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Gianfranco Donelli *Editor*

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Novel Polycationic Photosensitizers for Antibacterial Photodynamic Therapy

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Abstract

Antibacterial photodynamic therapy (APDT) is a promising method of treating local infected foci, in particular, surgical and burn wounds, trophic and diabetic ulcers. Photodynamic inactivation (PDI) is able to effectively destroy bacterial cells without them developing resistance in response to treatment.

This work was dedicated to the study of photophysical and antibacterial properties of new photosensitizers (PS) based on polycationic phthalocyanines and synthetic bacteriochlorins for photodynamic inactivation of *P. aeruginosa* bacteria and their biofilms. Gram-negative bacteria P. *aeruginosa* are often found in infected wounds, presumably in biofilm state and are characterized by rather low susceptibility to APDT, which is a problem. PS were studied for possible aggregation at various concentrations by means of absorption and fluorescence spectroscopy. The results of studies of the $ZnPcChol_8$, $(3\text{-}PyHp)_4BCBr_4$ and $(3\text{-}PyEBr)_4BCBr_4$ in water and serum confirm the assumption of a low degree of their aggregation at high concentrations.

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Consequently, their photodynamic efficiency is high enabling to use these PS at high concentrations to sensitize pathological foci for APDT.

It was shown that all the investigated PS had a high efficiency of photodynamic inactivation of Gram-negative bacteria *P. aeruginosa*, as well as their biofilms. Tetracationic hydrophilic near-infrared photosensitizer $(3\text{-PyEBr})_4\text{BCBr}_4$ with reduced molecule size had significantly higher efficacy of photodynamic inactivation of *P. aeruginosa* biofilms compared with other studied photosensitizers.

Keywords

Aggregation · Antibacterial photodynamic therapy; cationic photosensitizer · Bacterial biofilms · Bacteriochlorins · Luminescence

Abbreviations and Symbols

APDT	antibacterial photodynamic therapy
PDI	photodynamic inactivation
PS	photosensitizer
P. aeruginosa	Pseudomonas aeruginosa
S. aureus	Staphyllococcus aureus
A. baumannii	Acinetobacter baumannii
K. pneumoniae	Klebsiella pneumoniae
ZnPcChol_8	zinc octakis(cholinyl) phthalocyanine
$(3\text{-PyEBr})_4\text{BCBr}_4$	*meso*-tetrakis[1-(2′-bromoethyl)-3-pyridyl] bacteriochlorin tetrabromide
$(3\text{-PyHp})_4\text{BCBr}_4$	*meso*-tetrakis(1-heptyl-3-pyridyl)bacteriochlorin tetrabromide
Pc	phthalocyanine
Da	unified atomic mass unit or Dalton
CFU/ml	Colony Forming Units per milliliter
MBC	Minimal Bactericidal Concentration
Φ_F	quantum output of fluorescence
Φ_Δ	quantum yields of singlet oxygen
DNA	deoxyribonucleic acid
LB	Luria-Bertani medium

1 Introduction

Infected long-term non-healing complicated wounds of the skin and mucosa, trophic ulcers, pressure sores, ulcers of diabetic feet, represent serious problems for their treatment, especially in the case of resistant and multi-resistant pathogens. Approximately 1% of the population in both developed and developing countries faces these diseases (Bjarnsholt et al. 2011). In recent years, evidence has been obtained of the association of chronicity of wounds with biofilms, as well as with the presence of polymicrobial communities in the wound (Newton H et al. 2017). The exopolymer matrix of biofilms protects bacteria, in particular, from the effects of drugs and host immune defence factors, which complicates treatment. The main pathogens of wound infections are *Pseudomonas aeruginosa* (*P. aeruginosa*), *Staphyllococcus aureus* (*S. aureus*), *Acinetobacter baumannii*, *Klebsiella pneumoniae*, which can occur in the form of microbial associations. Antibiotic treatment in the case of a mature biofilm often gives only a temporary result, and even extensive debridement of the wound in combination with skin transplantation does not lead to rapid healing. Experts raise the question of the need for more effective methods of treating wounds (Bjarnsholt et al. 2011).

Antibacterial photodynamic therapy (APDT) is a promising way to treat infected surgical and burn wounds, trophic and diabetic ulcers (Park et al. 2011; Bertoloni et al. 1992; Percival et al. 2014) using light irradiation of lesions. The activation of photosensitizer (PS) by light, accompanied by the formation of singlet oxygen and other reactive oxygen species, leads to

multiple oxidative destruction of different subcellular structures of pathogens. Photodynamic process is able to effectively inactivate bacterial cells without developing resistance in response to the treatment, and, thanks to local irradiation, does not affect the normal microflora of the patient, as opposed to antibiotics (Nakonieczna et al. 2010; Tavares et al. 2010). The resistance to PS and APDT has not been detected even after 20 consequent cycles of the bacterial flora partial destruction followed by its regrowth (Hamblin and Hasan 2004; Vera et al. 2012). Almost all pathogenic microorganisms, including antibiotic-resistant strains of bacteria, are susceptible to APDT (Maisch 2015).

Chronic wound infections are often accompanied with biofilm formation, which are usually multi-species and include both Gram-positive and Gram-negative bacteria (Almeida et al. 2011). An effective PS must kill both Gram-positive and Gram-negative bacteria in planktonic as well as in biofilm state. Gram-positive and Gram-negative bacteria have fundamental differences in structure and charge of their cell wall, so they differ in sensitivity to drug effects. The sensitivity of Gram-negative bacteria to APDT is much lower compared to Gram-positive bacteria. The cell wall of Gram-positive bacteria has a relatively high degree of porosity. It is not a barrier for penetration by most PS, the molecular mass of which usually does not exceed 1500–1800 Da. The cell wall of Gram-negative bacteria prevents the penetration of large molecules and facilitates the resistance to many drugs (Nikaido 1994). Therefore, the inactivation of Gram-negative bacteria is the most important and difficult target for photodynamic treatment of infected foci (Maisch 2007).

Only polycationic PS with a rather small molecular mass effectively interact with Gram-negative bacteria. The efficiency of bacteria photodynamic inactivation depends on the properties of PS molecules – molecular mass (Meerovich et al. 2018), charge (number of cationic groups) (Simões et al 2016), photodynamic properties, in particular, the singlet oxygen quantum yield. The most likely mechanism ensuring the association of cationic PS with microbial cells is the electrostatic interaction of positively charged molecules with negatively charged sites of the cell walls.

Many studies of the photodynamic inactivation of Gram-negative bacteria were carried out using cationic phthalocyanines and bacteriochlorins

The cationic phthalocyanines are described in literature, carrying the positively charged substituents (Minnock et al. 1996; Griffith et al. 1997; Soncin et al. 2002; Vecchio et al. 2013; Roncucci et al. 2014; Ke et al. 2014; Spesia and Durantini 2013; Cakir et al. 2015; Colak et al. 2016; Zheng et al. 2013; Mantareva et al. 2013; Osifeko et al. 2015; Di Palma et al. 2015; Lourenço et al. 2015; Rocha et al. 2015; Segalla et al. 2002).

To ensure proper bacteria inactivation it is necessary to use high concentrations of PS during sensitization. The aggregation of tetrapyrrole molecules (van Lier and Spikes 1989; Moan 1984; Chowdhary et al. 2003) leads to a decrease in the intensity of absorption, the lifetime of the excited state of the PS, the quantum yield of fluorescence, as well as a change in the shape of the spectral absorption and fluorescence contours. The Coulomb repulsion of cationic PS molecules can partially diminish the aggregation. An increase in the number of substitutions (n) leads to a decrease of phthalocyanine aggregation degree in aqueous solutions and, consequently, to an increase of the quantum yield of fluorescence and singlet oxygen (Makarov et al. 2007, 2009; Strakhovskaya et al. 2009; Zhang et al. 2015). The rise of cationic phthalocyanines bactericidal photoactivity with n increase may be due both to the dissociation of inactive dimer complexes and increasing the efficiency of bacteria binding. As a result of our study it was found that octacationic zinc octakis(cholinyl)phthalocyanine $ZnPcChol_8$ has the highest bactericidal photoactivity (Kuznetsova et al. 2006; Yakubovskaya et al. 2015).

In bacterial biofilms, bacteria are additionally protected by a matrix, which significantly reduces the efficiency of photodynamic inactivation, primarily due to the fact that the matrix limits the diffusion of PS molecules and oxygen to bacteria.

The components of the matrix bind a significant part of photosensitizer molecules, including cationic ones, since the volume of the matrix is significantly larger than the total volume of bacteria. Therefore, a significant part of the light is absorbed by the sensitized matrix, consequently, a lot of photodynamic acts are realized at a distance from bacteria exceeding the free path of singlet oxygen (>1 μm), so it doesn't reach the bacterial cells.

It should be taken into account that the depth of the foci infected by *P. aeruginosa* can reach 12–15 mm (Bjarnsholt et al. 2011). Therefore, for the proper photodynamic effect on such foci, it is necessary to use PS, the excitation of which is carried out in the 720–850 nm spectral range – so called "biological tissue transparency window". Moreover, bacteria *P. aeruginosa* produce a number of pigments (in particular, pyocyanin, pyoverdin, pyorubrin, pyomelanin) in the course of their life in wounds (El-Fouly et al. 2015). The presence of these pigments in wound discharge leads to a large absorption in the 660–740 nm spectral range. Therefore, APDT with photosensitizers of the red spectral range may be ineffective due to the large losses of exciting light caused by absorption by both pigments and hemoglobin in host tissues. Thus, for the proper APDT of such foci, it is better to use the cationic PS excited in the 750–850 nm spectral range. That is why synthetic cationic derivatives of bacteriochlorins with intense absorption in near infrared spectral range of 760–850 nm are very promising as PS for APDT. Technological approaches proposed in (Mass and Lindsey 2011; Huang et al. 2010; Schastak et al. 2008, 2010; Dudkin et al. 2013a, b, 2014) allow the cationic derivatives of synthetic bacteriochlorins to be created with charges number from +4 to +8. The studies conducted on tetracationic and octacationic derivatives (Dudkin et al. 2013a, b) with a molecular weight of about 1500–1800 Da showed (Meerovich et al. 2016; Tiganova et al. 2017) that the PS can be used for the photodynamic inactivation (PDI) of Gram-positive bacteria *S. aureus* and Gram-negative bacteria *P. aeruginosa*. For these PS the values of their Minimal Bactericidal Concentration (MBC) were determined, which completely prevented the growth of bacteria.

This work was dedicated to the study of photophysical and antibacterial properties of new PS based on polycationic synthetic derivatives of phthalocyanines and bacteriochlorins designed for PDI of Gram-negative *P. aeruginosa* bacteria and their biofilms.

2 Materials and Methods

2.1 Synthesis of Photosensitizers

In our study, the original photosensitizers for photodynamic inactivation of bacteria and bacterial biofilms based on polycationic tetrapyrrole derivatives were synthesized in Organic Intermediates and Dyes Institute (Russia): zinc octakis(cholinyl)phthalocyanine $ZnPcChol_8$ with molecular mass 1678 Da, tetracationic bacteriochlorin derivatives: *meso*-tetrakis[1-(2′-bromoethyl)-3-pyridyl]-bacteriochlorin tetrabromide $(3\text{-PyEBr})_4BCBr_4$ with molecular mass 1374 Da and *meso*-tetrakis(1-heptyl-3-pyridyl)-bacteriochlorin tetrabromide $(3\text{-PyHp})_4BCBr_4$ with molecular mass 1339 Da (Fig. 1).

They showed high effectiveness in photodynamic inactivation of antibiotic resistant strains of Gram-negative bacteria (Vorozhtsov et al. 2006a, b; Meerovich et al. 2018).

The proposed method of synthesis of $ZnPcChol_8$ is presented in Fig. 2. The synthesis includes the chloromethylation of unsubstituted metal phthalocyanine and subsequent treatment of chloromethylated product with tertiary amines. In the case of diamines the resulting product was treated with methyl iodide doubling the number of cationic centres in the molecule.

The starting zinc phthalocyanine (ZnPc) was synthesized with yield of around 70% by heating the mixture of zinc acetate, *o*-phthalonitrile, N, N-dimethylaminoethanol and dimethylsulfoxide at 125–127 °C with subsequent dilution of chilled reaction mass by methanol, filtration of product and its multiple treatment with hot methanol

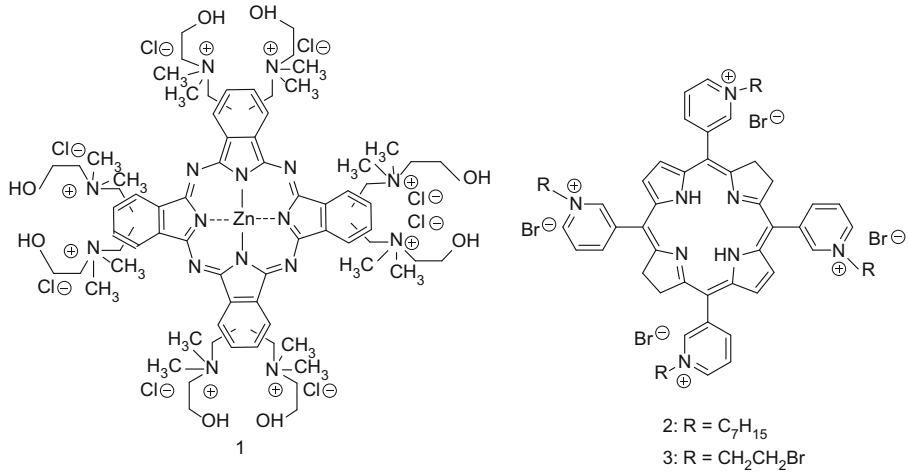

Fig. 1 The structures of the studied PS: 1 – $ZnPcChol_8$ (left); 2 – $(3\text{-}PyHp)_4BCBr_4$; 3 – $(3\text{-}PyEBr)_4BCBr_4$ (right)

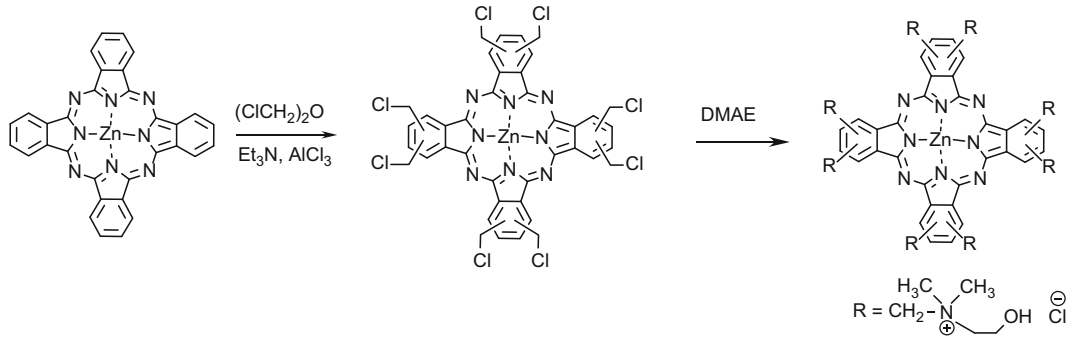

Fig. 2 The scheme of the synthesis of $ZnPcChol_8$

(Yuzhakova et al. 2005). We have developed the method of chloromethylation of ZnPc using a mixture of aluminum chloride, paraform and thionyl chloride at a temperature of 85–95 °C. This method allows high yield (above 90%) preparation of chloromethylated ZnPc with 7–8 substituents (Yuzhakova et al. 2010).

Tetracationic bacteriochlorin derivatives have been synthesized by alkylation of *meso*-tetra (3-pyridyl)bacteriochlorin with 1,2-dibromethane or heptyl bromide, respectively, in nitromethane in an inert atmosphere for 2 h (Makarova et al. 2018; Nevonen et al. 2018) (Fig. 3).

Compared to the tetracationic bacteriochlorins synthesized and studied by us earlier (Dudkin et al. 2014, Tiganova et al. 2017), the new PS have reduced molecular mass due to a decrease in the length of the alkylene chain from 4 to 2 methylene units in hydrophilic $(3\text{-}PyEBr)_4BCBr_4$ and from 11 to 7 carbon atoms in alkyl groups for amphiphilic $(3\text{-}PyHp)_4BCBr_4$.

2.2 Photophysical Studies of PS

The studies of the hydrophilic substances $ZnPcChol_8$ and $(3\text{-}PyEBr)_4BCBr_4$ were carried out in aqueous solutions, amphiphilic substance $(3\text{-}PyHp)_4BCBr_4$ was solubilized in 4% dispersion of Kolliphor ELP (BASF).

New PS were investigated *in vitro* for possible aggregation at various concentrations by means of absorption and fluorescence spectroscopy. The

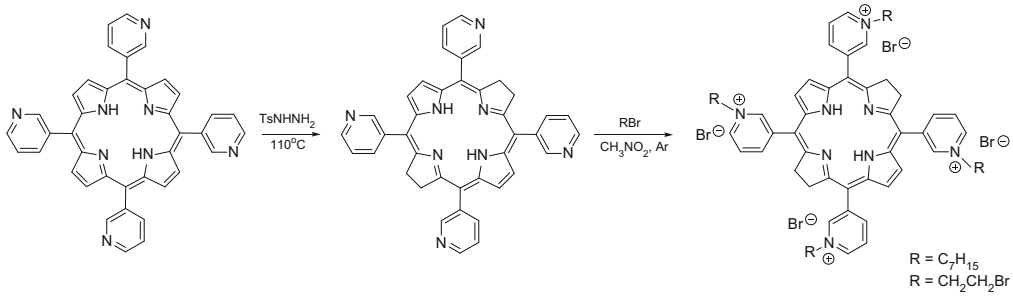

Fig. 3 The scheme of the synthesis of tetracationic bacteriochlorin derivatives

PS absorption in the concentration range from 0.001 mM to 0.1 mM was studied using a two-beam spectrophotometer "Hitachi U-3410" (Hitachi, Japan). Spectral-fluorescence studies were performed using a spectrum analyser "LESA-01-Biospec" (BIOSPEC, Russia). The fluorescence was excited by CW laser radiation at the wavelength of 532 nm (the second harmonic of the neodymium laser) coinciding with the Q_2-band of bacteriochlorin, and He-Ne laser with wavelength of 632.8 nm. To analyse the changes in the shape of the fluorescence spectral band of the PS, the measurements were performed in the cells of various lengths (1 mm and 10 mm). The shapes of the fluorescence spectral contours were calculated by dividing the intensity of the spectral signals by the intensity of their spectral maxima (so called "normalized fluorescence intensity").

For the evaluation of luminescence lifetime of PS the Hamamatsu streak-camera with a time-resolution was used. It comprised a laser source Picosecond Light Pulser "PLP-10" (Hamamatsu, Japan) with fibre optic output generating at the wavelength of 637 nm with a pulse duration of 65 ps and a polychromator with fibre optic input and Semrock optical filter "LD 01–785/10–12.5" or Omega Optical "660LP RapidEdge" inlet that transmits only the spectral region of luminescence band of bacteriochlorins or phthalocyanines (Bystrov et al. 2016). The image of the output slit of the polychromator was projected on the photocathode of the streak-camera, which included Streak scope "C10627–13" (Hamamatsu, Japan) with an adjustable time scan from 1 ns to 10 ms in the direction perpendicular to the image of the slit. The image from the output screen of Streak scope was received by the input of Digital CCD Camera "C9300–508". The synchronous delay generator "C10647–01" with a repetition rate of up to 1.6×10^7 Hz, synchronized the launch of the laser source and a Streak scope, allowing registering fluorescence signal with a spectral decomposition, and time base. The time-correlated counting method of single photons was used in the measurement process. The signal was recorded on a 5 ns time scan. The received signal was approximated by the function of two exponents with high accuracy of the approximation (5%).

2.3 Microbiological Studies

The microbiological studies were carried out on *P. aeruginosa* 32 and *P. aeruginosa* 21 clinical isolates. Bacteria were cultivated in LB-broth and 1% LB-agar (Difco, USA).

For the study of the photoinactivation of planktonic bacteria 18 h stationary phase broth culture was diluted 1:20 in LB broth and incubated 1.5 h at 37 °C with aeration. Bacterial suspension in saline was prepared with the initial content of bacteria 1×10^8 CFU/ml (Colony Forming Units per ml) by optical density. PS at different concentrations in water solutions were added in equal volumes to Eppendorf tubes with aliquots of bacterial suspension. After incubation with PS at 37 °C bacterial suspensions were centrifuged, supernatant was discarded and bacteria were resuspended in saline, aliquots of

100 μl from all samples and control (without PS) were placed in 96-well flat-bottom plates, one of them was irradiated, the other served as an unirradiated ("dark") control, every point in triplicate. The "SPD-M-685 LED" and "SPD-M-760 LED" light sources (BIOSPEC, Russia) at the wavelength of 685 and 760 nm were used for irradiation. "Coherent labmax" meter was used to control the power density. After irradiation, five 10-fold dilutions were prepared from every well and 20 μl from every dilution were plated on Petri dishes with LB agar. A day after, colonies were counted, amount of viable bacteria was estimated as CFU/ml, and means of 3 plates were plotted as a graph. It was estimated for standard conditions: incubation of bacteria with PS for 30 min, the dose of light 20 J/cm^2.

For estimation of bactericidal activity of PS on biofilms, the bacteria were grown for 20 h at 37 °C in 100 μl of LB in the wells of 96-well flat-bottom plates. Culture medium was replaced with water solution of PS at the desired concentration (every point in triplicate) and water for control and after 1 h incubation in the dark at 37 °C, PS was replaced with 100 μl of saline. Irradiation was performed with light dose 100 J/cm^2. To disaggregate biofilms, 11 μl of multienzyme substance ENZYMIX (BFR LABORATORIES, Russia) was added to every well for 10 min. The same procedures were performed for dark control plate. Five 10-fold dilutions were prepared from every well and 20 μl from every dilution were placed on Petri dishes. A day after, colonies were counted and the amount of viable bacteria was estimated as CFU/ml.

For spectral analysis of pigments production by biofilm, bacteria were grown in 20 ml of LB broth in Petri dishes for 1 or 3 days. Then the content of dishes was transferred into centrifuge tubes, vigorously vortexed and centrifuged at 7000 RPM for 15 min. Supernatant was filtered through 0,22 μ Millipore filter and vigorously shaked for good aeration before measurement.

Fluorescent microscopy technique was used for visualization of PDI results. The 18 h stationary broth culture was diluted 1:20 with fresh LB and added to glass cover slips. Young biofilms were formed in 3 h at 37 °C, then they were washed with distilled water and 50 μM of PS in water solution was added (the same volume of water was added for control). After 30 min of incubation in the dark, biofilms were washed 3 times with distilled water and irradiated with LED light sources mentioned above, except for the dark control. The glasses with biofilm were stained with Live/Dead Biofilm Viability Kit (Invitrogen Corp., USA) as specified, for 15 min. Pictures were taken and analyzed using fluorescent microscope Nikon H600L (Eclipse Ni) (Nikon Corp., Japan) using 20× objective, with subsequent overlay of images acquired with green and red filters.

3 Result and Discussion

3.1 Photophysical Studies

The absorption spectra of $ZnPcChol_8$, $(3\text{-}PyHp)_4BCBr_4$ and $(3\text{-}PyEBr)_4BCBr_4$ (Fig. 4) with a maximum wavelengths of about 677, 763 and 759 nm had a narrow band (34 nm for $ZnPcChol_8$, 28 nm for $(3\text{-}PyHp)_4BCBr_4$ and $(3\text{-}PyEBr)_4BCBr_4$).

