. The meropenem-resistant and colistin-resistant isolates were maintained in the presence of the appropriate antibiotic at below the minimal inhibitory concentration (MIC).

2.3 Antibacterial Profiling

2.3.1 Inhibition of Bacterial Growth

MIC values were determined as previously described (Balouiri et al. 2016). Briefly, most of the bacterial test strains were grown for 18 h, whereas *M. smegmatis* and the yeast *C. albicans* were grown for 48 h. The cultures were subsequently diluted in CAMB medium to a final concentration of 5×10^5 cells/mL (most bacteria), 1×10^5 cells/mL (*M. smegmatis*), or 1×10^6

Table 1 Properties of the synthetic *H. axyridis* AMPs

AMP	Sequence	Size	MW (g/mol)	pI	Charge	G
Col1 BR022 Coleoptericin-type	SLQPGAPNFPIPGQEKQEGWKFDPSLTRGEDGNTLGSINIHHTGPNHEVGANWDKVIRGPGKAKPTYSIHGSWRW	75	8256.01	9.31	1.4	−0.985
Col4 BR023 Coleoptericin-type	SLQPGAPNFPMPGQGKQEGWKFDPSLTRGEDGNTLGSINIHHTGRNHEVGANWDKVIRGPGKAKPTYSIHGSWRW	75	8261.1	10.26	3.4	−1.017
Col6 BR021 Coleoptericin-type	SLQPGAPKLPYAWSRKQEGWKFDPSLTRGEDGNTLGSINIHHTGRNHEVGANWNKVIRGPGKAKPTYSIHGSWRW	75	8433.31	10.73	6.4	−1.032
Col15 Coleoptericin-type	SLQPGAPKLPYAWSGKQEGWKFDPSLTRGEDDNTLGSINIHHTGPNHEVGANWDKVIRGPGKAKPTYSIHGSWRW	75	8334.13	9.71	2.4	−0.98
ColLA	DTEGWKVQPNINRDQDGNTAGSVRVQKDFGNHEVHAGASKVFSGPNRGEPSYNVGATFNW	60	6544.91	6.08	−0.8	−1.037
ColLC	DSEGWKVQPNINRDQDGNTAGSVRVQKQLGNHEVHAGASRVFSGPNRGGPSYNVGATFNW	60	6465.86	9.69	1.2	−0.98

The AMP properties molecular weight (MW), isoelectric point (pI), and net charge at pH 7 (charge) were calculated using software provided at http://pepcalc.com/
G, GRAVY score, total hydropathy values of all the amino acids divided by the size (Kyte and Doolittle 1982)

cells/mL (*C. albicans*). Peptides and the control antibiotics tetracycline, gentamicin, meropenem, and colistin were dissolved in sterile water. The final test concentrations were 1,024–0.031 μg/mL for the peptides and 64–0.002 μg/mL for the control antibiotics. Testing was conducted in lidded 384-well plates in a test volume of 20 μL per well at 37 °C, 85% relative humidity, and 180 rpm. After incubation for 18 h, microbial growth was quantified by measuring the turbidity at 600 nm for most of the bacterial strains and *C. albicans*, and by luminometric ATP quantification using the BacTiter-Glo assay kit (Promega, Fitchburg, WI, USA) for *M. smegmatis*. Growth inhibition was calculated with respect to blank and growth control values, and the lowest AMP/antibiotic concentrations associated with no visible growth represented the MIC (the MIC of the control antibiotics was used to confirm the integrity of each assay). Experiments were performed as triplicates. To obtain preliminary results for AMP-colistin interaction studies, the MIC values of the AMPs were determined in the presence of 0.075 μg/mL colistin. To investigate effects of other polymyxin derivatives on the activity of the AMPs, the MICs of the AMPs were determined in the presence of sub-inhibitory concentrations (1/8 MIC) of each derivative.

2.3.2 Checkerboard Assay

The checkerboard dilution test of the AMPs with colistin was conducted in 96-well round-bottom microtiter plates in a final volume of 100 μL per well and a final bacterial density of 5×10^5 cells/mL. We set up a tenfold 1:2 serial dilution series of colistin in the range 320–0.31 μg/mL along each row from column 1–10 and a sixfold 1:2 serial dilution series of the AMP in the range 320–5 μg/mL down each column from row A to G on one assay master plate. Horizontal wells H1 to H11 were used for MIC testing of colistin and vertical wells A12 to G12 for MIC testing of the AMPs. We transferred 10 μL of each dilution from the assay master plate to an assay plate and added 90 μL of each bacterial suspension. Lidded plates were incubated for 18 h at 37 °C and 85% relative humidity shaking at 180 rpm and bacterial growth/growth inhibition was monitored visually. Experiments were performed in duplicates. The fractional inhibitory concentration (FIC) and the FIC_{index} of each AMP-colistin combination were calculated using the following formulae:

FIC for compound A= MIC of compound A in combination/MIC of compound A.

FIC for compound B = MIC of compound B in combination/MIC of compound B.

FIC_{index} = FIC A+ FIC B.

$FIC_{index} \leq 0.5$ indicate synergy. $FIC_{index} > 4$ indicates antagonism.

2.4 Toxicity Studies

2.4.1 Hemolysis of Human Erythrocytes

The hemolytic activity of the AMPs was tested in a 96-well round-bottom microtiter plate in a final volume of 100 μL. Erythrocytes were isolated from fresh citrate-stabilized blood from human donors by repeated centrifugation (5 min at 500x g) and washing with PBS. To obtain the final suspension, the isolated erythrocytes were diluted 1:50 in PBS. The peptides were dissolved in sterile water, and we prepared a threefold 1:2 dilution series in the concentration range 2048–256 μg/mL in a volume of 50 μL. We then added 50 μL of the erythrocyte suspension to each well, and the lidded test plates were incubated at 37 °C and 85% relative humidity for 5 h, shaking at 180 rpm. The erythrocytes were then pelleted and 80 μL of the supernatant was transferred to a new 96-well microtiter plate to quantify the released hemoglobin by turbidity measurement at 540 nm. The percentage hemolysis caused by the peptides was calculated relative to the values of the blank and positive control (Triton X-100).

2.4.2 Cytotoxicity Assay Based on ATP Quantification and the Uptake of Neutral Red

The toxicity of the AMPs toward the human hepatocellular carcinoma HepG2 HB-8065 (ATCC) cells was assessed by using the CellTiter-Glo ATP Monitoring Kit (Promega)

and by quantifying the ability to store the dye neutral red (NRU-solution, Sigma-Aldrich, St Louis, MI, USA). The assay was conducted in 96-well microtiter plates in a test volume of 200 µL. Peptides were tested in an eightfold 1:2 dilution series and a final concentration range of 400–1.56 µM. HepG2 cells were maintained in DMEM-F12 medium containing 1% nonessential amino acids, 1% sodium pyruvate, and 10% heat-inactivated fetal calf serum at 37 °C and 5% CO_2. Prior to each test, 100 µL of culture medium was added per well (each containing about 20,000 cells) and the plates were incubated for 16 h as above. The peptides were diluted in culture medium to obtain appropriate concentrations and were added to the wells as six replicates. Ketoconazole was used as a positive control for toxicity and PBS was used as the blank. After incubation for 24 h as above, cell viability was calculated either by cell lysis and subsequent luminometric quantification of the ATP concentration in each sample or by measuring the amount of neural red taken up by the cells. NRU uptake was measured at 540 nm (Tecan Genios Pro) after 3 h incubation with NRU solution and subsequent cell lysis. The stated no observed effect concentration (NOEC) values refer to the highest sample concentration with a cell viability >80%.

2.4.3 Inhibition of the Human Ether-a-go-go-Related Gene Potassium Channel

The effect of the coleoptericins and coleoptericin-like AMPs on the human ether-a-go-go-related gene (hERG) potassium channel was investigated using an automated patch-clamp method as described by (Houtmann et al. 2017). Peptides were diluted in a fivefold 1:3 dilution series at a final concentration range of 30–0.12 µM in extracellular medium (150 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose, 0.06% Pluronic F-68, 0.3% residual DMSO). The hERG channel was constitutively expressed in Chinese hamster ovary cells (CHO hERG Duo®, B'SYS GmbH, Witterswil, Switzerland). CHO cells were grown at a concentration of 8×10^6 CHO cells/mL in QPlates® (Sophion/Biolin Scientific, Ballerup, Denmark) in Ex-Cell® animal component-free CHO medium (Sigma-Aldrich) supplemented with 25 mM HEPES, 100 U/mL penicillin-streptomycin, and 0.004% soybean trypsin inhibitor. To each well, we added extracellular medium containing the desired concentration of AMPs. The peptide-hERG interaction was quantified by recording the tail current following repolarization of the hERG channels using a QPatch HTX station (Sophion/Biolin Scientific). The half-maximal inhibitory concentrations (IC_{50}) were determined using the values from three replicates of the AMP concentration series with respect to the terfenadine citrate positive control and extracellular medium (blank).

2.5 Stability Studies

2.5.1 Plasma Stability

Peptides were incubated at a final concentration of 5 µM in human, mouse, and rat plasma. After incubation at 37 °C for 0, 1, 4, and 24 h, 100 µL of the plasma samples were mixed with ethanol containing 0.5% (v/v) NH_3 to interrupt interactions between the AMPs and plasma proteins, and the latter were precipitated by centrifugation at 1735 x g for 20 min. Each 10 µL of the supernatant was analyzed in triplicates for the presence of Col6, Col1, or ColLC by LC-MS^2 (Q Exactive hybrid quadrupole-Orbitrap device, Thermo Fisher Scientific) using an AERIS Peptide 3.6 µm XB-C18 50 × 2.1 mm column (Phenomenex, Aschaffenburg, Germany). Acetonitrile and water solvents (supplemented with 0.1% formic acid) were used in an ascending acetonitrile gradient (flow rate = 500µL/min). The stability of each peptide was determined by comparing the peptide-specific ion peaks in the sample with the corresponding blank controls.

2.5.2 Metabolic Stability

The in vitro metabolic stability of the AMPs was determined using HMCS3S cryopreserved human hepatocytes (Thermo Fisher Scientific), which were stored in liquid nitrogen, thawed in cryopreserved hepatocytes recovery medium

(Thermo Fisher Scientific), and diluted to 5×10^5 cells/mL in William's E medium (Sigma-Aldrich) containing 0.001% dexamethasone and 4% cell maintenance supplement pack B (Thermo Fisher Scientific). Peptides were incubated in duplicates at 38 °C, 10% CO_2, and a final concentration of 1 μM for 0, 15, 30, 60, 90, and 120 min. After each time point, incubation was terminated by the addition of acetonitrile, the hepatocytes were removed by centrifugation, and the samples were analyzed by LC-MS^2 to detect the remaining peptides. The scaled predicted hepatic clearance for humans, as well as the extraction ratio, was calculated based on the peptide half-life assuming a liver weight of 25.71 g/kg body weight, hepatocellularity of 99×10^6 cells/g liver, and a hepatic blood flow of 1.24 L/h/kg (Poulin et al. 2012).

3 Results

3.1 Antimicrobial Activity Against Reference Strains

We investigated the potential antimicrobial activity of the coleoptericin and coleoptericin-like AMPs by testing Col1 and ColLC against selected Gram-positive bacteria (*S. aureus* ATCC 25923, *S. aureus* ATCC 33592, *Staphylococcus epidermidis* ATCC 35984, *Enterococcus faecium* DSM 17050, and *Listeria monocytogenes* DSM 20600) and Gram-negative bacteria (*E. coli* ATCC 25922, *Klebsiella pneumoniae* DSM 30104, *A. baumannii* ATCC 19606, *P. aeruginosa* ATCC 27853, and *Proteus mirabilis* DSM 4479), as well as *M. smegmatis* ATCC 607 and the yeast *C. albicans* FH2173 (Table S2). All peptides substantially lacked activity (MIC ≥1,024 μg/ml). In addition, Col6 was tested against *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *K. pneumoniae* DSM 30104, *A. baumannii* ATCC 19606, and *P. aeruginosa* ATCC 27853. Weak activity was observed against *E. coli, K. pneumoniae*, and *A. baumannii* (MIC = 32 μg/ml). There was no observed activity against *P. aeruginosa* (MIC = 256 μg/ml) or *S. aureus* (MIC >1,024 μg/ml). Three closely related natural derivatives of the aforementioned AMPs – namely, Col4 (Col1-derivative), Col15 (Col6 derivative), and ColLA (ColLC derivative) – were tested against *E. coli* ATCC 25922, and no antimicrobial activity was observed at concentrations up to 1,024 μg/mL (data not shown).

3.2 Interaction with Membrane-Disrupting Compounds

Given that the selected coleoptericin and coleoptericin-like AMPs play an important role in the *H. axyridis* immune system (Schmidtberg et al. 2013; Vilcinskas et al. 2013) but did not exhibit antimicrobial activity when tested alone, we hypothesized that they naturally act in combination with other insect-derived membrane-disrupting peptides. The peptide-based antibiotic colistin is known for its ability to disrupt bacterial membranes, so we tested Col1, Col6, and ColLC in combination with sub-MIC concentrations of colistin in order to explore this hypothesis. We therefore exposed selected Gram-positive bacteria (*S. aureus* ATCC 25923, *S. epidermidis* ATCC 35984, *E. faecium* DSM 17050, and *Listeria monocytogenes* DSM 20600) and Gram-negative bacteria (*E. coli* ATCC 25922, *E. coli* RKI 131/08, *E. coli* RKI 6A-6, *K. pneumoniae* DSM 30104, *K. pneumoniae* RKI 93/10, *K. pneumoniae* RKI 19/16, *A. baumannii* ATCC 19606, *A. baumannii* RKI 19/09, P. *aeruginosa* ATCC 27853, and *P. aeruginosa* RKI 93/12) to the AMP-colistin combination (Table 2). Against Gram-positive isolates, colistin-resistant isolates, *P. aeruginosa*, and one clinical *K. pneumoniae* isolate, the MIC of the AMPs were not affected. In contrast, the MIC of the weakly active Col6 decreased by 8–16-fold to 4 μg/mL for *E. coli* and *K. pneumoniae*, and by two–fourfold to 8 μg/mL for *A. baumannii*. The MIC of Col1 and ColLC decreased by at least 128-fold to 4–8 μg/mL for *E. coli, K. pneumoniae*, and a clinical isolate of *A. baumannii*. Against the wild-type *A. baumannii* strain, the MIC of Col1 was reduced by 16-fold to 32 μg/mL, whereas the MIC of ColLC was not affected. To investigate the interaction between colistin and the AMPs in

Table 2 Activity of the *H. axyridis* peptides in combination with colistin

	MIC (μg/ml)							
	CST	MEM	Col1		Col6		ColLC	
Strain	CAMB	CAMB	CAMB	+CST	CAMB	+CST	CAMB	+CST
E. coli ATCC 25922	0.5	0.13	>1024	4	64	4	>1024	4
E. coli RKI 131/08	0.5	>64	>1024	4	32	4	>1024	4
E. coli RKI 6A-6	8	0.13	>1024	>1024	128	8	>1024	>1024
K. pneumoniae DSM 30104	0.5	0.25	>1024	8	32	4	>1024	8
K. pneumoniae RKI 93/10	0.5	>64	>1024	>1024	64	nd	>1024	>1024
K. pneumoniae RKI 19/16	64	0.25	>1024	>1024	>1024	>1024	>1024	>1024
P. aeruginosa ATCC 27853	0.5	1	>1024	>1024	256	512	>1024	>1024
P. aeruginosa RKI 93/12	1	64	>1024	>1024	>1024	>1024	>1024	>1024
A. baumannii ATCC 19606	1	2	512	32	32	8	>1024	>1024
A. baumannii RKI 19/09	0.5	64	512	4	16	8	>1024	4

MIC values were determined in cation-adjusted Mueller-Hinton broth (*CAMB*) and in CAMB supplemented with 0.075 μg/ml colistin (+*CST*). MIC values of the control antibiotics colistin (*CST*) and meropenem (*MEM*) are listed
nd not determined

more detail, checkerboard assays against *E. coli*, *P. aeruginosa*, and *A. baumannii* were carried out using different dilutions of colistin paired with different dilutions of Col1 or ColLC. We observed AMP-colistin synergy for all combinations ($FIC_{index} \leq 0.5$), but the synergy was more pronounced for Col1 than ColLC (Fig. 1a, b). Further checkerboard assays were prepared with the addition of 150 mM NaCl or 1.25 mM $CaCl_2$. The presence of NaCl reduced the FIC_{index} by two–fourfold (Fig. 1c, d). The AMP-colistin interaction was non-synergistic in the presence of 1.25 mM $CaCl_2$ when tested against *P. aeruginosa*.

3.3 Interaction Between AMPs and Polymyxin Derivatives

We conducted a preliminary structure-activity relationship (SAR) study on the AMP-colistin interaction by testing Col1, Col6, ColLC, and the naturally occurring derivatives Col4 (derivative of Col1), Col15 (derivative of Col6), and ColLA (derivative of ColLC) combined with sub-MIC concentrations (1/8 MIC) of polymyxin derivatives against *E. coli* ATCC 25922. The natural derivatives Col4, Col15, and ColLA were inactive against *E. coli* ATCC 25922 (MIC >1,024 μg/mL). Eight different polymyxin B and seven different polymyxin E (colistin) derivatives (some unpublished) were tested. Although the activity of ColLA was not affected by the polymyxin derivatives, the activity of ColLC was reduced by at least 256-fold in the presence of colistin E2 (0.032 μg/mL) to 2 μg/mL and by at least 16-fold in the presence of polymyxin B (0.063 μg/mL) to 32 μg/mL (Table 3). In the presence of polymyxin B (0.063 μg/mL) or colistin E2 (0.032 μg/mL), the activity of the coleoptericins was enhanced (16-fold for Col6 and at least 128-fold for Col1, Col4, and Col15) to MIC values of 2–4 μg/mL. Furthermore, the activity of Col1, Col4, and Col15 was enhanced at least 4–16-fold by colistin E1 (0.063 μg/mL), the inactive polymyxin B decapeptide derivative A000160918 (32 μg/mL), and the colistin decapeptide analog A000500146A (0.125 μg/mL) to 32–128 μg/mL. In contrast, the activity of Col6 was only enhanced twofold by the inactive polymyxin B decapeptide derivative A000160918 (32 μg/mL) and both eightfold by colistin E1 (0.063 μg/mL) and the colistin decapeptide analog A000500146A (0.125 μg/mL), resulting in MIC values of 4 and 16 μg/mL. The other ten derivatives we tested did not affect the MICs of the AMPs (data not shown).

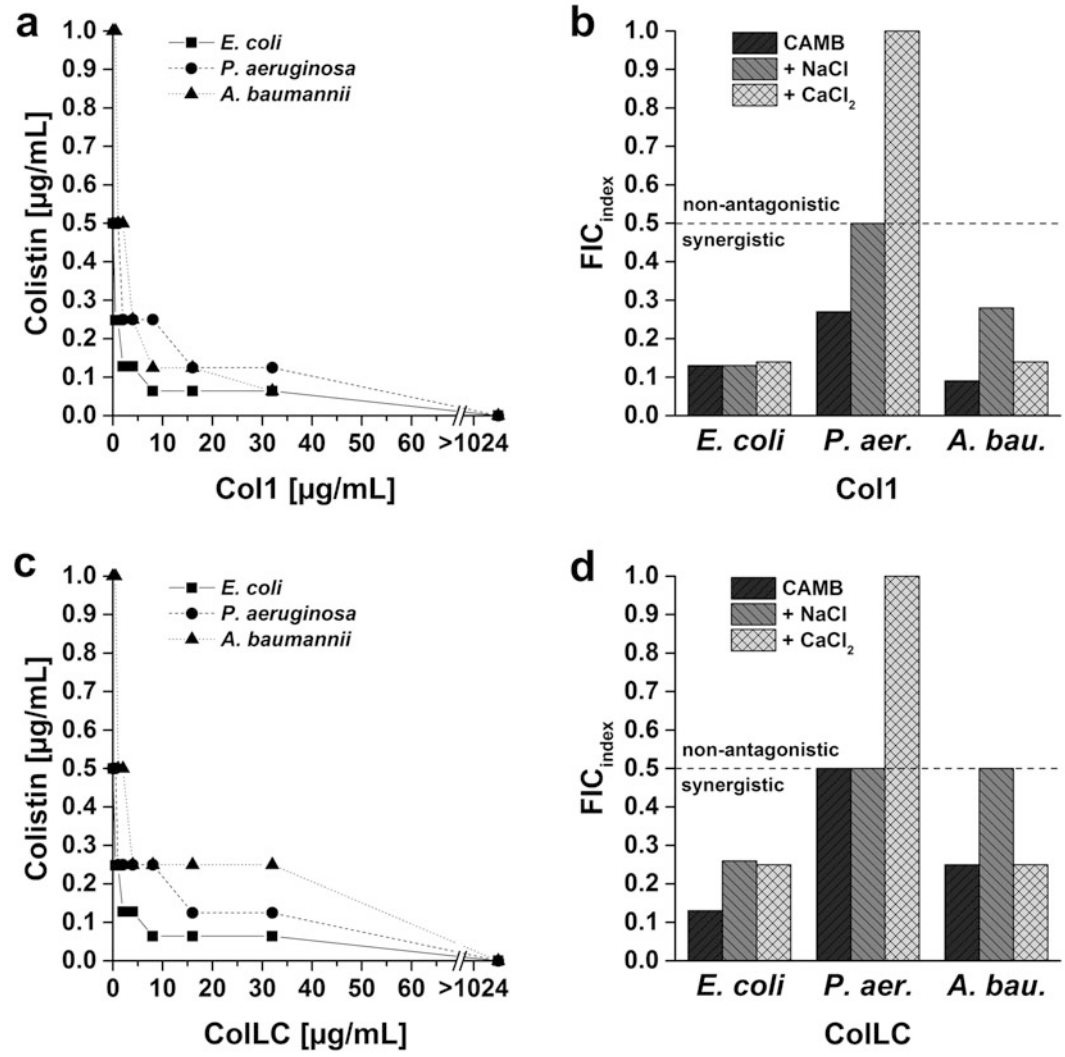

Fig. 1 Interaction of colistin with (**a**) Col1 and (**c**) ColLC in cation-adjusted Mueller-Hinton broth (*CAMB*) against *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *A. baumannii* ATCC 19606 depicted as isobolograms. Resulting FIC_{index} values were calculated for (**b**) Col1 and (**d**) ColLC in CAMB, in CAMB adjusted to 150 mM NaCl (*+NaCl*), and in CAMB adjusted to 1.25 mM $CaCl_2$ (*+$CaCl_2$*) for *E. coli*, *P. aeruginosa* (*P. aer.*), and *A. baumannii* (*A. bau.*). FIC_{index} values below 0.5 indicate synergy

Table 3 Activity of *H. axyridis* peptides in combination with polymyxin derivatives

Supplement		MIC [µg/mL]					
Cpd. Name	MIC/used conc. [µg/mL]	Col1	Col4	Col6	Col15	ColLA	ColLC
–		>256	>256	32	>256	>256	>256
Colistin E2	0.25/0.032	4	4	2	4	>256	2
Polymyxin B	0.5/0.063	4	2	2	4	>256	32
Colistin E1	0.5/0.063	64	32	4	64	>256	>256
A000500146A	1/0.125	64	64	4	64	>256	>256
A000160918	256/32	128	64	16	64	>256	>256

MIC values against *E. coli* ATCC 25922 were determined in cation-adjusted Mueller-Hinton broth (CAMB) and in CAMB supplemented with sub-MIC concentrations (1/8 MIC) of different polymyxin derivatives. MIC values of the supplemented polymyxin derivatives as well as the used sub-MIC concentrations are listed

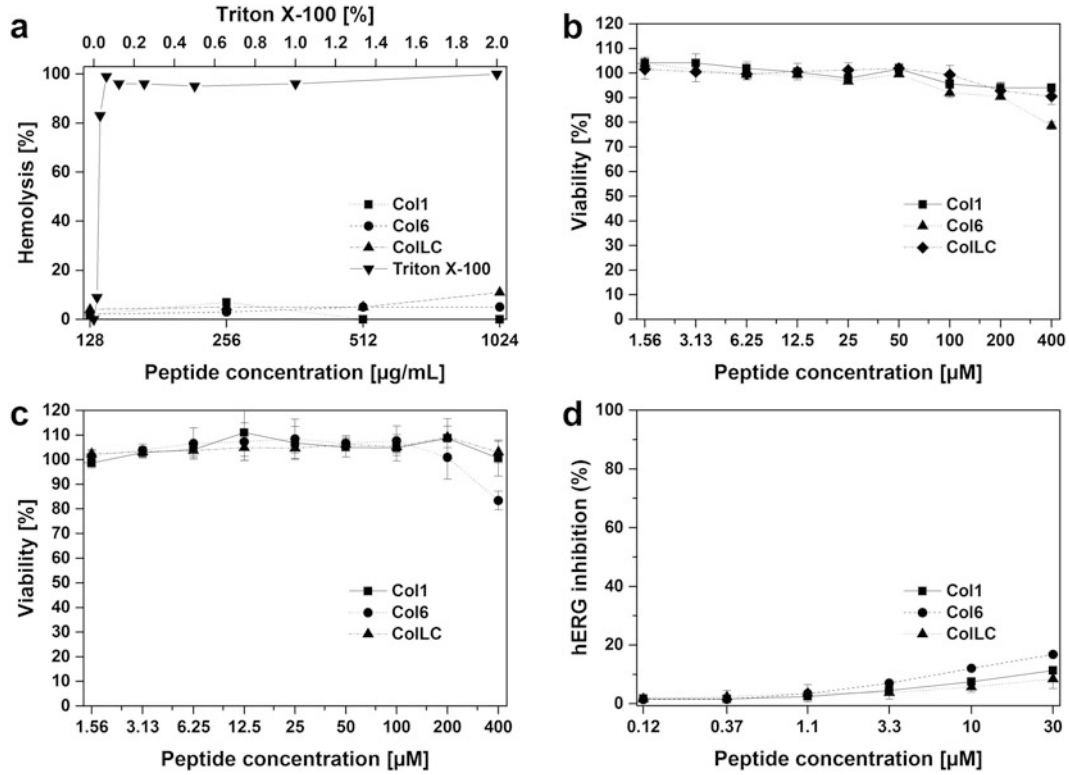

Fig. 2 Toxicity profiling of the *H. axyridis* coleoptericins Col1, Col6, and the coleoptericin-like AMP ColLC. (**a**) Hemolytic activity against human erythrocytes. Cytotoxic effects of on HepG2 cells were evaluated by measuring (**b**) neutral red uptake and (**c**) the concentration of ATP. (**d**) Inhibitory effects against the important off-target human ERG potassium channel

3.4 Toxicity Studies

The suitability of the coleoptericins and coleoptericin-like AMPs as adjuvants to minimize the dose of colistin for systemic administration in humans was investigated by toxicity assessment. First we tested the ability of Col1, Col6, and ColLC to disrupt the membrane of human erythrocytes (Fig. 2a). None of the peptides displayed hemolytic activity up to a concentration of 512 µg/mL. Next, we tested the toxicity of Col1, Col6, and ColLC toward HepG2 human hepatocellular carcinoma cells (Fig. 2b, c). The NOEC (cell viability >80%) was 100–400 µM (843–3,304 µg/mL), indicating that the peptides can be considered as nontoxic. To broaden the toxicity profile of the peptides, we used QPatch technology to test the antagonistic activity of Col1, Col6, and ColLC against the hERG potassium channel, an important off-target in the development of drugs for systemic administration in humans. No target-specific activity was observed, with IC_{50} values >30 µM (Fig. 2d).