The shape of the absorption spectra did not change with increasing concentration. The bathochromic shifted dimer band (Makarov et al. 2009) was absent in $ZnPcChol_8$ spectrum, as well as in $(3\text{-}PyEBr)_4BCBr_4$ and $(3\text{-}PyHp)_4BCBr$ spectra even at high concentrations. The absorption dependences on PS concentration was linear (Fig. 5). It agreed with extinction values determined at low concentrations. The experimental studies showed that the signs of aggregation in the absorption spectra behaviour of the studied octacationic phthalocyanine $ZnPcChol_8$ and tetracationic bacteriochlorin derivatives $(3\text{-}PyEBr)_4BCBr_4$ and $(3\text{-}PyHp)_4BCBr_4$ were not observed in the wide range of concentrations, up to 0.2 mM.

The results of PS absorption studying suggest that the degree of their aggregation at high concentrations was rather low.

As was shown in (Juzenas et al. 2004), the fluorescent properties of PS are more sensitive to aggregation than absorption properties. The fluorescence spectra of studied polycationic

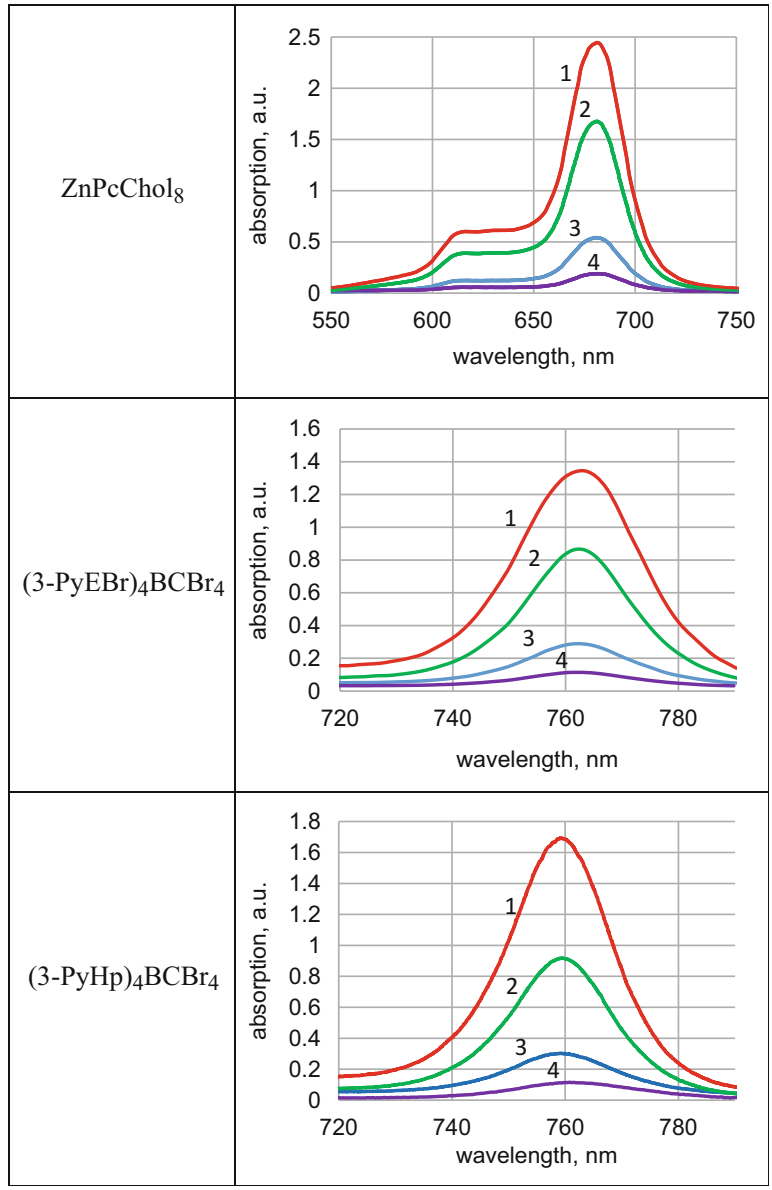

Fig. 4 The absorption spectra of $ZnPcChol_8$, $(3\text{-PyEBr})_4BCBr_4$ and $(3\text{-PyHp})_4BCBr_4$ at various concentrations: $ZnPcChol_8$: 1–0.15 mM; 2–0.1 mM; 3–0.03 mM; 4–0.01 mM. $(3\text{-PyEBr})_4BCBr_4$ and $(3\text{-PyHp})_4BCBr_4$: 1–0.2 mM; 2–0.1 mM; 3–0.03 mM; 4–0.01 mM

phthalocyanine and bacteriochlorin derivatives at concentrations of 0.005 mM are shown in Fig. 6.

The studied PS had a significant (>40%) overlap of the absorption and fluorescence spectral contours. Therefore, the process of reabsorption could affect their fluorescent properties at high concentrations (Fonin et al. 2014). So, fluorescent properties (the shape of the spectral contour of the fluorescence band, sublinear dependence of fluorescence intensity on concentration as well as value of the excited-state lifetime) at high concentrations could be caused by reabsorption, as well as aggregation (Dhami et al. 1995; Changenet-Barret et al. 2013). However, reabsorption, as a rule, did not affect the photodynamic efficiency, in contrast to aggregation, which reduces the effectiveness of PDT (Moan 1984). Therefore, it was important to assess which of the

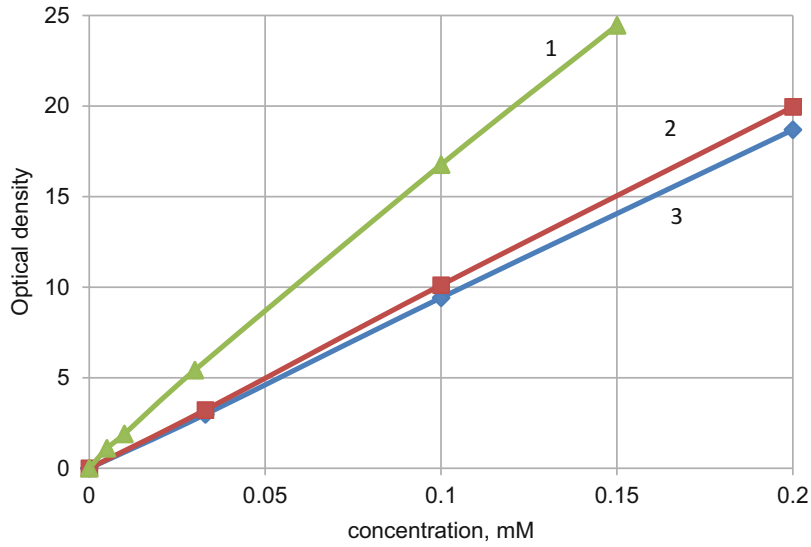

Fig. 5 The dependence of optical density of $ZnPcChol_8$ and $(3\text{-PyEBr})_4BCBr_4$ aqueous solutions (1,2), and $(3\text{-PyHp})_4BCBr_4$ in Kolliphor ELP nanodispersion (3), on their concentration

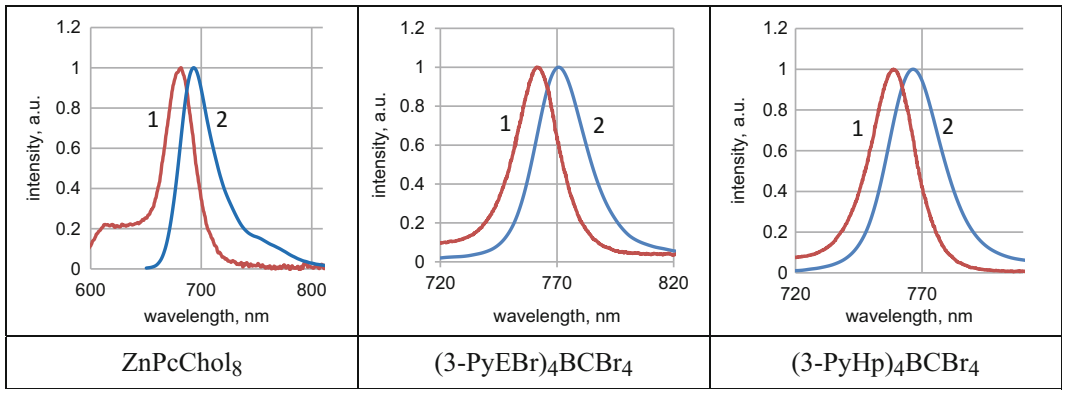

Fig. 6 The spectral contours of absorption (1) and fluorescence (2) bands of the studied PS at a low (0.005 mM) concentration

two phenomena dominates in the studied PS, especially at high concentrations. The signs of reabsorption in spectral-fluorescent studies, in contrast to the signs of aggregation, depended on the length of the cell (Juzenas et al. 2004). Therefore, spectral-fluorescent studies were carried out in cells 1 mm and 10 mm in length to separate the contributions of these phenomena and to evaluate the degree of aggregation of studied PS.

The shapes of the fluorescence spectra of $ZnPcChol_8$, $(3\text{-PyEBr})_4BCBr_4$ and $(3\text{-PyHp})_4BCBr_4$ at different concentrations in cells of various length are shown in Fig. 7.

Analysis of the spectra $ZnPcChol_8$ showed that an increase of cell length from 1 to 10 mm at low (0.005 mM) PS concentration does not affect the spectral shape (Fig. 8, A, spectra 1,2). This led only to a slight (1.8 nm) shift of the spectral maximum due to reabsorption, the fluorescence band remained narrow. At high concentrations (0.05 mM), a significant long-wavelength shift was observed, which depended on the length of the cell (5.8 nm – in a 1 mm long cell and 8.2 nm in a 10 mm cell). The shape of the spectra remained approximately the same. However, the half-width of the fluorescence

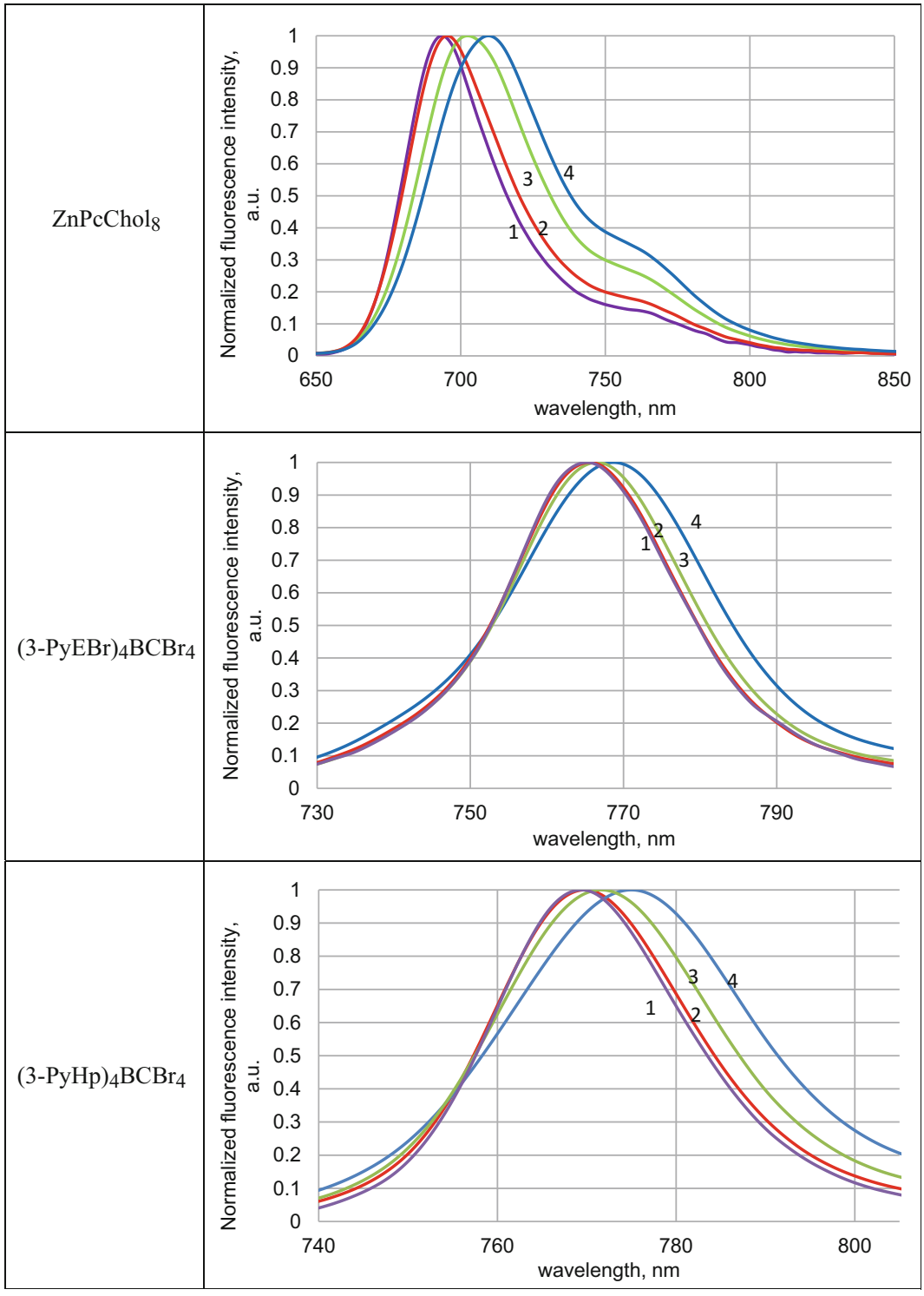

Fig. 7 The normalized fluorescence intensity (the shapes of the fluorescence spectra) of $ZnPcChol_8$, $(3\text{-PyEBr})_4BCBr_4$ and $(3\text{-PyHp})_4BCBr_4$ in cells of different lengths (1,3–1 mm; 2,4–10 mm) with concentrations: 1, 2–0.005 mM; 3,4–0.05 mM

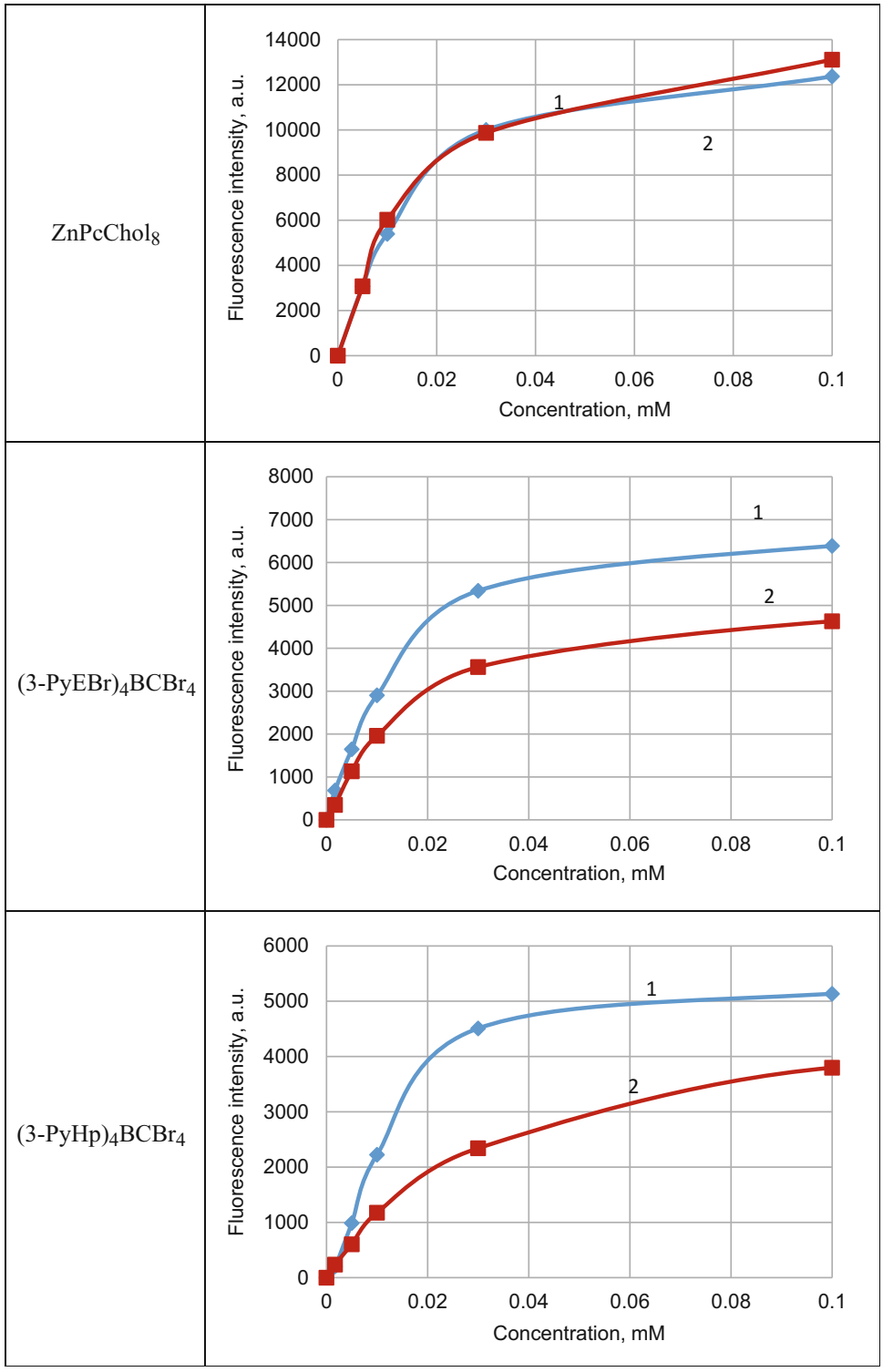

Fig. 8 The dependence of integral fluorescence intensity of $ZnPcChol_8$, $(3\text{-}PyEBr)_4BCBr_4$ and $(3\text{-}PyHp)_4BCBr_4$ on their concentration: (1) in water; (2) in serum

band increased: in the 1 mm cell – by 6.7 nm, and in the 10 mm cell – by 8.2 nm.

The analysis of the $(3\text{-PyEBr})_4BCBr_4$ and $(3\text{-PyHp})_4BCBr_4$ spectra showed that an increase of cell length from 1 to 10 mm at low (0.005 mM) PS concentration did not affect the spectral shape. This led only to a slight (0.3–0.4 nm) shift of the spectral maximum due to reabsorption, the fluorescence band remained narrow (27 nm). At high concentrations (0.05 mM, approximately corresponding to the PS concentration in blood 1 h after intravenous administration), a long-wavelength shift was observed, which depended on the length of the cell (at 2.2 nm – in a 1 mm long cell and at 5.6 nm in a 10 mm long cell). The shape of the spectra remained approximately the same. However, the half-width of the fluorescence band increased: in the 1 mm cell – by 3.3 nm, and in the 10 mm cell – by 6.9 nm.

This suggests that the observed phenomena at a high concentration are mainly associated with reabsorption and a weak aggregation (Dhami et al. 1995; Changenet-Barret et al. 2013; Juzenas et al. 2004).

The observed changes for $(3\text{-PyHp})_4BCBr_4$ were less than for $(3\text{-PyEBr})_4BCBr_4$. A long-wavelength shift of the spectral maximum of the fluorescence band occurs due to reabsorption at high concentrations (0.05 mM). The displacement depends on the length of cell (at 1.5 nm in a 1 mm cell and 3.4 nm in a 10 mm cell). The half-width of the fluorescence band in the 1 mm cell increased by 1.1 nm, and in the 10 mm cell – by 4.3 nm. The increase was much less than for $(3\text{-PyEBr})_4BCBr_4$. So, the observed changes occurring in $(3\text{-PyHp})_4BCBr_4$ at a high concentration are mainly associated with reabsorption; a weak aggregation takes place too but with a significantly smaller contribution (since the observed broadening is smaller).

It is known that aggregation of photosensitizers is lower in serum than in water (Tominaga et al. 1997). Therefore, some spectral fluorescence studies were carried out both in water and serum.

In the $ZnPcChol_8$ studies, the presence of two components in the solution was assumed: a monomeric PS with a lifetime in the range of 2.4–2.9 ns, and the second fluorescent component, possibly fluorescent dimer, with a significantly shorter lifetime (0.4–0.8 ns). In water, the share of the component with long lifetime, estimated by the number of photons with this lifetime among the total number of fluorescent photons, is about 94%. This allowed us to conclude that $ZnPcChol_8$ in the water does not aggregate in the studied range.

In the studies of $(3\text{-PyEBr})_4BCBr_4$ the presence of two components in the solution was also assumed: a monomeric PS with a lifetime in the range of 1.9–2.9 ns and its fluorescent dimer with a significantly shorter lifetime (<1 ns). In water, the share of the dominant component was about 74%. In serum, the lifetime was 2.4 ns; the share of the dominant component was higher (about 80–85% in the range below 0.1 mM). This correlated with the data (Tominaga et al. 1997) that in the blood plasma the aggregation of tetrapyrroles was lower in comparison with aggregation in water.

In the studies of the nanostructured $(3\text{-PyHp})_4BCBr_4$ fluorescence in water, the component with an average lifetime of 2.8 ns was dominant; its share was approximately 86%. In serum, the dominant component with an average lifetime of about 2.9 ns was almost 100%. This result was in good agreement with the data (Usacheva et al. 2001) that nanostructured PSs aggregate less in the aqueous environment at high concentrations.

Thus, the results of the fluorescence lifetime study also confirm that the aggregation of studied cationic PS at high concentrations (up to 0.1 mM) was low, especially in serum.

The results of integral fluorescence intensity study of $(3\text{-PyEBr})_4BCBr_4$ and $(3\text{-PyHp})_4BCBr_4$ depending on their concentration in water (1) and serum (2) are shown in Fig. 8.

The dependence of the fluorescence intensity of the $ZnPcChol_8$ on its concentration in water (1) was close to linear up to 0.03 mM; at higher concentration it became sublinear. The fluorescence intensity in serum was approximately the same as in water. The absence of aggregation in the selected concentration range was indirectly confirmed by the fact that, in studies in serum, there was practically no change in the shape of the fluorescence spectral band or an increase in its intensity.

The dependences of the integral fluorescence intensity of the $(3\text{-PyHp})_4BCBr_4$ nanodispersion as well as $(3\text{-PyEBr})_4BCBr_4$ solution on the concentration of PS were close to linear up to 0.03 mM in blood serum. At higher concentration they became sublinear. The same tendencies were observed for $(3\text{-PyHp})_4BCBr_4$ and $(3\text{-PyEBr})_4BCBr_4$ in water; their fluorescence intensity in water were lower in comparison with the fluorescence intensity in serum by approximately 1.3–1.4 and 1.5–2 times, respectively.

These results also suggest that in the most important for the APDT concentration range of <0.1 mM there were signs of both reabsorption and weak aggregation (aggregation is indicated by a lower fluorescence intensity in water compared to serum).

Thus, the results of spectral-fluorescence studies of the $(3\text{-PyHp})_4BCBr_4$ and $(3\text{-PyEBr})_4BCBr_4$ in water and serum confirmed the assumption of a low degree of their aggregation at high concentrations (Tominaga et al. 1997). Consequently, in the studied concentration range of these PSs, their photodynamic efficiency was high. This makes it possible to use these PS at high concentrations to sensitize pathological foci for APDT.

3.2 Light Absorption by Pigments in Non-sensitized P. aeruginosa Biofilm

P. aeruginosa bacteria produce pigments during biofilm growth, so culture medium becomes coloured. Analysis of spectral absorbance of culture medium of two clinical isolates revealed that the absorption of their pigments (presumably, pyocyanin) during the growth of a biofilm was practically negligible 5 h after the start of growth (data not shown). By hours 24 (by day 1), an intense band appeared in the absorption spectrum with a maximum at 690–700 nm, with a half-width of about 60 nm. The growth of this band continued for at least 3 days, its intensity depended on the strain of *P. aeruginosa*. The absorption spectra of pigments in the culture medium of two clinical isolates of *P. aeruginosa* 32 (A) and *P. aeruginosa* 21 (B) are shown in Fig. 9.

The optical density of pigments in culture medium of *P. aeruginosa 32* biofilms grown in LB broth reached high values at 690 nm (up to 1.8), which is close to the optical density of a variety of PS at effective concentration which are excited in this spectral region (in particular, $ZnPcChol_8$). It should be borne in mind that the concentration of pyocyanin in the spectrum of wound discharge during *P. aeruginosa* growth in the wound is much higher compared with biofilm growth in LB broth (El-Fouly et al. 2015).

4 Antibacterial Efficiency of the Studied Cationic PS

To assess the antibacterial efficiency of the studied cationic PS, viability of planktonic bacteria after photodynamic action was estimated under standard conditions (time of incubation of bacteria with PS was 30 min, light dose density of 20 J/cm^2). Dependence of viable bacteria amount on PS concentration is shown in Fig. 10. All photosensitisers tested had high efficiency against planktonic bacteria *P. aeruginosa* 32, which were completely inactivated after incubation with low concentrations of PS and irradiation: MBC of $(3\text{-PyEBr})_4BCBr_4$–2 μM, $(3\text{-PyHp})_4BCBr_4$–4 μM, $ZnPcChol_8$–5 μM). $(3\text{-PyEBr})_4BCBr_4$ had the lowest molecular weight (1374 Da), $ZnPcChol_8$ – the highest molecular weight among the studied PS (1678 Da). We suppose that smaller molecules have higher chance of penetration into the bacteria and this is a reason for the high efficacy of these PS. As was shown in (Usacheva et al. 2001), if PS is better at penetrating into the bacterial cell, the effectiveness of cell inactivation is higher. The high antibacterial efficacy of the studied PS is due to the fact that they are effectively associated with Gram-negative bacteria *P. aeruginosa* due to the large (4–8) number of cationic substituents and do not aggregate even at high concentrations.

These PS had significantly lower MBC for planktonic Gram-negative bacteria *P. aeruginosa* as compared with MBC of PS (>6 μM) proposed and investigated before (Meerovich et al. 2016; Tiganova et al. 2017).

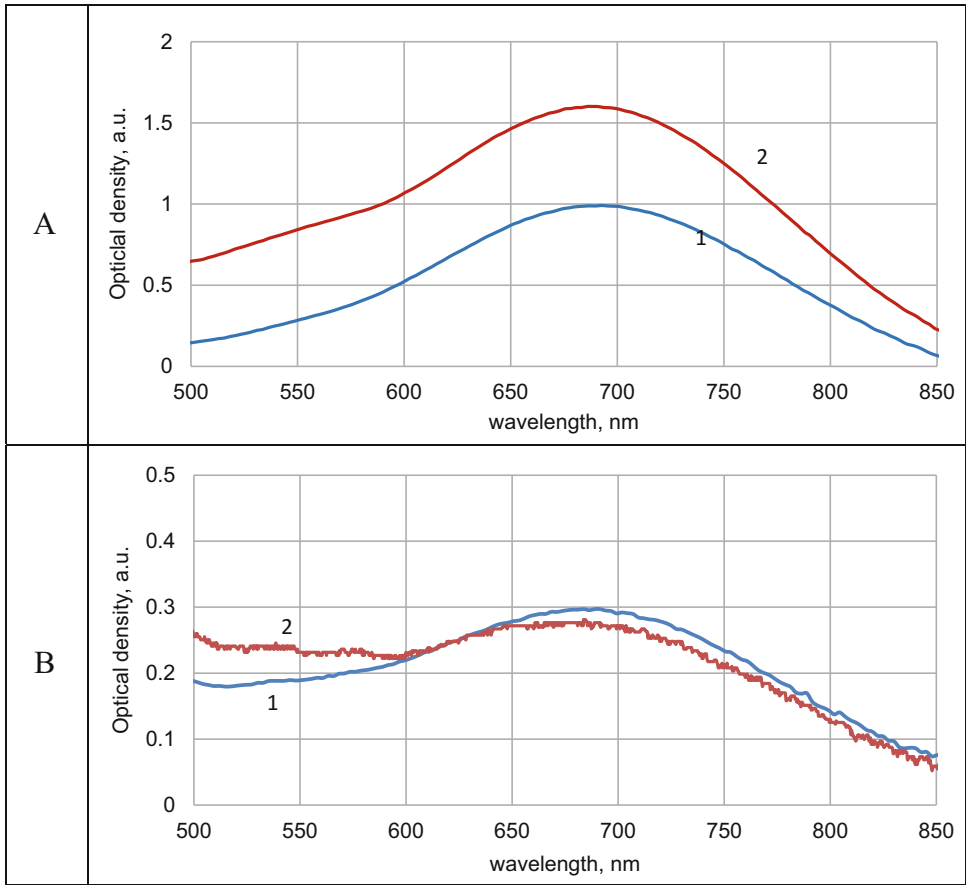

Fig. 9 The absorption spectra of pigments in the culture medium during biofilm growth of clinical isolates of *P. aeruginosa* 32 (**a**) and *P. aeruginosa* 21 (**b**): (1) by 24 h (by day 1) after the start of growth); (2) by 72 h (by day 3) after the start of growth

4.1 Photodynamic Inactivation of Bacterial Biofilms

Bacteria in biofilms are less susceptible to different influences, including PDI, so, higher concentrations of PS, a longer time of sensitization of biofilms and more light energy are need for their substantial inactivation.

The results of photodynamic inactivation of *P. aeruginosa 32* biofilms after 1 h incubation with the studied PS and irradiation with light dose of 100 J/cm^2 are shown in Fig. 11.

Inactivation of bacteria in biofilms depends on PS concentration during incubation and reached about 5 orders of magnitude with $(3\text{-PyEBr})_4\text{BCBr}_4$ and ZnPcChol_8. $(3\text{-PyEBr})_4\text{BCBr}_4$ had higher efficacy of photodynamic inactivation of *P. aeruginosa* biofilms compared with other studied photosensitizers. We suppose that smaller molecules of $(3\text{-PyEBr})_4\text{BCBr}_4$ have higher chance of penetration into the *P. aeruginosa* bacteria and more effectively diffuse through the matrix of the biofilm. Light loss for the excitation of this PS is less compared to ZnPcChol_8, since its excitation is carried out at 760 nm. These are the reasons for the high efficacy of $(3\text{-PyEBr})_4\text{BCBr}_4$.

The effectiveness of the *P. aeruginosa* bacteria inactivation in biofilms using ZnPcChol_8 was lower, since the molecules of ZnPcChol_8 have a molecular mass of 1678 Da and a lower probability of penetration into bacteria, and they are

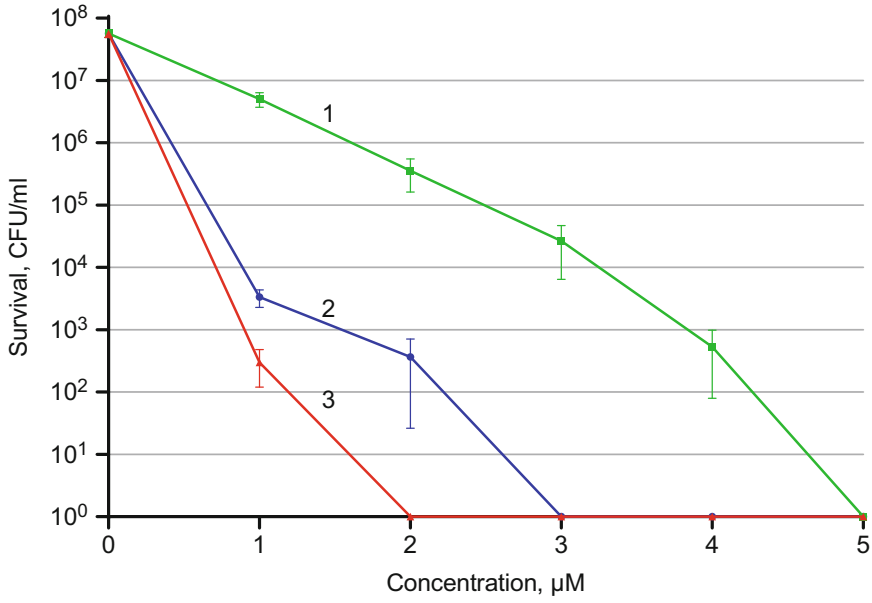

Fig. 10 Photoinactivation of planktonic bacteria *P. aeruginosa 32* depending of PS concentration: 1 – $ZnPcChol_8$; 2 – $(3\text{-}PyHp)_4BCBr_4$; 3 – $(3\text{-}PyEBr)_4BCBr_4$

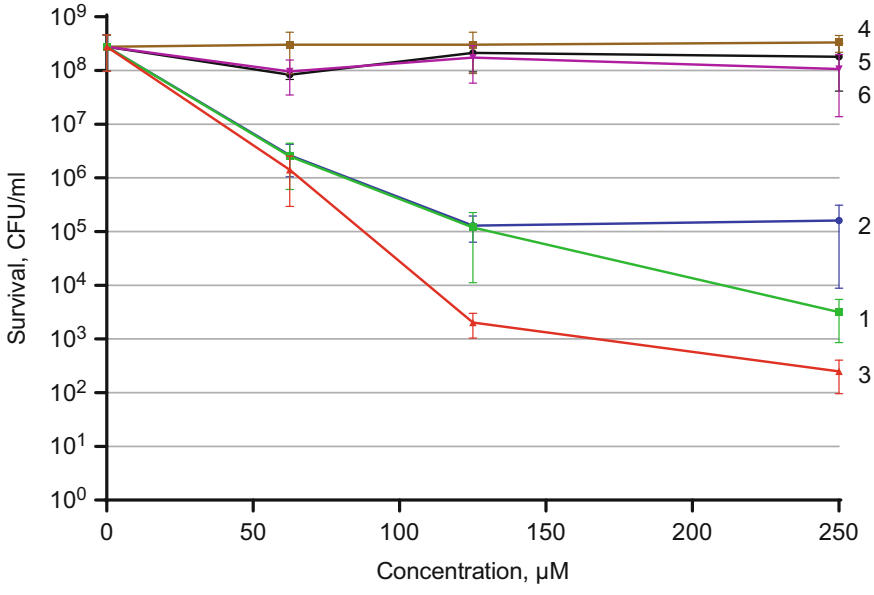

Fig. 11 Survival of *P. aeruginosa* in biofilms after PDI depending of PS concentration: 1, 2, 3 – after irradiation; 4, 5, 6-control without irradiation; 1, 4 – $ZnPcChol_8$; 2, 5 – $(3\text{-}PyHp)_4BCBr_4$; 3, 6 – $(3\text{-}PyEBr)_4BCBr_4$

excited in the spectral range of 680 nm, where the loss of exciting light due to the absorption of pigments in a biofilm is much higher.

The lower efficiency $(3\text{-}PyHp)_4BCBr_4$ in Kolliphor nanodispersion can be associated with a slower diffusion rate of Kolliphor nanoparticles through the matrix of biofilms.

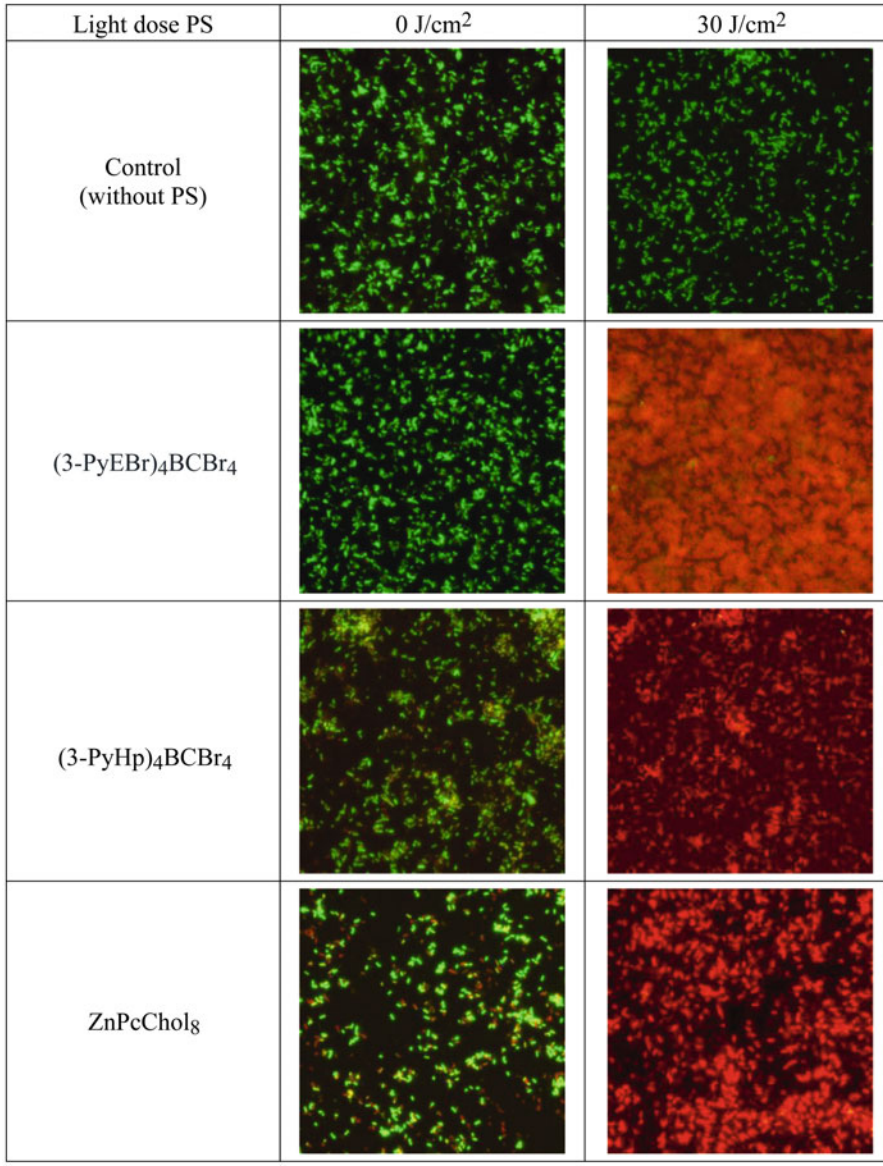

Fig. 12 The fluorescent microscopy images of *P. aeruginosa* biofilm before (left) and after (right) PDI using studied cationic PS

Fluorescent microscopy of biofilms after PDI and Live/Dead staining visualized the death of bacteria, or increasing of permeability of membranes, which is the first step of cell damage by reactive oxygen species after PDI. Damaged cells had red fluorescence due to staining with propidium iodide, which cannot enter the cells with intact membranes. We used a young biofilm because a mature one contains a large amount of extracellular DNA, which is also stained by propidium iodide and interferes visualization of bacteria. The results of fluorescent microscopy studies of *P. aeruginosa* 32 biofilms with studied PS and irradiation at 30 J/cm^2 are shown in Fig. 12. Live/Dead staining was not quantitative, but these pictures confirm that PDI using studied cationic PS provided effective inactivation of *P. aeruginosa* bacteria in biofilms.

5 Conclusion

Novel photosensitizers based on the derivatives of polyacationic phthalocyanines and synthetic bacteriochlorins have a high efficiency in photodynamic inactivation of Gram-negative bacteria *P. aeruginosa* as well as their biofilms *in vitro*. Tetracationic hydrophilic photosensitizer $(3\text{-PyEBr})_4\text{BCBr}_4$ with reduced molecular mass has higher efficacy of photodynamic inactivation of *P. aeruginosa* biofilms compared with other studied PS.

Studying the photophysical properties of PS in a wide range of concentrations demonstrated a low aggregation of these derivatives in water and serum. Consequently, their high photodynamic efficiency may be expected in in animal models enabling the use of these PS at high concentrations to sensitize pathological foci for APDT.

Some clinical isolates of *P. aeruginosa* produce pigments (in particular, pyocianine) in substantial amounts, which can significantly diminish light energy if excitation of PS occurs near 700 nm, thus protecting the bacteria from PDI. Photodynamic treatment using new bacteriochlorins with excitation at 760 nm, studied in this work, can overcome this difficulty; it facilitates the possibility of treating deep infected lesions *in vivo*.

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Graphene Oxide Coatings as Tools to Prevent Microbial Biofilm Formation on Medical Device

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Abstract

The clinical challenge on surface engineering of medical devices to prevent microorganisms adhesion and biofilm formation, has become an essential aspect for medical implants. Antibacterial properties of Graphene Oxide (GO) have been demonstrated across a broad spectrum of bacteria, and the different mechanisms of action with which this nanomaterial interacts with the microbial surface have been elucidated in detail. Innovative protective coatings based on graphene film and hydrogel could represent an innovative solution for the prevention of nosocomial pathogens colonization on implantable device. This brief review mainly focuses on the applications of graphene in nanomedicine with a particular deepening on the antibacterial properties of GO and GO-based nanomaterials. In order to evaluate the possible future applications of GO as an anti-biofilm coating material for medical devices, studies on the ability of graphene coated surface to prevent microbial adhesion are also discussed. A concise review on *in vitro* toxicity and *in vivo* safety is also presented.

Keywords

Anti-bacterial surface coating · Drug resistance · Graphene oxide · Healthcare · Medical device

1 Introduction

Health care-associated infections (HAI) represent a major public health threat in hospital setting. According to World Health Organisation (WHO), the definition of HAI is: "Health care-associated infections, or "nosocomial" and "hospital" infections, affect patients in a hospital or other health-care facility, and are not present or incubating at the time of admission. They also include infections acquired by patients in the hospital or facility but appearing after discharge and occupational infections among staff" (Boev and Kiss 2016). They are considered the sixth leading cause of death, with an incidence from 4% to 10% (Cloutier et al. 2015). Medical devices, such as

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catheters, cardiac pacemakers, joint prosthesis, prosthetic heart valves and dentures, represent one of principal source of nosocomial infection (Percival et al. 2015; Sabir et al. 2017; Boisvert et al. 2016). Microorganisms are able to adhere to biotic and abiotic surfaces, producing biofilm, composed of cells embedded in a self-produced matrix of extra-cellular polymeric substances (EPS). The main components of EPS are proteins, polysaccharides and extracellular DNA (Del Pozo et al. 2018; Ciofu et al. 2017). The biofilm formation proceeds through distinct stages, in the last stage the cells detach from the matrix and disseminate. Biofilm allows microorganisms to survive long period on the surfaces as they are in a starvation state, with low nutrient need (Kumar et al. 2017); therefore, the cells in biofilm are more resistant to the host immune response and to antimicrobial therapies making these infections hard to be treated, as antibiotics usually act against planktonic cells that are actively reproducing.

The main microorganisms isolated from medical devices health-care associated infections are gram-positive and gram-negative bacteria, such as *Staphylococcus aureus*, *Enterococcus faecalis, Escherichia coli*, *Pseudomonas aeruginosa, Klebsiella pneumoniae, Acinetobacter baumanii* and yeasts, particularly, *Candida* species (Sohail et al. 2018; Jain et al. 2019; Alcántar-Curiel et al. 2018; Touil et al. 2018; Percival et al. 2015; Sabir et al. 2017).

Research focuses on the development of new coating material that can prevent the adhesion and subsequent biofilm formation of bacteria and yeast on medical devices (Francolini et al. 2017, Cyphert and von Recum 2017, Swartjes et al. 2015). In recent years, the use of nanotechnologies has become an interesting approach to prevent: the use of metal or carbon nanoparticles appears to be simple, safe, not expensive and it is possible to overcome the problem of the antibiotic resistance (Polívková et al. 2017; Karahan 2018). Several antimicrobial nanoparticles have been identified, like iron oxide, zinc oxide, copper oxide but till now only silver nanoparticle (AgNps) is currently under clinical trial for evaluation as antimicrobials (Bao et al. 2011; Allahverdiyev et al. 2011; Srividya et al. 2017; Karahan 2018). Therefore, side-effects, such as AgNps accumulation in the tissue and subsequent inflammation that can be toxic for human body, have been reported (Karahan 2018).

Among the carbon nanomaterial, graphene is a two-dimensional ultra-thin nanomaterial composed only of hybridized-sp^2 carbon atoms, arranged in a hexagonal structure. Andrej Gejm and Konstantin Novosëlov, from the University of Manchester, synthetized graphene for the first time in 2004 (Novoselov et al. 2004). It is the first two-dimensional crystalline material ever produced. This material presents several physical properties: stretchability, electrical conductivity, huge surface area and high thermal conductivity (Palmieri et al. 2016; Karahan 2018; Compton and Nguyen 2010).

Since the synthesis, several derivatives have been studied, such as Graphene Oxide (GO) and reduced GO (rGO). Particularly, GO is a precursor of large-scale synthesis of graphene and it is prepared by the oxidation of graphite, making a single monomolecular layer of graphite heavily oxygenated, bearing hydroxyl and epoxide functional groups on their basal planes, in addition to carbonyl and carboxyl groups located at the sheet edges. The presence of these functional groups makes GO sheets strongly hydrophilic (Zhu 2010; Dreyer 2010).

Numerous are the potential applications of GO in biomedicine, such as biological and molecular imaging, drug/gene delivery, cancer therapy, tissue scaffold and antibacterial agent (Yousefi et al. 2017; Depana et al. 2011; Yao et al. 2012; Xia et al. 2019; Pulingam et al. 2019).

In this report, we provide a review of the studies conducted on the antimicrobial activity of GO, especially on the ability to inhibit the microorganism adhesion and biofilm formation on solid phase graphene oxide.

2 GO Antimicrobial Properties

The antimicrobial activity of GO has been widely reported, even if most of the studies focus on the activity of GO in solution while only few reported

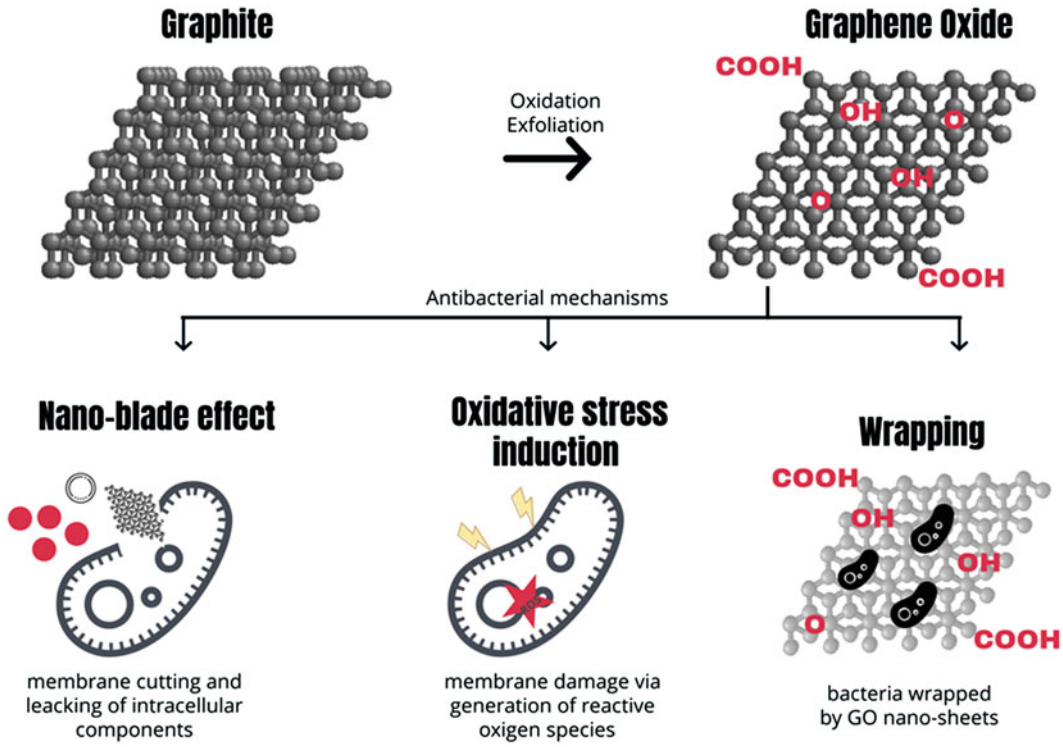

Fig. 1 Schematic representation of the three different GO antibacterial mechanisms of action

the activity of GO as coating material in solid phase (Zou 2016; Yousefi et al. 2017; Krishnamoorthy 2012).