3.5 Stability Studies

The metabolic stability of the coleoptericins and coleoptericin-like AMPs was tested in human hepatocytes. Col1, Col6, and ColLC were considered to be stable. The half-life of Col1 was 1,240 min in hepatocytes, resulting in a scaled human predicted hepatic clearance (hCL_{SP}) of 0.0672 L/h/kg and a human hepatic extraction ratio (Eh) of 12.1%. Col6 and ColLC showed no instability, preventing the calculation of hCL_{SP} and Eh values. The plasma stability of Col1,

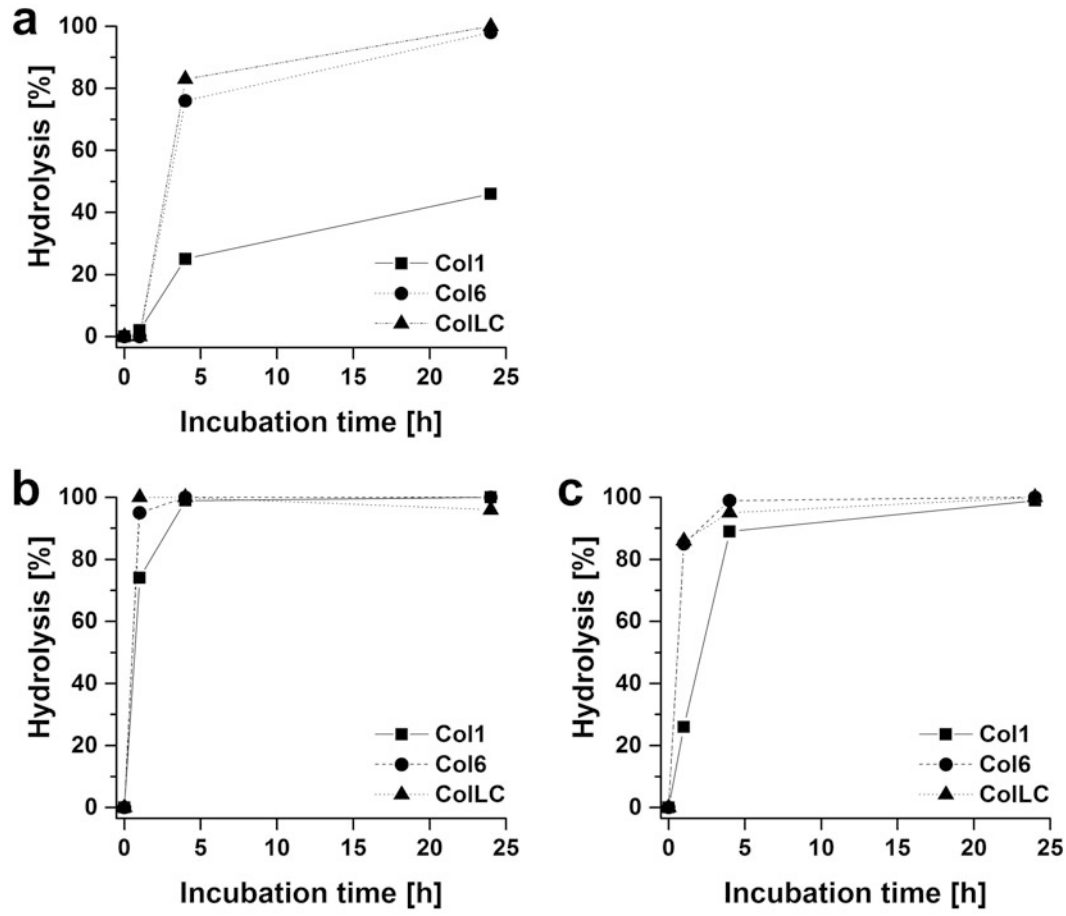

Fig. 3 Stability of the coleoptericins and coleoptericin-like AMPs Col1, Col6, and ColLC in plasma. Values indicate the percent hydrolysis of the *H. axyridis* peptides in (**a**) human, (**b**) mouse, and (**c**) rat plasma

Col6, and ColLC was tested with incubation periods of 1, 4, and 24 h (Fig. 3). All peptides were hydrolyzed after 4 h in all three plasma types (human, mouse, and rat). After 1 h, the peptides remained stable only in human plasma.

4 Discussion

Coleoptericins and coleoptericin-like peptides are glycine- and proline-rich AMPs that are structurally similar to the attacins but are found only in beetles (Mylonakis et al. 2016). They have been reported to operate in the control of endosymbionts rather than pathogen killing (Login et al. 2011; Masson et al. 2016). The deduced amino acid sequences of the *H. axyridis* coleoptericins include a signal peptide for extracellular localization, a furin cleavage site, and a mature peptide of ~75 amino acids (Vilcinskas et al. 2013). We selected two *H. axyridis* coleoptericins and one coleoptericin-like peptide for biological profiling against human pathogens based on several promising characteristics: (i) the number of genes encoding coleoptericins and coleoptericin-like peptides has expanded much more in *H. axyridis* than in native ladybirds, suggesting the peptides have undergone rapid functional diversification (Vilcinskas et al. 2013), (ii) Col1 is upregulated more than 10,000-fold in response to injected bacteria (Vilcinskas et al. 2013), (iii) Col1 is

expressed more strongly in invasive populations of *H. axyridis* than in noninvasive populations, and (iv) RNAi silencing of Col1 makes *H. axyridis* more susceptible to the entomopathogen *P. entomophila* but resistance can be restored by the injection of synthetic Col1 along with the bacteria (Gegner et al. 2018).

Surprisingly, the three AMPs showed little or no activity against human pathogens when tested alone. However, having previously shown that these coleoptericins potentiate the activity of *H. axyridis* c-type lysozymes against bacteria (Beckert et al. 2015), we postulated that their binding to intracellular targets in bacteria requires the simultaneous presence of membrane-disrupting compounds. The molecular mechanism underlying the potentiating functional interactions among insect-derived AMPs to increase their combined potency against Gram-negative bacteria was elucidated by combining abaecin and hymenoptaecin from the bumblebee *Bombus terrestris* (Rahnamaeian et al. 2015). The authors provided evidence that hymenoptaecin compromises the *E. coli* membrane in a manner that enables abaecin to enter the bacterial cell and interact with the bacterial chaperone DnaK, an evolutionarily conserved central organizer of the bacterial chaperone network.

To exploit the potentiating activity of coleoptericins for the development of new therapies, we tested coleoptericins and coleoptericin-like peptides combined with the peptide-based antibiotic colistin, which is used mostly as a reserve antibiotic due to its negative side effects (Falagas et al. 2005; Kelesidis and Falagas 2015). We confirmed that the coleoptericins and coleoptericin-like peptides were potentiated in the presence of colistin, increasing their activity against human pathogens, even including Gram-negative MDR clinical isolates. However, the potentiating effects with colistin were only observed against colistin-sensitive isolates. In preliminary experiments we also combined the peptides with the antibiotics meropenem, gentamicin, tobramycin, tigecycline, and rifampicin but did not observe effects on the resulting MIC values of the test bacterial strain. This supports the theory that colistin compromises the cell envelopes of Gram-negative bacteria and allows the coleoptericins to reach their intracellular targets. Similar effects were observed for hymenoptaecin from the bumblebee *Bombus terrestris*, which compromises the cell envelop of Gram-negative bacteria for abaecin (Rahnamaeian et al. 2015). Based on the experiments with colistin, we anticipated that mixtures of polymyxin B and the *H. axyridis* AMPs would also inhibit selected human pathogens. Notably, the polymyxin B nonapeptide is known to compromise the membranes of Gram-negative bacteria (Dixon and Chopra 1986; Vaara et al. 1984), but we found that it did not have any effect in combination with the AMPs, which is contrary to a pure membrane compromising role of the polymyxins in the polymyxin-AMPs interaction. To obtain preliminary SARs on the AMP-colistin interaction, we tested Col1, Col6, and ColLC as well as three derivatives of the coleoptericins and coleoptericin-like AMPs (Col4 (derivative of Col1), Col15 (derivative of Col6), and ColLA (derivative of ColLC)) combined with sub-MIC concentrations (1/8 MIC) of various polymyxin derivatives against *E. coli* ATCC 25922. While the activity of coleoptericins was similarly potentiated by colistin E2, colistin E1, polymyxin B, and two other polymyxin derivatives, the activity of ColLC was only potentiated by colistin E2 and polymyxin B. ColLA did not show activity in any tested combination. The coleoptericin-like AMPs clearly differ by length from the coleoptericins, and there are also significant charge differences (Fig. S1). Using the cobalt algorithm for alignment, it is noticeable that, on position 16, the coleoptericins are positively charged, whereas the coleoptericin-like AMPs are negatively charged. At positions 41, 58, 70, and 74, the coleoptericins are positively charged and the coleoptericin-like AMPs uncharged and at positions 39 and 50 it is vice versa. Furthermore, at positions 23 the coleoptericins are negatively charged and the coleoptericin-like AMPs are uncharged. Since bridging of the cell envelope is dependent on the charge of the compounds, this charge differences could explain the different

interaction patterns of the coleoptericins and the coleoptericin-like AMPs with the polymyxins. Charge may also explain why ColLA has not shown activity in any tested combination with the polymyxins. Unlike all other AMPs, ColLA has a negative charge at alignment position 43 while all others are uncharged at this position.

Because the antibacterial activity of various AMPs is known to be compromised by high concentrations of salt (Chu et al. 2013; Huang et al. 2011; Maisetta et al. 2008), we carried out checkerboard assays combining colistin and the *H. axyridis* AMPs under standard conditions in CAMB and in parallel in the same medium adjusted to 150 mM NaCl or 1.25 mM $CaCl_2$, approximately representing the salt concentration in human plasma (Li et al. 2016; Walser 1961). These assays revealed minimal salt sensitivity, so we proceeded to profile the AMPs under the rigorous standards of the pharmaceutical industry to assess whether these AMPs could be suitable as adjuvants in combination with colistin for systemic antibiotic therapy. One of the greatest barriers to the systemic use of AMPs is their potential toxicity to eukaryotic cells, particularly erythrocytes (Kang et al. 2014), which is associated with their high net charge and hydrophobicity (Laverty and Gilmore 2014; Teixeira et al. 2012). Col1, Col6, and ColLC showed neither hemolytic activity against erythrocytes nor toxicity toward HepG2 cells, which probably reflects the relatively low charge and hydrophobicity of these peptides (Table 1). Instability in body fluids is another vulnerability of AMPs for systemic administration (Chung et al. 2015; Diao and Meibohm 2013). We found that the *H. axyridis* AMPs were stable in human hepatocytes ($t_{1/2} > 1{,}200$ min) but unstable in human, mouse, and rat plasma. Overcoming the proteolytic degradation of AMPs or prolonging their half-life in serum is challenging because the activity of AMPs depends on their tertiary structure, and this limits the extent of chemical modifications to enhance stability (Rao et al. 2005). Indeed, strategies such as PEGylation, dendrimerization, pro-peptide administration, and cyclization can all extend the peptide half-life but must not inhibit the biological function (Brunetti et al. 2016; Knappe et al. 2010; Lam et al. 2016; Pini et al. 2005). The use of D-enantiomers can also extend the peptide half-life, but activity is lost (Casteels and Tempst 1994) reflecting the stereospecific nature of coleoptericin interactions with intracellular targets (Krizsan et al. 2015; Login et al. 2011). The low plasma stability of the natural peptides is incompatible with systemic in vivo delivery, but they could nevertheless serve as chemical scaffolds for the development of more stable analogs. In conclusion, due to their high in vitro therapeutic index and their potentiating activity with colistin against MDR Gram-negative bacteria, coleoptericins and coleoptericin-like peptides may be useful as leads for the development of adjuvants for topical delivery or administration by inhalation. Due to their multi-target activity in combination with polymyxins, and the resulting lower doses of polymyxins, coleoptericins and coleoptericin-like AMPs could prevent the emergence of pathogen strains that are resistant against polymyxin antibiotics.

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Conflict of Interest The authors declare no conflict of interest.

Appendix

Table S1 Overview of the test strains and their culture conditions

Test strain	Properties	Culture medium	Temperature [°C]
Gram-negative bacteria			
E. coli ATCC 25922	Type strain	CAMB	37
E. coli RKI 131/08	Clinical isolate	CAMB	37
E. coli RKI 6A-6	Clinical isolate	CAMB	37
K. pneumoniae DSM 30104	Type strain	CAMB	37
K. pneumoniae RKI 93/10	Clinical isolate	CAMB	37
K. pneumoniae RKI 19/16	Clinical isolate	CAMB	37
P. aeruginosa ATCC 27853	Type strain	CAMB	37
P. aeruginosa RKI 93/12	Clinical isolate	CAMB	37
A. baumannii ATCC 19606	Quality control strain	CAMB	37
A. baumannii RKI 19/09	Clinical isolate	CAMB	37
P. mirabilis DSM 4479	Type strain	CAMB	37
Gram-positive bacteria			
S. aureus ATCC 25923	MSSA	CAMB	37
S. aureus ATCC 33592	MRSA	CAMB	37
S. epidermidis ATCC 35984	Clinical isolate	CAMB	37
E. faecium DSM 17050	VRE	CAMB	37
L. monocytogenes DSM 20600	Type strain	CAMB	37
M. smegmatis ATCC 607	Wild type	BHI + 1% Tween-80	37
C. albicans FH2173	Wild type	CAMB	28

CAMB cation-adjusted Mueller-Hinton broth, *BHI* brain heart infusion medium, *MSSA* methicillin-sensitive *S. aureus*, *MRSA* methicillin-resistant *S. aureus*, *VRE* vancomycin-resistant enterococci

Table S2 Activity of the *H. axyridis* AMPs against reference strains

	MIC (μg/ml)		
Strain	Col1	Col6	ColLC
S. aureus ATCC 25923	>1024	>1024	>1024
S. aureus ATCC 33592	>1024	nd	>1024
S. epidermidis ATCC 35984	>1024	nd	>1024
E. faecium DSM 17050	>1024	nd	>1024
L. monocytogenes DSM 20600	>1024	nd	>1024
E. coli ATCC 25922	>1024	32	>1024
K. pneumoniae DSM 30104	512	32	>1024
A. baumannii ATCC 19606	512	32	>1024
P. aeruginosa ATCC 27853	512	256	>1024
P. mirabilis DSM 4479	>1024	nd	>1024
M. smegmatis ATCC 607	>1024	nd	>1024
C. albicans FH2173	>1024	nd	>1024

MIC values were determined in cation-adjusted Mueller-Hinton broth (CAMB)
nd not determined

Fig. S1 Alignment of the coleoptericins and the coleoptericin-like AMPs. Amino acid differences that result in charge differences of coleoptericin-like to coleoptericins are marked in red font. Amino acid differences that affect charge differences between the coleoptericin-like AMPs ColLA and ColLC are marked in green font

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Real-Time Antimicrobial Susceptibility Assay of Planktonic and Biofilm Bacteria by Isothermal Microcalorimetry

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Abstract

Most antimicrobials currently used in the clinical practice are tested as growth inhibitors against free-floating microorganisms in a liquid suspension, rather than against sessile cells constituting biofilms. Hence, reliable, fast, and reproducible methods for assessing biofilm susceptibility to antimicrobials are strongly needed. Isothermal microcalorimetry (IMC) is a nondestructive sensitive technique that allows for the real-time monitoring of microbial viability in the presence or absence of antimicrobial compounds. Therefore, the efficacy of specific antimicrobials, alone or in combination, may be promptly validated supporting the development of new drugs and avoiding the administration of ineffective therapies. Furthermore, the susceptibility of both planktonic and biofilm cells to antimicrobials can be conveniently assessed without the need for elaborated staining procedures and under nontoxic working conditions. Quantitative data regarding the antimicrobial effect against different strains might be collected by monitoring the microbial cell replication, and, more importantly, a dose-dependent activity can be efficiently detected by measuring the delay and decrease

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in the heat flow peak of the treated samples. A limitation of IMC for anti-biofilm susceptibility test is the inability to directly quantify the non-replicating cells in the biofilm or the total biomass. However, as IMC is a nondestructive method, the samples can be also analyzed by using different techniques, acquiring more information complementary to calorimetric data. IMC finds application also for the investigation of antibiotic eluting kinetics from different biomaterials, as well as for studying bacteriophages activity against planktonic and biofilm bacteria. Thus, the wide applicability of this ultra-sensitive and automated technique provides a further advance in the field of clinical microbiology and biomedical sciences.

Keywords

Antimicrobial susceptibility assay · Biofilm · Isothermal microcalorimetry · Medical microbiology · Real-time analysis

Abbreviations

CAMHB +2.5% LHB	Cation Adjusted Müller Hinton Broth supplemented with 2.5% lysate horse blood
CFUs	Colony-forming units
GC	Growth control
HA	Hyaluronic acid
HA/Levo	Levofloxacin-loaded hyaluronic acid hydrogel
HA/PBS	Phosphate buffered saline/ hyaluronic acid hydrogel
H_{tot}	Total heat produced
IMC	Isothermal microcalorimetry
k	Growth rate constant
λ	Lag phase
MBBC	Minimum biofilm bactericidal concentration
MBEC	Minimum biofilm eradicating concentration
MBPC	Minimum biofilm preventing concentration
MHIC	Minimum heat inhibiting concentration
$MHIC_b$	Minimum heat inhibiting concentration for biofilm
MIC	Minimum inhibiting concentration
P_{max}	Maximum heat flow peak
PBS	Phosphate buffered saline
S. pyogenes	*Streptococcus pyogenes*
T_{max}	Time of the maximum heat flow peak
TTD	Time to detection
TSA	Trypticase soy agar
TSA+2.5% LHB	Trypticase soy agar supplemented with 2.5% lysate horse blood

1 Introduction

The evaluation of antimicrobial susceptibility is a crucial procedure in the development of new drugs, as well as in the prediction of the therapeutic outcome during the treatment of an infection. Determining the minimum inhibiting concentrations (MICs) against planktonic microorganisms represents the starting point to estimate the efficacy of antimicrobial agents with the aim of successfully manage acute infections (Bjarnsholt 2013). However, since 65–80% of human infections is caused by pathogens in the form of biofilms (Coenye and Nelis 2010), the difficulty of employing conventional susceptibility tests raised the need for the development of biofilm susceptibility assays (Ciofu et al. 2017; Percival et al. 2015).

Biofilms consist of complex aggregations of microbial cells embedded within a self-produced matrix which adhere to each other and to living or abiotic surfaces (Bjarnsholt et al. 2013). Biofilm microorganisms are rather different from their planktonic counterparts in terms of metabolic status and display a significantly higher resistance to the host immune response and antibiotic treatment (Zimmerli et al. 2004), ultimately causing chronic persisting infections.

Although no "gold standard" is currently available to reveal the presence of microbial

biofilm from samples collected within clinical settings, various techniques have been developed for the analysis of biofilm-embedded cells, such as crystal violet, alamar blue (Di Luca et al. 2017), resazurin (Dalecki et al. 2016), and confocal laser scanning microscopy (Di Luca et al. 2017), as well as methods based on biofilm dislodging, culture plating, and colony counting. Nevertheless, most of these methods either requires the use of toxic reagents or implies scarce reproducibility and time-consuming procedures. In addition, many of them do not allow to perform a real-time monitoring of the drug activity and to use the sample for further analysis using different methods. Thus, highly sensitive and accurate methods for the real-time analysis of biofilm are required.

IMC is a nondestructive method which allows for the monitoring in the microwatt range of any exothermic or endothermic reaction related to physical and chemical process in the tested sample. All chemical and biological processes either generate or consume heat, which can be measured by IMC as heat flow. Indeed, IMC enables a precise real-time monitoring of the heat flow related to the microbial metabolism, which might proportionally correlate to the growth rate of the tested microorganism (Braissant et al. 2010, 2013) at any time point. Recent literature also reported on the convenient combination of IMC with another noninvasive and automated technique for the investigation of metabolic profiles belonging to mature biofilms of fast- and slow-growing bacteria (Solokhina et al. 2017). Moreover, previous studies showed the suitability of this nonconventional technique as an analytical method to assess the antimicrobial activity of different compounds against several pathogens (Gonzalez Moreno et al. 2017; Bormann et al. 2017; Oliva et al. 2014) and parasites in both their planktonic and biofilm forms (Gonzalez Moreno et al. 2017; Furustrand Tafin et al. 2013; Wenzler et al. 2012). Then, the ability of resorbable and degradable biomaterials to prevent biofilm formation of various bacterial strains (Butini et al. 2018) and to treat an already established biofilm infection (Casadidio et al. 2018) was also investigated by IMC. In addition, further studies reviewed the use of this sensitive technique for biofilms research applied to various field (Buchholz et al. 2010a) and investigated the ability of chip calorimetry in evaluating the activity of antimicrobials on biofilms (Buchholz et al. 2010b). Of note are also the application of IMC for investigating the metabolism of biofilms grown on zirconia and titanium surfaces (Roehling et al. 2017) and for quantifying the antimicrobial efficacy of implant coatings (Braissant et al. 2015b).

Among others, *Streptococcus pyogenes* is one of the pathogens that might be isolated from hematogenous implant-associated infections due to its ability to spread and form biofilm (Gonzalez Moreno et al. 2017).

Here, we described the use of IMC to evaluate in real time the susceptibility of planktonic and biofilm *S. pyogenes* to levofloxacin. In addition, we reported the procedure to test the capability of antimicrobial agents to prevent biofilm growth on porous glass beads. We defined the minimum heat inhibiting concentration (MHIC) as the minimum concentration of antibiotic able to suppress the metabolic heat production of planktonic bacteria and the minimum biofilm bactericidal concentration (MBBC) as the lowest concentration that strongly reduced biofilm cells viability. As IMC is a noninvasive technique that allows to reuse the sample for further analysis, the minimal biofilm eradicating concentration (MBEC) was also evaluated by sonication of biofilms formed on the beads and plating of sonication fluids for colony counting.

2 Materials and Methods

2.1 Storage and Culture of Bacterial Strain

Stocks of *Streptococcus pyogenes* (strain ATCC 19615) were prepared and maintained in cryovial bead preservation system at −80 °C. The bacterial strain was cultivated on trypticase soy agar (TSA) supplemented with 5% defibrinated sheep blood for 18 h at 37 °C under 5% CO_2 atmosphere.