Three main GO antimicrobial activities (Mangadlao 2015), are reported and represented in Fig. 1:

1. GO sheets sharp edges can physically interfere with microorganism by cutting the membrane with subsequent intracellular leakage of cytoplasmic constituent and death of the microorganism; this mechanism is called nano-knife or nano-blade effect (Akhavan and Ghaderi 2010; Pham et al. 2015; Castrillón 2015);
2. GO can induce oxidative stress, this phenomenon is caused by a chemical reduction of graphene sheets by bacteria (Gurunathan 2012; Salas et al. 2010; Liu et al. 2011)
3. GO can wrap and isolate microorganisms from the environment so that they cannot find nutrition, stopping proliferation; this mechanism has been mainly observed in solution phase rather than in GO coated surfaces (Palmieri et al. 2017; Perreault et al. 2015).

It has been reported how different solvents, the method used to prepare the graphene oxide, the size, the dispersion state and other factors influence the antimicrobial activity of GO.

In a study published in 2017, Palmieri et al. investigated the activity of GO in different solvents, such as sodium chloride (NaCl), magnesium chloride ($MgCl_2$), ultra-pure water and Phosphate-buffered saline (PBS) and in the growth media LB (Luria Bertani broth). GO was used at concentrations ranging from 3 up to 200 μg/mL. After GO incubation with *Escherichia coli* and *Staphylococcus aureus* the antimicrobial effect of GO was assessed by the colony counting method. This work clearly shows how the solution and the different GO concentrations affect the antibacterial properties:

in water, for example, GO is highly stable, presenting a homogenous solution and the main mechanism is nano-blade, cutting the bacteria membranes. In the solution containing electrolytes, the GO quickly aggregates, and the main antibacterial effect is the bacteria wrapping. Interestingly, when GO is dispersed in nutrient media such as LB, it creates aggregates that cannot be resuspended by vortex. The antimicrobial effect was visible in a concentration-dependent manner after 5 h incubation but disappeared after 24 h, when the number of bacteria saturated the GO available in solution (Palmieri et al. 2017). In addition to the antimicrobial properties that GO possess in solution, GO has also been extensively studied for its ability to inhibit microbial adhesion and biofilm formation.

3 Antimicrobial Activity of GO-Coated Surfaces

GO material can be used to coat surfaces, to obtain anti-adhesion and anti-biofilm activity either alone or in combination with other substances.

Different methods have been used to prepared GO-based coating, such as spin coating and electrospinning, vacuum filtration, electroless plating, phase inversion, electrophoretic deposition, hydrogel self-assembly/crosslinking, wet chemical reduction, solvent evaporation and solutions casting (Hu et al. 2010; Duan et al. 2015; Liu et al. 2014; Kumar et al. 2016; Carpio et al. 2012; Nine et al. 2015).

Preliminary studies based on GO-coated surfaces were conducted in 2010. Hu and colleagues created GO and rGO paper by vacuum filtration and tested it against *E. coli*. They either measured the metabolic activities of *E.coli* in the presence of GO nano-sheets *via* a luciferase-based ATP assay and the viability, counting the colony forming unit. Data collected demonstrated a decreased metabolic activity and viability of the bacteria after the interaction with GO paper (almost 85% of viability loss after 2 h incubation using 85 μg/ml GO) while the rGO paper was less effective against bacteria (Hu et al. 2010). The authors further analysed the biocompatibility of GO. Trasmission Electron Microscopy (TEM) analysis suggests that GO can be internalized within A549 cells *via* endocytosis. The biocompatibility assays showed that GO nanosheets at 20 g/mL exhibited no cytotoxicity to A549 cells after 2 h incubation and a slight decrease in cell viability after 24 h. GO nanosheets at higher concentration (85 g/mL) displayed an increased cytotoxicity (50%) within 24 h.

Another study (Akhavan and Ghaderi 2010), focuses on Go and rGO surfaces formed by electrophoretic deposition on stainless steel substrates. A mechanism of membrane disruption has been reported also in this case, but rGO appeared to be more active than GO. The better antibacterial activity of the rGO was motivated by its more sharpened and charged edges with a consequent major bacterial cell membrane damage caused by direct contact. The authors claim that the Gram-negative *E.coli*, characterized by an outer membrane was more resistant to membrane impairment caused by the rGO than the Gram-positive *S. aureus*. In 2015, Perrault et al. explored the different antimicrobial mechanism of GO nano-sheet both in suspension and on GO-coated surfaces, analysing the impact of nano-sheet size on antimicrobial activity. They demonstrated that, on GO-coated surfaces, the higher antimicrobial activity is exploited on GO-smaller sheets size, through a mechanism of bacterial cells oxidation, especially when sizes of 0.10 and 0.01 μm^2 are tested, obtaining 50% and 30% of cell viability, respectively. The main antibacterial effect is due to the bacterial cell oxidation, confirmed by the author by measuring the percentage of glutathione (GSH) oxidation that increases when the sheets size is decreasing. In suspension, the higher antimicrobial activity was obtained using larger GO sheets: in particular, the number of viable bacteria is 0.5% when 0,65 μm^2 sheets size are used. The GO sheet area inhibited bacterial development by a cell entrapment mechanism, so the largest sheets are more able to wrap the bacteria (Perreault et al. 2015). A paper, published in 2017, highlights the importance of the orientation of the GO sheets for its antimicrobial

activity. The antibacterial activity of GO films with different alignments of the sheets was measured using *E. coli* as a model. Bacteria were deposited for 3 h on the HEMA-GO (Polyhydroxyethylmethacrylate-GO) film. The vertically aligned sharp edges have enhanced antibacterial activity, showing decreased cell viability (56%) compared to random aligned (75.3%) and planar aligned sheets (81.8%) (Lu 2017). Authors showed that either a mechanism of membrane perturbation and oxidative stress contributed to the anti-bacterial activity of the film.

Another way to obtain GO coated surfaces is to mix with hydrogel to obtain a uniform and stable antimicrobial surface. In a paper published in Papi et al. 2016, Papi et al. described a hydrogel made of GO and agar and its activity against *S. aureus*, *E. coli*, and the yeast *Candida albicans*. Particularly, they used the laser induced super cavitation technology to reproduce on the surface of the GO-hydrogel the natural antimicrobial pattern of the *Cancer Pagurus.* This formulation showed anti-microbial activity due to the GO blade effect already described and the anti-adhesive pattern reproduced on the surface of the GO hydrogel (Papi et al. 2016).

3.1 Inhibition of Microbial Adhesion and Biofilm Formation on GO-Coated Surface

As already described, biofilm related infections represent a crucial point in clinical setting (Singhai 2012).

Anti-biofilm activity of graphene oxide has been described in the last few years. Di Giulio et al., investigated the anti-biofilm activity of GO against the principal wounds pathogens: *P. aeruginosa*, *S. aureus* and *C. albicans* (Di Giulio et al. 2018). They analysed the effect of GO in the inhibition biofilm formation and in the ability to interfere with mature biofilm. For the evaluation of the inhibition, microorganisms were put in contact with 50 mg/L of GO in 96 wells-flat bottom plate for 24 h and the biofilm biomass was quantified, reflecting a significant reduction in the wells treated with GO for the microorganisms under study; the greatest reduction was observed for *S.aureus*. GO appears to be able to act also in mature biofilm, possibly penetrating in the extra-cellular matrix of biofilm; indeed, author revealed a reduction of the biomass after the exposure of the mature biofilm to 50 mg/L of GO and a detachment of the cells from the biofilm. An interesting study was conducted by Zhang et al. 2018. The effect of GO on *P. aeruginosa* biofilm was evaluated after short (4 h) and long-term (7 days) exposure. GO at 3 different concentrations (10,20,40 mg/L) was used to evaluate the inhibition of biofilm formation. The bacteria were put in suspension with the different GO concentrations and then incubated in a 96-wells plate to evaluate the biofilm production. A biofilm biomass reduction, more evident at 40 mg/L concentration, was observed. The authors therefore decided to analyse the effect obtained after 7 days of exposure (20 mg/L GO), no differences compared to the control have been detected in biomass production, suggesting that GO may not have long-term effect. The effect of GO on quorum sensing (QS) of *P.aeruginosa,* implicated in the biofilm formation of this bacteria, have been therefore explored. No impact on the expression of QS-related genes has been detected after either short and long-term exposure but GO affects biofilm formation by absorbing QS signals or related products (such as proteases) in the short term exposure but the altered biofilm formation appears to be restored during the long-term treatment, as the level of proteases and other products increase and any-change detected in the short term exposure are not present after 7 days, indicating the need to analyse the effect on the biofilm in long period with further experiments.

Song et al. tried to determine the anti-biofilm mechanism of graphene oxide, analysing the GO activity against *E.coli* and *Bacillus subtilis* biofilm formation. Bacteria were inoculated in 24-wells plate using different concentrations of GO (0, 10, 20, 40, 80, 160 mg/L) for 48 h and the biofilm biomass quantified by crystal violet staining. They found that at low GO concentration (10 mg/ml) the biofilm formation was enhanced compared to the control, while at 20 and 40 mg/ml of GO no significant differences

have been detected; the biofilm biomass decreased substantially when high GO concentration (160 mg/ml) was tested. The authors speculated that, at low GO concentration, the cell not in contact with GO might utilize the released cytoplasmatic materials as nutrients for growth, promoting the biofilm formation. The amount of oxidised GSH was furthermore quantified. About 25% of GSH was oxidized in the presence of 10 mg/L GO, and the loss of oxidation increased with the increasing GO concentrations, indicating the oxidative stress one of the anti-biofilm mechanism of GO (Song et al. 2018).

Few studies reported the activity of GO coated surface against microorganism adhesion and biofilm formation. In 2017, Thampi and collaborators deposited GO on polycarbonate urethane (PCU) membrane by a simple method of electrospraying, creating a thin layer of GO surrounding the membrane (GOPCU). PCU is a widely used material for medical devices. They measured the adhesion of the Gram-positive *S. aureus* and the Gram-negative *Pseudomonas aeruginosa* to PCU and GOPCU surfaces, detecting, by plate count of viable bacterial colony, a reduction of adhesion of 85.5% and 63.5%, respectively. Authors generically attribute this antibacterial activity to various factors such as size (without actually giving precise information about it), or formation of reactive oxygen species (ROS) by surface functional groups, as already described by Perrault in 2015. GOPCU appears to be biocompatibility, as the hemocompatibility assay conducted showed no adhesion or aggregation of platelets on GOPCU and proliferation assay on fibroblast mammalian cells showed a good percentage of survival (Thampi et al. 2017).

In a paper published in 2017 (Yadav et al. 2017), GO coated surfaces where prepared using two different methods, the traditional Hummer's method and an improved method that improves the efficiency of the oxidation process providing a greater amount of hydrophilic oxidized graphene material. This result was achieved by excluding the $NaNO_3$ and increasing the amount of $KMnO_4$ regularly used for the traditional Hummer's method.

These two methods of graphene deposition, resulting in two different surfaces that differ for their nano-sheets size, morphology and exposition of functional groups. They examined the rate of biofilm formation of the bacteria *E. coli* and *S. aureus* either by crystal violet assay and microscopy imaging, revealing that both the GO formulations inhibit cell adhesion and biofilm formation.

The GO concentration and the different methods of deposition impact on the ability to inhibit biofilm formation in Gram-positive and Gram-negative bacteria, maybe due to the different cell wall, resulting in different interaction between the microorganism and the surface (Yadav et al. 2017). To explore the physical and chemical mechanism underlying the anti-bacterial activity of the GO surfaces tested, the oxidation of GSH was evaluated. The percentage loss of glutathione increased with the increase of GO concentration that led to ROS mediated oxidative stress.

In another study, GO was used to coat PVDF (Polyvinylidene fluoride) membrane by vacuum-filtration. Also in this study, the microorganisms taken as models are the Gram-positive *S. aureus* and the Gram-negative *E. coli.* Bacteria were incubated in contact with the surface for 2, 4 and 6 h. The bactericidal and anti-adhesive effect was evaluated by colony forming unit quantification, live/dead assay and the biofilm formation rate quantified by crystal violet assay (Farid et al. 2018) revealing low rate of survival and cell growth on GO surfaces. The bactericidal effect increases with the increasing time of incubation. Crystal violet quantification revealed inhibition of biofilm formation. The anti-biofilm activity displayed+ has been attributed by the authors to physiochemical properties of the surface: GO membrane has a hydrophilic surface compared to the PVDF membrane leading to a minor adsorption of substances released from the bacteria after exposure with GO that can represent nutrient source for bacteria. Moreover, the exposure of carboxylic functional groups of the surface determines a negatively-charge GO surface that can inhibit the initial adhesion of negative-charged *S.aureus* and *E.coli* due to electrostatic repulsion.

Biofilm was further monitored under continuous flow condition over 48 h. The lower biofilm formation formed on GO coated PVDF membrane compared to pristine PVDF was related mainly to the formation of a "wrinkled surface with sharp exposed nano-sheet edges which serve as nano-blades that strongly impair the cell's outer membranes by piercing or laceration. The thickness of biofilm formation on GO coated membrane was 56.5 μm in contrast to the thickness of the biofilm developed on the virgin PVDF membrane, 110,3 μm. Moreover, the stability of the biofilm was evaluated washing the membrane after measuring the thickness, that revealed to be high on the untreated membrane (106,2) and low on the GO treated membrane (4,8 μm).

The authors investigated whether the mechanical disruption or the chemical oxidation could be the mechanism responsible for GO bactericidal effect. To examine the physical destruction of the membranes, the intra-cytoplasmic material released upon membrane destruction has been quantified and the cell integrity analysed by TEM. Loss of membrane integrity and release of intracellular components confirmed the physical destruction of GO surfaces after interaction with GO. The GO-induced oxidative stress has been confirmed by measuring the increased intracellular level of ROS in *E.coli* and *S.aureus* following contact with GO surfaces. Authors speculate that the intracellular ROS induction is mainly attributed to the presence of oxygen-containing functional groups on the surface of GO (Farid et al. 2018).

3.2 GO Functionalization

GO can be combined with other substances either to enhance its antimicrobial activity or to improve the stability of the coating. Different studies evaluated the antimicrobial properties of GO mixed with nanoparticles, such as silver (Xie et al. 2017), zinc oxide (Jones et al. 2008; Chen et al. 2016), gold (Hussain et al. 2014), titanium dioxin (He et al. 2013) or with other polymers, such as chitosan (Konwar et al. 2016; Chen et al. 2013) or antibacterial substances like lysozyme (Bera et al. 2018) or curcumin (Bugli et al. 2018).

Silver nanoparticle is one of the most studied nanoparticles as it presents good features, such as broad antimicrobial activity, high resistance to oxidation and high-thermal conductivity (Yousefi et al. 2017, De Faria et al. 2014). In Zhao et al. 2018 Zhao et al., synthesize a polyethyleneimine (PEI)-modified and AgNP-decorated GO nanocomposite (GO–PEI–Ag) with a size of around 5 nm that displayed antimicrobial activity against Gram-negative *E.coli*, *Acinetobacter baumanii*, *Shigella sonnei*, the Gram-positive bacteria *S. aureus* and the fungi *Aspergillus fumigatus* and *C. albicans*. This composition can kill the adhered bacteria resulting in a complete biofilm formation inhibition. Cell membrane disruption and intra-cytoplasmic leakage appear to be the main inhibition mechanism. Moreover, this formulation showed long-term stability compared to GO-Ag and biocompatibility as HeLa cells treated for 8hs and 24 h using 10, 20 and 50 μg/mL of GO−PEI − Ag were 86.1, 81.4, and 80.2% of viability, respectively, for the 8 h group and 80.0, 77.9, and 72.0%, respectively, for the 24 h group (Zhao et al. 2018).

GO can be also combined with antibacterial substances that can enhance its efficacy in killing and inhibiting bacteria adhesion, such as lysozyme, one example is the work of Duan et al. They prepared a GO-lysozyme surface resulting in a effective activity against *Escherichia coli* (Duan et al. 2015).

In 2016, an antimicrobial film composed by chitosan-iron oxide coated GO has been realized; specifically, iron oxide coated GO nanomaterial was prepared by a modified co-precipitation method and, in a second step, chitosan hydrogel was incorporated thought hydrogen bonding and electrostatic interaction. The antimicrobial activity was compared to the individually synthesized chitosan GO and chitosan iron oxide hydrogel against methicillin-resistant *S. aureus* (MSRA), *S. aureus*, *E. coli* and *Candida albicans*; authors noticed an improved activity of chitosan-GO nanocomposite respect to the individual former type of films. This mixed GO appeared also to be biocompatible as the haemolysis test conducted on

human erythrocytes displayed low to mild haemolytic activity (0,37%) and, moreover, the cytotoxicity assay on L929 fibroblast cell line showed a much higher viability (80–93%) compared to the negative control. (Konwar et al. 2016).

Curcumin is a yellow–orange polyphenol compound that possesses a wide range of pharmacological activities due to its anti-inflammatory, anticarcinogenic, and anti-infectious properties. The major issue in curcumin-based therapies is the poor solubility of this hydrophobic compound and the cytotoxicity at high doses. Bugli et al. (2018) load the curcumin on the surface area of GO and demonstrated its effectiveness against *Staphylococcus aureus* MRSA. Curcumin (CU) is a hydrophobic compound, it is able to spontaneously absorb on GO surface by π-π stacking interactions, without the need of chemical agents, leading to the creation of a 'green' and safe antimicrobial solution. GO and curcumin showed a synergic effect as MIC of GO alone ranges from 2.35 to 18.75 μg/ml, MIC of curcumin alone against MRSA ranges from 125 to 256 μg/ml while the MIC of the GO-CU combination are between 1.06 and 2.8 μg/ml. Furthermore, this formulation has no cytotoxic effects on eukaryotic cells.

GO-CU nanocomposite was investigated by the same group (Palmieri et al. 2018) for its ability to inhibit biofilm formation of the fungus *C. albicans,* often isolated from medical devices related infections. While GO seems to not have effect in the adhesion of Candida cells on GO-coated plastic discs, GO-CU shows a partial inhibition of adhesion and poor biofilm formation of *C.albicans*. Furthermore, PEG (polyethylene glycol) was used to coat GO discs either alone or with curcumin. The GO-CU-PEG nanocomposite showed anti-adhesion property and the ability to act as drug release surface: CU is released in the media when the coating is in ddH_2O showing anti-fungal effect.

Frigols and collaborators recently presented a zinc alginate graphene-oxide film (Frigols et al. 2019) with strong anti-bacterial activity against *S. aureus* and *S. epidermidis* enhanced by the release of Zn^{2+} in the solution. Although the antibacterial properties of this innovative material can only be attributed to the presence of Zn^{2+}, GO stabilizes and promotes long-term ion release, confirming the potential drug-release technology of the graphene oxide-based coating.

Table 1 shows some studies focusing on the antimicrobial properties of surfaces covered with graphene with different coating methods and types of functionalization. Anti-biofilm activity tested microbial species and biocompatibility are also revised in the table.

4 Go-Based Antibacterial Materials Safety Profile in Mammals

With the perspective to promote the clinical use of GO, it is fundamental to evaluate the short- and long-term cytotoxicity profiles of GO towards eukaryotic cells. Despite the benefits that GO possesses for antibacterial applications, such as high surface area and exceptional solubility in aqueous environments, its biocompatibility is at the moment controversial. In the biological environment, GO-based nanomaterials with their peculiar physico-chemical surface are straight exposed to direct contact with cells and biological macromolecules. In recent years several publications (Zhang et al. 2016; Ou et al. 2016; Wang et al. 2011) have deepened the potential toxic effects of GO in mammalian cells and animals. Zhang B. and co-workers (Zhang et al. 2016) highlighted a widespread toxic effect of GO in mammalian systems due to cell membrane disruption and lysosomal and mitochondria dysfunction with massive ROS generation. GO toxicity was closely linked to three different parameters: GO concentration, lateral size and surface functionalization. These factors are, not surprisingly, the same ones that influence the antibacterial properties of GO (Ou et al. 2016).

4.1 GO Concentration

GO dose-dependent toxicity in cells lines and animals has been demonstrated in recent years

Table 1 Antimicrobial activity of GO

Material	Coated surface	Surface coating method	Microorganism tested	Biofilm inhibition	Biocompatibility	GO concentration tested	References
GO/rGO	Paper	Vacuum filtration	*E.coli*	none	Adenocarcinomic alveolar basal epithelial cells A549 cells (20 μg/ml)	20, 85 μg/ml	Hu et al. (2010)
GO/rGO	Stainless steel	Electrophoretic deposition	*E.coli* *S.aureus*	none	none	Not indicated	Akhavan and Ghaderi (2010)
PVK-GO	Indium tin oxide (ITO).	Electrophoretic deposition	*E.coli*	yes	none	1 mg/ml	Santos et al. (2011)
PLA/PU/ GO	Fibrous paper	Electrospinning	*E.coli* *S.aureus*	yes	Osteoblastic cell line MC3T3-E1 (GO: 3, 5%)	GO:3, 5%	An et al. (2013)
GO	Silicon dioxide (SiO_2)	Spin-coating	*Halomonas spp.*CAM2	yes	none	Not indicated	Parra et al. (2015)
GO-Lys	Water membrane	Phase inversion	*E.coli*	none	none	512, 256, 128, 64, 32 μg/ml	Duan et al. (2015)
GO	Mixed cellulose filter	Vacuum filtration	*E.coli* *S.aureus*	none	none	200 μg/ml	Perreault et al. (2015)
Ag-HAP-GO	Titanium	Electro-deposition	*E.coli, S.aureus*	none	PBMC	Not indicated	Janković et al. (2015)
GO	None –study in suspension	–	*E.coli* *S.aureus*	none	None	2 to 200 μg/ml	Palmieri et al. (2016)
Agar-GO	Plastic disc	Hydrogel self-assembly	*E.coli, S. aureus, C. albicans*	none	none	1 mg/ml	Papi et al. (2016)
GO	Polycarbonate urethane (PCU) membrane	Electrosprying	*S. aureus* *P.aeruginosa*	none	L-929 mammalian fibroblast cells	Not indicated	Thampi et al. (2017)
GO	96-well plate	Drop-casting and slow oven drying	*E.coli* *S.aureus*	yes	none	50, 200 μg/ml	Yadav et al. (2017)
GO-HEMA	Glass substrates	Magnetic fields-UV irradiation	*E.coli*	none	none	200 μg/ml	Lu et al. (2017)
GO	PVDF membrane	Vacuum-filtration	*E.coli* *S.aureus*	yes	none	100 μg/ml	Farid et al. (2018)

(continued)

Table 1 (continued)

Material	Coated surface	Surface coating method	Microorganism tested	Biofilm inhibition	Biocompatibility	GO concentration tested	References
GO–PEI–ag	None- in suspension	–	*E.coli* *S.aureus* *A.baumanii* *Shigella sonnei* *Candida albicans* *Aspergillus fumigatus*	Yes	Epitelian cervix adenocarcinoma cells HeLa cells (10,20,50 µg/ml)	1 to 128 µg/ml	Zhao et al. (2018)
Chitosan-iron oxide GO hydrogel	Hydrogel	Gel-casting Co-precipitation	*E.coli, S.aureus* *S.aureus MRSA* *C.albicans*	none	Human erythrocytes L929 fibroblastic cells	Not indicated	Konwar et al. (2016)
GO-CU	None- in suspension	_	*S.aureus MRSA*	none	3 T3 fibroblast cell line (GO: 50 µg/ml CU: 200,300 µg/ml)	GO:1 mg/ml CU: 100,200,300 µg/ml	Bugli et al. (2018)
GO-CU GO-PEG GO-CU-PEG	Plastic disc Central venous catheters	Drop-casting	*C.albicans*	yes	VERO kidney's epithelial monkey cell line (PEG 5%)	GO:1 mg/ml CU:2 mg/ml PEG:5%,10,20%	Bugli et al. (2018)
GO-Zn^{2+}-SA1 and SA2	Glass petri dish	Chemical route	*S.aures* *S.epidermidis* *MRSE*	no	Human keratinocyte HaCaT cells (1% diluited GO-Zn^{2+}-SA1 and SA2)	1%w/w GO	Frigols et al. (2019)

When available, the safe concentration of GO resulted in the biocompatibility test has been indicated ().Abbreviations: *GO* graphene oxide, *GO–PEI–Ag* polyethyleneimine (PEI)-modified and AgNP-decorated GO nanocomposite, *PVK* polyvinyl-N-carbazole, *PVDF* Polyvinylidene Difluoride, *HAP-GO* silver graphene oxide hydroxyapatite, *PBMC* peripheral blood mononuclear cells, *CU* curcumin, *PEG* polyethylene glyco, *lPLA\PU* Polylactic Acid/Polyurethane, *Lys* Lysozyme, *SA* sodium alginate, *MRSA* methicillin-resistant *Staphylococcus aureus*, *MRSE* methicillin-resistant *Staphylococcus epidermidis, Hema* Hydroxyethyl methacrylate

by numerous studies. The main toxic effects result in cell apoptosis, liver and kidney lesion and lung fibrosis. Wang et al. (Wang et al. 2011) analyzed GO cytotoxic effects on human dermal fibroblast cells (HDF). In this study, different concentration of GO, ranging from 10 to 100 μg/ml were exposed to HDF for 24 h. Significant increase in cytotoxicity was observed at 50 μg/ml with decreased cell survival rate and increased apoptosis. GO sheets that enter the cells were found in the cytoplasm and very few within the nucleus. The internalized GO was mainly distributed inside the lysosomes and mitochondria and the size of GO sheets was between 100 and 200 nm. Another study from Lammel and co-workers (Lammel et al. 2013) showed that the exposure of HepG2 cells to both GO and carboxylated graphene nanoplatelets (CXYG) displayed a dose and time dependent increase in ROS production. The predominating size distribution of GO and CXYG determined by means of dynamic light scattering was 385 and 1,110 nm respectively. Both graphene-based nanomaterials penetrate into the cell by impairing the membrane phospholipid bilayer. These data were recently confirmed by a study from Duan and collaborators (Duan et al. 2017) in which they demonstrated that graphene sheets, both pristine and GO with a lateral sizes ranging from 200 nm to 700 nm, caused the formation of pores in the cytoplasmic membranes of A549 and Raw264.7 cells, consequently reducing cell viability. Other studies confirm the fact that the amount of cellular uptake increases with the exposure time and increasing dose. (Wang et al. 2011, 2015; Lee et al. 2017). In *in vivo* studies, dose and surface modification are considered the main parameters that modulate graphene-based nanomaterials bio-distribution and toxicity. One of the most basic pre-clinical *in vivo* studies demonstrated that GO, with an average size of 200 nm, if injected *via* tail-vein in mice at a concentration of 0.25 mg/kg does not affect lifetime, while a high GO dose of 0.4 mg/kg showed chronic toxicity (Wang et al. 2011).

4.2 GO Lateral Size

GO lateral size may influence its cellular uptake and bio-distributions in mammalian organs.

Gurunathan and collaborators (Gurunathan et al. 2019) profiled a significant size and dose dependent toxicity of different sized GO nanosheets with an average size of 100 and 20 nm on two different germ cell lines. Smaller flakes resulted more cytotoxic in a dose depending manner showing higher cellular internalization with loss of cell viability and cell proliferation. A study from Ma et al. (2015) highlighted that large GO flakes showed evident binding with plasma membrane and minor phagocytosis, eliciting robust activation of NF-kB pathway while small GO sheets were highlighted much more internalized into cell cytoplasm. Liao and coworkers investigated the effects of graphene on human erythrocytes and shown that 350 nm-sized graphene could induce strong hemolysis compared to 3 μm-sized graphene sheets (Liao 2015). Drastic membrane disruption by nanoscale sized graphene could be related to the strong electrostatic interactions between the graphene surface and the lipid bilayer of the erythrocyte membrane. On the other hand, low toxicity of microscale sized graphene sheets may be attributed to their lower overall surface areas.

4.3 GO Surface Functionalization

Recent perspective strategies for future biological applications of GO-based nanomaterials include several surface functionalization strategies to improve their safety profile. GO owns a strong hydrophobic surface that allow the formation of a protein corona in biological environment, thus minimizing the physical interactions between GO and cell membranes and reducing cytotoxicity (Hu et al. 2011). This study evaluated the effect of fetal bovine serum (FBS), a common component in cell culture medium, on GO cytotoxicity. At 1% concentrations of FBS, GO showed concentration-dependent cytotoxicity.

Interestingly, the cytotoxicity of GO was much lower at 10% FBS, the concentration usually employed in cell culture medium.

The influences of surface charge have also been highlighted in GO-induced toxicities because the electrostatic repulsion between GO and non-phagocytes plays an important role in particle internalization (Ou et al. 2016). Also, in *in vivo* studies, bio-distribution of graphene nanomaterials is influenced by surface functionalization which in turn modulates their toxicological profiles. (Kanakia 2014). In a study by Yang et al. (Yang et al. 2011), radiolabeled PEG functionalized GO and rGO were administrated intragastrically to female BALB/c mice and bio-distribution. After 4 h of injection, was mainly observed in stomach and intestine, disappearing 1-day post feeding. The same study highlighted the bio-distribution of the same PEG functionalized GO and rGO after intra peritoneal administration. Conversely to oral administration, intraperitoneal injection resulted in high accumulation of graphene nanoparticles on liver and spleen, still evident 30 days after administration.

5 Conclusion

Preventing the bacterial colonization of biomedical devices is the key for limiting the spread of hospital-acquired infections. Antibacterial coatings have become a very active field of research, strongly stimulated by the urgent and increasing need to identify innovative strategies instead of using traditional antibiotics. This review focuses on the antibacterial properties of GO, analyzing the different mechanisms of action exerted to impair bacteria integrity. The antibacterial mechanisms of graphene oxide have still to be completely understood, but most of the current findings and advances support its antibacterial efficacy. Most of the studies agree on the importance of precise physicochemical parameters that most influence the GO bacteriolytic and bacteriostatic properties, like the GO size, the GO concentration and the quantity of hydroxyl groups on the surface. These characteristics impact on the different interaction of GO with the microbial surface: cutting bacteria membranes by the sharp edges, inducing oxidative stress and wrapping bacteria between large GO sheets. Several studies reported in this article focus on the functionalization of the GO with different compounds, some with known antimicrobial activities but not usable individually for intrinsic toxicity (Xie et al. 2017), others highly hydrophobic that can be delivered by GO nano sheets (Bugli et al. 2018). It is now largely accepted that bacteria can attach to solid substrates, in sessile structured communities called biofilms, where they can persist for long periods, acting as a pathogens reservoir. In this regard, the prospect of exploiting the antimicrobial properties of GO to coat medical devices and prevent microbial adhesion represents a challenge of great impact on human health. Key topic of this brief review focused on GO-coated surface ability to interfere with microbial adhesion. Data on this subject are still rather limited and deriving from different methods of GO deposition on as many different surfaces. Overall, the results are promising and demonstrate an effective capacity of GO-coated surfaces to interfere with microbial adhesion and biofilm formation. Plastics are the most widespread material used for medical devices construction, for weight, cost, and performance purposes. To translate results obtained from such a considerable number of studies, it is mandatory to standardize the best method of covering specific materials used for medical devices (e.g. polyethylene, polypropylene, polyvinyl chloride, polycarbonate), accurately assessing the stability and biocompatibility. Further, the present review offers a concise overview of research on biosafety of GO towards mammals, both *in vitro* and *in vivo*. Toxicity of GO and graphene family nanomaterials is deeply reviewed in current literature, but the research is still in its infancy and it is hard to conclude the potential risks to human health associated with the use of such innovative nanomaterials. As an extraordinary material with remarkable and unique properties, we are convinced that graphene will have a significant impact on many aspects of our life, ranging from general biotech up to biomedical

applications, like "anti-biofilm devices". The enormous increase in research and development of graphene will replace most of the materials on the market so far.

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Quinolines and Quinolones as Antibacterial, Antifungal, Anti-virulence, Antiviral and Anti-parasitic Agents

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Abstract

Infective diseases have become health threat of a global proportion due to appearance and spread of microorganisms resistant to majority of therapeutics currently used for their treatment. Therefore, there is a constant need for development of new antimicrobial agents, as well as novel therapeutic strategies. Quinolines and quinolones, isolated from plants, animals, and microorganisms, have demonstrated numerous biological activities such as antimicrobial, insecticidal, anti-inflammatory, antiplatelet, and antitumor. For more than two centuries quinoline/quinolone moiety has been used as a scaffold for drug development and even today it represents an inexhaustible inspiration for design and development of novel semi-synthetic or synthetic agents exhibiting broad spectrum of bioactivities. The structural diversity of synthetized compounds provides high and selective activity attained through different mechanisms of action, as well as low toxicity on human cells. This review describes quinoline and quinolone derivatives with antibacterial, antifungal, anti-virulent, antiviral, and anti-parasitic activities with the focus on the last 10 years literature.

Keywords

Antibiotics · Antifungals · Anti-parasitics · Antivirals · Anti-virulence activity · Quinoline/quinolone derivatives

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1 Introduction

Antimicrobial drugs, structurally diverse molecules, can be natural products, semi-synthetic derivatives of natural compounds, or chemically synthesized compounds. The development of antimicrobials in general, first of all antibiotics, but also antivirals and antimalarials, revolutionized medicine in many ways, and as such is one of the greatest successes of modern

medicine. Unfortunately, time with these drugs is rapidly running out. Occurrence and global spread of resistance of bacteria, fungi, viruses, and protozoan parasites to available antimicrobial medicines threaten to send humanity back to pre-antimicrobial era. Therefore, there is an urgent need not just to develop novel antimicrobials but also to introduce into practice novel therapeutic options to fight against both drug-sensitive and drug-resistant pathogens.

A promising alternative to the classic antibiotic approach has recently been established and is known as anti-virulence therapy. Instead of targeting microbial viability, this alternative strategy aims to target pathogens' virulence machinery required to cause host damage and disease. Microbial virulence machinery includes plethora of virulence factors, which are diverse in structure, function, and localization. One of the most important virulent characteristics of both bacteria and fungi is their ability to form biofilms. Biofilms are multicellular communities enclosed in self-synthetized polymeric matrices attached to biotic or abiotic surfaces (Hall-Stoodley et al. 2004; Costa-Orlandi et al. 2017). According to the National Institutes of Health of the United States, more than 75% of microbial infections that occur in the human body are promoted by the formation and persistence of biofilms (Miquel et al. 2016). Biofilm confers an extreme capacity for persistence against phagocytosis, oxidative stress, nutrient/oxygen restriction, metabolic waste accumulation, interspecies competition, and most importantly, conventional antimicrobial agents (Moradali et al. 2017). Quorum sensing (QS), a cell-to-cell communication system, is a global regulatory system of virulence factor production and biofilm formation in both bacteria and fungi (Albuquerque and Casadevall 2012; Defoirdt 2018), with no homologous components in humans, thus its inhibition is considered the most attractive strategy for the development of anti-virulence agents.

Quinine (Fig. 1), quinoline alkaloid isolated from the bark of the *Cinchona* tree in 1820, used in the treatment of malaria played a historical role in the development of quinoline alkaloids as therapeutics. These quinoline based compounds have been isolated and identified from natural sources (plants, animals, and microorganisms), and many studies have documented their antitumor, antimalarial, antibacterial, antifungal, antiviral, antiparasitic and insecticidal, anti-inflammatory, antiplatelet and other activities (Shang et al. 2018). Quinoline and 4-quinolone (Fig. 1) moieties were used as a scaffold for drug development for more than two centuries (Heeb et al. 2011). The most successful drug based on quinoline scaffold is chloroquine (Fig. 1), which was specifically developed as antimalarial agent. Until today, numerous quinoline-based compounds and drugs were developed as antimalarial agents, designed to target all stages of parasite life-cycle.

In fact quinolines still serve as inexhaustible models for design and development of new semisynthetic or synthetic quinoline/quinolone antimicrobial agents, which are the focus of this review article.

2 Antibacterial Activity

Important group of antibacterial agents are synthetic antibiotics with 4-quinolone as core structure, which are used in the treatment of urinary tract and respiratory infections (Anderson et al. 2012). Today antibacterial 4-quinolones in clinical use include nalidixic acid, that is introduced to medical practice in 1964 (Bisacchi 2015), followed by ciprofloxacin, levofloxacin, norfloxacin, besifloxacin, and moxifloxacin (Fig. 2). Majority of them belong to fluoroquinolone chemotype. The quinolone antibiotics are very potent towards a wide range of Gram negative bacteria, with minimal inhibitory concentrations (MICs) in the ng/ml range, and are reasonably active against many Gram positive bacteria (MICs in the mg/ml range) (Anderson et al. 2012). Their antibacterial activity is based on inhibition of DNA replication through inhibition of DNA gyrase and topoisomerase IV activities to varying extents depending on the pathogen. In order to maintain antibacterial activity, positions C(3) (unsubstituted carboxyl group) and C(4) (keto group) in 4-quinolone ring should not be altered (Gualerzi et al. 2013).

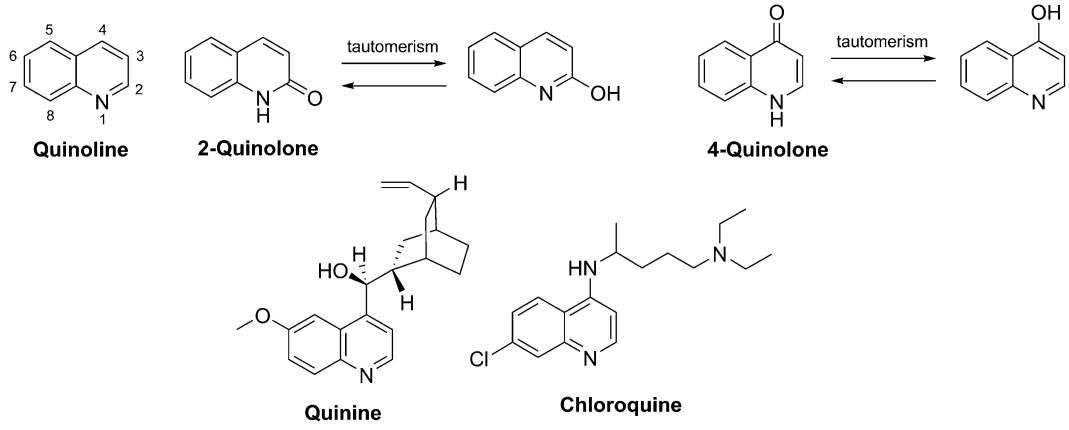


Fig. 1 Quinoline and quinolone scaffolds and their best-known drugs

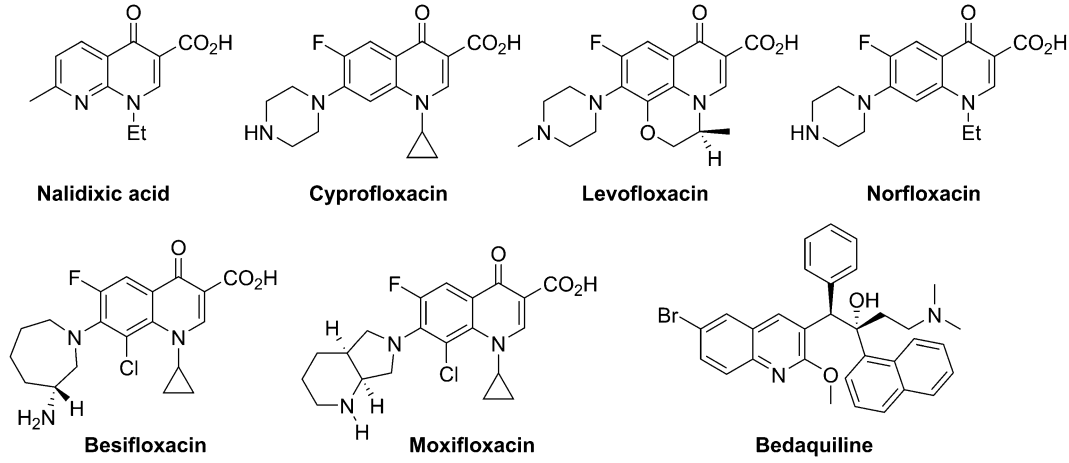


Fig. 2 Quinoline and quinolone antibiotics

After more than 40 years, in 2012, a new antibiotic with quinoline as a core pharmacophore developed to fight multidrug-resistant (MDR) mycobacteria, was granted accelerated approval by the United States Food and Drug Administration, process applicable only to the therapeutics that should fill an unmet medical needs (World Health Organization 2013). Unlike other members of quinolone antibiotics, new antibiotic bedaquiline (Fig. 2) imposes its antimycobacterial activity (MIC 0.06 μg/ml), through interaction with proton pump of the ATP synthase of *Mycobacterium tuberculosis* (Andries et al. 2005).

Using building-block approach in quest for novel antibacterial agents Dolan and colleagues (Dolan et al. 2016) combined structural moieties, such as functional groups bearing fluorine atoms, quinoline bicycles, saturated N-heterocycles, and thioureas, all of them recognized as motifs in antimicrobial agents, and synthetized a series of thiourea-containing compounds. Derivative **1** (Fig. 3) was the most active compound with MIC_{90} values of 7.90–10.52 μM, 10.52–15.78 μM, and 17.74 μM for *Escherichia coli*, *Staphylococcus aureus*, and methicillin resistant *S. aureus* (MRSA), respectively, but without any effect on *Pseudomonas aeruginosa*. Its antibacterial activities were in the line with MIC_{90} values obtained for vancomycin, well-known antibiotic of the last resort often used to

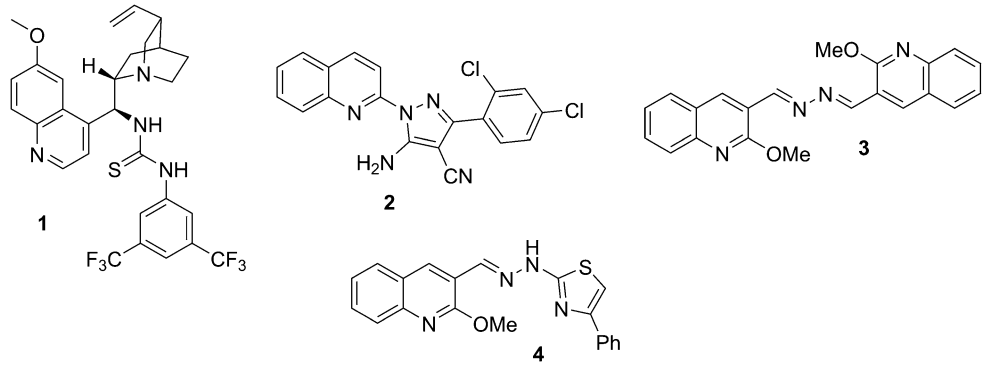


Fig. 3 Quinoline derivatives with antibacterial activity

treat drug-resistant infections. The derivative **1** was found to be non-toxic to *Galleria mellonella* larvae at concentrations of up to 1000 μg/ml. Hence, the quinine-thiourea structure, as found in compound **1**, has potential as a new class of non-toxic, anti-MRSA agent.

Synergistic effect of quinoline and pyrazole derivatives (also known for numerous bioactivities including antituberculosis, antiviral, and anti-inflammatory ones) on antimicrobial activity of three diverse series was assessed (El Shehry et al. 2018). Majority of derivatives exhibited antibacterial and antifungal activities, and the most promising derivative proved to be compound **2** (Fig. 3). Its MICs ranged from 0.12 to 0.98 μg/ml for *Shigella flexneri*, *Klebsiella pneumonia*, *Staphylococcus epidermidis*, and *Proteus vulgaris*, were in the range of ampicillin and gentamycin MICs, thus demonstrating that quinoline derivative bearing pyrazole motifs are interesting scaffolds for development of antimicrobial agents.

Similarly, antibacterial and antifungal activities of a novel quinoline series of Schiff bases and hydrazide derivatives containing moiety, synthesized *via* condensation of aromatic amines or hydrazines with 2-substituted quinoline-3-carbaldehydes was assessed (Hamama et al. 2018). Antimicrobial activities of quinoline derivatives **3** and **4** (Fig. 3; MIC values 30.6–93.7 μg/ml and 62.5–125 μg/ml, respectively) were even better than activity of ampicillin (MIC values 125–187.5 μg/ml) against *E. coli*, *P. aeruginosa*, *S. aureus* and *B. subtilis*. The same derivative exhibited similar antifungal activity (MIC values 4.5–7.8 μg/ml and 15.6–23 μg/ml) as clotrimazole (5.6–5.8 μg/ml). The activities of quinoline derivatives **3** and **4** are most likely due to their combination with another quinoline and thiazole moieties, respectively.

Several studies were focused on synthesis of different halogenated quinolines (HQ) and the assessment of bioactivities of the obtained derivatives, namely antibacterial and biofilm eradicating ability. In the first study, the HQ scaffolds or esters of halogenated 8-hydroxyquinoline derivatives were synthetized (Abouelhassan et al. 2014). Five brominated quinolines demonstrated more potent antibacterial activity (MICs 0.2–1.56 μM) against *S. aureus* and *S. epidermidis* strains in comparison to nitroxoline (MIC = 12.5–25 μM). In biofilm dispersion assay against MRSA isolate seven quinoline derivatives were very efficient (concentration at which 50% of pre-formed biofilms is disrupted; $BDIC_{50} \leq 5$ μM), with derivative **5** as the most potent (Fig. 4; $BDIC_{50} = 2.06$ μM). HQ compounds were less potent against *S. epidermidis*, although derivatives **6** and **7** exhibited excellent biofilm eradication potential (Fig. 4; $BDIC_{50} = 3.26$ μM and $BDIC_{50} = 5.56$ μM, respectively). Nitroxoline $BDIC_{50}$ values were 10.5 μM and 14.2 μM for MRSA and *S. epidermidis* biofilms, receptively. In the abovementioned study it has been revealed that the C(2)-position of the HQs is of crucial importance for their antibacterial profile against different bacterial species and their potential to

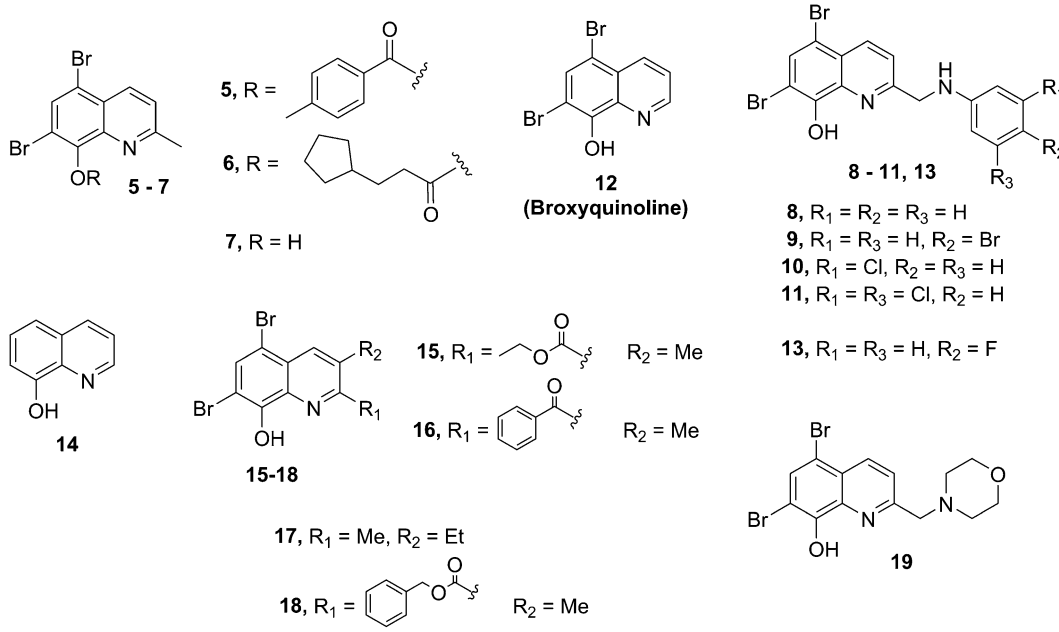


Fig. 4 Halogenated quinoline derivatives with antibacterial and antibiofilm activities

eradicate preformed biofilms. Therefore a series of C(2)-substituted analogues of the HQs were synthetized through reductive amination and tested for bioactivity (Basak et al. 2015). These derivatives demonstrated equipotent or slightly reduced antibacterial activities as parent compound **7** (Fig. 4) against clinical isolate MRSA, while products **8** (aniline derived) and **9** (4-bromoaniline derived) were the most potent analogues against methicillin-resistant *S. epidermidis* (MRSE), fivefold more potent than vancomycin (MIC = 0.78 μM) (Fig. 4). Derivatives **8** through **11** proved to be three- to six-fold more potent (MIC = 0.39–0.78 μM) against vancomycin-resistant *Enterococcus faecium* (VRE) than **7** (MIC = 2.35 μM) or linezolid (MIC = 3.13 μM). The best potency for MRSA biofilm eradication activities demonstrated **9** with minimum biofilm eradication concentration (MBEC) of 125 μM, being at least 16-fold more potent than vancomycin, daptamycin, or linezolid antibiotics (MBECs>2000 μM). Derivatives **8** (MBEC = 3.0 μM) and **10** (MBEC = 5.9 μM) were the most potent MRSE biofilm eradicators in this series, while **7** and **8** through **11** demonstrated potent biofilm eradication activities against VRE biofilms (MBEC = 1.0–1.5 μM) and as such were equipotent to linezolid, which is used to treat VRE infections. Haemolytic activity of the compounds has varied between 21.3% and less than 4%, as for **8** and **9**, indicating that analogues do not eradicate biofilms through the destruction of bacterial membranes, but through other mechanism. In addition, the authors have concluded that the HQs antibacterial activity is realised through a metal(II)-dependent mode of action possibly through the targeting of a metalloprotein critical to bacterial biofilm viability (Basak et al. 2015).