2.2 IMC

For isothermal microcalorimetric analysis, a TAM III-48 microcalorimeter (TA Instruments, New Castle, DE, USA) with a detection limit of heat production of 0.2 μW and equipped with 48 minicalorimeters was used. Sterile glass ampoules (4 ml volume) were sealed for air tightness and introduced into the minicalorimeters in the equilibration position. After 15 min, ampoules were lowered in the measuring position, and then heat flow (μW) and heat (J) were measured in real time.

2.3 Antimicrobial Assay Against Planktonic Bacteria by Real-Time IMC

An inoculum was prepared according to a McFarland standard turbidity of 0.5 (corresponding to ~10^8 Colony Forming Units (CFUs)/ml, $\lambda = 565 \pm 15$ nm) and diluted in Cation Adjusted Müller Hinton Broth supplemented with 2.5% lysate horse blood (CAMHB+2.5% LHB) to a final concentration of ~10^6 CFUs/ml (T0). The exact CFUs/ml was determined by plating 100 μl of tenfold serial dilutions of the initial inoculum (T0) on TSA supplemented with 5% defibrinated sheep blood and counting colonies after 18-h incubation at 37 °C under 5% CO_2 atmosphere. Then, twofold serial dilutions of 10× concentrations of levofloxacin (5 mg/ml, Sanofi) were prepared, and glass ampoules were filled with 2400 μl CAMHB+2.5% LHB, 300 μl 10× concentration of the diluted antibiotic, and 300 μl diluted bacterial suspension (T0) to a final concentration of ~1-5 × 10^5 CFUs/ml. One ampoule with 3000 μl CAMHB+2.5% LHB and another one with inoculated CAMHB+2.5% LHB (~1-5 × 10^5 CFUs/ml) were included as negative (sterility) and positive (growth) control, respectively. Ampoules were sealed for airtightness and inserted in the minicalorimeters, first in the equilibration position (15 min) and then in the measuring position. The analysis was carried out for 24 h at 37 °C, and the minimum heat inhibiting concentration (MHIC) was defined as the lowest antimicrobial concentration that inhibited the bacterial metabolic heat production during 24-h incubation in the microcalorimeter, thus resulting in an undetectable heat flow signal. Each experiment was performed in triplicate.

2.4 Antimicrobial Assay Against Biofilm Bacteria

2.4.1 Real-Time IMC

For biofilm formation on porous glass beads (diameter, 4 mm; pore size, 60 μm; porosity, 0.2 m^2/g), a microbial inoculum was prepared according to a McFarland standard turbidity of 1.0 and subsequently diluted 1:10 in Tryptic Soy Broth supplemented with 2.5% lysate horse blood (TSB + 2.5% LHB). Then, sterile porous glass beads were incubated in the diluted bacterial suspension for 24 h at 37 °C. After incubation, beads with biofilm were carefully rinsed (3×) using sterile saline (0.9% NaCl) and exposed to twofold serial dilutions of antibiotic in glass ampoules filled with a final volume of 3000 μl fresh CAMHB+2.5% LHB (1 bead/1 ampoule). One ampoule containing 3000 μl CAMHB+2.5% LHB and one sterile bead and another one with 3000 μl CAMHB+2.5% LHB and a bead with untreated biofilm were included as negative (sterility) and positive (growth) control, respectively. IMC analysis was run for 24 h at 37 °C. The minimum heat inhibiting concentration for biofilm ($MHIC_b$) was defined as the lowest antimicrobial concentration that completely inhibited the heat production related to the viability of biofilm cells during 24-h incubation in the microcalorimeter, so resulting in an undetectable heat flow signal. Each experiment was performed in triplicate.

2.4.2 Sonication of Beads and Colony Counting

To determine the exact number of CFUs/ml on the glass bead after 24-h incubation, beads with biofilm were sonicated for colony counting. Briefly, washed beads were transferred to

Eppendorf tubes filled with 1 ml phosphate buffered saline (PBS; pH 7.4, 10 mM) and vortexed for 30 s. Afterward, beads were sonicated for 1 min in a bath sonication instrument at 40 kHz and 0.2 W/cm^2 and finally vortexed for 30 s. Fifty microliters of tenfold serial dilutions of the sonication fluid were plated on TSA supplemented with 5% defibrinated sheep blood, and colonies were counted after 18-h incubation at 37 °C under 5% CO_2 atmosphere and expressed as CFUs/ml. Each experiment was performed in triplicate.

2.4.3 Evaluation of the Reduction/Eradication of Sessile Cells

By Sonication and Colony Counting

To evaluate the reduction/eradication of biofilm cells after IMC, ampoules containing biofilm showing no heat production and ampoules containing untreated biofilms (growth control) were opened, beads were carefully rinsed (3×) using sterile saline to remove any trace of antimicrobial agent, and sonication/colony counting was performed as described above (Sect. 2.4.2.). The minimum biofilm eradicating concentration (MBEC) was defined as the lowest antimicrobial concentration required to kill sessile cells (0 CFUs/bead on plate counts).

By IMC

Ampoules containing biofilm on beads showing no heat production and ampoules containing untreated biofilms (growth control) were opened, and beads were carefully rinsed (3×) using sterile saline to remove any trace of antimicrobial agent and incubated in ampoules filled with 3000 µl fresh CAMHB+2.5% LHB. One ampoule containing 3000 µl CAMHB+2.5% LHB and one sterile bead and another ampoule with 3000 µl CAMHB+2.5% LHB and a bead with untreated biofilm were included as negative control (sterility) and positive (growth) control, respectively. IMC analysis was carried out for 48 h at 37 °C. The minimum biofilm bactericidal concentration (MBBC) was defined as the lowest antimicrobial concentration that strongly reduced the number of viable bacterial cells within the biofilm, therefore leading to undetectable heat flow values. In this analysis, the heat monitored was related to the metabolic reactivation of cells within biofilm during 48-h incubation in fresh medium.

2.5 Biofilm Prevention Assay

An inoculum was prepared according to a McFarland standard turbidity of 0.5 and diluted in CAMHB+2.5% LHB to a final concentration of ~10^7 CFUs/ml (T0). The exact CFUs/ml was determined by plating tenfold serial dilutions of the initial inoculum (T0) and counting colonies after 18-h incubation at 37 °C under 5% CO_2 atmosphere, as described above. Twofold serial dilutions of 10x concentration of levofloxacin were prepared, and test tubes were filled with 2400 µl CAMHB+2.5% LHB, 300 µl 10× concentration of the diluted antibiotic and 300 µl diluted bacterial suspension (T0) to a final concentration of ~1-5x10^6 CFUs/ml. Finally, one sterile porous glass bead was added to each tube. One tube with 3000 µl CAMHB+2.5% LHB and one sterile bead and another one with inoculated CAMHB+2.5% LHB (~1-5 × 10^6 CFUs/ml) and one sterile glass bead were included as negative (sterility) and positive (growth) control, respectively. After 24-h incubation, beads were carefully rinsed (3×) with sterile saline and incubated in sterile glass ampoules with 3000 µl CAMHB+2.5% LHB. One ampoule with 3000 µl CAMHB+2.5% LHB and one sterile bead and another one with 3000 µl CAMHB +2.5% LHB and a bead with untreated biofilm were included as negative control (sterility) as positive (growth) control, respectively. The IMC analysis was carried out at 37 °C for 48 h, defining then the minimum biofilm preventing concentration (MBPC) as the lowest antimicrobial concentration that prevented the formation of biofilm on the glass beads, thus leading to an undetectable heat flow signal during 48-h incubation in fresh medium.

2.6 Formulation of Levofloxacin-Loaded Physical Hydrogel, Drug Release by Agar Diffusion Assay, and Antibiotic Activity by IMC

Levofloxacin-loaded physical hydrogels, tested as antibiotic delivery reservoirs, were formulated in microcalorimetric glass ampoules dissolving hyaluronic acid (HA) (hyaluronic acid sodium salt, Sigma-Aldrich, Germany) in levofloxacin solution (5 mg/ml) to a final concentration of 15% w/v (final volume 300 µl). Upon mixing, ampoules containing hydrogels were incubated at 37 °C. As a control, HA physical hydrogels were formulated dissolving HA in PBS (pH 7.4, 10 mM) at a final concentration of 15% w/v.

Upon gelification, 1200 µl PBS buffer (pH 7.4, 10 mM) were added on top of the hydrogels, and ampoules were statically incubated at 37 °C. At different time points, 60 µl of release buffer were sampled and replenished. The concentration of active levofloxacin released was evaluated by agar diffusion assay against *S. pyogenes* (strain ATCC 19651), as previously reported (Butini et al. 2018; Casadidio et al. 2018). Briefly, a bacterial inoculum was prepared according to a McFarland standard turbidity of 0.5 (~1-5 × 10^8 CFUs/ml, λ = 565 ± 15 nm). Then, a sterile cotton swab was dipped into the bacterial suspension to evenly streak the surface of a CAMH agar plate supplemented with 5% defibrinated sheep blood. Next, a 6 mm hole was punched on the plate and filled with 60 µl of sampled release buffer. After 20 ± 4 h incubation, bacterial growth's inhibition halos were measured, and the concentration of active levofloxacin was calculated according to a calibration curve. Each experiment was performed in triplicate.

The real-time microcalorimetric analysis of the antimicrobial activity of levofloxacin eluted by the physical hydrogel was monitored for 24 h at 37 °C. Briefly, ~1–5 × 10^5 CFUs/ml of *S. pyogenes* were inoculated in CAMHB+2.5% LHB and incubated in glass ampoules together with levofloxacin-loaded hydrogels (final volume bacteria+gel 1500 µl). As controls, bacterial cells in the same concentration were incubated in CAMHB+2.5% LHB with HA/PBS gel and without gel, whereas a negative control consisting in HA/PBS gel was incubated with sterile medium. Each experiment was performed in duplicate.

2.7 Data Analysis

IMC data analysis was accomplished using the manufacturer's software (TAM Assistant; TA Instruments, New Castle, DE, USA). Resulted data were expressed as heat flow (µW) versus time (h) and as heat (J) versus time (h). Figures were plotted using GraphPad Prism 6.00 (GraphPad Software, La Jolla, CA, USA). IMC time to detection (TTD, h) was defined as the time between the insertion of the ampoule into the minicalorimeter and the exponentially increasing heat flow production exceeding the threshold of 10 µW (Trampuz et al. 2007). The maximum heat flow peak (P_{max}, µW), the time of the maximum heat flow peak (T_{max}, h), and the total heat produced (H_{tot}, J) were defined as the highest value of the heat flow-time curve, the time at which the P_{max} was detected and the cumulative amount of heat produced during the whole experiment, respectively. IMC data were converted into microbiologically relevant information such as growth rate constant (k, h^{-1}) and lag phase (λ, h) by deriving according to growth models, as previously reported (Yang et al. 2007; Howell et al. 2012; Braissant et al. 2013).

3 Results

3.1 Antimicrobial Assay Against Planktonic Bacteria

Antimicrobial activity of levofloxacin was tested in real time against planktonic *S. pyogenes* (strain ATCC 19615). Figure 1 shows the recorded heat flow (µW) (Fig. 1a) produced by *S. pyogenes* at each time point due to exothermic metabolic processes and the total heat (Fig. 1b), which is the cumulative amount of heat (J) produced over the experimental time. This parameter expresses the area under the heat flow curve, and it is indeed

obtained by the mathematical integration of the instantaneous heat curve. The shape of the total heat curve is similar to the bacterial growth curve. Indeed, the total heat represents a proxy for bacterial replication and reaches a maximum value as the bacterial metabolic activity starts to diminish. The incubation with levofloxacin determined a dose-dependent reduction of heat produced by bacteria, as compared to the metabolic activity of the untreated control (GC). The MHIC of levofloxacin against planktonic *S. pyogenes* was 1 μg/ml.

Moreover, the thermokinetic parameters of *S. pyogenes* growth during incubation with various concentrations of levofloxacin are listed in Table 1. The growth rate constants (k) gradually decreased with increasing concentrations of antibiotic from 1.35 ± 0.01 h^{-1} to 0 h^{-1}, when bacteria were incubated with levofloxacin ranging from 0.125 to 1 μg/ml, respectively. *S. pyogenes* growth was completely inhibited during the monitoring time when the concentration of antibiotic reached 1 μg/ml, showing an inhibitory ratio (I) of 100%. A growth inhibition exceeding the 50% was already observed after treatment with 0.25 μg/ml levofloxacin.

3.2 Antimicrobial Assay Against Biofilm Bacteria

The activity of levofloxacin was also tested in real time against 24-h-old biofilms of *S. pyogenes*

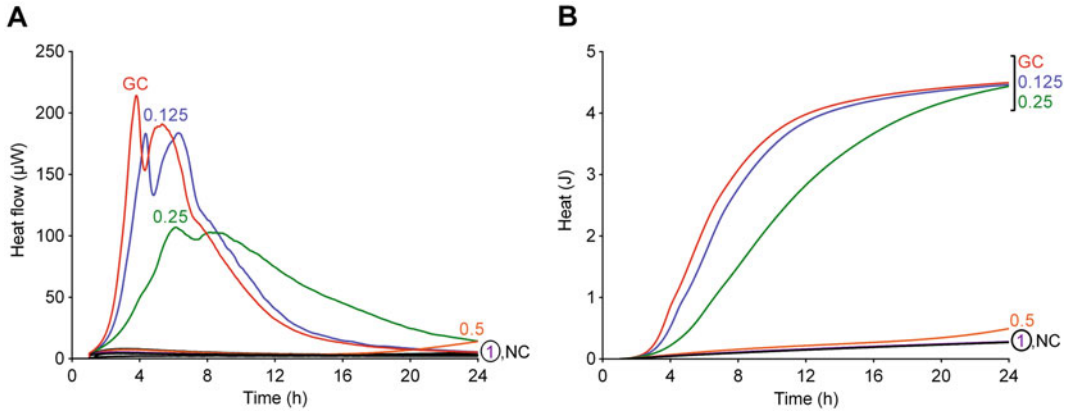

Fig. 1 Microcalorimetric analysis of planktonic *S. pyogenes* (ATCC 19615) co-incubated with different concentrations of levofloxacin. (**a**) Heat flow and (**b**) heat plot. Numbers represent concentrations of levofloxacin (μg/ml). Circled value represents the MHIC. *GC* growth control, *NC* negative control. Representative data of replicated experiments are reported

Table 1 Parameters of *S. pyogenes* growth in the presence of increasing concentrations of levofloxacin

C (μg/ml)	*k* (h^{-1})[a]	R^2	*I* (%)[b]
0	1.87 ± 0.38	0.9995 ± 0.0001	0.00
0.125	1.35 ± 0.01	0.9998 ± 0.0005	24.72 ± 13.75
0.25	0.75 ± 0.03	0.9991 ± 0.0000	57.91 ± 8.64
0.5	0.06 ± 0.08	0.9983 ± 0.0000	96.23 ± 5.34
1	0.00	–	100.00 ± 0.00

Data are expressed as mean ± SD, n = 3
k (h^{-1}): growth rate constant
R^2: correlation coefficient
I (%): inhibitory ration
[a] $\ln W_t = \ln W_0 + kt$
[b] $I = [(k_0\text{-}k_c)/k_0]\cdot 100\%$

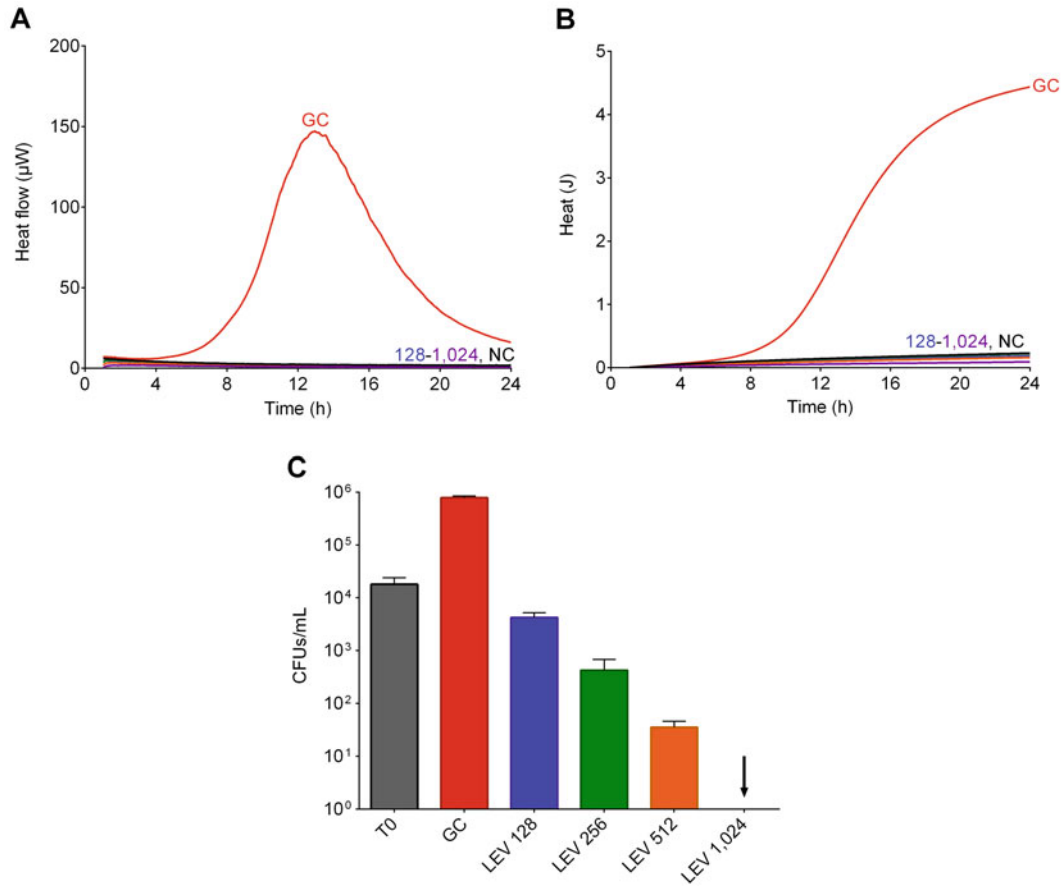

Fig. 2 Microcalorimetric analysis of *S. pyogenes* (ATCC 19615) biofilm co-incubated with different concentrations of levofloxacin. (**a**) The curve corresponds to the instantaneous heat produced by viable bacteria present in the biofilm of the growth control (GC) and (**b**) to the total heat produced during the whole experiment. The absence of heat production corresponds to biofilm co-incubated with antibiotic. Numbers represent concentrations of levofloxacin (μg/ml). *GC* growth control, *NC* negative control. Representative data of replicated experiments are reported. (**c**) Evaluation of biofilm survival after anti-biofilm treatment by CFUs counting of the sonicated beads. Arrow indicates the MBEC. T0, CFUs/ml on glass beads before anti-biofilm treatment; *GC* biofilm growth control, *NC* negative control; (mean ± SD, $n = 3$)

(strain ATCC 19615). As shown in Fig. 2, all the tested concentrations of levofloxacin (ranging from 128 to 1024 μg/ml) inhibited the replication of bacteria from the biofilm, resulting in a suppression of the heat production during 24-h incubation in the microcalorimeter (Fig. 2a and b). Therefore, the $MHIC_b$ was $\leq$128 μg/ml.

Then, the analysis of viable bacteria attached on the beads was performed by colony counting after bead sonication and plating of the sonication fluids. As shown in Fig. 2c, an increase of ≈ 2 $\log_{10}$ CFUs/ml was observed in the GC samples, as compared to the CFUs/ml calculated after sonication of the bead before the treatment (T0). Moreover, a dose-dependent reduction of *S. pyogenes* CFUs/ml was observed for all samples treated with levofloxacin, as compared to the GC (Fig. 2c). A concentration of 1024 μg/ml levofloxacin was able to kill all sessile cells, as no colonies were observed after sonication and plating (plating detection limit = 20 CFUs/ml).

In order to confirm the data observed by colony counting, a set of beads was washed after 24-h treatment with antibiotic and inoculated in fresh medium (without any antibiotic) for a second round of calorimetric analysis. As shown in

Fig. 3, a heat signal was observed for all the samples pre-treated with levofloxacin ranging from 128 to 512 μg/ml, suggesting that residual bacteria were present on the beads and therefore replicated in fresh medium. By contrast, biofilm pre-treated with 1024 μg/ml levofloxacin showed no heat production.

As reported in Table 2, a longer lag phase (λ) of ~20 h, ~25 h, and ~35 h was observed when biofilm was treated with increasing antibiotic concentrations (from 128 to 512 μg/ml, respectively), as compared to the growth lag phase displayed by the untreated biofilm (~5 h), suggesting a gradually decreased number of viable bacteria left on the beads. This increase in the lag phase obtained from IMC data analysis was also consistent with the bactericidal effect of the drug observed after plating the sonication fluid and counting bacterial colonies. Additionally, Table 2 reports also on the P_{max} (μW), T_{max} (h), and H_{tot} (J) related to the metabolic activity of viable bacteria in the biofilm after the antibiotic treatment. Similarly, values of maximum heat flow peaks progressively increased, while their corresponding T_{max} decreased, when biofilms were treated with more diluted antibiotic doses. The total heat (after 48 h) produced from the samples treated with 128 and 256 μg/ml of levofloxacin did not vary deeply from the growth control (4.13 ± 0.72, 4.04 ± 0.16, and 4.05 ± 0.13 J, respectively). However, higher concentrations of levofloxacin (512 and

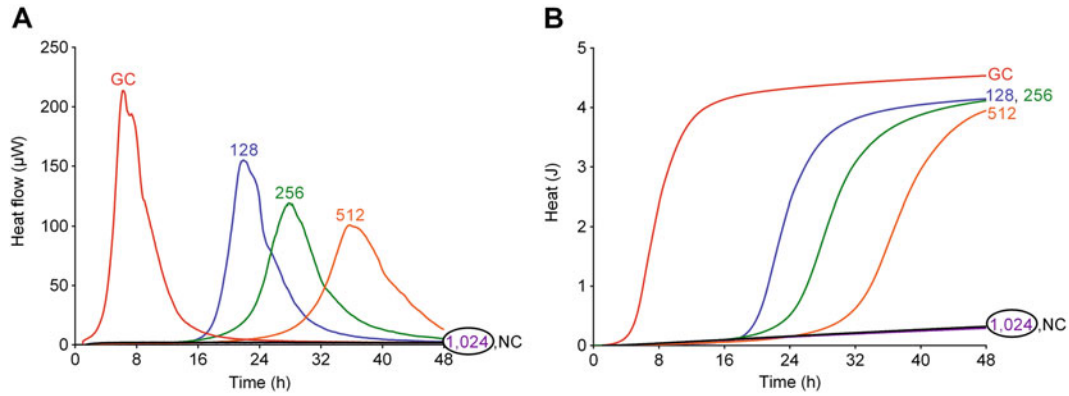

Fig. 3 Microcalorimetric analysis of *S. pyogenes* (ATCC 19615) biofilm treated with different concentrations of antibiotic. Each curve shows (**a**) the heat produced by viable bacteria present in the biofilm after 24 h of antibiotic treatment or no treatment (GC) and (**b**) to the total heat produced during the whole experiment. Numbers represent concentrations of levofloxacin (μg/ml). Circled value represents the MBBC. *GC* growth control, *NC* negative control. Representative data of replicated experiments are reported

Table 2 Microcalorimetric parameters of *S. pyogenes* biofilm pre-treated with levofloxacin

	Levofloxacin (μg/ml)				
	0	128	256	512	1024
P_{max} (μW)	214.84 ± 1.79	136.72 ± 18.07	128.86 ± 24.68	81.86 ± 67.97	2.00 ± 0.23
T_{max} (h)	6.35 ± 0.17	25.70 ± 3.34	31.28 ± 3.86	33.96 ± 13.41	26.49 ± 17.32
H_{tot} (J)	4.13 ± 0.72	4.04 ± 0.16	4.05 ± 0.13	2.83 ± 2.01	0.28 ± 0.03
TTD (h)	2.93 ± 0.46	19.40 ± 2.14	23.33 ± 3.33	29.66 ± 17.39	–
λ (h)	5.50 ± 0.79	20.80 ± 2.59	25.37 ± 1.27	35.83 ± 8.51	–

Data are expressed as mean ± SD, n = 3
P_{max} (μW): the maximum heat flow peak
T_{max} (h): time of the maximum heat flow peak
H_{tot} (J): total heat produced
TTD (h): time to detection
λ (h): lag phase duration

1024 μg/ml) resulted in a decrease in the total heat produced (2.83 ± 2.01 and 0.28 ± 0.03 J, respectively). Lastly, the time needed to reach the detection threshold of 10 μW (TTD) was longer when biofilms were treated with increasing amounts of drug (from ~20 h to ~30 h, respectively, after treatment with 128 and 512 μg/ml of drug). The treatment with 1024 μg/ml of levofloxacin resulted in a deep reduction of bacterial cell viability. Indeed, the heat flow value never exceeded 10 μW, thus remaining undetectable during the 48 h monitoring.