In the following study Basak and colleagues (Basak et al. 2016) derivatised broxyquinoline (**12**, Fig. 4) through multi-step synthetic routes to achieve highly diverse HQ analogues alkylated and aminated at the C(2)-position. Among 39 synthetized derivatives, **13** (Fig. 4) emerged as a highly potent anti-planktonic compound and eradicating agent against MRSA (MIC = 0.39 μM, MBEC = 7.8–93.8 μM), MRSE (MIC = 0.39 μM, MBEC = 5.9 μM) and VRE (MIC = 0.78 μM, MBEC = 1 μM) biofilms, when compared to vancomycin (MRSA: MIC = 0.59 μM,

MBEC>2000 μM; MRSE: MIC = 0.78 μM, MBEC>2000 μM; VRE: MIC>100 μM, MBEC = 150 μM). With haemolytic activity <5% and low cytotoxicity, this compound certainly represent a promising lead for further development of useful treatments against persistent infection caused by Gram positive bacteria (Basak et al. 2016).

Abouelhassan and colleagues (Abouelhassan et al. 2015) tested whether phytochemicals, typically considered as a safe, could potentiate antibacterial activity of halogenated derivatives of 8-hydroxyquinoline (**14**, Fig. 4). They have shown that gallic acid, which itself does not act as antibacterial agent, potentiate several derivatives from authors' libraries described in previous studies against different *S. aureus* strains, by lowering their MICs at least fourfold and, in some cases, up to 11,800-fold. On the other hand, gallic acid in combination with antibiotics of different classes, including quinolone, has not potentiated their antibacterial activity, thus indicating that antibacterial mechanisms of the HQs differs from those of conventional antibiotics. It was also demonstrated that gallic acid in combination with compound **7** potentiate MRSA biofilm eradication fourfold.

For further development of 8-hydroxyquinolines derivatives microwave-enhanced Friedländer synthesis protocol was used in order to synthesize the HQ compounds with C(2)- or C(2)- and C(3)- substituted positions (Garrison et al. 2017). Several derivatives exhibited significant antibacterial activity. Compounds **15** and **16** (Fig. 4) with MICs against MRSA isolate of 0.39 μM and 0.59 μM, respectively, were similar to vancomycin (MIC = 0.59 μM) or better than daptamycin (MIC = 4.69 μM) and linezolid (MIC = 3.13 μM). Compounds **15**, **17**, and **18** exhibited MICs of 0.10 μM, 0.15 μM, and 0.10 μM, respectively, against MRSE, and as such were more efficient than vancomycin (MIC = 0.78 μM), daptamycin (MIC = 12.5 μM) and linezolid (MIC = 3.13 μM). Among all, derivative **18** demonstrated the highest potency against VRE (MIC = 0.3 μM). When tested for eradication potency, compound **16** proved to be one of the most potent biofilm eradicators ever reported against both MRSA (MBEC = 3.9–23.5 μM) and MRSE (MBEC = 1.0 μM), while compounds **15** and **18** exhibited promising biofilm eradication activities against MRSA (MBEC = 31.3 μM) and VRE (MBEC = 1.5 μM), respectively. Since negligible haemolytic activity was observed for those most promising derivatives (<8% at 200 μM) it seems that the HQs could be a promising class of compounds capable of treating biofilm associated infections.

These studies demonstrated great potentials of numerous HQ derivatives as antibacterial agents and biofilm eradicators, so further investigations have been focused on development of the HQ analogues with improved water solubility while maintaining potent biofilm eradication properties against major human pathogens (Basak et al. 2018; Huigens 2018). Conducting diverse synthetic modification at the C(2)-position of the HQ scaffold in order to enhanced water solubility, a new compound that had lower ClogP value (3.44) than parent compound **7**; ClogP = 4.19) was developed. This derivative, with morpholine moiety at the C(2)-position **19** (Fig. 4), was the most effective eradicator of MRSE biofilm (MBEC = 2.35 μM) but showed no activity on MRSA biofilms. Therefore, although HQ could play a critical role in the development of next-generation antibacterial therapeutics, at the moment it is still work in progress.

Massoud and colleagues (Massoud et al. 2013) described antibacterial activity of six new Ag (I) compounds with quinoline-derived ligands tested against 15 different MDR bacteria isolated from diabetic foot ulcers and compared them to antibacterial activity of silver sulfadiazine used clinically to prevent infections in burns and wounds. Compound $[Ag(8\text{-nitroquinoline})_2]NO_3 \times H_2O$ showed activity similar to topical antibiotic against clinical isolates, being active against all strains and having slightly better average silver efficiency than silver sulfadiazine (5 *vs.* 6 μg Ag/ml).

Promising candidates for development of new drug are dimeric molecules since they exhibit

some unique properties in comparison to corresponding monomer, such as enhanced biological activity. In the last three decades, numerous quinoline and quinolone dimers were assessed for their biological activities, including antimicrobial, and quite recently very detailed review article has been published on the subject (Chu et al. 2019); hence, quinoline/quinolone dimeric molecules are not reviewed here.

It is worth noting that bacterial responses to antibiotics (not just 4-quinolone) are concentration-dependent. At high concentrations, antibiotics exhibit their antimicrobial activities on susceptible cells, but at subinhibitory concentrations they can induce diverse biological responses in bacteria. At these non-lethal concentrations, bacteria may perceive antibiotics as extracellular signals and thus trigger different cellular responses, which may include an altered antibiotic resistance/tolerance profile (Bernier and Surette 2013). Bacterial responses to subinhibitory concentrations of antibiotics and mechanisms of their responses vary depending on antibiotic and species, thus we advise readers for literature search on this topic.

3 Antifungal Activity

Fungal infections have become an everyday problem, but also a serious threat to human health due to the development of resistant strains causing weak and unsatisfactory therapeutic response to known antifungals. Although the collection of antifungal drugs is broad, the most commonly used agents have major drawbacks such as side effects and high level of toxicity. Together with the emerging resistance, these drawbacks restrict the number of medicines which can be used to treat such infections. Thus, there is a clear need for development of novel more effective antifungal agents with a broad-spectrum activity, better pharmacokinetic profile and low toxicity. As eukaryotic organisms, fungi share numerous conserved pathways with their human hosts, therefore only a few drug targets can be exploited to selectively kill these pathogens. Enzymes involved in the synthesis of cell wall polysaccharides are one of the most popular targets for development of antifungal drugs.

A quinoline scaffold can be found in many classes of biologically active compounds which are used as antifungals (Musiol et al. 2010). The unmodified quinoline exhibits relatively high activity against some fungal strains at nontoxic concentrations, which is a clear advantage in the context of designing of novel antifungal drugs. The fungistatic activity of 8-hydroxyquinoline (**14**, Fig. 4) and its metal complexes has been known since the early 1920s and these compounds are still broadly used in healthcare. Relatively simple quinoline modifications have been widely investigated in order to obtain better antifungal activity and some of these compounds are still in use. Previous efforts in the development of quinoline-based antifungals have been extensively reviewed earlier (Musiol et al. 2010), thus here we focus on the achievements from last 10 years.

A series of small HQ molecules exhibiting a potent antifungal activity against *Candida albicans* and *Cryptococcus neoformans* through intracellular mode of action have been synthetized (Zuo et al. 2016). HQ analogues with bromine and chlorine halogens **(7, 20)** (Figs. 4 and 5) inhibited *C. albicans* growth with MIC of 100 nM which is four to eight times more potent than the best-known antifungal agents amphotericin B and itraconazole, while several analogues inhibited *C. neoformans* at MICs of 50–780 nM. Importantly, the HQ analogues could eradicate mature *C. albicans* and *C. neoformans* biofilms with MBEC of 6.25–62.5 μM. The same HQ analogues also showed a range of antibacterial activities inhibiting their planktonic and biofilm forms, suggesting that these compounds could be a promising scaffold for the development of therapeutics against mixed-species and/or biofilm-associated infections. The biological activity of the HQs depended on the nature of the groups attached to the C(5)- and C(7)-positions of quinoline ring. The presence of chlorine, bromine or iodine atoms in the C(5)- or C(7)- positions of the

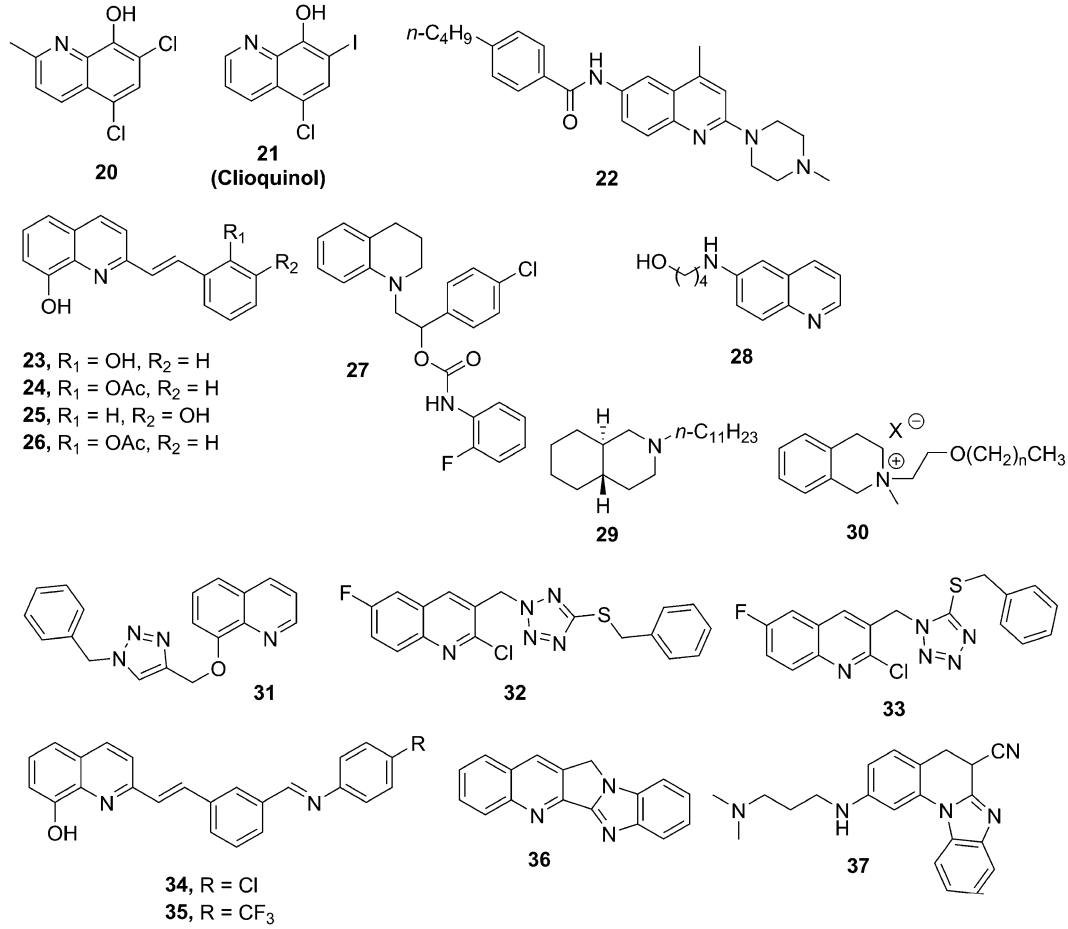


Fig. 5 Quinoline derivatives with antifungal activity

compounds was related to strong antifungal activity, which was not observed with the fluorinated compounds. Although the observed antifungal activity can be attributed to the presence of halogens, those substituents can also cause higher toxicity of the compounds. Bromoquinol (broxyquinoline; **12**, Fig. 4) is identified as one of the most potent compounds with antifungal activity during screening of 40,000 drug-like molecules from diverse chemical compound libraries against *Aspergillus fumigatus* (Ben Yaakov et al. 2017). Its activity was blocked by iron, copper or zinc supplementation, which suggested that it interferes with the utilization of these metals. It was demonstrated that bromoquinol induces oxidative stress and apoptosis in *A. fumigatus*. Bromoquinol significantly reduced mortality rates of *Galleria mellonella* infected with *A. fumigatus*, but was ineffective in a murine model of infection.

Clioquinol (**21**, Fig. 5) is a dihalogenated 8-hydroxyquinoline and antiseptic drug effective against multidrug resistant *Candida* (Pippi et al. 2018). It blocks hyphal development thus preventing biofilm formation. Clioquinol also reduces the metabolic activity of sessile *Candida* but the susceptibility was lower compared to planktonic cells (0.031–0.5 μg/ml required to inhibit 50% planktonic cells and 4–16 μg/ml to inhibit 50% preformed biofilms). Clioquinol, as well as other 8-hydroxyquinoline derivatives such as 8-hydroxy-5-quinolinesulfonic acid and 8-hydroxy-7-iodo-5-quinolinesulfonic acid effectively inhibited growth of *Candida* spp.,

Microsporum spp. and *Trichophyton* spp. (Pippi et al. 2017) with MIC values ranging from 0.031–2 μg/ml, 1–512 μg/ml, and 2–1024 μg/ml, respectively.

Most of the known quinoline drugs have a side chain on the C(4)- or C(8)-position of the quinoline scaffold. However, other positions also provide opportunities for the design of novel bioactive compounds. Two subseries of 2,6-disubstituted quinolines, consisting of 6-amide and 6-urea derivatives exhibit fungicidal activity against *C. albicans* with minimal fungicidal concentration (MFC) values lower than 15 μM (Delattin et al. 2012). The 6-amide derivatives displayed the highest fungicidal activity against *C. albicans* and a bit lower activity against *C. glabrata* (MFC < 50 μM) (**22** as the most potent, Fig. 5). Some of the 6-amide derivatives and the 6-urea derivatives could also eradicate *C. albicans* biofilms by inducing accumulation of endogenous reactive oxygen species.

Styrylquinolines (**23**–**26**, Fig. 5) are a novel group of quinoline drugs that have p53-independent antiproliferative activity and antiviral properties. They were also found to have antifungal activity and to decrease the activity of ABC multidrug transporters in *C. albicans*. They also show synergistic activity with first-line drug fluconazole (Szczepaniak et al. 2017).

Novel phenylcarbamate derivatives with tetrahydroquinoline were designed as inhibitors of HIV-1 reverse transcriptase (RT) also showed antifungal activity against *C. albicans* and *A. niger* (Chander et al. 2016). The most active derivative (**27,** Fig. 5) displayed antifungal activity against *C. albicans* almost comparable to fluconazole with MIC value of 8 μg/ml.

The 7-chloroquinoline moiety was extensively studied mainly because of its antimalarial properties. Duval and co-workers synthetized 7-chloroquinolin-4-yl arylhydrazone derivatives and addressed their antifungal activity against several *Candida* species and two yeasts species of *Rhodotorula* (Duval et al. 2011). The active derivatives showed MIC and MFC values in the range of 25 μg/ml and 50 μg/ml, which was the activity comparable with the fluconazole. Four of these hydrazones potently inhibited enzymatic processes in *C. albicans* at sub-antifungal concentrations, showing enzymatic repression of phospholipase and aspartyl proteases, which are the most frequent enzymes produced by *C. albicans* (de Azambuja Carvalho et al. 2016). Notably, the compounds exhibited low cytotoxicity against mouse fibroblasts (NIH/3 T3 cell line) at sub-antifungal concentrations.

On the way to N-functionalized 3-, 5-, 6- and 8-aminoquinolines, were obtained and their antifungal activity was assessed against *C. albicans*, *Rhodotorula bogoriensis* and *A. flavus* (Vandekerckhove et al. 2015). Several compounds displayed antifungal activity against all three microorganisms tested, while derivative **28** (Fig. 5) was selected as the most potent one, exhibiting activity toward the *A. flavus* strain comparable to amphotericin B.

Evaluation of antifungal activity of *N*-alkyl tetra and decahydroisoquinolines showed that the activity of these compounds depends on the length of the alkyl chain, with an optimum of about 10–12 carbon atoms, whereas longer or shorter alkyl chains lead to a decrease or complete loss of activity (Krauss et al. 2014). The *trans*-decahydroisoquinolines showed high antifungal activity, comparable to the reference antifungal drug clotrimazole with MIC values against *C. glabrata* between 2.4 and 25 μg/ml. The decahydroisoquinoline **29** (Fig. 5) was found to inhibit the enzyme D14-reductase in *C. glabrata*, while an additional inhibition of the downstream enzyme D8,7-isomerase was not excluded. The same group of researchers synthetized a series of (±)-*trans*-*N*-alkylperhydroquinolines, which also showed high antifungal activity with MIC values against *C. glabrata* between 5 and 50 μg/ml (Krauss et al. 2015). The activity of the most potent derivative was comparable to drug clotrimazole. The maximum of activity was found with the derivatives having C10 or C12 alkyl chains, while shorter alkyl chains led to a decrease in activity, as already found for other *N*-alkyl heterocycles. In comparison to *N*-alkyl perhydroisoquinolines, perhydroquinoline compounds showed similar antifungal activity, but higher cytotoxicity against a human cell line. Both perhydroquinolines and perhydroisoquinolines

targeted the same enzyme in ergosterol biosynthesis ($\Delta^{8,7}$-isomerase), but the latter chemotype showed higher selectivity.

The lipid-like choline and colamine analogues based on 1,2,3,4- tetrahydro(iso)quinoline have been developed as a part of compound libraries of lipid-like systems that combine two fragments: hydrophilic – 1,2,3,4-tetrahydro(iso)quinolinium pharmacophore system, and lipophilic – long chain alkyl substituent (**30**, Fig. 5), that helps the molecule in its passive transport across lipophilic barriers and plasma membranes *in vivo* (Zablotskaya et al. 2017). Tetrahydroisoquinoline derivatives and compounds possessing substituents with chain length of 10 or 11 carbon atoms showed the strongest antifungal activities with MIC values between 8 and 64 μg/ml. Tetrahydroisoquinolinium heptyl and decyl derivatives **30** exhibited high antifungal action as compared with corresponding tetrahydroquinolinium analogues. These compounds exhibited stronger antifungal activity than fluconazole.

Quinoline based 1,2,3 triazoles emerged as a group of very potent antifungal compounds with IC_{50} values (half maximal inhibitory concentration) for the most active derivative **31** (Fig. 5) of 0.044 μg/ml against *C. albicans*, 12.02 μg/ml against *C. glabrata*, and 3.60 μg/ml against *C. tropicalis* (Irfan et al. 2015; Irfan et al. 2017). Moreover, these antifungal concentrations were not cytotoxic. The presence of these compounds affected the secretion of extracellular hydrolytic enzymes (proteinases and phospholipases). The antifungal target for these triazoles was plasma membrane as suggested by altered cell membrane, reduced plasma membrane H^+ATPase activity, and significant inhibition in ergosterol biosynthesis in the presence of these compounds.

A series of 2,5 and 1,5-regioisomers of tetrazole with quinoline and benzylthio substituents, were synthesized (Shaikh et al. 2017). The designed compounds consist of a quinoline nucleus, which serves as pharmacophore and the non-heterocyclic part such as phenyl group separated by a tetrazole bridge. The antifungal activity of synthesized compounds was tested against *A. fumigatus* and *C. albicans* strains and the obtained MIC values for the most active compounds **32** and **33** (the 2,5- and 1,5-isomer with fluoro substituent at the C(6)-position) (Fig. 5) were between 2.5 and 25 μg/ml. All the derivatives having halogens as substituents showed better activity than other derivatives. The active compounds came to be inhibitors of dihydrofolate reductase and N-myristoyl transferase, and exhibited no cytotoxic effects.

New hybrid analogues containing 7-chloro-4-aminoquinoline and 2-pyrazoline N-heterocyclic fragments were synthesized and evaluated for antifungal activity against *C. albicans* and *C. neoformans* (Montoya et al. 2016). The compounds displayed stronger activity against *C. neoformans* with the most active derivative showing MIC_{50} values of 15.5 and < 3.9 μg/ml against *C. albicans* and *C. neoformans*, respectively.

In an attempt for development of novel antimicrobial agents, three series of quinoline derivatives bearing pyrazole moiety were synthesized (El Shehry et al. 2018). The most active compound **2** (Fig. 3) showed fourfold potency of amphotericin B in inhibiting the growth of *A. clavatus* (MIC 0.49 μg/ml) and *C. albicans* (MIC 0.12 μg/ml), respectively. The same compound showed equipotent activity to amphotericin B in inhibiting the growth of *A. fumigatus* (MIC 0.98 μg/ml).

New quinoline derivatives (Khan et al. 2019) bearing vinyl benzylidene imine with substituted aniline at the C(2)-position showed antibiofilm and antifungal activity. The antibiofilm activity of compounds **34** and **35** (Fig. 5) against *C. albicans* (IC_{50} values 66.2 and 51.2 μM, respectively) were similar to activity of fluconazole (IC_{50} = 40.0 μM). Compound **34** exhibited slightly lower antifungal activity (MIC = 94.2 μg/ml) than fluconazole (MIC = 50.0 μg/ml).

Benzimidazole fused pyrrolo[3,4-b] quinoline (Villa et al. 2019) compound **36** (Fig. 5) exhibited potent antifungal activity against *C. albicans*, *C. parapsilosis*, *C. tropicalis*, *C. glabrata*, and *C. neoformans* with the MIC values ranging from 0.0625 to 2 μg/ml. Fungal strains that are resistant to fluconazole were also inhibited by **36** with the MIC ranging from 0.25 to 0.5 μg/ml.

These compounds also showed antibiofilm activities without apparent toxicity to mammalian cells. A fluorescent benzimidazo[1,2-α] quinoline was identified within a set of nine benzimidazole derivatives as bifunctional *Candida* spp. biofilm detector and eradicator (de Souza et al. 2016). Compound **37** (Fig. 5) was the most active derivative showing antifungal activity against different *Candida* strains including *C. tropicalis*, *C. albicans*, and *C. parapsilosis* with MIC values in the range of 4 μg/ml against *C. albicans* and 32 μg/ml against the other isolates. Spraying of **37** over *Candida* sp. biofilm contaminated surface enabled detection of the biofilms under UV light. At the same time, **37** showed the potential to eradicate the detected biofilms. Benzimidazole **37** thus has a good potential to enable the usage of disinfected medical and surgical instruments in clinical and surgical procedures contributing to increased safety for patients.

As described, quinoline scaffold can be considered a valuable pharmacophore to be used for a simple synthesis and development of novel antifungal agents with the superior efficacy to commonly used antifungal drugs. The structural diversity of synthetized compounds can provide high and selective activity achieved through different modes of action, as well as low cytotoxicity on human cells.

4 Anti-virulence Activity

P. aeruginosa produces more than 50 different alkylquinolines (Deziel et al. 2004) of which 2-heptyl-3-hydroxy-4-quinolone (PQS) and its direct precursor 2-heptyl-4-hydroxyquinoline (HHQ) are PQS pathway autoinducers regulating production of several virulence factors such as pyocyanin and hydrogen cyanide, as well as biofilm formation. Alkylquinolines signalling has so far been detected in *P. aeruginosa* and certain *Burkholderia* and *Alteromonas* species (Diggle et al. 2006; Vial et al. 2008).

The majority of bacterial QS inhibitors (QSIs) have been designed based on the core structures of autoinducers. Quinolone-based compounds are expected to exhibit species-specific QSI activity against *Pseudomonas,* some *Burkholderia* and *Alteromonas* species.

Following a ligand-based drug design approach, a set of HHQ and PQS analogs were synthesized by variyng the side chain and introducing substituents into the benzene moiety of the quinolone molecule. PQS analogues with hydroxy group showed PqsR agonistic activity in *P. aeruginosa*, and this effect was circumvented by synthetizing a series of HHQ analogs (Lu et al. 2012). Highly potent PQS inhibitors have been synthetized by introducing modifications in benzene ring and 3-alkyl substituents achieving almost complete PqsR inhibition at 5–10 μM concentrations (**38**, Fig. 6) and reduction of virulence factor pyocyanin production by 74% at 3 μM. Introduction of polar groups at the C(3)-position of derivative **39** resulted in novel compounds with enhanced anti-virulence activity, with the most active derivative **40** inhibiting pyocyanin production at IC_{50} of 3.8 μM (Lu et al. 2014a). The QSI activities of these compounds were confirmed in *in vivo* experiments (Lu et al. 2014b).

A 4-quinolone isostere quinazoline was designed as highly potent competitive inhibitor of PqsR with derivatives **41–43** being the most active ones (Fig. 6) (Ilangovan et al. 2013). A simple isostere replacement (OH for NH_2) changed quinolone isostere quinazoline activity from potent agonistic to potent antagonistic. Derivative **41** inhibited pyocyanin production, virulence genes expression, aminoquinoline biosynthesis, and biofilm development.

A series of 4-aminoquinoline derivatives have been described as dual *P. falciparum* and botulinum neurotoxin inhibitors (Opsenica et al. 2012; Videnovic et al. 2014; Solaja et al. 2008) as well as ligands in complexes with anticancer activities (Nikolić et al. 2015). Following the drug repurposing approach several 4-aminoquinoline-based molecules with QSI activities have been further developed. Within a series of 31 derivatives of antiprotozoal 4-aminoquinolines compounds with PqsR antagonistic activity were identified inhibiting receptor activity by 70–85% at 10 μM (Soukarieh et al. 2018). Derivatives **44** and **45** inhibited pyocyanin production, while compound **44**, inhibited also

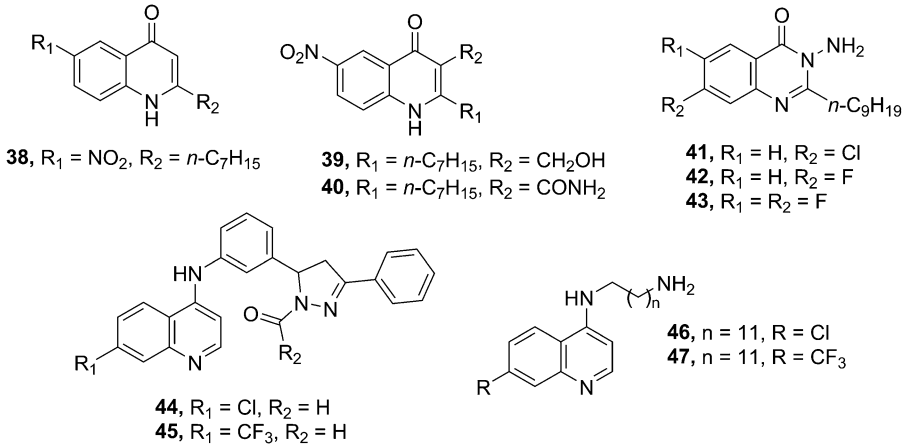


Fig. 6 Quinoline and quinolone derivatives with anti-virulence activity

HHQ, PQS, and 2-heptyl-4-hydroxyquinoline N-oxide (HQNO) production, and biofilm formation (Fig. 6). The efficacy of these compounds depended on the strains of *P. aeruginosa* (*P. aeruginosa* PA14 vs. *P. aeruginosa* PAO1).

Recently, PQS inhibitory activity of long-chain 4-aminoquinoline derivatives (Fig. 6) has been reported (Aleksić et al. 2017). Within this series, derivatives **46** and **47** with C12 alkyl chain at C(4)-position and chlorine at C(7)-position were the most active anti-virulence compounds inhibiting biofilm formation in *P. aeruginosa* with IC_{50} values 63 μM and 69 μM, respectively, and pyocyanin production with IC_{50} values of 40 and 2.5 μM, respectively. The same compounds also inhibited biofilm formation in *Serratia marcescens* with the same efficacy.

PQS, HHQ and some of the 4-quinolone derivatives can modulate interspecies and interkingdom interactions (Fernández-Piñar et al. 2011; Reen et al. 2011, 2012, 2015), which makes this chemotype even more attractive for further development of QSI.

Long chain amide derivatives of 2-amino-4-quinolone showed ability to inhibit biofilm formation in *P. aeruginosa* and *S. aureus* (Espinosa-Valdes et al. 2019). The most active compounds were those with an alkyl chain of more than 12 carbon atoms and in general they were more active against biofilm formation in *P. aeruginosa* than in *S. aureus*. The most active compounds **48** and **49** (Fig. 7) also inhibited pyocyanin production between 62.6 and 68.2% at concentration of 20 μM. None of the tested compounds affected bacterial growth suggesting that observed activity of the derivatives could be through modulation of QS.

A library of quinoline amino alcohol derivatives have been evaluated as biofilm inhibitors against the Gram-negative pathogen *Vibrio cholerae* (Leon et al. 2015). The most potent compound was *meta*-substitued pentafluorosulfonyl derivative **50** (Fig. 7) with $BIFC_{50}$ of 4.4 μM and $BDIC_{50}$ value of 7.4 μM. The antibiofilm activity of this compound was observed at concentration that did not affect bacterial growth (MIC = 78.1 μM) suggesting that mode of antibiofilm action was different from its bactericidal activity. This compound also exhibited cytotoxicity to HeLa cells with IC_{50} value of 7.27 μM, thus it can be considered as active component for functionalization of materials for medical use rather than for systemic application.

Chloroquine (CQ, Fig. 1) has been tested in combination with antifungal drugs fluconazole, voriconazole, amphotericin B, and caspofungin for ability to inhibit *C. albicans* planktonic forms and biofilms (Shinde et al. 2013). No synergistic activity of CQ and antifungals has been observed against planktonic growth. Alone fluconazole and voriconazole didn't affect formation

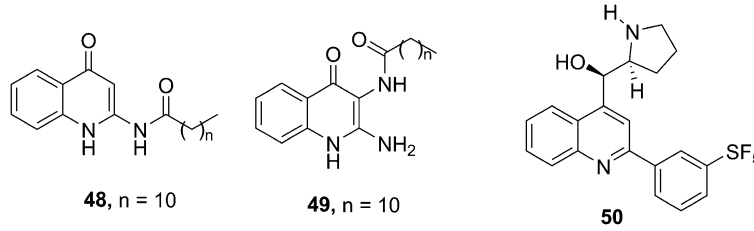


Fig. 7 Quinoline and quinolone derivatives with antibiofilm activity

of *C. albicans* biofilms even at high concentrations, but in a combination with 250 μg/ml CQ, antibiofilm activity was observed at fluconazole concentration of 4 μg/ml and voriconazole concentration of 0.25 μg/ml. Combinations of CQ with amphotericin B, or CQ with caspofungin showed no synergistic activities against biofilm formation. Antifungal drugs fluconazole and voriconazole showed no effect on mature *C. albicans* biofilms, while amphotericin B and caspofungin inhibited mature biofilms but high concentrations were required (0.5 and 1 μg/ml, respectively). In the presence of 250 μg/ml CQ, mature biofilms were disrupted as a result of synergistic activity with fluconazole and voriconazole, while combination with amphotericin B and caspofungin showed low effect against mature biofilms. CQ can be developed as a promising partner molecule of antifungal drugs for combination therapy against *C. albicans* biofilm.

5 Antiviral Activity

Viral infections have concerning effects on the health of human populations worldwide. The successful treatments of these infections require highly effective antiviral drugs, which together with the growing spread of resistance require new therapeutic agents. A lot of efforts has been done on antiviral properties of quinolines and quinolones and their structural analogues against human immunodeficiency virus (HIV), but their antiviral activity was also demonstrated against human cytomegalovirus (HCMV), SARS corona virus, Zika virus, Chikungunya virus, hepatitis C virus (HCV), and Ebola virus (Luthra et al. 2018; Plantone and Koudriavtseva 2018; Al-Bari 2015; Delvecchio et al. 2016; Barbosa-Lima et al. 2017; Loregian et al. 2010). Chloroquine shows antiviral effects by inhibiting pH-dependent steps of the replication of several viruses including members of the flaviviruses, retroviruses, and coronaviruses (Savarino et al. 2003).

Human immunodeficiency virus type 1 (HIV-1) integrase (IN), HIV-1 RT and HIV-1 protease are the essential enzymes for retroviral replication, and represent the important targets for interrupting the viral replication cycle and thus development of novel antiviral therapeutics. Reverse transcriptase and protease inhibitors, which have been used in retroviral therapy, cannot achieve complete suppression and there is a risk for resistance development. The best-studied effects of CQ are those against HIV replication, and its analogues have been used in clinical trials as investigational anti-retroviral agents in humans with HIV-1/AIDS (Al-Bari 2015). Approved **51** (Fig. 8), later called elvitegravir, is the first quinolone-based anti-HIV drug, exhibiting potent inhibitory activity against integrase-catalyzed DNA strand transfer (Sato et al. 2006). Recently, a series of quinolone-3-carboxylic acids have been synthetized as HIV-1 integrase inhibitors featuring a fluorine atom at C(5) position (He et al. 2013). The most active compound **52** (Fig. 8) exhibited activity against both wild-type and the mutant virus, with an EC_{50} value of 0.032 and 0.082 μM, respectively. Another series of quinolone-3-carboxylic acids have been synthesized by introducing different hydrophobic groups at N(1), C(2), C(7), and C(8) positions (Hajimahdi et al. 2016). Most of the compounds of this group showed anti-HIV activity without cytotoxicity at concentration of 100 μM. The

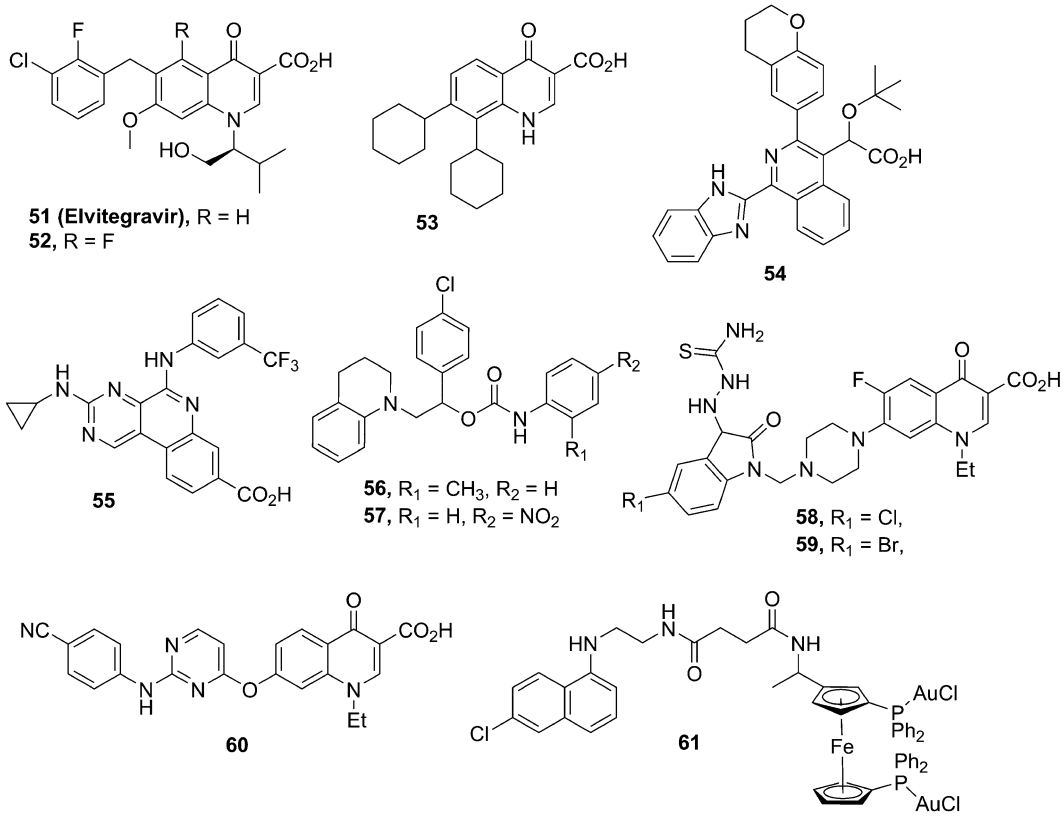


Fig. 8 Quinoline and quinolone derivatives with anti-HIV activities

most active compound **53** (Fig. 8) showed anti-HIV activity with an inhibition rate of 84%. Docking study revealed that the anti-HIV activity of this group of compounds might involve a metal chelating mechanism. Several other quinoline analogues have been synthetized, which exhibit low micromolar inhibitory potency against HIV-1 IN as recently reviewed by Wadhwa et al. (Wadhwa et al. 2018). A series of multi-substituted quinolines, with the focus on the substitution pattern of the 4-phenyl moiety and incorporation of heteroaromatic or polycyclic substituents at the C(4)-position, were prepared and examined for their ability to trigger HIV-1 IN multimerisation *via* binding to an allosteric site (Jentsch et al. 2018). 4-(4-Chlorophenyl)quinoline and 2,3-benzo[b][1,4]dioxine showed highest potency with EC_{50} values (half maximal eradication concentration) of 0.1 and 0.08 μM, respectively. A series of isoquinoline analogues with allosteric IN inhibitory activity has been recently reported (Wilson et al. 2019). Compound **54** (Fig. 8) was highly potent with EC50 of 1.1 μM.

Protein kinase CK2 plays a role in the stimulation of RT and protease. Substituted pyrimido [4,5-c]quinoline ATP competitive inhibitors of protein kinase CK2 showed a good antiviral activity with the compound **55** (Fig. 8) having IC_{50} values as low as 80 nM against HIV-1 viruses with an excellent therapeutic index (Pierre et al. 2011).

Using the ligand based drug design approach the 1-(4-chlorophenyl)-2-(3,4-dihydroquinolin-1(2H)-yl)ethyl phenylcarbamate derivatives were designed as inhibitors of HIV-1 RT (Chander et al. 2016). The most active compounds **56** and **57** (Fig. 8) inhibited the RT activity with IC_{50} 8.12 and 5.42 μM, respectively. Cytotoxicity and anti-HIV activity of these compounds were evaluated on T

lymphocytes. Both compounds exhibited potent anti-HIV activity with EC_{50} values 11.10 and 2.76 μg/ml, for **56** and **57** respectively, while showing a very good safety index.

A molecular-hybrid approach is a powerful tool in the design of new molecules with improved affinity and efficacy and as such was applied for development of novel antiviral agents involving quinoline scaffold. The fluoroquinolone-isatin hybrids **58** and **59** (Fig. 8) showed inhibition on the replication of HIV-1 in human cells with EC_{50} of 11.3 and 13.9 μg/ml, and the selectivity index values were > 5. The promising antiviral properties of isatin and fluoroquinolone hybrids have been extensively discussed recently (Xu et al. 2019). Further, a series of diarylpyrimidine–quinolone hybrids was synthesized and evaluated against both wt HIV-1 and mutant viral strains (Mao et al. 2015). The most active hybrid **60** (Fig. 8) displayed an EC_{50} value of 0.28 ± 0.07 μM against HIV-1 by targeting RT.

HIV infects multiple cells in human body, but its replication starts after infecting CD4 lymphocytes (T-cell or CD4- cell). The treatment of proliferating CD4 T-cells with antiproliferative activity agents reduces the ability of these cells to support HIV replication. Combination therapy involving cytostatic compounds such as hydroxychloroquine or hydroxyurea with proven anti-retroviral drugs as didanosine decreases viral replication and increases the CD4 count in antiretroviral-naive HIV patients thus helping to control the infection. A novel di-gold(I) complex of ferrocene–quinoline (**61**, Fig. 8) was investigated for cytostatic behaviour as well as antiviral activity (Gama et al. 2016). Di-gold quinoline derivative showed inhibition of virus infectivity by 83% at concentration of 10 μg/ml and cytostatic activity with significant S and G2/M phase cell arrest, which make it a good candidate for use in HIV-1 infection as a virostatic agent.

Although quinoline/quinolone derivatives showed promising antiviral effects in *in vitro* studies, published clinical studies evaluating the effects of chloroquine/hydroxychloroquine administration, alone or in combination with other drugs, in HIV infected subjects reported different effects in terms of immune activation, viral load, and CD4 counts (Savarino and Shytaj 2015). The outcome of the studies depended on applied doses, duration of the treatments or the drug exposure, or on the individual differences in drug metabolism and distribution.

6-Aminoquinolones with amino group at the C (6) position of the bicyclic quinolone ring system, specifically inhibit HIV replication (Cecchetti et al. 2000). On the other side, **62** (Fig. 9), which is characterized by a cyclopropyl group at the N(1) position and a 4-(2-pyridyl)-1-piperazine moiety at the C(7) position showed specific activity against HCMV by inhibiting transactivation activity of immediate-early 2 (IE2), a multifunctional factor essential for viral replication (Loregian et al. 2010; Mercorelli et al. 2014). The target of this compound is different from that of the anti-HCMV drugs currently approved for clinical use. **62** demonstrated activity against laboratory strains of HCMV but also against clinical isolates and virus strains resistant to clinically relevant anti-HCMV agents. Importantly, this compound does not show cross-resistance with ganciclovir, acyclovir, cidofovir, and foscarnet, which is important advantage for its potential clinical use, since drug-resistant HCMV strains often emerge after long-term treatment with antiherpetic drugs.

Most of the current clinical compounds and approved drugs against HCV target non-structural (NS) proteins NS3, NS5A, and NS5B. 3-Heterocyclyl quinolones have been described as a series of allosteric-site (NNI-2) inhibitors of the HCV NS5B polymerase (Kumar et al. 2012). The most potent compounds in this series were **63** and **64** (Fig. 9), with EC_{50} below 250 nM. Derivatives of 1H -quinazolin-4-one were reported as allosteric HCV NS5B thumb pocket 2 (TP-2) inhibitors (Hucke et al. 2014) with picomolar antiviral potency in genotype (gt) 1a and 1b ($EC_{50} = 120$ and 110 pM, respectively) and with $EC_{50} \leq 80$ nM against gt 2−6.

A group of 6-aminoquinolone derivatives demonstrated inhibitory activity against NS5B

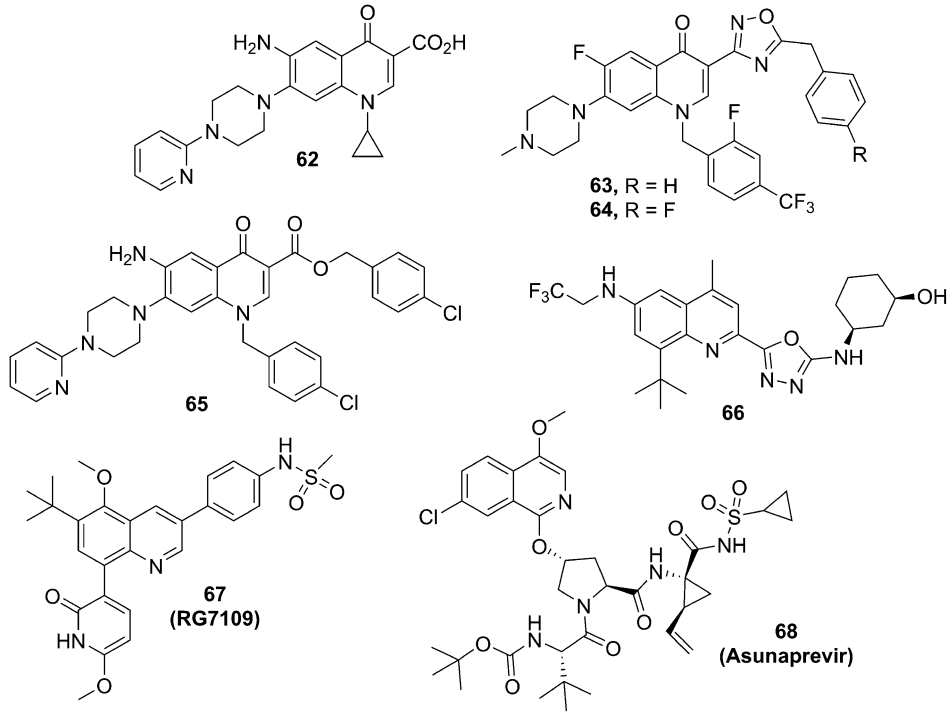


Fig. 9 Quinoline and quinolone derivatives with anti-HCMV and anti-HCV activities

polymerase (Manfroni et al. 2014). The most active compound of this group was 6-amino-7-[4-(2-pyridinyl)-1-piperazinyl]quinolone derivative **65** (Fig. 9) with an IC_{50} value of 0.069 μM. Its selective antiviral effect was EC_{50} 3.03 μM, while cytotoxicity was not observed.

Derivatives of 2-oxadiazoloquinoline were designed to inhibit HCV NS4B, which is integral membrane protein believed to act primarily as an endoplasmic reticulum-localized scaffold for the assembly of the replicase complexes needed for HCV RNA replication (Phillips et al. 2014). Compound **66** (Fig. 9) from this series showed exceptional activity against different replicons with EC_{50} values ranging between 0.08 nM and 3.1 nM.

The compound **67** (**RG7109**, Fig. 9), was synthetized as a result of an effort to develop a combination therapy against HCV consisting of only direct-antiviral agents. The standard therapy of HCV included pegylated interferon α (Peg-IFN) and ribavirin (RBV), however this therapy was effective against gt 2 or 3, but gt 1 did not respond well, with SVR (absence of detectable HCV RNA in blood serum for 24 weeks after treatment withdrawal) rates of <50% even after 48 weeks long therapy. Upon FDA approval of HCV protease inhibitors the treatment of gt 1 included Peg-IFN and RBV and a protease inhibitor, which improved the SVR rates up to 90% with gt 1 naïve patients. Interferon-associated side effects limit treatment success in a large number of patients. With the aim to develop a combination therapy consisting of only direct-antiviral agents, Talamas and co-workers identified a bicyclic template with potent activity against the NS5B polymerase containing 3,5,6,8-tetrasubstituted quinoline core **67**, which was selected for advancement to clinical development (Talamas et al. 2014).