3.3 Biofilm Prevention Assay

The evaluation of the biofilm preventing activity of levofloxacin is represented in Fig. 4, whereas Table 3 reports on the corresponding parameters of P_{max} (μW), T_{max} (h), H_{tot} (J), and TTD. The heat flow observed during 48 h IMC monitoring is related to the metabolic activity of viable bacteria attached on the beads during the co-incubation with the antibiotic. By contrast, the absence of heat flow after 48 h would correlate with the lack of viable cells attached to the porous bead and, consequently, with no biofilm formation on the abiotic surface. An alternative explanation could be that specifically concentrated antimicrobials, giving an undetectable heat flow signal, might have timely suppressed free-swimming microbes before surface colonization, thus avoiding biofilm development. As observed for *S. pyogenes* (strain ATCC 19615), a concentration of levofloxacin ≥256 μg/ml did not determine the total reduction of heat flow, even though a noteworthy decrease in heat production and a significant temporal shift thereof could be clearly appreciated. Hence, our results suggest that, despite the high antibiotic dose tested, levofloxacin did not successfully prevent the formation of biofilm on the beads.

As reported in Table 3, P_{max} of newly formed biofilms after non-exposure and exposure to levofloxacin concentrated up to 32 μg/ml did not vary profoundly (P_{max} ranged between 254.82 ± 6.52 and 219.81 ± 20.96 μW). Differently, when bacteria were co-incubated with drug concentrations ranging from 64 to 256 μg/ml, P_{max} of the different curves decreased to values lower than 200 μW (P_{max} between 169.96 ± 27.22 and 64.38 ± 37.73 μW). The time at which P_{max} were observed differed without following regular shift, suggesting a certain extent of variability among newly developed biofilms. By contrast, the H_{tot} after 48-h monitoring showed similar values among drug-exposed and unexposed biofilms (around ~4.65 J), except for biofilm grown during incubation with the highest tested concentration (256 μg/ml), which

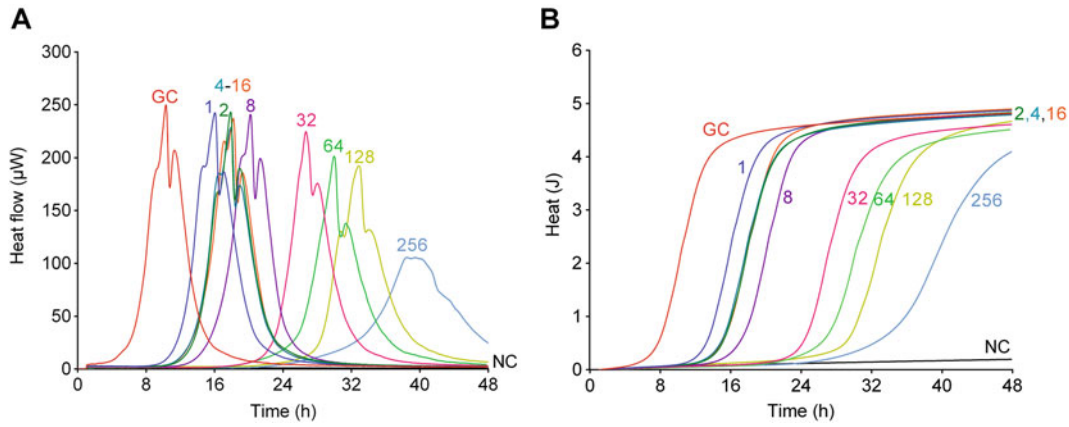

Fig. 4 Microcalorimetric analysis of levofloxacin preventing *S. pyogenes* (ATCC 19615) biofilm formation. Each curve shows (**a**) the heat produced by adherent cells on glass beads formed during the 24 h co-incubation with levofloxacin and (**b**) the total heat produced during the whole experiment. Numbers represent concentrations (μg/ml) of levofloxacin. *GC* growth control; *NC* negative control. Representative data of replicated experiments are reported

Table 3 Microcalorimetric parameters of *S. pyogenes* cells attached on glass beads after exposition of planktonic cells to levofloxacin (biofilm prevention assay)

	Levofloxacin (µg/ml)									
	0	1	2	4	8	16	32	64	128	256
P_{max} (µW)	254.82 ± 6.52	219.81 ± 20.96	234.86 ± 13.39	242.54 ± 16.04	227.89 ± 20.05	232.97 ± 26.29	232.28 ± 7.91	169.96 ± 27.22	171.07 ± 37.69	64.38 ± 37.73
T_{max} (h)	10.51 ± 0.15	18.37 ± 3.23	18.24 ± 3.59	23.30 ± 4.94	28.43 ± 7.14	29.85 ± 13.51	21.22 ± 5.20	23.70 ± 6.68	34.75 ± 2.24	45.07 ± 5.69
H_{tot} (J)	4.87 ± 0.05	4.79 ± 0.11	4.77 ± 0.22	4.60 ± 0.16	4.56 ± 0.21	4.46 ± 0.57	4.79 ± 0.16	4.55 ± 0.07	4.50 ± 0.20	2.27 ± 1.65
TTD (h)	4.73 ± 0.46	12.14 ± 2.27	12.68 ± 3.53	17.23 ± 4.83	22.25 ± 7.33	23.06 ± 12.40	15.52 ± 5.38	15.90 ± 5.54	26.90 ± 2.67	32.91 ± 5.62

Data are expressed as mean ± SD, n = 3
P_{max} (µW): the maximum heat flow peak
T_{max} (h): time of the maximum heat flow peak
H_{tot} (J): total heat produced
TTD (h): time to detection

produced a H_{tot} of 2.27 ± 1.65 J. The analyzed heat flow curves exceeded the threshold of 10 μW in the first half of the monitoring. Exception was observed for the heat values given by biofilm newly formed during incubation with 128 and 256 μg/ml levofloxacin, which indeed reached the detection limit after 26.90 ± 2.67 and 32.91 ± 5.62 μW, respectively.

3.4 Formulation of Levofloxacin-Loaded Physical Hydrogel, Drug Release by Agar Diffusion Assay, and Antibiotic Activity by IMC

The simultaneous drug loading and hydrogel formulation was successfully achieved upon addition of levofloxacin solution to HA and mixing at room temperature. The biomaterial fully swelled within a time span of 24 h (Fig. 5a), and a burst release of ~1 mg/ml levofloxacin in the release medium was observed (Fig. 5b) within the first 8 h of incubation at 37 °C. As depicted in the microcalorimetric curves, when levofloxacin-loaded gels (HA/Levo gel) were incubated with planktonic *S. pyogenes* (strain ATCC 19615) for 24 h, the bacterial metabolism was completely inhibited, resulting in undetectable heat flow values (Fig. 5c and d). By contrast, free-swimming bacteria incubated with control HA-gels formulated with PBS (HA/PBS gel without antibiotic) showed an unaltered metabolic activity similar to what observed for microbial cells incubated with neither material nor pure levofloxacin (GC).

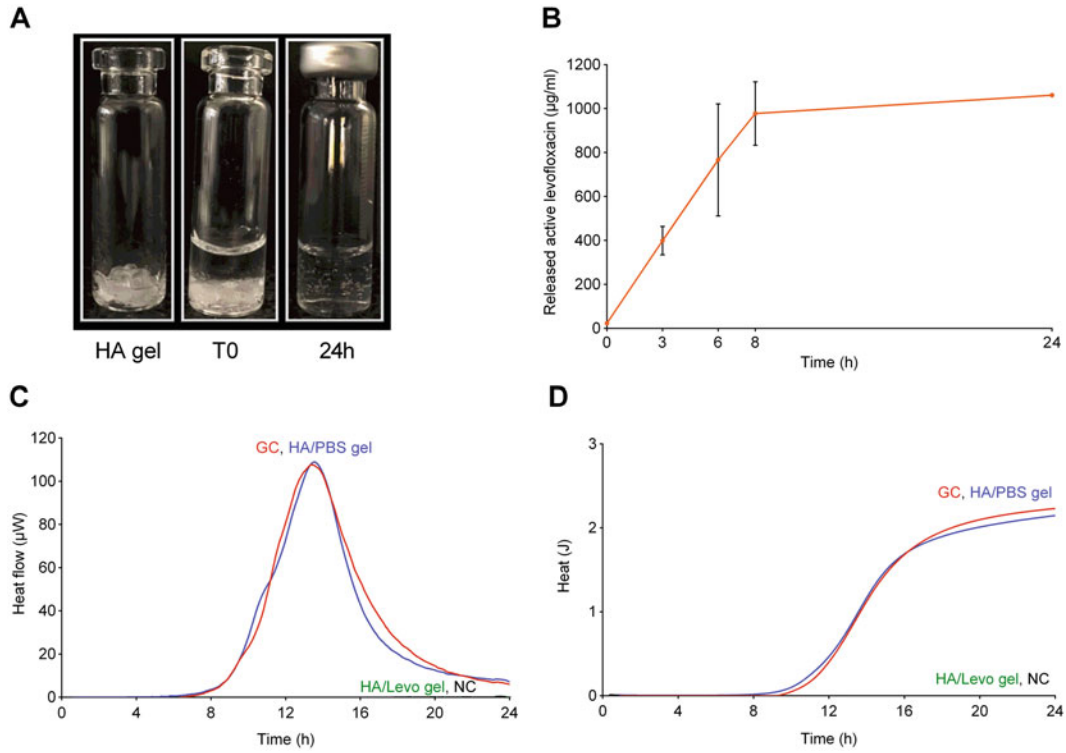

Fig. 5 Formulation of levofloxacin-loaded physical hydrogel and drug release studies. (**a**) Gross picture of drug-loaded HA hydrogel after gelification (HA gel), upon addition of release buffer on top of the hydrogel (T0) and after a 24-h release at 37 °C. (**b**) Released active levofloxacin (μg/ml) during 24 h incubation in release buffer at 37 °C. Data are expressed as mean±SD, $n = 3$. (**c**) Heat flow and (**d**) heat curves resulted from 24-h real-time IMC analysis of antimicrobial activity of levofloxacin released from hydrogels against planktonic *S. pyogenes* (ATCC 19615). *GC* growth control, *HA/PBS gel* PBS/hyaluronic acid hydrogel (control gel without levofloxacin), *HA/Levo gel* levofloxacin-loaded hyaluronic acid hydrogel, *NC* negative control. Representative data of replicated experiment are reported

4 Discussion

The analysis of the antimicrobial susceptibility in planktonic and sessile cells is a crucial step in either the development of a new drug or the establishment of an effective therapy (Balouiri et al. 2016). Therefore, a high-throughput system for either screening the antimicrobial efficacy of different molecules or evaluating the minimal concentration able to kill or reduce pathogenic microorganisms is desirable.

Here, we have reported on IMC, a nonconventional approach to investigate the susceptibility of *S. pyogenes* (strain ATCC 19615) to levofloxacin by employing a 48-channel microcalorimeter. IMC is a fast and simple method to evaluate in real time the viability of both free-floating and sessile bacteria during and after the treatment with an antibiotic. The IMC relies on the continuous measurement of the heat instantaneously produced by metabolically active cells. Hence, the effect of antimicrobials may be evaluated in terms of metabolism/growth inhibition, as long as the active compound is co-incubated with the tested strain (Figs. 1, 2a and b), or bactericidal activity, when treated samples are examined for the presence of viable/replicating bacteria after removal of the antimicrobial agent (Figs. 3 and 4). Due to its high sensitivity, IMC can detect low numbers of bacterial cells (detection limit 10^4–10^5 CFUs/ml) that would be otherwise undetectable even using standard optical density (600 nm) measurements (detection limit 10^7–10^8 CFUs/ml) (Braissant et al. 2015a). Although it is not considered a standardized method yet, IMC techniques have been demonstrated to generate data in agreement with those obtained after performing standard conventional tests (Gonzalez Moreno et al. 2017; Butini et al. 2018; Di Luca et al. 2017; Mihailescu et al. 2014; Oliva et al. 2014). This holds true also with our results (Fig. 2c and 3), where colonies count after the anti-biofilm treatment showed a dose-dependent activity of levofloxacin consistent with the effect observed on the heat production of samples treated under the same conditions.

Moreover, IMC is a noninvasive method that allows to collect the samples after the measurement and to then proceed with further analysis, such as plating for colony counting.

As opposed to standard methods to assess planktonic antimicrobial susceptibility like macro-broth dilution, agar disc diffusion (CLSI 2018), and E-test, which are performed at end point, IMC immediately generates data about dynamic processes, as microbial cells replicate during the co-incubation with the active agents. In addition, the aforementioned tests are not specifically designed to evaluate the effective concentration of antimicrobials able to eradicate a sessile community of microorganisms involved in most infections.

By using IMC alone and in combination with sonication, plating, and colony-counting procedures, we defined three parameters related to the anti-biofilm activity of an antimicrobial compound: namely, the $MHIC_b$, MBBC, and MBEC. All these values can be used to evaluate and compare the anti-biofilm activities of different compounds. Antimicrobial agents displaying lower values are therefore more efficient in the anti-biofilm treatment. Moreover, further descriptive parameters can be inferred by calorimetric analysis when sub-inhibiting concentrations of antibiotics are tested, such as P_{max} (μW), T_{max} (h), H_{tot} (J), and TTD (h), which are related to the metabolic activity of viable bacteria in the biofilm. Given that one bacterial cell can generate a heat of ~2 pW (Higuera-Guisset et al. 2005), a real-time estimation of the amount of metabolically active cells in the biofilm might be made. As an example, the number of CFUs calculated at the maximum peak of heat flow (P_{max}), which approximately corresponds to the end of the exponential growth phase, may provide important data on the amount of active biofilm cells remained on the porous glass bead, in our case, after the anti-biofilm treatment. Concurrently, information regarding the time at which the logarithmic phase ceases (T_{max}) could also be easily recruited. Nevertheless, the connection between heat flow and cell count must be considered with care, as it exists only at early growth stages (Fan et al. 2008). Indeed, decreases in heat production following P_{max} can be mostly related not to a decrease in

cell number but rather to a reduced metabolic rate, a possible switch from aerobic to anaerobic processes or the gradual depletion and sequential use of carbon sources (Braissant et al. 2013). Lastly, data on the cumulative heat (H_{tot}) produced by the tested microorganism could be used both as end point datum and as a real-time parameter. In fact, H_{tot} can be employed to calculate the overall percentage reduction of the total heat produced by treated strains (compared to an untreated control), as well as to monitor the switch from biomass building (initial slope) to biofilm maintenance phases (plateau) during the analysis (Astasov-Frauenhoffer et al. 2012). The time to detection (TTD), namely, the temporal interval lying between the experiment start and the exponentially increasing heat flow production exceeding the threshold of 10 μW (Trampuz et al. 2007), also provides real-time information on the metabolic change from lag to log phase of microbial replication. Deeper analysis for acquiring microbiologically and pharmacologically relevant data has also been described and applied (Braissant et al. 2013). Indeed, as we also reported for planktonic and biofilm *S. pyogenes* treated with a fluoroquinolone, the calculated growth rate (k) and lag phase (λ) provide valuable basis for comparison between metabolic differences and bacterial cell counts of treated and untreated samples. The tendency of an antimicrobial compound to act in a more microbiostatic or microbicidal manner could be therefore efficiently investigated (Astasov-Frauenhoffer et al. 2014), thus promptly supplying trustful therapeutic guidelines for the management of clinical cases.

The relevance of studying the effect of antimicrobial sub-inhibitory concentrations on biofilms remarkably emerged in the recent years. Indeed, similarly to the induction of resistance mechanism in planktonic cells, sub-$MHIC_b$ may foster biofilm formation (Rachid et al. 2000), rather than inhibit it. These data can be also obtained by analyzing and comparing the activity of sub-inhibitory antimicrobial concentrations by IMC (von Ah et al. 2009) and evaluate the amount of heat produced.

In general, IMC allows for a fast and reliable investigation of biofilm-forming strains, without the need for expensive disposable materials or toxic reagents. Moreover, compared to standardized methods for microbial biofilms studies such as crystal violet assay, resazurin fluorescence dye (Dalecki et al. 2016), and quantification assays based on surface scraping, the microcalorimetric method involves neither biofilm staining nor physical harsh manipulation.

As reported by different authors, a critical step in testing antibiotic is to avoid contamination (Mah 2014). Indeed, in the majority of susceptibility tests, it is difficult to detect the presence of a contaminant microorganism, which might ultimately alter the assay results. Conversely, the outcomes obtained from microcalorimetric measurements enable differentiating among different microorganisms, since each microbial strain displays a "fingerprint" in the form of a characteristic shape of the heat flow curve monitored in real time. A contaminated sample could be therefore more easily identified.

The main advantage of microcalorimetry is to accommodate any type of sample that can fit in ampoules specifically designed depending on the instrument (in our case, 4 ml ampoules). To test implants and other biomedical materials, this feature allows for the insertion of a label-free solid sample into a microcalorimetric ampoule, as we demonstrated by conveniently testing a biphasic gentamicin-loaded calcium sulfate/hydroxyapatite bone graft substitute in our recent work (Butini et al. 2018). Similarly, further studies also highlighted the advantageous application of IMC for the investigation of bacterial adhesion and biofilm formation on titanium and zirconia implant surfaces (Roehling et al. 2017) and for the monitoring of antimicrobial properties of coatings or porous materials (Braissant et al. 2015b). Moreover, following a procedure similar to what recently described (Casadidio et al. 2018), here we confirmed the suitability of this technique for an easy formulation of antimicrobial-loaded hydrogels and, subsequently, a real-time monitoring of microbial response to the released agent. Ordinarily assessed through specifically adjusted agar diffusion methods (Marchesan et al. 2013; De Giglio et al. 2011) requiring several hours of incubation, the real-time analysis of the antimicrobial activity of loaded jellified materials still faces many

difficulties. Moreover, the poor mechanical properties of physical hydrogels (i.e., hyaluronic acid hydrogels) might represent a challenging step in antimicrobial susceptibility tests on inoculated agar plates, as the lack of structural stability given by the absence of chemical cross-links could hinder the analysis. Hence, the use of IMC proved to provide a fair improvement in these experimental procedures, as it allows for undemanding polymer dissolution in glass ampoules, simultaneous drug loading and following minimal workload on the gel network.

However, IMC also carries some limitations, mostly related to critical steps within the operating procedure. As highlighted in the experimental protocol, special attention must be paid to ampoules sealing and cap shaping prior to insertion in the minicalorimeters to avoid evaporation and subsequent errors in the measurement. Understandably, this essential step prevents gas and medium exchanges inside the closed ampoules, therefore impairing oxygen and nutrients availability to the inoculated cells. Hence, it is necessary to carefully evaluate the data obtained after more than 5 days of long incubation in the minicalorimeters. Another critical step in sample preparation is the washing of biofilm formed on glass beads that is performed to remove any trace of planktonic bacteria and active molecules. During liquid aspiration in washing steps, the contact between vacuum aspirator and the bead could damage the biofilm structure, thus leading to an erroneous interpretation of the outcomes (e.g., heat flow detection of planktonic bacteria diffusing out from the altered biofilm matrix or false biofilm eradication due to aspiration of residual attached cells). Thus, careful positioning of the aspirating pipette on the side of the test tube slightly inclined is appropriate. Lastly, in addition to drawbacks related to the experimental procedure, the cost of the instrument might be a heavy limiting factor in the choice of application of this technique.

In general, as isothermal microcalorimeters (as other types of calorimeters) record the net heat flow of all processes producing or consuming heat in an IMC ampoule (also non-specific signals), it is important to include a negative control recording heat flow from ampoules containing only the medium and/or material without any specimen.

In our previous works, we have used this method to evaluate the antimicrobial activity of different compounds (including antibiotics (Gonzalez Moreno et al. 2017), bacteriophages (Tkhilaishvili et al. 2018) and antimicrobial peptides (Bormann et al. 2017)) against different planktonic and biofilm-embedded microorganisms, such as bacteria (Gonzalez Moreno et al. 2017), fungi (Furustrand Tafin et al. 2013), and parasites (Wenzler et al. 2012). Moreover, IMC demonstrated to be suitable for investigating the ability of different materials to prevent biofilm formation of different bacterial strains (Butini et al. 2018).

In conclusion, IMC is a nondestructive technique that permits the real-time analysis of microbial viability in the presence or absence of compounds with an antimicrobial activity. The susceptibility of planktonic and, more importantly, biofilm cells to antimicrobials can be conveniently assessed without using time-consuming procedures and potentially harmful reagents. Hence, the wide applicability of this ultra-sensitive method provides further advances in the field of clinical microbiology and biomedical sciences, fostering the scientific research toward the development of new drugs and antimicrobial biomaterials, besides supporting physicians in choosing effective antimicrobial therapies in the daily clinical practice.

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Authors' Contribution MEB, MGM, and MDL conceived and designed the work. MEB and MGM performed the experiments. MGM analyzed the data with the contribution of MEB, AT, and MDL. MDL and MEB drafted the manuscript, with the contribution of MGM, MC, AK, and TT. All authors reviewed and revised the first and final drafts of this manuscript.

Declaration of Interest None.

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An Innovative Strategy for the Effective Reduction of MDR Pathogens from the Nosocomial Environment

Elisabetta Caselli, Maria D'Accolti, Irene Soffritti, Luca Lanzoni, Matteo Bisi, Antonella Volta, Filippo Berloco, and Sante Mazzacane

Abstract

Antimicrobial resistance (AMR) is currently one of the main concerns for human health.

Due to its rapid increase and global diffusion, several common microbial infections might become not curable in the future decades, making it impossible to apply other lifesaver therapies, such as transplant or chemotherapy.

AMR is frequently observed in hospital pathogens, due to selective pressure exerted by antibiotic use, and consistently with this, in the recent years, many actions have been proposed to limit AMR spread, including hygiene measures for hospital professionals and a wiser antibiotic usage.

Indeed, the hospital environment itself represents a reservoir of pathogens, whose control was so far addressed by conventional sanitation procedures, which however cannot prevent recontamination and might further favour the selection of resistant strains.

Here we report the results collected by studying an innovative sanitation strategy based on the use of probiotic bacteria, capable of reducing in a stable way the surface load of pathogens and their AMR. Collected data suggest that this system might contribute significantly to AMR control and might be thus considered as one of the tools for AMR and infection prevention and control.

Keywords

Antimicrobial resistance · Healthcare-associated infections · Hospital environment · Probiotics · Sanitation strategy

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1 Introduction

Antimicrobial resistance (AMR) is currently one of the main concerns for human health.

Due to the rapidity of its diffusion and growth in the last decades, our current period has been

named as the "post-antibiotic" era (Bragg et al. 2018), and accordingly with recent observations, it has been hypothesized that AMR might kill more than cancer within 2050 (O'Neill 2014).

Several common infections with microbial aetiology might in fact become totally not curable, leading to the impossibility to apply other lifesaver therapies, including transplants or chemotherapy. AMR is particularly severe in the hospital environment, due to the selective pressure exerted by the wide use of antimicrobials, and in this context is tightly associated to the severity of the so-called healthcare-associated infections (HAIs). HAIs represent a major safety concern, both for patients and for hospital professionals, continuously increasing at an alarming rate, 3–20 times higher in emerging economies compared to western countries, being poverty a crucial yet neglected step towards preventing AMR (Revelas 2012; Alp and Damani 2015; Alividza et al. 2018). HAIs increase morbidity and mortality, as well as costs, and more is needed to fight this problem and augment hospital safety (Sydnor and Perl 2011; Glance et al. 2011).

In Europe, up to four million new patients develop an HAI per year (Cassini et al. 2016; ECDC 2013), highlighting the high burden of HAIs and the need for increased efforts for their prevention and control. About one out of twenty patients acquire an HAI while receiving health care, and this directly leads to at least 37,000 deaths every year (ECDC 2013). Among HAI-associated pathogens, those presenting one or more drug-resistances are more prevalent compared to community-acquired germs, and their AMR further threatens the outcome of infected patients (Ferjani et al. 2015; OECD and Union 2016; Cassini et al. 2016; Suetens et al. 2013).

A few bacterial genera cause up to 90% of HAIs, namely *Staphylococcus* spp. (both *S. aureus* and coagulase-negative staphylococci), *Enterococcus* spp. (including *E. faecalis* and *faecium*), Gram-negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae* and *oxytoca*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Enterobacter* spp., *Proteus* spp.) (Sievert et al. 2013; Hidron et al. 2008; Weiner et al. 2016). All of them have been included in the ESKAPE group, representing the leading cause of nosocomial infections throughout the world (Rice 2008; Bush and Jacoby 2010).

Notably, a high proportion of such microbes show a multidrug-resistant (MDR) phenotype in the hospital environment: methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant Enterococci (VRE), extended-spectrum cephalosporin-resistant, carbapenem-resistant or MDR *Enterobacteriaceae* (*K. pneumonia/oxytoca*, *E. coli*, *Enterobacter spp.*, and *A. baumannii)* (ECDC 2017b). Consequently, WHO included them in the so-called "dirty dozen", a global priority list of antibiotic-resistant bacteria, subdivided in critical, high or medium priority depending to their drug resistance and thus for the need to develop new effective drugs to counteract their infection (WHO 2017). Some Gram-negative strains show a particularly high AMR when isolated from device-associated HAIs (Weiner et al. 2016), and certain strains display an almost total resistance to third-generation cephalosporins, fluoroquinolones, and carbapenems (Weiner et al. 2016; Messina et al. 2014; Parajuli et al. 2017; Banerjee et al. 2018; Cassini et al. 2019). Although reported data show some differences in patterns of resistance in different countries, likely reflecting the existing epidemiology and types of drugs administered, the increasing frequency of MDR phenotypes together with the lack of new antimicrobials increase AMR burden worldwide.

Consistently with this, in the recent years actions have been taken to limit AMR spread and fight the growing diffusion of resistant germs and associated infections (WHO 2015). These include improving awareness of AMR through education and training, strengthening the evidence base by surveillance and research, increasing sanitation and infection prevention measures, optimizing the use of antimicrobials in humans and animals, investing in new medicines, diagnostic tools, prevention by vaccine and other interventions (WHO 2015; ECDC 2017a).

The over-use of antibiotics is, in fact, one the major causes for the observed exponential increase of AMR, as it has been reported that on any given day one out of three patients receives at

least one antimicrobial (ECDC 2013). The consumption of specific antimicrobials for treatment of MDR microorganisms has almost doubled between 2011 and 2014 in Europe (ECDC 2017b), where it is calculated that around 25,000 patients die annually as a result of infections sustained by resistant bacteria. Antibiotic stewardship interventions have been thus included in the global action plans against AMR worldwide (WHO 2015; ECDC 2017a), and defined in international consensus procedures (Monnier et al. 2018; Klepser et al. 2017), with the aim of limiting general antibiotic abuse and to specifically address the use of some antimicrobials.

Similarly, as hands are an effective vehicle for microbes transport and transmission between surfaces and subjects, hand hygiene has been recognized as a key defence in the fight against AMR (ECDC 2017a; Europe 2017). Many studies evidence that hand hygiene is affecting pathogen transmission (Gould et al. 2017) and combination strategies including hand hygiene compliance are therefore indicated as possibly effective preventive measures (Haque et al. 2018).

However, also the environment itself can be a huge reservoir of resistant pathogens, especially in the hospital, and notably this aspect was included as one of the key points in the 2017 EU plan against AMR (ECDC 2017a).

So far, the elimination of pathogens from hospital environment was addressed by conventional sanitation procedures, which however allow recontamination phenomena, that are ultimately the reason for the persistence of microbial contamination (Rutala and Weber 2014; Almatroudi et al. 2016). Furthermore, chemical-based sanitation can favour the selection of resistant strains, a highly undesirable "side effect" associated with this type of cleaning, that might worsen pathogens' AMR. Disinfectant-induced resistance may be directed either against disinfectants themselves (Caini et al. 2013; Cornejo-Juarez et al. 2015) but even against antibiotics, as recently reported for Chlorhexidine induction of resistance against Colistin antibiotic, considered as a last-resort drug for treatment of difficult-to-treat infections sustained by MDR Gram-negative bacteria (Bock et al. 2016; Wand et al. 2017).

In addition, several recent studies have highlighted the role of dry-surface biofilms (DSBs) in the persistence and transfer of contaminant germs on hospital surfaces, as they can protect microbes harboured inside biofilm, rendering sanitation procedures less effective and potentially playing a critical role in HAI transmission (Chowdhury et al. 2018; Almatroudi et al. 2016). Recent reports showed that DSBs are much more widespread in ICU than previously recognized (Costa et al. 2019), representing more than 90% of microbial contamination on ICU surfaces tested after terminal cleaning, with over 50% of them containing MDR bacteria (Hu et al. 2015; Johani et al. 2018). Recent studies performed by scanning electron microscopy and culture-based methods on DSBs from the hospital environment showed a prevalence of Gram-positive bacteria, mainly belonging to *Staphylococcus* genus and including a high proportion of MRSA (Ledwoch et al. 2018). This data indicate that commonly used disinfectants are not effective against bacteria within DSBs and that to achieve the purpose of reducing HAI rates it would be crucial considering also the potential impact of sanitation strategies on DSBs removal.

In the search for effective methods, we studied the application of a probiotic-based sanitation system (Probiotic Cleaning Hygiene System, PCHS), inspired by the studies on the human microbiome, where it is accepted that rather than attempting to eliminate all the pathogens, it is more effective replacing bad microbes with good ones, to restore a positive balance thus preventing the colonization by potential pathogens.

Among the microorganisms potentially useful toward this aim, probiotics appear particularly suitable, being beneficial microbes for our health, capable to 'fill the void', thus disadvantaging the colonization by pathogens (WHO & FDA 2001; Hill et al. 2014). Notably, probiotics have been shown effective in reducing the occurrence of different nosocomial infections, including diarrhoea, necrotizing enterocolitis (Giamarellos-Bourboulis et al. 2009), upper respiratory infections (Banupriya et al. 2015), and infections in surgical patients (Rayes et al. 2002; Sommacal et al. 2015; Rayes et al. 2012).

Based on that, we have been studying an innovative sanitation strategy based on the addition of spores of probiotics belonging to the *Bacillus* genus to eco-sustainable detergents.

Here we report some of the most significant results obtained by the probiotic-based method on the remodulation of the surface microbiome and AMR control in the hospital environment, and discuss the merits of such alternative option.

2 Probiotic-Based Sanitation and its Impact on Surface Pathogen AMR

Bacteria belonging to *Bacillus* genus are gram-positive rods able to form spores and present ubiquitously in soil, water, and human and animal gut. They are considered safe except for two well recognizable species (*Bacillus anthracis* and *Bacillus cereus*) (EFSA 2010).

Bacillus spores have a long history of safe application in humans (Fig. 1), including: food preparation (for example *Bacillus subtilis var. natto* for traditional Japanese soy food), agriculture (*B. subtilis*-based antifungal preparations) (Leyva Salas et al. 2017), aquaculture (Vaseeharan and Ramasamy 2003), veterinary (Cutting 2011; Mingmongkolchai and Panbangred 2018; Xu et al. 2018), human therapy of the gut (Mazza 1994; Lopetuso et al. 2016; Ghelardi et al. 2015) and pharmaceutical industry (Ripert et al. 2016). Some *Bacillus* strains were shown to have specific antimicrobial activity against *Helicobacter pylori* (Pinchuk et al. 2001), *Campylobacter spp.* (Sorokulova et al. 1997), and more recently against *S. aureus*, via signalling interference (Piewngam et al. 2018).

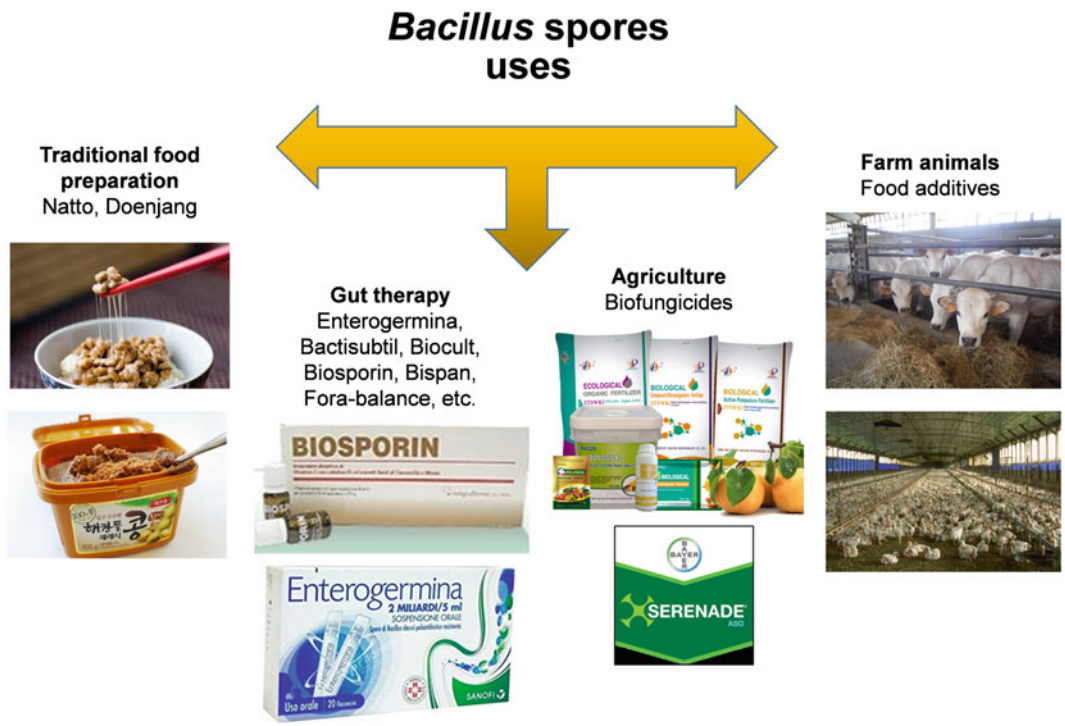
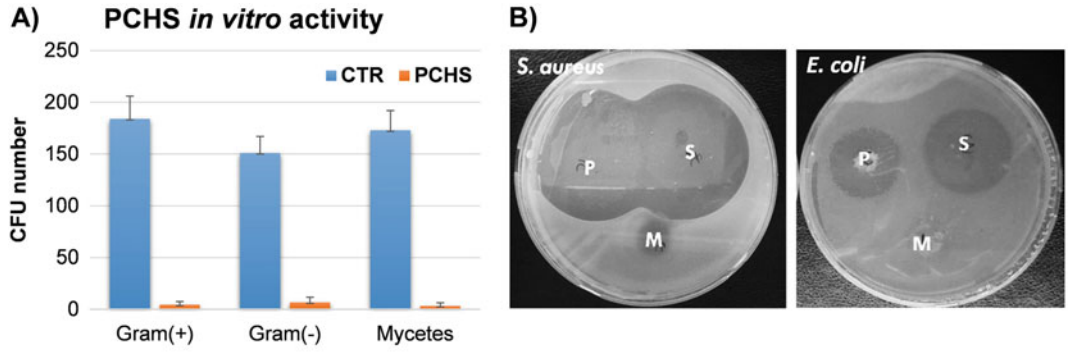


Fig. 1 ***Bacillus* spores uses.** Some of the most frequent uses of *Bacillus* spores are represented, including preparation of traditional oriental food (Japanese Natto, Korean Doenjang, based on soybeans fermented by Bacillus bacteria); prevention and therapy of gut diseases, through replenishment of beneficial gut flora (as an example *Bacillus clausii* of Enterogermina, or *Bacillus subtilis 3* of Biosporin); use as bio fungicides in agriculture; use as food additives in farm animals, to prevent diseases and increase livestock wellness

Indeed, we recently observed that PCHS-derived *Bacillus* species also inhibit the growth of environmental microbes derived from ancient paintings, suggesting a very generalizable use (Caselli et al. 2018c).

Notably, *Bacillus* spores are resistant to many physical-chemical factors, thus rendering them particularly suitable for addition to concentrated detergents. When diluted in water at work concentration and applied on surfaces, spores can germinate originating the vegetative bacteria, which are responsible for cleaning and hygienizing action. Three species are included in PCHS cleanser, namely *B. subtilis, B. pumilus, B, megaterium*, selected for their ability to remove organic dirt by their enzymatic activity and to counteract both bacteria and mycetes growth by competitive exclusion (Caselli et al. 2016b). In particular, unpublished results obtained by us *in vitro*, prior to their use on field, showed that PCHS-*Bacillus* could inhibit almost completely the growth of Gram-positive and Gram-negative bacteria, as well as of mycetes (Fig. 2a), and that their antimicrobial activity was at least in part attributable to the production of antibacterial compounds, as shown by the ability of PCHS *Bacillus*-conditioned medium to inhibit bacterial growth (Fig. 2b, c). Also, the peculiar enzymatic



C)

Group	Species	Percentage of growth inhibition		
		B. subtilis	*B. pumilus*	*B. megaterium*
Gram (+)	*S. aureus*	94±5%	93±6%	29±9%
	S. pyogenes	97±2%	95±3%	35±12%
Gram (-)	*E. coli*	57±6%	53±9%	28±6%
	P. aeruginosa	62±10%	42±11%	19±4%
	K. pneumoniae	52±12%	44±10%	20±6%
	Proteus	55±9%	39±10%	22±5%
	E. cloacae	92±5%	75±9%	31±7%
	S. marcescens	78±15%	49±7%	21±3%

Fig. 2 In vitro activity of PCHS Bacillus. (**a**) Growth inhibition of target Gram-positive or Gram-negative bacteria, and mycetes. Target microorganisms were cultured on Plate Count Agar (PCA) alone (CTR) or together with PCHS derived *Bacilli* (PCHS). Colony forming units (CFUs) of target microbes were counted after 24 h. Results refer to the following target microbes: *S. aureus* (Gram-positive), *E. coli* (Gram-negative) and *C. albicans* (Mycetes). Results represent the mean value ± SD of triplicate samples in over twenty independent experiments. (**b**) Bacterial growth inhibition by antimicrobial compounds produced by PCHS-derived *Bacillus* species in stab overlay assays; *Bacilli* were grown for 24 h on PCA plates (P, *B. pumilus*; S, *B. subtilis*; M, *B. megaterium*), then removed by scraping, and plates used to seed *S. aureus* and *E. coli*: typical inhibition areas were observed after 24 h. (**c**) *Bacilli* were grown in Luria broth for 14 h, then removed by 0.45 μm filtration, and conditioned media were used for the growth of the indicated target bacteria. Bacterial growth was evaluated by spectrophotometric reading at 660 nm and compared to that obtained in unconditioned Luria broth medium. Results represent mean inhibition values ± SD of triplicate samples in three independent experiments

activity of the PCHS-*Bacillus* strains resulted effective in degrading bacterial biofilm *in vitro* (personal unpublished observations), suggesting that PCHS might have anti-biofilm properties on treated surfaces, potentially useful for DSB attack and removal. However, due to the importance of this action and to the difficulty of counteracting DSB persistence, this aspect should deserve future detailed studies on field, in the hospital environment.

The application of the *Bacillus*-based PCHS system on field, performed starting from 2012 in several different public and private healthcare structures, confirmed its antimicrobial activity in all enrolled settings, where PCHS was either directly compared to conventional chlorine-based cleaning in similar wards (Vandini et al. 2014), or analyzed in the same wards in pre-post intervention studies (Caselli et al. 2016b, 2018a).

Compared to conventional sanitation, PCHS was shown not only to decrease pathogen load up to 90% more than chlorine sanitation, but also, more importantly, to maintain the decrease stable during time compared to what obtained with chemicals (Fig. 3a) (Vandini et al. 2014; Caselli et al. 2016b). All samplings were in fact performed after at least 7 h from sanitation, a time sufficient to allow recontamination to occur

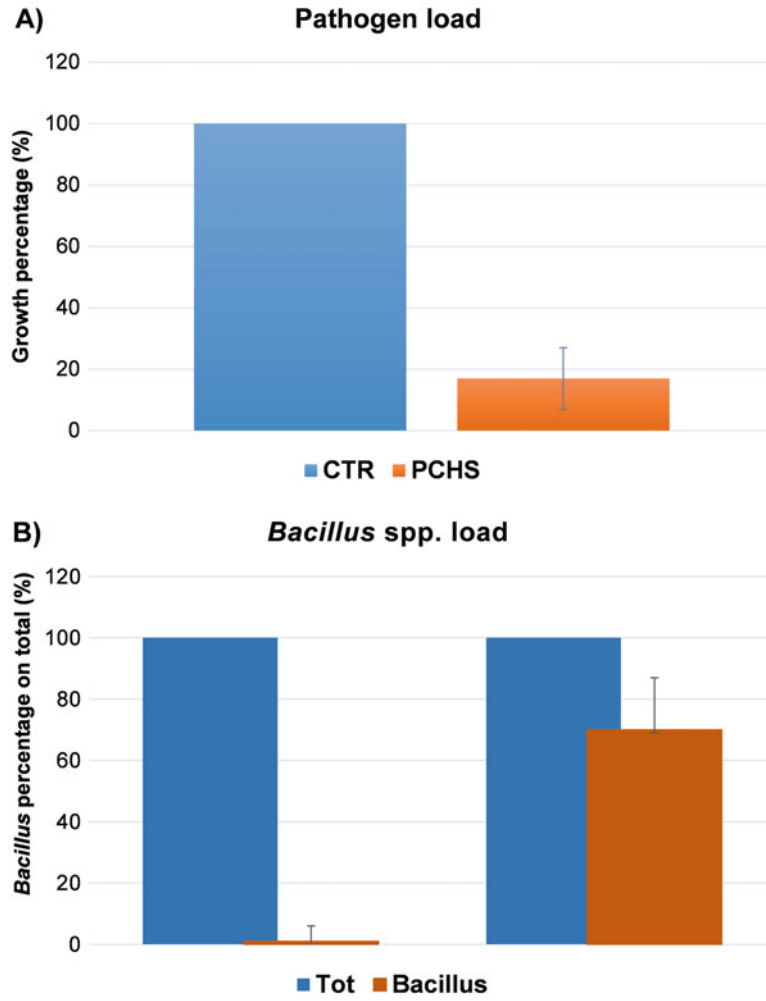


Fig. 3 Remodulation of contaminating surface microbiome by PCHS sanitation. (**a**) Global load of six HAI-associated pathogens, as measured in over 32,000 environmental samples collected in duplicate and analyzed by CFU counts on Rodac plates; results are expressed as mean value ± SD (growth percentage) (Caselli et al. 2018a; Caselli et al. 2016b). (**b**) PCHS-*Bacillus* replacement of pathogens on treated surfaces, as measured in over 32,000 samples collected in duplicate and analyzed by real-time PCR for total bacterial load or specific for *Bacillus* genus (Caselli et al. 2016b, 2018a). Results are expressed as mean value ± SD for total bacterial load, quantified by *panB* qPCR (Tot), and PCHS-*Bacillus* percentage, quantified by spo0A qPCR (Bacillus)

and to evaluate the regrowth of microbes when using conventional disinfectants (Vandini et al. 2014). Notably, all the monitored microbial species resulted significantly decreased following the introduction of PCHS sanitation, including *Staphylococcus* spp., *Enterobacteriaceae* spp., *Acinetobacter* spp., *Pseudomonas* spp., *Candida* and *Aspergillus* spp., and sporogenic *C. difficile* (Vandini et al. 2014; Caselli et al. 2018a; Caselli et al. 2016b), which is instead highly persistent when using conventional disinfectants. In particular, *C. difficile* levels dropped below the detection limit of the used analysis methods in 4 weeks of PCHS application, remaining stably low in the following weeks (Vandini et al. 2014). In parallel, PCHS-*Bacilli* arrived to represent about 70% of the total surface microbiota after 1 month of application, confirming the actual replacement of most of the original surface pathogens (Fig. 3b) (Caselli et al. 2016b).

In parallel, considering the risk of increasing the AMR concern associated with the use of some cleansers in the hospital environment, the AMR characteristics of the contaminating population were assessed continuously before and after the introduction of PCHS system in all the healthcare structures enrolled in the studies. To this purpose, all environmental samples collected since 2014 were regularly analyzed for the presence of resistance (R) genes by a real-time PCR microarray capable of detecting and quantifying simultaneously 84 R genes, thus defining the resistome of the contaminating population (Caselli et al. 2016b, 2018a). In addition, we analysed the presence of the *mcr-1* gene, coding for the transferable resistance against colistin (*mcr-1* plasmid driven colR), as it represents a growing threat for difficult-to-treat infections sustained by Gram-negative carbapenem-resistant bacteria. Analyses were performed both by molecular and biological assays (microdilution). Last, as all settings revealed prominent contamination by *Staphylococcus* spp., in consideration of the importance of *S. aureus* in HAI onset, all bacterial isolates belonging to such bacterial species were also analysed by standard Kirby-Bauer antibiograms, to have a confirmation of the resistance features at the phenotype level.

As expected, such analyses evidenced the presence of several R genes harboured by surface microbiota when using conventional sanitation, interestingly reflecting the specific selective pressure exerted by the use of certain antibiotics in specific settings. Detected resistances included resistance against aminoglycosides, fluoroquinolones, macrolides, methicillin, vancomycin, and class-A, class-C and class-D β-lactamases (Caselli et al. 2016b, 2018a).

Consistently with the high level of Staphylococcal contamination detected in all settings, the most prevalent R gene was *mecA*, coding for methicillin resistance (42.7% of all detected R genes), followed by genes *ermC* (26.9%) and *msrA* (26.6%), coding respectively for resistance against erythromycin and macrolides (Caselli et al. 2016b, 2018a).

Globally, the microarray results, collected in seven hospitals in two independent studies (Caselli et al. 2016b, 2018a), showed that PCHS did not select any resistant strain, rather determining a profound and significant decrease of the population R genes ($P_c < 0.01$). The decrease was observed in all enrolled hospitals, independently R genes prevalence prior to PCHS introduction, and was very evident particularly for the most prevalent R genes, arriving at minus 3 Logs compared to the pre-PCHS amount detected on surfaces (Fig. 4a).

Similarly, the analyses of *S. aureus* isolates by conventional antibiograms showed a global 72.4% decrease of MDR strains *S. aureus* isolates when using PCHS (Caselli et al. 2019), as well as a 71.7% reduction of *mcr-1* positive Gram-negative bacteria with MDR phenotype (Caselli et al. 2019) (Fig. 4b).

The general decrease observed, upon PCHS introduction, in all the resistances originally present in the contaminating population suggests that PCHS sanitation likely acts with a non-specific mechanism. This, consistently with the

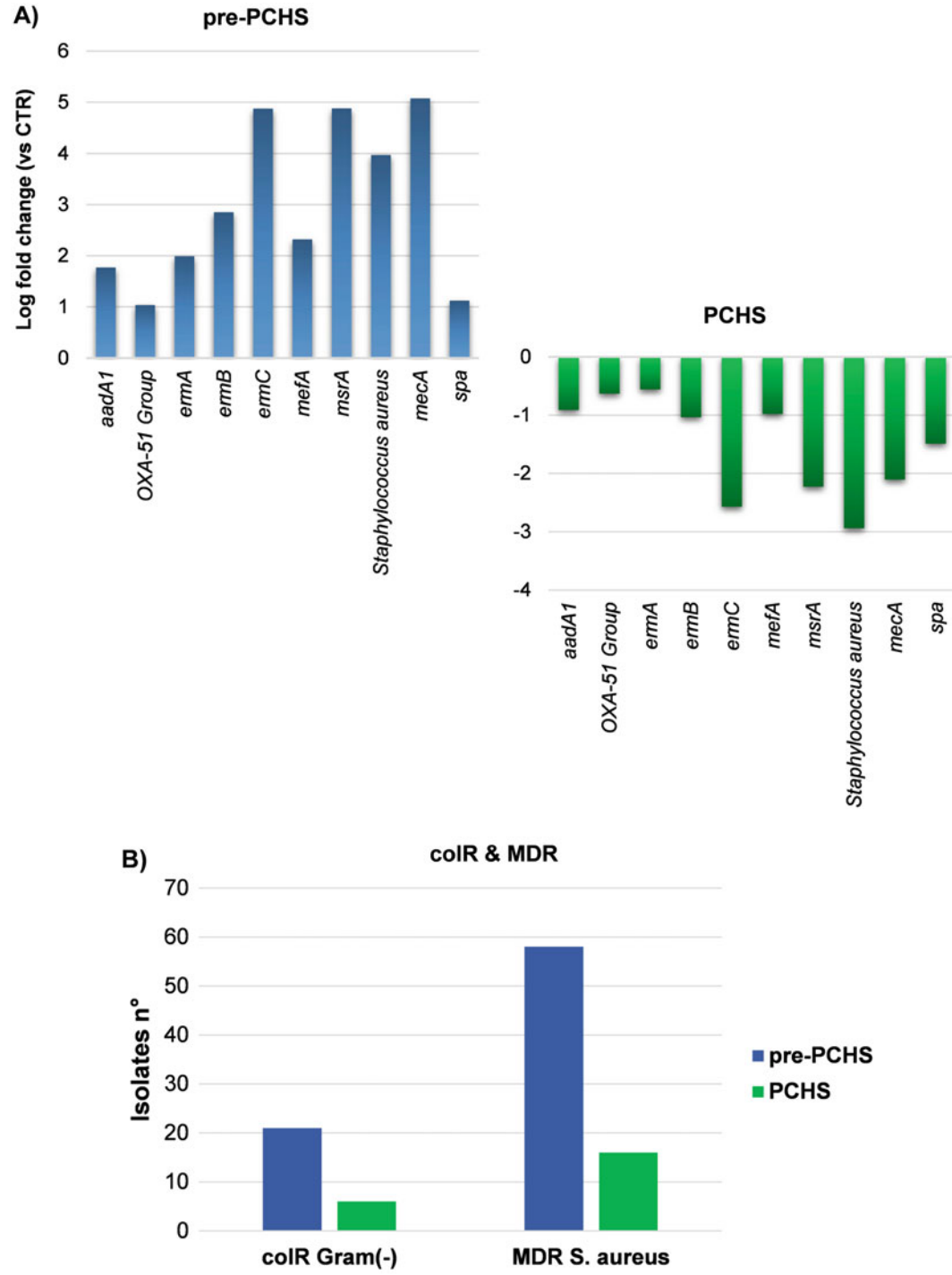


Fig. 4 PCHS impact on antimicrobial resistance of contaminating microbiome of hospital surfaces. (**a**) Global reduction of most prevalent R genes in the resistome of the contaminating microbiome, as measured by real-time PCR microarray in over 1,200 environmental samples (Caselli et al. 2016b, 2018a, b, 2019); pre-PCHS results are expressed as

competitive exclusion mechanism, would modulate the whole microbiome, reducing the number of potential pathogens and consequently the amount of those pathogens that are drug-resistant among them. Considering that resistant pathogens are a fraction of the total ones, a 90% reduction of whole pathogens can lead to a virtual disappearing of resistant ones. However, importantly, there is no certainty of their total eradication, and suspending PCHS sanitation might cause pathogens reappearance (Vandini et al. 2014) as well as AMR re-emergence.

3 Conclusions

Due to the ever-increasing AMR in the last 50 years, in the absence of new antibiotic discovery, the humankind must now face a major health concern, in the form of MDR microorganisms. Urgent actions are needed to avoid the risk of a post-antibiotic era.

Beside those directed toward the responsible use of antibiotics in human and animal healthcare (antibiotic stewardship), it has been recently pointed out the important role of environmental hygiene, as a possible tool for limiting AMR spread (ECDC 2017a, b).

Toward this aim, we studied the potential use of an eco-friendly and sustainable sanitation system, based on the use of mild cleansers containing spores of probiotics belonging to the *Bacillus* genus.

Their use resulted associated with a stable remodulation of hospital microbiome and an even more pronounced modulation of microbiome AMR (Caselli et al. 2016b, 2018a; Caselli 2017), meanwhile resulting safe for use, being genetically stable (Caselli et al. 2016b, 2018a; Vandini et al. 2014) and devoid of infectious risk in hospitalized patients (Caselli et al. 2016a).

Notably, the PCHS-induced decrease of pathogens and of their drug-resistance features impacted importantly on HAI incidence, which was reduced of 52% compared with the use of conventional sanitation (Caselli et al. 2018a), significantly affecting also antimicrobial consumption and related costs (Caselli et al. 2019), and underlying the role of environmental hygienization in preventing pathogens, AMR and HAI spread.

Taken together, these data support the hypothesis that a sanitation based on the principle of competitive antagonism between beneficial and pathogenic microbes can highly affect the healthiness of the hospital environment, disadvantaging the spread of virulent and drug-resistant pathogens. This without additional costs for the healthcare structures.

On the other hand, we are aware that constant monitoring of treated environments is needed, not only when using biological sanitation strategies, as microbiological and molecular analyses can grant the optimization of the procedures and their safety of use, allowing taking immediate countermeasures when needed.

Also, future studies should address the elucidation of PCHS anti-biofilm activity on field, focussing especially on its ability to remove dry-surface biofilms (DSBs), as those are extremely persistent on frequently hand-touched surfaces in healthcare settings, not removed by chemical disinfectants, and potentially impacting on transmission of pathogens such as Staphylococci, that are regularly found inside DSBs.

However, based on the data collected so far, we hope these innovative strategies will open new perspectives in the fight against AMR and infections in hospitals, improving health sustainability.

Fig. 4 (continued) log fold change compared to control values, whereas PCHS results are expressed as fold-changes compared to pre-PCHS data. (**b**) Global reduction of *mcr-1* plasmid-driven colR in Gram-negative bacterial isolates, and of MDR S. aureus isolates. Results refer to environmental samples collected in seven Italian hospitals and are expressed as the total number of isolates during pre-PCHS and PCHS periods of the study (Caselli et al. 2019)

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Novel Therapies for Biofilm-Based *Candida* spp. Infections

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Abstract

The presence of fungal infections continue to grow worldwide, mostly in immunosuppressed patients, and in individuals with continued antimicrobial treatments. *Candida* spp. are the most common yeasts involved in these disorders, being associated with a high rate of antifungal resistance and an increased ability to form biofilms, which make the treatment of these infections difficult. This review aims to present and discuss the main biofilm-related infections cause by several *Candida* spp. and novel therapies that are currently available in the clinical, scientific and academic environment. New drugs with promising antifungal activity, natural approaches (*e.g.* probiotics, essential oils, plant extracts, honey) and a final consideration on alternative methodologies, such as photodynamic therapy are presented and discussed.

Keywords

Biofilms · *Candida* spp. · Drugs · Essential oil · Honey · Infection · Photodynamic therapy · Plant extract · Prebiotic · Probiotic · Resistance

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1 Biofilm-Related Infections

According to the US National Institutes of Health, microbial biofilms account for 65% of nosocomial infections and over 80% of chronic infections (Jamal et al. 2018). Despite the importance, biofilms remain poorly understood and effective strategies to combat biofilm infections remain limited. Biofilms are defined as complex communities of microorganisms that grow in matrix encased microcolonies of extracellular polymeric substances (EPS) which can be surface-associated or populations that self-aggregate (e.g. cystic fibrosis) (Kundukad et al. 2016; Rodrigues et al. 2017; van Wolferen et al. 2018; Wang 2019).

These characteristics help biofilm microorganisms survive and thrive in diverse environments including the human host. Dangerous biofilm infections include those associated with medical devices like catheters (Stickler 1996; Yoshikawa et al. 2015), prosthetic joints (Gristina and Costerton 1985; Tunney et al. 1999), and cardiac devices (Herzberg and Meyer 1998; Otto 2008), as well as lung infections associated with cystic fibrosis (Singh et al. 2000; Sønderholm et al. 2017), periodontal disease (Schaudinn et al. 2009; Rodrigues et al. 2019a, b), and burns or chronic wounds (Thompson et al. 2008; Rybtke et al. 2011).

The biofilm antibiotic tolerance must not be confused with antibiotic resistance. Microorganisms within a biofilm are typically tolerance and or resistant to antibiotics, but generally become more susceptible to the treatment if the biofilm is disrupted (Vincent et al. 2009). Chronic infections associated with biofilm growth have a much slower evolution than acute infections. The symptoms associate to them are frequently unclear and these chronic infections are very challenging, or even impossible to treat with antibiotics. The associated chronic inflammation is usually characterized by an adaptive inflammatory response, controlled by mononuclear leucocytes, IgG antibodies and, frequently, continuous recruitment of polymorphonuclear leucocytes (PMNs). Chronic infections are very frequent in immunosuppressed patients or conditions that cause deficiencies in the primary defensive barriers (innate immunity) (Sardi et al. 2013). The same happens with opportunistic or nosocomial infections, which commonly turn into life threatening, systemic infections. Treating chronic, recurrent infections associated with the biofilm mode of growth is a challenge even for modern antibiotics due to the 10–1000-fold increased tolerance of sessile cells compared to free-swimming cells (Hall-Stoodley et al. 2004; Hall-Stoodley and Stoodley 2005). Chronic infections cycle through extended asymptomatic periods following treatment, and recurrence of acute symptoms. Biofilms are thought to play an important role in this cycle as they regrow from remaining untreated cells. In addition to their implication in chronic infection, biofilms can damage tissue and elicit tissue inflammation and serve as a *nidus* for spread of infection (Hall-Stoodley et al. 2004; Hall-Stoodley and Stoodley 2005).

Biofilm association with chronic infection is due in part to evasion of host immune detection and clearance, evading aspects of both innate and acquired, humoral defence systems. Complement activation has been shown to be lower in biofilms compared to planktonic cells (Jensen et al. 1993; Duggan et al. 2015) and phagocytosis has been impeded by the surrounding EPS matrix (Rodgers et al. 1994; Morán et al. 1998). Furthermore, dormant persister cell populations within biofilms have been shown to evade macrophage clearance at even higher rates (Mina and Marques 2016; Wuyts et al. 2018). As a result of this poor immune clearance, we must rely more on antimicrobials for clearing biofilm infections. However, biofilms are also inherently tolerance to many antimicrobials' drugs and therapies.

Although the specifics of biofilm development differ from species to species, certain general features are accepted to be characteristic to all biofilms, including their innate tolerance to antimicrobials. Challenges in treating biofilms stem from their unique chemical, physical and phenotypic properties (Costerton et al. 1999; Stoodley et al. 2002). Key traits impacting tolerance have been reviewed extensively including their protective extracellular matrix (Stewart 1996; Pierce et al. 2017), slow growth and persister cell formation (Brooun et al. 2000; Lewis

2008; Wuyts et al. 2018), and cell-to-cell communication that facilitates regulation of biofilm-specific phenotypes (O'Toole and Kolter 1998; Sauer et al. 2002; Petrova and Sauer 2009; Polke et al. 2018). Physically, the biofilm matrix aids surface attachment and structural integrity of biofilms and acts to slow penetration of antimicrobials. Biofilm maturation also creates altered microenvironments that increase biofilm heterogeneity and therefore tolerance to drugs that rely on metabolic activities of the sessile cells. Biofilm microorganisms also use efflux pumps and degradative enzymes to pump out or degrade antimicrobials that are capable of penetrating the matrix. The highly regulated "biofilm phenotype" of mature sessile cells is also implicated in affording biofilm-specific protective phenotype (Costerton et al. 1999; Khemiri et al. 2015). Many infections are also polymicrobial, adding to biofilm heterogeneity and cell-to-cell communication which can confound antimicrobial strategies further. Within these microbial communities, increased rates of horizontal gene transfer have been observed and could play a role in the rise and spread of genetic antimicrobial resistance (Li et al. 2002; Wang et al. 2002; Sultan et al. 2018). As such, treatment of biofilm infections represents an important and tough challenge.

The prevalence of (invasive) fungal infections continues to grow due to the constant use of immunosuppressive and broad spectrum antimycotic therapy particularly in severely immunocompromised patients (Pfaller and Diekema 2007; Brown et al. 2012; Rodrigues et al. 2017). *Candida albicans* is, generally, the most frequently isolated species in yeast infections, yet there has also been a rise of non-*Candida albicans Candida* (NCAC) species, such as *Candida glabrata, Candida parapsilosis* and *Candida tropicalis* (Lass-Flörl 2009). *Candida* spp. have several important virulence factors that impact the ability to adhere to medical devices and host cells, which often leads to the formation of biofilms (Brown et al. 2012; Rodrigues and Henriques 2017a, b). Presently, echinocandins are considered the first-line antifungals for treating systemic candidiasis (Pappas et al. 2015; McCarty and Pappas 2016), but both breakthrough infections and acquired resistance mutations in certain *Candida* spp. have been reported, making the management of candidiasis a growing challenge (Sanguinetti et al. 2015; Pfaller et al. 2016).

Attempts have been made in both finding or developing new antimicrobial drugs as well as interventions to exploit weaknesses in biofilm development. High-throughput screening of both synthetic antimicrobial peptides and other natural compounds is being done to identify potential new antimicrobial compounds with better effects on sessile cells. Drugs such as photodynamic antimicrobial dyes are also being developed as novel targeted approaches to recalcitrant biofilms (Wainwright 1998; Bandara et al. 2017). Strategies exploiting aspects of biofilm development include preventing initial surface attachment of cells and biofilm formation, inducing dispersion of mature sessile cells, or use of enzymes and tactics to degrade or weaken the matrix and biofilm architecture. Interventions for preventing biofilm formation are trying to affect surface chemistries to prevent attachment (Li et al. 2018) or blocking communication molecules such as c-di-GMP from regulating attachment and matrix production (Hall and Lee 2018). Late stage interventions have focused on using enzymes to degrade the biofilm matrix (Kerrigan et al. 2008), destabilizing the biofilm architecture using treatments such as DNAses (Whitchurch et al. 2002; Tetz et al. 2009; Gunn et al. 2016), or using environmental cues and cell-to-cell signals to disperse and disaggregate mature biofilms (Kaplan 2010; Marques et al. 2015; Petrova and Sauer 2016; Goodwine et al. 2019). Some of these strategies make use of what we know about biofilm development, using biofilm cell-to-cell signals or regulatory mechanisms to intervene on the unique biofilm specific phenotype that imparts antimicrobial and immune tolerance (Kerrigan et al. 2008; Davies and Marques 2009; Morales et al. 2013; Pierce et al. 2015; Goodwine et al. 2019).

Although much of our early biofilm understanding has been built on bacterial biofilms, this review focuses on fungal biofilms, specifically those associated with *Candida* spp. Fungal biofilm infections, especially those of the *Candida* genus, are emerging as important

nosocomial infectious agents, especially in immunosuppressed populations (Ramage and Williams 2013; Bongomin et al. 2017; Rodrigues et al. 2017). Clinically relevant *Candida* biofilm infections include those associated with cardiac devices, prostheses, catheters and mucosal or endothelial tissues (Elving et al. 2002; Kojic and Darouiche 2004; Nett 2016). Here we review these common *Candida* biofilm infections and novel therapeutic compounds and strategies that promise to address the challenges of biofilm tolerance to antifungals.

2 *Candida* spp. Biofilms

Typically, in the biofilms, the EPS matrix accounts for almost 90% of the biofilm dry mass while the remaining volume is constituted by the microorganisms (ca. 10%) (Garrett et al. 2008; Kundukad et al. 2016). Its composition and structure define the physicochemical properties of biofilms as well as contribute to the key properties such as antibiotic resistance and processes including detachment (Kundukad et al. 2016). Extracellular polysaccharides are the major component of the biofilm matrix, which crosslink with eDNA to stabilize the framework of biofilms (Li et al. 2018).

Biofilm formation can be recognized as the following stages: initial attachment of the microorganism to a surface; propagation and development of the characteristic biofilm structure; and finally, detachment (Joo and Otto 2012; Kumar et al. 2017). During propagation the production of EPSs is increased, providing a way to allow the movement of nutrients and the elimination of waste by-products into and from the biofilm (Kumar et al. 2017; van Wolferen et al. 2018). În detachment single cells or cell aggregates are disseminated (Kumar et al. 2017). Biofilms provide protection from drugs (e.g. antibiotics), homecare products (e.g. disinfectants), and resistance to extreme environmental conditions (e.g. nutrient deficiency) (Garrett et al. 2008; Kundukad et al. 2016; Zhang et al. 2019), therefore detachment provides a mechanism for generating susceptible cells.

Candidiasis are among the most predominant opportunistic fungal infections. In fact, *Candida* biofilm growth is considered to be the predominant reason *Candida* cells display antifungal resistance (Bruder-Nascimento et al. 2014; Silva et al. 2017; Rodrigues et al. 2019a, b). The genus *Candida* is currently comprised of 150 species, although only a few of them, such as *C. albicans*, *C. tropicalis, C. glabrata, C. dubliniensis*, *C. krusei* and *C. parapsilosis* are human pathogens (Pappas 2010). *Candida* spp. typically colonize humans as biofilms. Moreover, biofilm formation is an important virulence attribute of *Candida* spp., as the biofilm cells show a greater resistance to antifungals and host defenses (Alcazar-Fuoli and Mellado 2014; Rodrigues and Henriques 2018).

2.1 Mucosal and Systemic *Candida* spp. Biofilm-Related Infections

Many studies associate *C. albicans* biofilms with the occurrence of several dental disease like denture stomatitis (Darwazeh et al. 2010; Lazarin et al. 2014; Cavalcanti et al. 2015) and oral candidiasis (Park et al. 2014; Schlecht et al. 2015; Matsubara et al. 2016a, b). Since the 1980s the studies of oral infections by *C. albicans* have increased due to the escalation in HIV infection and the AIDS epidemic (Williams and Lewis 2011). Todate, pseudomembranous candidiasis is the most common oral opportunistic infection associated with HIV+ and other immunocompromised individuals such as cancer patients and patients with Sjogren's syndrome (Tsui et al. 2016). Pseudomembranous candidiasis, also known as thrush, is characterized by creamy white lesions on the palate, buccal mucosa and tongue and may extend into the pharynx (oropharyngeal candidiasis) (Fidel 2011).

In oral health, commensal biofilm bacteria and fungi modulate the host immune systems using their polymeric and hydrated matrix as the first-line defense against pathogens (Kavanaugh et al. 2014). Any breakdown in this biofilm defense favors local infections (oral candidiasis, gingivitis and periodontitis, dental caries and endodontic

infections, mucositis, and periimplantitis) (Krom et al. 2014; Ortega et al. 2015; Scannapieco and Cantos 2016; Chevalier et al. 2018). On host mucosal tissue, *Candida* biofilms can be formed as part of the commensal microflora (Williams et al. 2013). The combination of *C. albicans* and *Streptococcus* spp. increases the virulence in invasive candidiasis, periimplantitis or early childhood caries (Diaz et al. 2012; Falsetta et al. 2014). On the other hand, oral *Actinomyces* (Guo et al. 2015) and *Lactobacillus* (Vilela et al. 2015; Ribeiro et al. 2017) species can inhibit *C. albicans* proliferation, virulence and biofilm formation.

The ability of *C. albicans* to adhere to host surfaces is a prerequisite for successful colonization and persistant infection. Adhesins such as agglutinin-like sequences (ALS) and hyphal wall proteins (Hwp1) aid the attachment of *C. albicans* to receptors on host tissues and are regulated in biofilm cells (Sundstrom et al. 2002; Williams and Lewis 2011). In addition, *C. albicans* extracellularly releases lipases, estereases, proteinases, phospholipases and hemolysins that are involved in host tissue invasion and nutrient acquisition facilitating *Candida* pathogenesis (Tsui et al. 2016; Rodrigues et al. 2019a, b).

Similar to the oral cavity, *C. albicans* is a resident in the vaginal microbiome and can cause vaginitis during dysbiosis (Tsui et al. 2016; Rodrigues et al. 2019a, b). At least once in their lifetime, approximately 75% of all women of childbearing age are afflicted by vulvovaginal candidiasis and 5–8% of these women suffer from recurring episodes (Harriott et al. 2010; Peters et al. 2014). Harriott et al. (2010) were the first report an *in vivo C. albicans* biofilm in an immunocompetent animal model of vaginitis. Particularly, *C. albicans* strains defective in hyphae formation display significantly reduced vaginitis symptomatology indicating a requirement for hyphae in the pathogenesis of this disease (Harriott et al. 2010). Other properties such as estrogen production and microbiota disruption have been proposed to play major roles inrecurrent vulvovaginal candidiasis (Tsui et al. 2016). *Candida* biofilm architecture observed using *in vivo* and *ex vivo* vaginal mucosal mouse models present clear yeast, hyphae, and extracellular matrix (Harriott et al. 2010).

Gastrointestinal and urogenital tracts are also common sites where *Candida* species colonize and cause opportunistic infections (Falagas et al. 2006). Symptomatic candiduria is seen in patients with cystitis, epididymorchitis, prostatis, pyelonephritis and renal candidiasis (Behzadi et al. 2010). Biofilms need to grow at an interface, which can either be cell culture (biotic), air-liquid interface (abiotic), or solid material (Chevalier et al. 2018). Urinary catheters are on such surface that is susceptible to *C. albicans* biofilms (Seddiki et al. 2013). Urinary tract infections in catheterized patients, candidal biofilms on hemodialysis and peritoneal dialysis catheters are associated with a *Candida* infection rate of up to 20% (Raaijmakers et al. 2007; Mathé and Van Dijck 2013). *Candida* biofilms obtained from patients with infected intravascular catheters also confirm the presence of yeast, hyphae and extracellular matrix (Ramage et al. 2005). In a rat urinary catheter model, *C. albicans* biofilm formation occurred over 24–48 h, followed by pyuria, acute cystitis, and bladder tissue invasion with hyphae (Nett et al. 2014). Moreover, in this same model a *C. albicans* mutant lacking adhesins displayed compromised biofilm formation and consequently virulence (Nett et al. 2014).

Cells released from *Candida* biofilms can migrate into the bloodstream and cause systemic infections which are associated with high mortality, especially inimmunocompromised patients (Finkel and Mitchell 2011; Mathé and Van Dijck 2013; Rodrigues et al. 2019a, b). Phospholipases secreted by *C. albicans* that disrupt host cell membranes are implicated in systemic *Candida* infection. Consequently, the deletion of class B phospholipases have been implicated in attenuation of systemic infection (Leidich et al. 1998; Theiss et al. 2006; Rodrigues et al. 2019a, b). Once a *Candida* biofilm is formed on an implanted medical device, it has the potential to seed disseminated bloodstream infections and lead to invasive systemic infections of tissues and organs (Nobile and Johnson 2015). In fact, candidemia is the fourth most common bloodstream infection,

annually affecting more than 250,000 individuals worldwide and causing more than 50,000 deaths (McCarty and Pappas 2016).

As a member of the healthy microbiota, *Candida* spp. asymptomatically colonizes the gastrointestinal tract, reproductive tract, oral cavity, and skin of most humans. Superficial *Candida* spp. fungal infections can affect the skin, hair and nails (Otašević et al. 2019). Once *C. albicans* overgrow they can cause superficial mucosal and dermal infections, such as thrush, vaginal yeast infections, and diaper rash, as well as more severe hematogenously disseminated infections (Nobile and Johnson 2015). *C. albicans* is the most common fungal species associates with biofilm infections of medical devices, like urinary and central venous catheters, pacemakers, mechanical heart valves, joint prostheses. Increased use of these devices has led to an increased incidence of *Candida* spp. infections (Tsui et al. 2016).

Candida auris is an emerging multidrug-resistant fungal pathogen causing nosocomial and invasive infections. This yeast was firstly isolated in Japan in 2009 from ear discharge. It is associated with high mortality rates and is frequently misidentified by commercially available phenotypic identification platforms (Kordalewska et al. 2017). Studies have shown that chlorhexidine washing and appropriate use of disinfectants can possibly eradicate *C. auris* from patients and hospital environment (Biswal et al. 2017). Conversely, another report showed biofilm grown cells with reduced susceptibility to clinically relevant concentrations of chlorhexidine and hydrogen peroxide, with complete eradication achieved only using povidone iodine. Authors concluded that *C. auris* appears to show more biofilm-specific resistance than other *Candida* spp. (Kean et al. 2018). More studies are still needed in order to clarify *C. auris* susceptibilities. Novel approaches might be particularly interesting to apply in this MDR species.

Finally, *Candida* spp. endocarditis is uncommon but often fatal especially in the damaged vascular endothelium of native heart valves in patients with pre-existing cardiac disease (Baddley et al. 2008). The primary lesion or thrombus develops on the surface of the heart valve and then become colonized with *Candida* spp. cells leading to embolization (Donlan and Costerton 2002).

3 Innovative Treatments

Due to the rising number of *Candida* spp. strains with elevated drug resistance leads there is an increased need for alternative therapies to current antifungal agents. Many avenues are presently being investigated, including the development of novel antifungal compounds, the use of probiotics, prebiotics, or symbiotics, the exploitation of the antifungal properties of plant derivatives and honey, and the use of photodynamic therapy. An outline of the latest advances in these approaches is provided in the next sections.

3.1 New Compounds with Promising Antifungal Activity

The rise of *Candida* spp. resistance, highlights the need for new molecules, with new targets and new sources for the treatment of candidiasis (Rodrigues et al. 2018). Several new drugs, drug associations or new drug activities are presented in Table 1.

Nanotechnology is an alternative approach focused on designing drugs with extended persistence and controlled release. Perera et al. (Perera et al. 2015) evaluated the encapsulation of citric acid into a Mg-Al-layered double hydroxide. The authors reported an improved topical antifungal formulation against *C. albicans,* and *C. glabrata*, but not *C. tropicalis*. Additionally, silver nanoparticles loaded with amphotericin B showed promise forimproving antifungal efficiency of amphotericin B against *Candida* spp. (Leonhard et al. 2017).

In a very different approach, an exploration of the defence mutualisms between social insects and microorganisms uncovered that the symbiotic nature of endophytic microorganisms leads to metabolic interactions that promote production of bioactive compounds (Casella et al. 2013). Authors have indicated the symbiosis of *Pseudallescheria*

Table 1 New drugs, drug association and new activities reported in the last years, related to *Candida* spp. biofilms

New drug, drug association, new drug activity	Effect	References
Aldehydes, hydrazones and hydrazines	Good results on against *Candida* spp. membrane	Casanova et al. (2012)
N-acyldiamines	Antifungal activity against *C. albicans*, *C. parapsilosis, C. tropicalis*, and *C. glabrata*	Ferreira et al. (2014)
1,3-thiazolidin-4-one nucleus and their N-benzylated derivatives	Comparable/higher biological activity of fluconazole, amphotericin B, clotrimazole, ketoconazole, miconazole, tioconazole;	Carradori et al. (2017)
	Low cytotoxic effects	
Mannich base-type eugenol derivatives	Effective against *C. albicans*, *C. glabrata*, and *C. krusei;*	Abrão et al. (2015)
	IC_{50} values below those of fluconazole	
Isolation of a new polyene macrolide antibiotic (from the fermentation broth of *Streptomyces* species)	Activity against *C. albicans*, *C. krusei* and *C. glabrata* (and other fungi and bacteria)	Vartak et al. (2014)
cis-2-decenoic acid (from *Pseudomonas aeruginosa*)	Induces the dispersion or even inhibiting *C.albicans* biofilm development	Davies and Marques (2009)
(1-(4-ethoxyphenyl)-4-(1-biphenylol-2-hydroxypropyl)-piperazine)	Significantly decreases *C. albicans* virulence by interfering with morphological transition;	Zhao et al. (2018)
	No cytotoxicity against human cells at a micromolar level	
Chemical modifications in glucosides compounds	Fungistatic activity, three-fold higher than fluconazole; promising fungicidal activity against *C. glabrata*	de Souza et al. (2015)
β-peptide structural mimetics of natural antimicrobial α-peptides	Prevention of the formation of *C. albicans*, *C. glabrata*, *C. parapsilosis* and *C. tropicalis* biofilms	Raman et al. (2015)
Concomitant use of epigallocatechin gallate (EGCG) and miconazole, fluconazole or amphotericin B	Can low the dosages of antifungal drugs needed to treat infections (preventing possible adverse effects and the emergence of resistant strains)	Ning et al. 2015)
Chloramphenicol	Antifungal activity, comparable to caspofungin, ketoconazole, and metronidazole	Joseph et al. (2015)
	Almost no activity against all *C. albicans* tested, *C. famata*, *C. glabrata*, and *C. haemolonei*	
Thioether, sulfone, triazole, amide	The triazole linker seems to generally provide optimal activity against *Candida* spp.	Fosso et al. (2018)
Cerium (lanthanide)	Induces severe cellular metabolic activity impairment and membrane damage in planktonic and sessile *Candida* spp. cells	Silva-Dias et al. (2014)
	Efficiently prevent biofilm formation both *in vitro* and *in vivo*, and to almost eradicate fixed biofilms when applied at high concentrations	
	Possible application as an antifungal agent to prevent the formation of biofilm-associated infections in clinical settings, for example, by catheter coating	
N,N′-Diaryl-bishydrazones (fluconazole derivatives)	Very good antifungal activity against a broad spectrum of filamentous and non-filamentous fungi	Thamban Chandrika et al. (2018)
	The leading candidate, 4,4′-bis((E)-1-(2-(4-fluorophenyl)hydrazono)ethyl)-1,1′-biphenyl, shows less hemolysis at concentrations at or below that of voriconazole, and it is fungistatic, with no cytotoxicity	

(continued)

Table 1 (continued)

New drug, drug association, new drug activity	Effect	References
27 new fluconazole derivatives	Broad-spectrum antifungal activity. All compounds display an MIC value of <0.03 μg/mL against at least one of the fungal strains tested, low hemolytic and cytotoxicity than approved for amphotericin B	Shrestha et al. (2017)
	The best derivative shows to inhibit of the sterol 14α-demethylase enzyme involved in ergosterol biosynthesis, as fluconazole	

boydii and *Nasutitermes sp* led to the production of tyroscherin and N-methyltyroscherin, which have antifungal activity against *C. albicans* and *C. parapsilosis* (Nirma et al. 2013). The same authors discovered the new compounds ilicicolinic acids A, C, and D and ilicicolinal isolated from *Neonectria discophora* SNB-CN63 isolated from a termite nest, with *in vitro* antifungal activity against *C. albicans* and *C. parapsilosis* (Nirma et al. 2015). Casella and colleagues have described three active fungal extracts, which resulted in the isolation of eight compounds with antifungal and cytotoxic potential against *C. albicans* ATCC10213 (Casella et al. 2013). It is important to note that these studies are still preliminary and need to be further explored, but they might be a good source of new antifungal drugs. Other components have also been analysed with good results (Ramage et al. 2014; Borghi et al. 2015). Acetylcholine was important in the pathogenesis of fungal infections in *Galleria mellonella,* by inhibiting *C. albicans* yeast–to-hyphae transition and biofilm formation; promoting cellular immune response; and regulating the antifungal defences to limit sepsis induced damage of host tissues (Rajendran et al. 2015). Additionally, carbohydrate derived fulvic acid proved to be fungicidal against *C. albicans* planktonic and sessile cells at similar concentrations, with results similar to those found with caspofungin (Morrell et al. 2005; Sherry et al. 2012). Furthermore, myriocin displays direct antifungal activity against *C. albicans* biofilms with progressive reduction in biofilm biomass and metabolic activity. Additionally, lipid raft formation and filamentation were strongly reduced (Martin and Konopka 2004; Lattif et al. 2011; de Melo et al. 2013; Sharma et al. 2014).

3.2 Natural Approaches

3.2.1 Prebiotics, Probiotics and Symbiotics

Prebiotics are "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health" (Gibson and Roberfroid 1995; Davani-Davari et al. 2019). Sugars and dietary fiber are examples of prebiotics, which is in contrast to probiotics such as lactic acid bactera (Gibson and Roberfroid 1995; Davani-Davari et al. 2019). However, because sugars increase the risk of oral pathologies (e.g. dental caries), this alternative is not without its own side effects (Ng et al. 2016). A significant rise in the *Candida* spp. colony forming unit (CFU) count was recently demonstrated when adding only 2% of glucose in the media (Rodrigues and Henriques 2017a, b). In the Turku sugar studies (a series of clinical trials) Larmas et al. (1974, 1976) reported that xylitol significantly reduced the colony counts of oral *Candida* spp.. Likewise, others (Pizzo et al. 2000; Abu-Elteen 2005) have later confirmed that the presence of xylitol inhibits the adhesion of *Candida* spp. to the mucosal surfaces. Xylitol, a sugar with an alcohol group, is normally not assimilated by lactobacilli. Yet, authors have indicated that 36% of human Lactobacilli strains were able to metabolize xylitol (Almståhl et al. 2013). Kojima et al. (2016) also indicated a reduction in the growth of *C. albicans* ATCC18804 in the presence of xylitol, xylose, and arabinose, when compared with glucose.

Probiotics are living microorganisms that offer health benefits to their hosts, when administered/consumed in the adequate amounts (FAO/WHO

2001; Gourbeyre et al. 2011). *Lactobacillus* and *Bifidobacterium* are the most commonly used probiotic organisms (Sanders 2008; Gourbeyre et al. 2011). These microorganisms were initially thought to be exclusively used for the management of the intestinal diseases (e.g. diarrhoea, inflammatory bowel disease and lactose intolerance complications), nonetheless further studies suggested broader health benefits. Among them, the most important are general immunological stimulation, acid and bile tolerances or antagonistic activity against pathogens in several tracts (Fuller 1989; Sanders 2008; Gourbeyre et al. 2011). For safety reasons, probiotics need to be of human origin, and cannot transmit any antibiotic resistance genes (Sanders 2008). Probiotics have been reported as a good prophylactic and therapeutic alternative agent for the management of candidiasis (Krasse et al. 2006; Hatakka et al. 2007; Haukioja 2010; Vivekananda et al. 2010; Jørgensen et al. 2012; Mendonça et al. 2012; Cagetti et al. 2013; Demirel et al. 2013; Kumar et al. 2013; Li et al. 2014; Ishikawa et al. 2015; Matsubara et al. 2016a, b; Ng et al. 2016; Bandara et al. 2017). Naturally, most of the studies are related to oral and gastrointestinal candidiasis. The exact mechanisms of action for probiotics against *Candida* spp. are still uncertain. It is believed that if facilitates restoration of a natural healthy microbiome, responsible for anti-*Candida* activity (Sanders 2008). Restoration starts with a co-aggregation of probiotic and fungal cells to inhibit fungal colonization, followed by the assembly of antimicrobial and anti-biofilm compounds; next, competition for available nutrients and adhesion sites, *quorum sensing* chemicals' production that lead to down regulation of toxin production by the fungi and, finally, modulation of the humoral and cellular immune system of the host (Reid et al. 2011; Bandara et al. 2017). At large, reports support that administration of single or multiple strains of probiotic bacteria, alone or in combination with antifungals drugs can diminish the *Candida* spp. colonization, increase antifungal activity and alleviate signs and symptoms of candidiasis (Reid et al. 2011).

Authors (Maekawa et al. 2016) showed that *Lactobacillus pentosus* S-PT84 obstructed *in vitro* mycelial growth of *C. albicans* TIMM1768, demonstrating an *in vivo* prophylactic effect against oral candidiasis in mice. In general, regarding the *in vivo* animal studies, the results remain debatable. Some reports propose a local and systemic beneficial effects of probiotics (Wagner et al. 1997; Elahi et al. 2005; Matsubara et al. 2012), while others signpost inconclusive effects (Zavisic et al. 2012). These differences in the results may be from discrepancies in the administration technique employed. Matsubara et al. (Matsubara et al. 2012) suggested that the effects of probiotics may be better than the antifungal drugs in the reduction of oral candidiasis. The capacity of probiotics in combating *Candida* spp. biofilms was found to be both species and strain-specific (Kojima et al. 2016; Matsubara et al. 2016a, b). For therapeutic purposes, the key factor for the effectiveness of probiotics against *Candida* spp. is the selection of the appropriate strain(s) of probiotic(s) (Kojima et al. 2016; Matsubara et al. 2016a, b). A high variation of the genome size (1.23–4.91 Mb) and GC content (Caufield et al. 2015) confers diverse properties to *Lactobacillus* (Kõll et al. 2008; Tiihonen et al. 2010). Specific strains have been linked to positive effects on *Candida* spp. infections (Ng et al. 2016). James and colleagues (James et al. 2016) have evaluated combinations of *Lactobacillus plantarum* SD5870, *Lactobacillus helveticus* CBS N116411 and *Streptococcus salivarius* DSM 14685, reporting that co-incubation with probiotic supernatants or live probiotics reduced *C. albicans* biofilm formation and size. Several *C. albicans* genes involved in the yeast–hyphae transition, biofilm formation, tissue invasion and cellular damage, were also reduced. Song et al. (Song and Lee 2017) described the antifungal biofilm activity against blastoconidia and *Candida* spp. when using probiotics *Lactobacillus rhamnosus* and *Lactobacillus casei*. The results demonstrated that these probiotics were ideal for the prevention and treatment of denture-related stomatitis. Zhao et al. studied different probiotics (Zhao et al. 2016). In their report, *Bacillus subtilis* R0179 was found to have a significant antifungal

effect against *C. albicans* and *C. parapsilosis* but not for *C. krusei*. The authors also acknowledged a preliminary antifungal mechanism of *B. subtilis* R0179 and identified iturin A, an antifungal agent.

Additionally, there are probiotic antimicrobial molecules that have a direct growth in inhibitory effect on *Candida* spp.. Organic acids (*e.g*. lactic acid, acetic acid), bacteriocins (*e.g*. bacteriocin L23 (Pascual et al. 2008), plantaricin (Sharma and Srivastava 2014) pentocin TV35b (Okkers et al. 1999), hydrogen peroxide, and uncharacterized low molecular weidgh compounds appear to be effective against the yeast form of *Candida* spp. (Chen and Hoover 2003). Lactobacilli can produce lactic acid that can reduce the metabolic activity of *Candida* spp. (Zalán et al. 2010; Köhler et al. 2012). Lactobacilli can also produce low molecular weight substances (*e.g*. reuterin (Talarico et al. 1988) reutericyclin (Nzle et al. 2000) and dyacetyl (Jay 1982)), which have an anti-*Candida* spp. effect in the yeast forms (Chung et al. 1989). Nonetheless, *Lactobacillus* do not produce efficient concentrations of hydrogen peroxide against *Candida* spp. (Shokryazdan et al. 2014). Also, anti-*Candida* spp. bacteriocins with activity in hyphal forms have not yet been identified (Calderone and Fonzi 2001; Douglas 2003).

The dietary intake of probiotics has also been related to a decline in the incidence of candidiasis. It has been reported by Miyazima and others (Miyazima et al. 2017; Hatakka et al. 2007; Jørgensen et al. 2012) that a daily consumption of cheese supplemented with *L. acidophilus* NCFM or *L. rhamnosus* Lr-32, reduced the colonization of oral *Candida* spp. in denture wearers, indicating a potential in reducing the risk of oral candidiasis. Remarkably, Oliveira et al. (Oliveira et al. 2016) showed that the when *Candida* spp. interacted with *L. rhamnosus,* proteinase and hemolysin expression was reduced. Additionally, the germ tube formation and biofilm formation capacity also declined due to alterations in susceptibility to antifungal drugs. Mendonça and collegues (Mendonça et al. 2012) demonstrated that *L. casei* and *Bifidobacterium breve* severely reduced *Candida* spp. colonization and increased a specific secretory immune response against these yeasts (increase of the anti-*Candida* IgA levels). Other authors have stated analogous results in IgA secretory responses (Van Houte et al. 1972; Wagner et al. 1997).

The use of probiotics in toothpastes have also been attempted. Authors (Mishra et al. 2016) have showed that probiotics are equally effective as 0.2% chlorhexidine digluconate rinse in decreasing *C. albicans*. Similarly, the use of a rinse with antifungals in comparison to antifungals with *Bifidobacterium longum*, *Lactobacillus bulgaricus* and *Streptococcus* thermophiles showed more reduced *Candida* spp. growth in the probiotic group compared to the control (Li et al. 2014). Amižić et al. (2017) assessed the antimicrobial activity of two probiotic toothpastes, one with *Lactobacillus paracasei* and the other with *Lactobacillus acidophilus*. The results revealed that the probiotic toothpastes had a higher inhibition effect on *C. albicans* and *S. salivarius* than toothpaste without probiotic. In these two cases and for the rest of the bacteria evaluated, toothpastes had stronger inhibition capacity than mouthrinses ($p \leq 0.05$). In another study, Bohora and Kokate (2017) revealed that *Lactobacillus* and *Bifidobacterium* can prevent the *E. faecalis* growth, but no effect was detected on *C. albicans*. Curiously, in the biofilm form, both had an antibacterial effect on pathogenic organisms.

A symbiotic is the combined use of prebiotics and probiotics. Symbiotics were first recommended for therapeutic applications for the intestinal tract (Gibson and Roberfroid 1995; Olveira and González-Molero 2016). Probiotics are challenge colonizers in adult oral cavities (Lazarevic et al. 2010; Tiihonen et al. 2010). However, symbiotics are considered to be more effective for oral applications than probiotics alone. Conversely, it is acknowledged that suitable symbiotic therapy may suppress the development of candidiasis, through activation of important host immune responses (Ng et al. 2016). Caufield et al. (2015) showed that children who were oral Lactobacilli carriers had similar Lactobacilli in their feces. Other clinical and

in vivo studies also describe a transition from symbiotic activity in the oral cavity to the intestine (Van Houte et al. 1972; Wagner et al. 1997; Mendonça et al. 2012). This transition could be responsible for the observed benefits of prebiotics and probiotics (e.g. immune stimulation and protection against infections).

3.2.2 Plant Extracts and Essential Oils

Despite efforts to discover and/or synthetize new chemical molecules, more efficient and effective than the existent ones, it is necessary to consider that these compounds are commonly associated to a wide variety of side effects. In fact, with the increasing use of synthetic molecules, natural therapies have become secondary. However, more recently natural matrices (namely, plant extracts rich in phenolic compounds) and correspondent biological properties have gained a special attention. Prior to the use of synthetic drugs, botanical formulations were used for several health conditions. Medicinal plants have been used since pre-historic times for therapeutic purposes (Silva and Fernandes Júnior 2010; Junio et al. 2011; Martins et al. 2015a).

Several studies have investigated the antifungal properties of phenolic enriched plant extracts, against *Candida* spp.. Due to the antifungal potential evidenced by some plant extracts, an increasing number of studies have evaluated the antifungal potential of phytochemicals, namely phenolic compounds, isolated from those matrices. Despite the existence of a tenuous variation on the antifungal activity against *Candida* spp., in general, plant extracts obtained using methanol: water extracts seem to be more effective against *Candida* spp. than the extracts prepared using a unique solvent. This antifungal activity may be directly correlated with the solubility of the active principles in the solvents used (Martins et al. 2015b). Furthermore, phenolics compounds are one of the most abundant bioactive molecules present in polar extracts, and there is evidence that these extracts and compounds affect fungal cells through interaction with the lipid bilayer of the cell membrane. They could act by disrupting the membrane integrity or by intercalation into cell wall and/or DNA. Despite those advances, for most plant species no extensive knowledge is available about the responsible phytochemicals for the observed biological effects, their mechanisms of action, therapeutic and prophylactic doses, synergism, antagonisms and other interrelations between them.

A number of secondary metabolites such as flavonoids, glycosides, terpenoids, tannins and alkaloids have been implicated in these antifungal effects (Cowan 1999; Dahanukar et al. 2000). Essential oils are particularly odorous volatiles (Gupta et al. 2010; Martín et al. 2010) and composed of an extraordinary mixture of compounds (Dorman and Deans 2000; Aleksic and Knezevic 2014). Their hydrophobicity is intimately related to fungicidal activity, as it is expected to promote interaction with the lipid bilayer of fungal cell membrane, amplifying permeability, which promotes release of cellular contents and therefore cell death (Prabuseenivasan et al. 2006). These products do not exceed 1% of the plant mass and are only found in 10% of the plant kingdom (Bowles 2003; Djilani and Dicko 2012). Plant extracts are also of complex composition and can be obtained from different plant organs. Because of this complexity and the impact of the solvent in extraction, different therapeutic effects are often obtained (Lapornik et al. 2005). Essential oils and plant extracts are considered promising antifungal agents due to their relative safety, broad tolerance and sustainability (Sawamura 2000; Ormancey et al. 2001; Sharanappa and Vidyasagar 2013). However, these compounds have high solubility and potential toxicity issues. Therefore, before clinical application, essential oils need to be subject to solubility studies in different pharmaceutical formulations, and also tested against different cell types (e.g. human dermal fibroblasts, keratinocytes, macrophages) over translational studies from experimental data to clinical use (García-Salinas et al. 2018).

Because of this promise and need, numerous plant essential oils and extracts have been further explored. Specifically, Martins et al. (2016) found that *Glycyrrhiza glabra* L. (licorice) had a remarkable anti-*Candida* spp. effect, namely against cells in suspension and biofilms. After determination of

its chemical composition, the authors stated that phenolic compounds seemed to be the main responsible constituents for the observed effects, namely related with its content in flavones (apigenin derivatives), flavanones (liquiritin derivatives) and a methylated isoflavone (i.e. formononetin-7-O-apiosylglucoside) (Martins et al. 2016). However, no significant effects were observed *in vitro* against *Candida* spp., indicating that the most abundant compounds were not the main contributors for the observed effects, but instead synergistic interactions with minor compounds. In fact, these effects were confirmed afterwards, by flow cytometry analysis and transmission electron microscopy (TEM) imaging. Licorice extract displayed a strong and irreversible candidacidal effect, primarily affecting cellular membrane permeability, altering electric potential of cells, inducing its disruption, culminating in a complete destruction of *Candida* spp. cells and organelles (Martins et al. 2016). Equally, extracts from *Juglans regia* (considered fungistatic), *Eucalyptus globulus*, *Pterospartum tridentatum* and *Rubus ulmifolius* were also effective against oral isolates of several *Candida* spp. (Martins et al. 2015b). Antimicrobial activity against *C. glabrata* oral isolates was detected for *Origanum vulgare*, *Lippia graveolens* and *Cinnamomum zeylanicum* essential oils (Pozzatti et al. 2008). Costa el al. (2017) described the anti-*Candida* activity of essential oils from leaves of *Hymenaea courbaril* var. *courbaril*, *Myroxylon peruiferum*, and *Vismia guianensis* from Brazil. The oils containing caryophyllene oxide, *trans*-caryophyllene, spathulenol, α-pinene, humulene epoxide II, demonstrated antifungal activity against *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. glabrata*, and *C. krusei*. Kumar et al. (2015) explain the synergistic anti-candidal activity on two asarones (∞ and β) from *Acorus calamus*. When combined with fluconazole, clotrimazole, and amphotericin B, minimal inhibitory concentrations necessary to inhibit the biofilm formation of *Candida* spp. were reduced. Similar results were demonstrated with a combination of amphotericin B and acteoside, from *Colebrookea oppositifolia* (Ali et al. 2011). Ramage and colleagues (Ramage et al. 2011) assessed the ability of mouth rinses with natural compounds to reduce *C. albicans* biofilms (Ramage et al. 2011). Listerine (Pfizer Consumer Health Care, UK) which contains eucalyptol, thymol, and menthol as natural active agents was able to reduce the viability of 48 h *C. albicans* biofilms by approximately 80%. In another study, Shino et al. (2016) disclosed that coconut oil was similar to ketoconazole in terms of antifungal activity on *C. albicans*. Saleem et al. (2016) stated that lichochalcone-A (a polyphenol found the roots of *Glycyrrhiza* spp.), can decrease *Candida* spp. biofilms *in vitro* and *in vivo*, surpassing fluconazole activity with no apparent toxicity. This compound was associated to a decline in the proteinases and phospholipase activities, highlighting the potential for topical treatment (Seleem et al. 2016). Szweda et at. (2015) reported that cinnamon oil was the most active out of a range of essential oils, presenting candidacidal activity against both planktonic and biofilm cultures of oral clinical isolates *C. albicans*, *C. glabrata*, and *C. krusei*. Finally, a common therapeutic treatment of superficial candidiasis is a topical application of calendula and commiphora plant extracts (Dalirsani et al. 2011).

3.2.3 Honey

Honey is a natural ingredient manufactured by bees, from the nectar of flowers or plants' secretions, converted in the upper aero-digestive tract of the same insects (Leticia Estevinho et al. 2011). After the fabrication, this sweet product is stored and matured in the honeycomb. The chemical composition of honey is variable depending on the botanical source (Leticia Estevinho et al. 2011). Several properties of honey have been reported. Important amongst them are: anti-inflammatory, antioxidant, bactericidal, bacteriostatic, antiviral, or anti-tumoral (Molan 2001; Theunissen et al. 2001; Lusby et al. 2005; Irish et al. 2006; Küçük et al. 2007; Bardy et al. 2008; Boukraa et al. 2008; Estevinho et al. 2008; Koc et al. 2009; Eteraf-Oskouei and Najafi 2013), giving this product a key role in traditional and modern medicine.

Several reports have described the antifungal properties of honey. Irish et al. (2006) assessed

floral and artificial honeys against *C. albicans*, *C. glabrata* and *C. dubliniensis* clinical isolates. *C. dubliniensis* was the most susceptible to honey, while *C. glabrata* was the least susceptible. Importantly, floral honeys had better antifungal activities when compared with the artificial honey.

Shokri and Sharifzadeh (2017), studied three honey samples from different regions of Iran and tested them against different oral *Candida* spp. isolates. All honeys displayed antifungal activities against fluconazole resistant *Candida* spp.. *C. krusei* and *C. glabrata* had lower susceptibility and *C. tropicalis* and *C. albicans* showed higher susceptibility to the honeys (Shokri and Sharifzadeh 2017). Finally, Estevinho et al. (2011) have also observed that synthetic honey solution was inferior in anti-fungal activity compared with natural honey. The Xmin – concentration that inhibited 10% of the yeasts growth – for the lavender honey ranged from 31.0% (*C. albicans*) and 16.8% (*C. krusei*) while synthetic honeys were both above 58%. The authors suggest this shows that the active antifungal agents are not sugar based. Further *in vitro* and *in vivo* assessments are still essential to fully measure the antifungal potential of honey. For *in vivo* applications, honey may be restricted to topical treatments. Still, honey may be used prophylactically to prevent more serious infections. A small number of studies have already confirmed the possibility of honey as a prophylactic product for *Candida* spp. infections. Honey also showed prevention of catheter exit site infection by coating catheters with honey, which was found to be at least as effective as povidone iodine (Quadri and Huraib 1999) or mupirocin (Johnson et al. 2005).

3.3 Other Approaches

3.3.1 Photodynamic Therapy

Photodynamic inactivation (PDI) seems to be an ideal new way to treat biofilm-associated infections because no microbial resistance observed with this therapy. In the literature it is also named as antimicrobial photodynamic therapy (aPDT) or photodynamic antimicrobial chemotherapy (PACT) due to its effect on microorganisms, or many times, only photodynamic therapy (PDT) commonly associated with oncological therapy.

PDI combines visible light of appropriate wavelength and a nontoxic dye, known as a photosensitizer (PS), resulting in production of reactive oxygen species (ROS) (Dai et al. 2012; Spagnul et al. 2015) (Fig. 1) that can kill the treated cells (da Silva et al. 2018). ROS are singlet oxygen ($^1O_2$), superoxide anion ($\bullet O_2^-$), hydroxyl radical (•OH), and hydrogen peroxide (H_2O_2) (Dai et al. 2012). Since the efficacy of PDI largely depends on the selection and administration of PS, the achievement of a clinically active PS is an immediate need (Khan et al. 2019). The irradiation light source is typically a laser (Černáková et al. 2017) or light-emitting diode (LED) light (Dovigo et al. 2010; Mahmoudi et al. 2018; Ghasemi et al. 2019).

Mahmoudi et al. (2018) indicated that the use of the PSs methylene blue (MB), toluidine blue O (azine – phenothiazinium, with absorption 600–680 nm), and indocyanine green (cyanine, 500–600 nm) with a light diode laser show significant results for the treatment of infectious diseases (Dai et al. 2012; Mahmoudi et al. 2018). In fact, MB has been widely used in PDI, however, the mechanisms of action (Type I or Type II) are defined by its state of aggregation (da Collina et al. 2018). Natural products such as hypericin, riboflavin or curcumin (Spagnul et al. 2015; Güzel Tunccan et al. 2018; Khan et al. 2019) have also shown antimicrobial effects. Other groups of photoactive dyes are porphyrines (600–650 nm, chlorine, bacteriochlorine, texaphyrine or aminolevulic acid – protoporphyrin IX) and xanthines (rose bengal 450–700 nm or rhodamines (500–600 nm) (Spagnul et al. 2015; Güzel Tunccan et al. 2018).

Several models have been used to test application of PDI on biofilms such as *in vitro* polystyrene plates (Trigo-Gutierrez et al. 2018) (Fig. 2), and *ex-vivo* human teeth (Eslami 2019) or mouse tongues (Černáková et al. 2015). *Galleria mellonella* larvae are also an interesting model for *in vivo* PDI efficacy assessment (Paziani

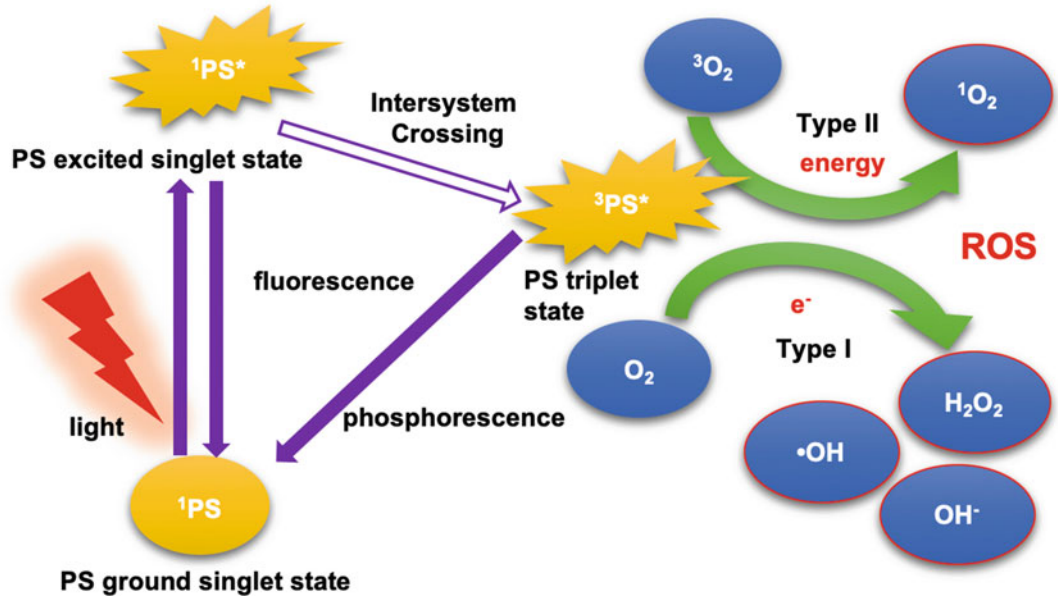

Fig. 1 Modified scheme of photodynamic inactivation (PDI) including the Jablonski diagram. Energy diagram illustrates the electronic states of a molecule and the transitions between them. After irradiation, PS initially absorbs a photon resulting in PS excited singlet state and this form can relax to the more long-lived triplet state. This triplet PS stadium can interact with molecular oxygen through two pathways; Type I and Type II, leading to the formation of ROS. (Modified according to (Dai et al. 2012; Spagnul et al. 2015))

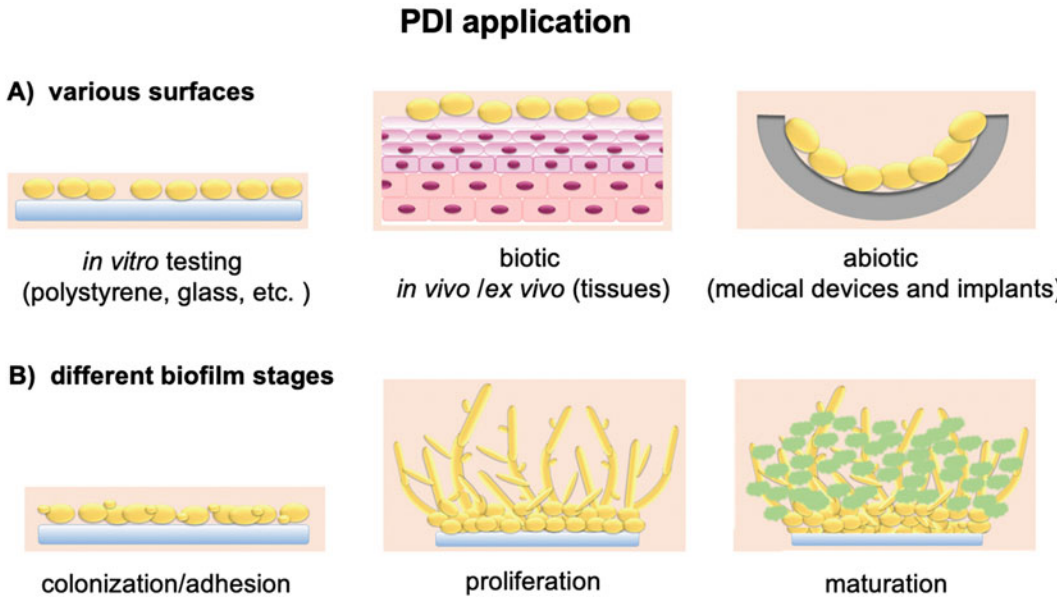

Fig. 2 Eradication of *Candida* spp. biofilm by photodynamic inactivation (PDI) is aim of many studies but they differ in (**a**) tested surfaces on which biofilm was developed or (**b**) in approach –regardless of PDI application time: to adhesion phase, after 24 h to adherent cells which are proliferating or to completely mature biofilm

et al. 2019). The methods for evaluation of PDI effect on biofilm inhibition are mainly colony-forming unit quantification (Dovigo et al. 2010), however, the metabolic activity of the biofilms can be measured by the XTT assay (Davies et al. 2016) as well as quantification of biomass with crystal violet staining and optical density values (Güzel Tunccan et al. 2018; Trigo-Gutierrez et al. 2018). Microscopy (confocal or scanning electron microscopy) can reveal another point of view for impact of PDI on yeast biofilms (Černáková et al. 2017; Güzel Tunccan et al. 2018). Černáková et al. showed that (Černáková et al. 2017) after PDI treatment, the percentage of survived biofilm cells was: 24.57, 23.46, and 22.29% for *C. albicans* SC5314 and 40.28, 17.91, and 5.89% for *C. albicans* CY 1123, respectively, compared with the controls.

Dovigo et al. (2010) evaluated the *in vitro* susceptibility of planktonic suspensions of *Candida* spp. to PDI induced by Photogem® showing efficacy against *C. albicans, C. dubliniensis* and *C. tropicalis*. Complete killing was observed for these species, while for *C. krusei* only the reduction in the viability was achieved (Dovigo et al. 2010). In another approach, MB activated with red LED light (576–672 nm) was compared to caspofungin. *C. albicans* and *C. parapsilosis* strains were tested at the beginning of biofilm formation and to 24-h after biofilm formation (Fig. 2) (Černáková et al. 2015). Davies et al. (2016) performed a study where *C. albicans, C. glabrata, C. tropicalis* and *C. parapsilosis* biofilms were exposed to PDI using another PS – porphyrin-based PS TMP-1363 -, but in this experiment, PS was combined with miconazole. The effect on *C. albicans* was additive, with *C. tropicalis* and *C. parapsilosis.* The combined treatment had a higher, but not entirely additive, cytotoxic effect: ATCC strains and *C. glabrata* 7531/06 were not sensitive, while the metabolic activities of the remaining three clinical isolates decreased to 64.2 ± 5.5%, compared to the controls (Davies et al. 2016). ROS production and cellular darmage was stimulated using irradiated toluidine blue on biofilms of *C. krusei* (da Silva et al. 2018).

Chloroaluminium phthalocyanine in cationic nanoemulsion have been investigated for PDI against *C. albicans, C. glabrata* and *Streptococcus mutans,* grown as multispecies biofilm formed in wells of a microtiter plate. After 30 min of incubation, biofilms were illuminated with red light fluorescence of 39.3 J/cm^2. PDI of the multispecies biofilm was confirmed, however, the total biomass of the biofilm was not affected by the treatment (Trigo-Gutierrez et al. 2018). The antimicrobial effects of calcium hydroxide $Ca(OH)_2$, triple antibiotic paste, PDT, toluidine blue, LED and a 940 nm diode laser were compared on the biofilm of *Enterococcus faecalis* and *C. albicans* in the root canal system of *ex-vivo* human teeth. Exposure led to reduced biofilm thickness (Eslami 2019). Another study with various dyes (rose bengal, riboflavin, and methylene blue) and light sources (LED and UVA) against staphylococcal and candidal biofilms indicated the *in vitro* efficacy of aPDT. These results indicate that PDT is a promising technique for the control of biofilm growth within intravenous catheters (Güzel Tunccan et al. 2018). PDT seems to be effective against dental caries and white spot lesions (Lacerda Rangel Esper et al. 2019) or as an adjunct for the decontamination of dental implant surfaces (Ghasemi et al. 2019). Also, biofilms of common pathogens were eradicated in infections of diabetic foot ulcers (Li et al. 2019). On the other hand, Freitas et al. summarized that pACT is not as effective against multi-species biofilms. Multi-species biofilms were more resistant to the antimicrobial, possibly due to their thickness and complexity (de Freitas et al. 2017).

3.3.2 *Quorum-sensing* Molecules

Microbial cells "talk" to each other releasing chemical signals. Self-inhibitory molecules can limit the cell number of dense communities such as biofilms (Barriuso et al. 2018; del Rosario et al. 2019). This method of microbial communication is called *quorum-sensing* (QS) and can regulate several behaviours in biofilm by cell-density-dependent manner. The process involves the production and detection of soluble QS molecules (Albuquerque and Casadevall 2012; Lohse et al.

2017). The existence of QS molecules in fungi was described almost two decades ago when the signalling function of farnesol (FAR) was revealed. FAR controls filamentation, yeast-to hyphae transition in *C. albicans* biofilms (Hornby et al. 2001; Ramage et al. 2002). Since that time, it has been further developed as a novel anti-biofilm therapeutic agent (Ramage et al. 2002). Subsequently, it was shown that this natural sesquiterpene alcohol plays multiple roles in *C. albicans* physiology (Albuquerque and Casadevall 2012; Ku and Lin 2016). FAR is a precursor in the isoprenoid/sterol pathway and can be cytotoxic at certain concentrations (Shirtliff et al. 2009). In fact, FAR inhibits hyphal growth by regulating the cyclic AMP (cAMP) signalling pathway in *C. albicans. CYR1* and *PDE2* regulate a pair of enzymes that are directly responsible for cAMP synthesis and degradation. The results indicate that *CYR1* and *PDE2* regulate the resistance of *C. albicans* biofilms to antifungals. FAR suppresses the resistance of *C. albicans* biofilms to antifungals by regulating the expression of the gene *CYR1*and *PDE2*, while *PDE2* regulation was subordinate to *CYR1* regulation (Chen et al. 2018). Also, FAR induces upregulation of the *MCA1* caspase gene and promotes apoptosis in *C. albicans* through caspase activation and ROS production. Therefore FAR is shown to play an important physiological role in the fungal cell life cycle related to adaptation and survival (Shirtliff et al. 2009). The reduction in *C. albicans* biofilm robustness in the presence of 200 μmol/L FAR-alone or in combination with fluconazole was observed by microscopy. The presence of this concentration of FAR downregulated all tested genes; *ERG20, ERG9,* and *ERG11*. FAR acted effectively only at higher concentrations against *C. albicans* biofilms (Dižová et al. 2018).

Tyrosol, another aromatic alcohol, was also found to be a *C. albicans* QS molecule linked to growth, morphogenesis and biofilm control (Albuquerque and Casadevall 2012). While FAR blocks morphological transformation from germ-tubes to the mycelial form (negative control), tyrosol has a positive effect – to accelerate this process (Chen et al. 2004; Alem et al. 2006). Action of tyrosol is more significant during the early and intermediate stages of biofilm formation stimulating hyphae production (Alem et al. 2006). The antifungal activity of the combination of tyrosol and FAR against *C. albicans* and *C. glabrata* in the planktonic and biofilm states was examined. *C. glabrata* biomass, metabolism of *C. albicans* and mixed biofilms, and cultivable cells of single biofilms showed reduction for the drug combination, indicating an additive effect (Monteiro et al. 2017). Another *in vitro* study demonstrated the specific anti-biofilm effect, of FAR and tyrosol, as tested in *C. albicans* standard strain and six isolates from dentures. FAR at 3 mM exerted a stronger action when added at the beginning of biofilm formation than when added to preformed biofilms. Similarly, tyrosol at 20 mM had a greater effect on biofilm at an early stage (Sebaa et al. 2019).

Biofilm formation and FAR production differs significantly not only between *Candida* spp. but within *C. albicans* strains as well. FAR concentration was the highest for *C albicans*, but also detected in *C. dubliniensis*, *C. tropicalis*, *C. parapsilosis*, *C. guilliermondii, C. kefyr, C. krusei* and *C. glabrata* biofilms (Weber et al. 2008; del Rosario et al. 2019). In *Saccharomyces cerevisiae*, two other QS, aromatic alcohols, phenylethanol and tryptophol were found to be regulate morphogenesis during nitrogen starvation conditions. Additionally, population density-dependent behaviors that resemble QS have been described in several other fungal species (Albuquerque and Casadevall 2012). Moreover, FAR acts in concentration-dependent-manner (Fig. 3), while higher concentration of FAR inhibits yeast-to-hyphae transformation – negative regulation of biofilm development, lower concentration stimulates filamentation and biofilm formation (Ramage et al. 2002; Dižová and Bujdáková 2017). In general, higher FAR concentrations (200–300 μM) are stressful for yeasts and can lead to apoptosis, but lower (about 40 μM) protect them from stress (Dižová and Bujdáková 2017) or modulate activity of efflux pumps in resistant strains (Sharma and Prasad 2011; Černáková et al. 2019). In combination they reduced the minimum *in vitro* inhibitory

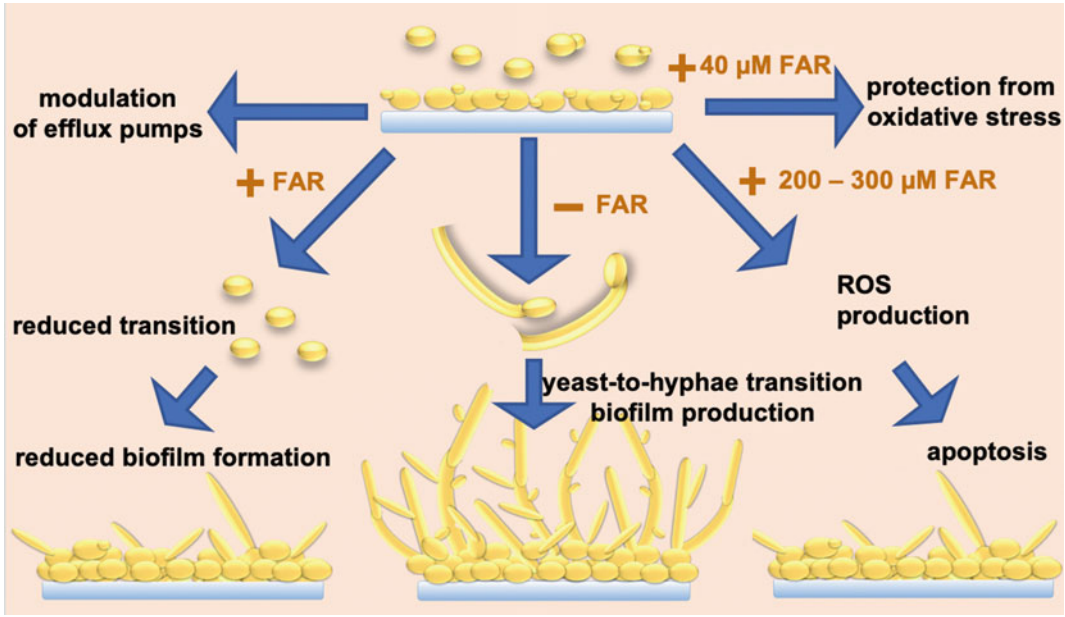

Fig. 3 Several effects of farnesol (FAR) on yeasts in concentration-dependent-manner. Scheme was created and modified according to review Dižová and Bujdáková (2017)

concentrations of amphotericin B, fluconazole, itraconazole, caspofungin against drug-resistant strains *C. albicans, C. parapsilosis and C. tropicalis* (Cordeiro et al. 2013). Also, FAR (at 60 and 100 μM) decreased the minimal inhibitory concentrations, from 8 to 4 μg/mL of fluconazole, when the resistant strain *C. albicans* CY 1123 was not cultivated with this drug (Černáková et al. 2019). Although fungal QS research is still in its infancy (Albuquerque and Casadevall 2012), results require further investigation of the role of FAR in the balance of the sterol biosynthetic pathway and how it interferes with cell viability (Cordeiro et al. 2013). Polke et al. (2018) assumed that despite the identification of various factors involved in FAR signalling, its exact mode of action remains largely unclear (Polke et al. 2018).

Studies also demonstrate a potential pharmaceutical application of FAR in oral hygiene against polymicrobial biofilms (Sebaa et al. 2019; Černáková et al. 2019). Chitosan nanogel contained FAR (300 μM) significantly decreased expression of *HWP1* and *SAP6* genes in *C. albicans* (Nikoomanesh et al. 2019). Results of del Rosario et al. (2019) support described the use of tyrosol, 2-phenylethanol and FAR as multi-species (*Rhodotorula mucilaginosa, C. tropicalis, C. krusei* and *C. kefyr*) biofilm inhibitors in beverages and findings inspire to design novel preservative and cleaning products for the food industry (del Rosario et al. 2019).

3.3.3 Coating Surfaces

Inhibitors of early yeast adhesion to different surfaces are important for innovative medical device manufacturing. Anti-biofilm drug development can also be useful for pre-therapeutic coatings of a diverse range of biomaterials (Vargas-Blanco et al. 2017; Palmieri et al. 2018). Proteases immobilized on a polypropylene surface reduced the adhesion of *C. albicans* biofilms. Therefore, they may be useful in developing anti-biofilm surfaces based on non-toxic molecules and sustainable strategies (Andreani et al. 2017). Treatment with Filastin significantly inhibited the ability of *C. albicans* to adhere to bioactive glass (cochlear implants, subcutaneous drug delivery devices and prosthetics), silicone (catheters and other implanted devices), and

dental resin (dentures and dental implants) (Vargas-Blanco et al. 2017). Similarly, the surface and wettability characteristics and the microbial biofilm interaction of graphene coating on titanium were evaluated. The transfer of graphene which was repeated two times on titanium via a liquid-free technique decreased biofilm formation for all species. The increased hydrophobicity of graphene films was correlated with the decreased biofilm formation for various species (Agarwalla et al. 2019). In another study, combination of graphene oxide-curcumin-polyethylene glycol coatings reduced fungal adhesion, proliferation and *C. albicans* biofilm formation (Palmieri et al. 2018). The action of clioquinol, an antiseptic drug effective against multidrug-resistant *Candida* spp. on biofilms in intrauterine devices was also investigated. The authors suggest that clioquinol could be applied as a coating to prevent morphological switching (Pippi et al. 2018). Also interesting is the use of various PS to modify the medical devices surface like silicone, polyurethane, polystyrene or cellulose acetate (Bujdáková 2016).

3.3.4 Antibodies/Peptides

A hot topic of anti-biofilm therapy development is antibody-derived peptides. Their ability to significantly combat *Candida spp.* biofilms is the basis for development of an alternative strategy that might protect against *Candida spp*. infections (Paulone et al. 2017; Carrano et al. 2019). In a very recent study, Carrano et al. (2019) evaluated the *in vitro* antifungal activity of antibodies against *C. albicans* germ tubes raised in a rabbit model of candidemia. The antibodies reduced *C. albicans* growth, and impaired its metabolic activity along with the ability to form *in vitro* biofilms (Carrano et al. 2019). Other research investigated the effects of a synthetic killer peptide (KP), known to be active *in vitro*, *ex vivo* and/or *in vivo* against different pathogens on *C. albicans* biofilms. KP treatment resulted in an increase in *C. albicans* oxidative stress response and membrane permeability. Additionally, biofilm-related gene expression was significantly reduced (Paulone et al. 2017). *Candida auris*, an emerging pathogen, needs to be controlled effectively due to its association with a high rates of mortality rate and antifungal resistance, as well as for its ability to form biofilms (Ledwoch and Maillard 2018). Dekkerová et al. (2019) demonstrated the presence of antigen, complement receptor 3-related protein (CR3-RP) on the surface of *C. auris* and point to the potential of an anti-CR3-RP polyclonal antibody in eradication of biofilms formed by this novel fungal pathogen (Dekkerová et al. 2019). Rudkin and colleagues (Rudkin et al. 2018) have prepared human anti-*Candida* monoclonal antibodies (mAbs) with diagnostic and therapeutic potential. The authors expressed recombinant antibodies from genes cloned from the B cells of patients suffering from candidiasis and declared morphology-specific, and high affinity binding to the cell wall. These mAbs showed interesting properties for diagnostics, strong opsonophagocytic activity of macrophages *in vitro*, and protection in a murine model of disseminated candidiasis (Rudkin et al. 2018).

3.3.5 Lock Therapy

For treatment of catheter-related infections, the systemic administration of antibiotics as well as local antibiotic locks into the catheter to sterilize the catheter are used. High doses of antimicrobial agents are poured in drops directly into the catheter and are subsequently locked inside for a specific length of time (from several hours to days (Bujdáková 2016). The development of optimal catheter lock solutions is challenge, and there is no clear recommendation on antibiotic locks that would specify the type and concentration of antimicrobial medication (Visek et al. 2019). An ethanol-based lock solution with 40% ethanol +60 IU heparin administered daily for 72 h has reported to be sufficient to almost eradicate the metabolic activity of bacterial and fungal biofilms, but future studies are needed to study cell regrowth after this therapy (Alonso et al. 2018). On the other hand, a combined lock solution of micafungin, ethanol and doxycycline was highly effective for the prevention of *C. albicans* biofilm formation but did not demonstrate an advantage over 20% ethanol alone (Lown et al. 2016). In another study, a solution containing

trimethoprim (5 mg/ml), ethanol (25%), and calcium EDTA (3%), was used against bacterial and fungal biofilms, in *in vitro* and *in vivo* (rabbit) catheter biofilm models. Results demonstrated efficacy against both adhesion-phase and mature biofilms (Chandra et al. 2018). Liposomal amphotericin B was more effective at eradicating *Candida* spp. biofilms (*C. albicans, C. glabrata, C. tropicali,s* and *C. parapsilosis*) in a 3-day course of systemic and lock therapy than micafungin. Therefore, it may be useful for the treatment of catheter-related *Candida* spp. biofilm infections, but further studies including a long treatment duration will be needed (Fujimoto and Takemoto 2018).

4 Concluding Remarks

Biofilm infections are documented for being more complex to treat than other infections, especially when linked to medical devices. Specifically, *Candida* spp. infections are becoming more recalcitrant to antifungal treatments, particularly to azole antifungals. Hence, it is essential to explore novel therapies and successfully replace the conventional antifungal programs. New compounds have been discovered in nature, others are entirely synthethized in laboratory or identified from symbiotic relations. It is expected that, in the near future, these new compounds will improve antifungal responses. Prebiotics, probiotics, and symbiotics seem to display promising results. In fact, they are intimately related to immunostimulatory effects in the host and the presence of these microorganisms have improved certain clinical disorders and reduced the prevalence of *Candida* spp.. However, they still need to be fully explored and tested. Plant extracts and essential oils are certified options to current drugs. Yet, it is critical to determine criteria for the therapeutic use of these plant derivatives. This will reduce variation in efficacy amongst samples as the result of several factors (e.g. time of harvesting or processing methods, genotype, cultivation area) (Prabhakar et al. 2008). Data to date suggests that the oxidizing photodynamic treatments, quorum-sensing molecules, antibodies or lock therapy methods have a huge potential for combating candidiasis, due to the unlikely development of resistance mechanisms to these procedures. Compared with conventional antifungals applied for candidiasis' treatments, all these unconventional remedies show improvement in certain clinical conditions and diminish the prevalence of *Candida* spp.. Nonetheless, these novel therapies have to be prudently tested in human clinical trials, and the toxicities of many of the compounds are yet to be assessed.

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Conflicts of Interest The authors declare no conflict of interest.

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