Isoquionoline-containing compound called asunaprevir (**68**) (Fig. 9), a tripeptidic acylsulfonamide inhibitor of the NS3/4A enzyme developed by Scola and co-workers (Scola et al. 2014a; b) went through phase III clinical trials for efficient treatment of HCV infection.

6 Derivatives with Anti-parasitic Activity

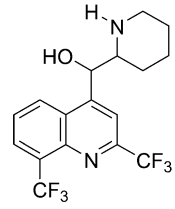
Parasitic diseases caused by protozoan species represent one of the major health challenges worldwide, affecting millions of people of all ages and social classes. The most threatened are children, pregnant women, immunocompromised individuals, and people living in developing countries. Consequences of illness burden not only patients and their families, their financial status, but also local and global economy. Taking into account the limitations of currently available drugs, the only proven strategy to fight against these diseases is development of the new therapeutics under clearly defined criteria (Nwaka and Hudson 2006).

6.1 Antimalarial Activity

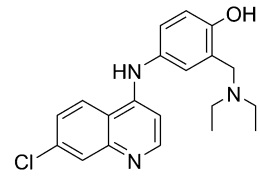
According to WHO annual report, in 2017 approximately 219 million cases of malaria occurred worldwide, resulting in 435,000 deaths (World Health Organisation 2019). Most cases were registered in African regions, followed by South-East Asia and Eastern Mediterranean regions. Malaria is caused by five *Plasmodium* species with *Plasmodium falciparum* as the most dangerous one, since it causes cerebral malaria and is the major cause of death.

Although many different pharmacophores have been developed during past decades, quinoline derivatives (Figs. 1 and 10) are still a backbone for development of novel antimalarial drugs (Barnett and Guy 2014). Actually, one of the most successful antimalarial therapeutics, a well-known quinoline derivative chloroquine, has been used for decades to threat this illness. The main mechanisms of action of CQ and its congeners (Figs. 1 and 10) are inhibition of hemozoin polymerization and release of a free toxic haem (Egan and Marques 1999). Among diverse antimalarials used in clinics CQ, considered as safe even for children and pregnant women, is the easiest to synthesize. Unfortunately, malaria parasite has developed resistance to most of antimalarial drugs including CQ. Structural modifications of this chemotype, which would include altering the nature of the quinoline core and side chain at C(4)-position, or synthesis of organic (Boudhar et al. 2016) or organo-metallic hybrids (Salas et al. 2013) present a good direction for obtaining novel derivatives with better activity against CQ resistant strains (Manohar et al. 2013).

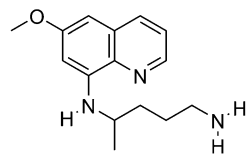
Introducing of steroidal structure fragment, various thiophen, benzothiophen and adamantyl groups, numerous derivatives of 4-aminoquinoline have been synthetized and tested for their antimalarial activity. Steroidal compounds reported by (Videnovic et al. 2014), which were developed based on the results of the previous study (Solaja et al. 2008), showed low nanomolar IC_{50} (50% inhibition of β-haematin formation) activities against CQ resistant and MDR *P. falciparum* strains. Among them derivatives **69–71** were the most active in the series (Fig. 11), being more active than mefloquine (Fig. 10) against MDR strains. High potency of these derivatives was demonstrated with three times better inhibition of hemozoin production than CQ. Derivatives bearing electron-rich thiophen or benzothiophen groups were designed to enhance activity against CQ resistant and MDR strains of *P. falciparum*, by additional π-interactions with hem (Opsenica et al. 2015). 4-Fluoro derivatives, **72** and **73**, were the most active in *in vivo* experiments showing curative activity (Fig. 11). Addition of aromatic ring, elongation of diaminoalkyl linker, and replacement of F-atom with CN-group (**74–76,** Fig. 11) enhance active against CQ resistant strain (Konstantinovic et al. 2017). The most active benzothiophene derivate **77** cured mice infected with *P. berghei* when dosed orally (Fig. 11). Derivatives with adamantyl-substituent, **78–81** (Fig. 11) (Terzic et al. 2016) were designed to increase lipophilicity of quinoline core and to provide amphiphilic structure (Wanka et al. 2013) and metabolic stability. These compounds demonstrated *in vitro* antimalarial activity at low nanomolar concentrations against CQ resistant and MDR strains, presenting the highest potency up today. This modification increased



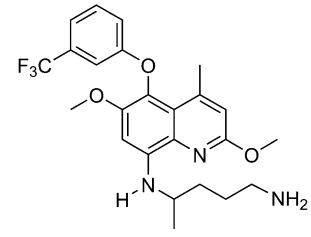
Mefloquine



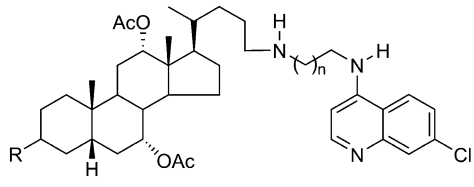
Amodiaquine



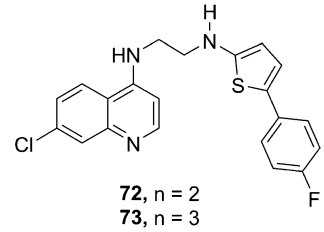
Primaquine



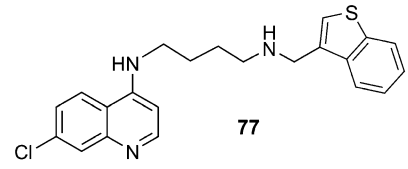
Tefenoquine



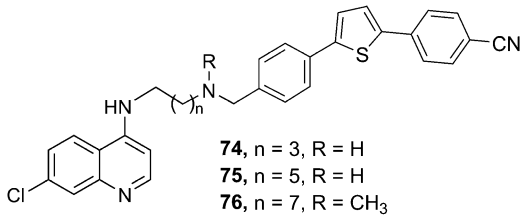
69, n = 2, R_1 = =O , R_2 = H
70, n = 1, R_1 = ····NH_2 , R_2 = CH_3
71, n = 1, R_1 = ····$N(CH_3)_2$, R_2 = CH_3



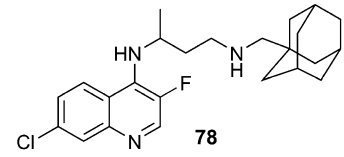
72, n = 2
73, n = 3



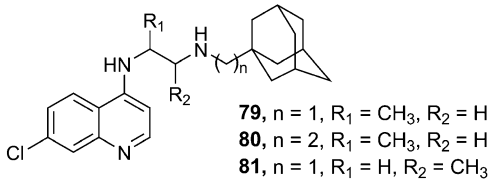
77



74, n = 3, R = H
75, n = 5, R = H
76, n = 7, R = CH_3



78



79, n = 1, R_1 = CH_3, R_2 = H
80, n = 2, R_1 = CH_3, R_2 = H
81, n = 1, R_1 = H, R_2 = CH_3

Fig. 10 The most-successful quinoline derived antimalarial drugs developed in the twentieth century

Fig. 11 Quinoline derivatives with antimalarial activity

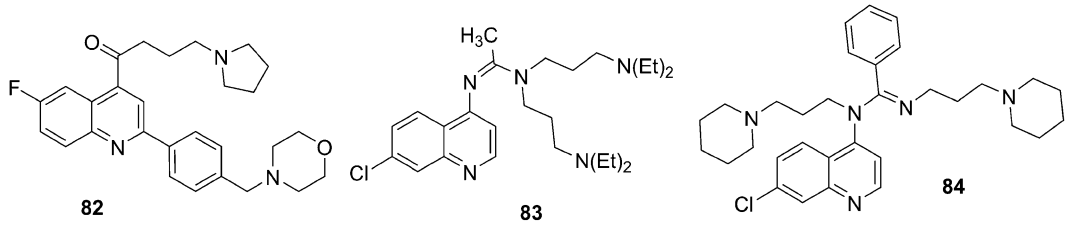


Fig. 12 Keto- and amidino-quinolines with antimalarial activity

microsomal metabolic stability over 60 min. Introducing the F-atom at C(3)-position attenuated *in vitro* activity against erythrocyte form of *P. falciparum*. However, five F-C(3) derivatives showed excellent *in vitro* and *in vivo* activity against liver stage of *P. berghei*, with **78** as the most active derivative with $IC_{50} = 0.1$ μM.

Changing the character of 4-amino group, or replacing it with different functional group, alters electronic properties of quinoline core. One of the such example is derivative **82** (Fig. 12) who contained carbonyl group attached to C (4) (Baragana et al. 2015). Compound **82** showed potent activity against multiple stages of the *Plasmodium* parasite life-cycle and exhibited excellent pharmacokinetic properties and an acceptable safety profile. This molecule having $EC_{90} = 2.4$ nM against MDR *P. falciparum* strain reduced parasitemia for 90% in mice infected with *P. berghei* after a single oral dose. Translation elongation factor 2, a protein responsible for the GTP-dependent translocation of the ribosome along mRNA and as such required for protein synthesis at all stages of the parasite life cycle was identified as the target. Therefore, with its potent activity against multiple stages of the parasite life cycle, high metabolically stability, novel mode of action, and excellent drug-like properties, **82** meets the key criteria for new antimalarial drug.

Few derivatives of yet another series of 4-amidinoquinolines, tested against CQ sensitive and CQ resistant strains, exhibited IC_{50} values less than 10 nM (Korotchenko et al. 2015). Introduction of the third substituent on the amidine-group provided both good activity and enhanced metabolic stability. Derivatives **83** and **84** (Fig. 12) showed activity with $IC_{50} = 1.9$–34 nM. However, those promising results were diminished with high resistant index (activity against resistant vs. activity against sensitive strains) ratio.

Amodiaquine (Fig. 10), well known and intensely studied drug, could replace CQ in antimalarial therapy (Olliaro and Mussano 2003). It is successful in treatment of CQ resistant strains but unfortunately when used prophylactically toxic metabolites are produced (Schlitzer 2007). Therefore, a series of *N*-pyridyl, *N*-1H-benzo[d]imidazole, N-benzo[d]oxazole, and N-benzo[d]tiazole were tested (Ongarora et al. 2015). Three derivatives (**85–87**, Fig. 13) showed *in vivo* efficacies in dose-depend manner with 99.5% parasitemia reduction among which derivative **85** was the most successful. 8-Aminoquinolines primaquine and tefenoquine (Fig. 10) are also known and promising antimalarial compounds (Waters 2011). Shared common disadvantage of these compounds is biotransformation of terminal amino-groups into aldehydes, which are toxic. To prevent such transformation and to maintain good antimalarial activity a new derivatives that contained aminoxy and oxime-groups were synthetized (Leven et al. 2019). The compounds were evaluated *in vitro* against asexual blood stages, liver stages, and sexual stages of *P. falciparum*. 8-Aminoquinolines bearing a 2-alkoxy and a 5-phenoxy substituent (**88–92**, Fig. 13) were the most active compounds, with the IC_{50} values in the range 0.95–1.3 μM, comparable to primaquine and tefenoquine. Derivatives **93** and **94** showed significant activity against asexual blood stage of parasites (Fig. 13). However, identification and toxic profile of potential metabolites remained inexplicable.

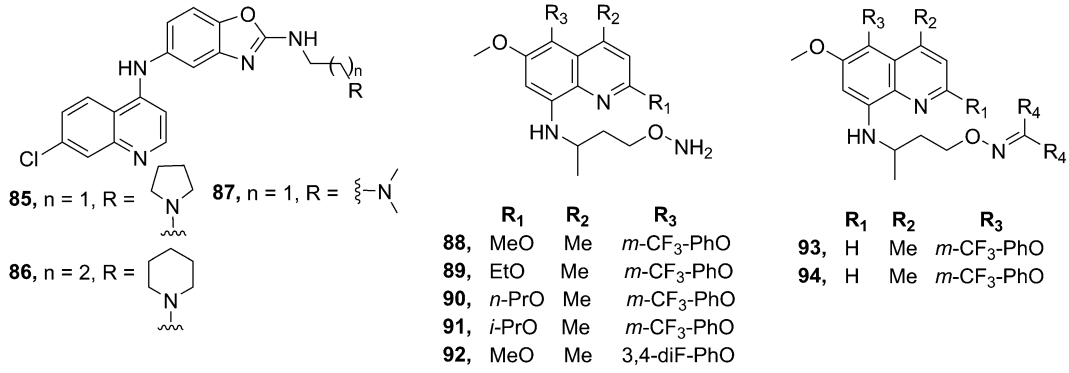


Fig. 13 4-Amino and 8-aminoquinolines with antimalarial activity

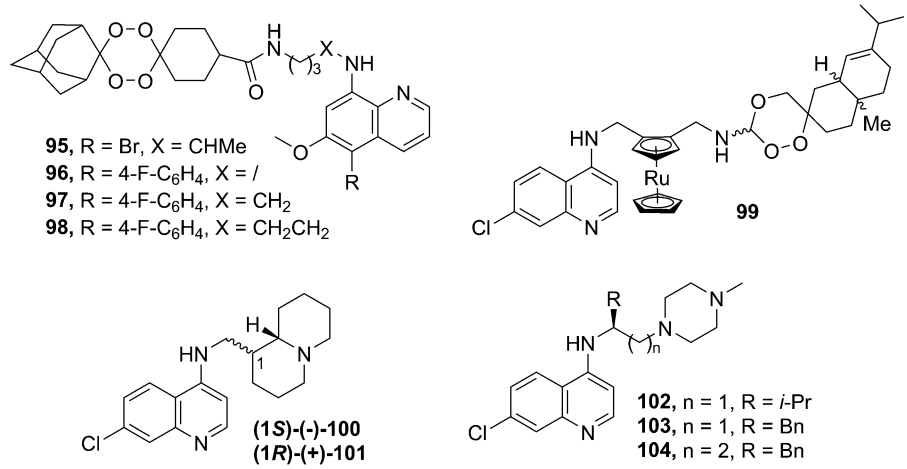


Fig. 14 Quinoline hybrides with antimalarial activity

Synthesis of hybrid compounds that contained two or more pharmacophore is a new promising approach in development of more successful antimalarials. It was expected that those hybrid-compounds cover distinct mechanism of actions, or affect different phase of plasmodium life cycle; hence, synergistic effect was anticipated.

1,2,4,5-Tetraoxane-8-aminoquinoline hybrids with various aryl or heteroaryl group as substituents at the metabolically labile C(5)-position of the 8-aminoquinoline moiety were tested *in vitro* as dual-stage antimalarial agents against *P. falciparum* CQ resistant strain (blood stage) and *P. berghei* (liver stage) (Capela et al. 2018). Derivatives **95–98** (Fig. 14) have improved metabolic stability, and proved to be efficient as dual-stage antiplasmodial agents with good metabolic stability, inhibiting the development of intra-erythrocyte forms of *P. falciparum* (EC_{50} = 15–17 nM) and *P. berghei* liver stage (EC_{50} = 1.11 μM).

Hybrid compound **99** (Fig. 14) containing a ruthenocene, 4-aminoquinoline, and a 1,2,4-trioxane motifs exhibited IC_{50} = 65.33 nM and IC_{50} = 62.0 nM against CQ resistant and MDR strain of *P. falciparum*, respectively (Martínez et al. 2017). As such derivative **99** was better than CQ, and the parent metal-free and quinoline-free trioxane, indicating possible route for designing new derivatives than could overcome resistance problem.

Hybrid derivative **100** and **101** (Fig. 14), contained 4-aminoquinoline and a quinolizidine (Sparatore et al. 2005) fragments displayed a very

attractive bioactivity profile (Basilico et al. 2017). Both racemic (1*R*/1*S*)-AM1 and enantiomers exhibited an antimalarial activity in low nanomolar range against sensitive and CQ resistant strains, as well as against MDR strain of P. falciparum. Racemic (1*R*/1*S*)-mixture showed remarkable antimalarial activity against ten different isolates of *Plasmodium vivax* with three times higher potency than CQ. Enantiomer **(1*S*)-100** displayed an additive effect when applied in combination with dihydroartemisinin. All forms, racemate and enantiomers, were *in vivo* active with no significant differences in potency among them.

Enantiopure chiral 4-aminoquinoline derivatives that contain piperazine were synthesized (Dola et al. 2017) among which derivatives **102–104** (Fig. 14) showed activities in the range of 5.03–39.39 nM against *P. falciparum* strains. Compound **102**, showed the best activity *in vivo*, and successfully cured mice infected with CQ resistant *P. yoelii* strain. This derivative has been identified as a preclinical candidate molecule, due to excellent physicochemical properties, an acceptable pharmacokinetic profile, and moderate metabolic clearance in liver microsomes.

Large number of examined quinoline derivatives has provided necessary information on structure-activity relationship and revealed new potential targets for the treatment of malarial parasite. However, although those results are highly valuable, new derivatives still have not brought much needed breakthrough in the therapy. Currently, in accordance with WHO recommendation, the most successful clinical treatments are combination therapies, which include established antimalarial drugs, such as artemisinin (Chico and Chandramohan 2011).

6.2 Antileishmanial Activity

Leishmaniasis is caused by *Leishmania* parasites and is transmitted to humans by the bite of infected female phlebotomine sandflies. The disease may occur in humans and animals, including those from the closest human surrounding. About 70 animal species and humans can be a reservoir of *Leishmania* and depending on the source it can be classified as zoonotic or anthroponotic. More than 20 different *Leishmania* species that infect mammals can cause infection in humans. There are four main forms of the disease: visceral leishmaniasis (also known as kala-azar), post-kala-azar dermal leishmaniasis, cutaneous leishmaniasis, and mucocutaneous leishmaniasis (World Health Organisation 2018). While cutaneous leishmaniasis is the most common form of the disease, visceral leishmaniasis is the most serious and almost always fatal if untreated. Based on the assumption that similar chemotypes could be active against related pathogens, broad series of quinoline-like structures (Woodring et al. 2015), and quinolines (Baragana et al. 2015; Devine et al. 2015; Konstantinovic et al. 2017; Solaja et al. 2008) were examined simultaneously as antimalarial, antileishmaniasis and antitrypanosomal agents, including application in combination therapy (Wijnant et al. 2017).

Ten out of thirty tested quinoline derivatives that contained adamantine or benzothiophen moiety exhibited IC_{50} values less than 1 μM against *Leishmania infantum* and *Leishmania tropica* promastigote stage (Konstantinovic et al. 2018). Two derivatives, **105** and **106** (Fig. 15), exhibited dose-dependent *in vivo* activity, and a high level of mice survival and reduction of parasites level – up to 99% after 50 mg/kg/day and 5 days treatment. Those two compounds induced production of nitric oxide by IFNγ-primed macrophages, but only when highest doses were used. Also, both derivatives induced a persistent increase of reactive oxygen species (ROS) at all tested doses.

During the search for new scaffolds that would be able to inhibit three protozoan pathogens, quinoline derivatives **107–109** (Fig. 15) tested for inhibitory activity against two life stages (promastigote and amastigotes) of *Leishmania major* (Devine et al. 2015). While derivatives **107–109** showed high activities against promastigote life stage, exhibiting EC_{50} values in the range 0.2–0.4 μM, only derivative **109** also showed activity against amastigotes life stage ($EC_{50} = 0.89$ μM).

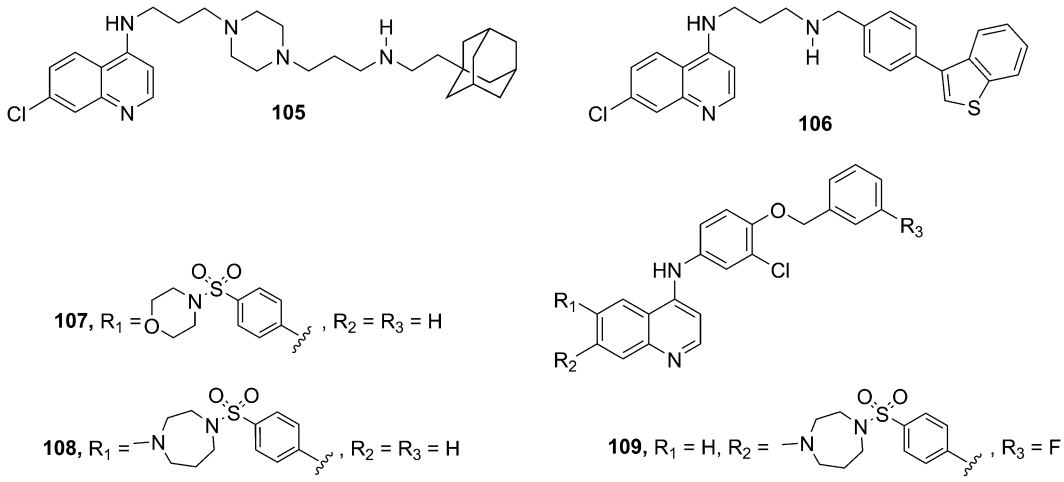


Fig. 15 4-Aminoquinolines with antileishmanial activity

Two derivatives of 4-aryloxy-7-chloroquinolines showed a strong inhibition of *Leishmania donovani* promastigote (IC_{50} = 11.43 μM and IC_{50} = 7.9 μM) and amastigote proliferation (IC50 = 1.01 μM and IC50 = 1.02 μM) (Valdivieso et al. 2018). Combinations of **110** or **111** (Fig. 16) with amphotericin B showed a synergistic anti-promastigote effect, while combinations of either **110** or **111** with miltefosine showed a synergistic anti-amastigote effect. Compounds showed deleterious effect on the mitochondrial electrochemical potential in IC_{50} concentrations, suggesting that rapid collapse of the parasite's mitochondrial membrane potential could be a mechanism of action.

A quinoline derivative **112** (Fig. 16) exhibited a strong effect against *Leishmania amazonensis*, inhibiting the growth of its promastigotes and intracellular amastigotes forms (Antinarelli et al. 2015; Antinarelli et al. 2018). The compound also affected the proliferation of amastigote-like forms of *L. amazonensis*, with IC_{50} = 7.0 μg/ml. Intramacrophage amastigotes treated with **112** showed a low capacity to reverse the effect of the compound in a drug-free medium. The strong effect on all parasite stages and the absence of reversibility of the action of **112** suggest that severe damage has been caused to the targets, and mechanism of its activity was preliminarily associated with mitochondrial dysfunction.

A quinoline derivative clioquinol (Fig. 5) inhibited *L. amazonensis* and *Leishmania infantum* promastigotes with EC_{50} = 2.55 μg/ml and EC_{50} = 1.44 μg/ml, respectively, and also showed inhibitory activity against axenic amastigotes of *L. amazonensis* (EC_{50} = 1.88 μg/ml) and *L. infantum* (EC_{50} = 0.98 μg/ml) (Tavares et al. 2018). Significant reductions in the percentage of infected macrophages after treatment and in the pre-treated assay using clioquinol were observed. Clioquinol induces morphological and biochemical alterations in parasites, including reduction in cell volume, loss of mitochondrial membrane potential, increase in the ROS production and rupture of the plasma membrane.

N-Aryl derivatives of 2- and 3-aminoquinoline were evaluated as antiproliferative agents against *Leishmania mexicana*, the etiological agent of leishmaniasis and *Trypanosoma cruzi* (Chanquia et al. 2019). Fluorine-containing derivatives **113** and **114** (Fig. 16) were more than two-fold more potent than geneticin against intracellular promastigote form of *L. mexicana*, both exhibiting IC_{50} values of 41.9 μM. The IC_{50} values of derivatives **113–115** (Fig. 16) were of the same order as drug benznidazole against epimastigote form of *T. cruzi*. The compounds were capable to inhibit the degradation of haem, inducing intracellular

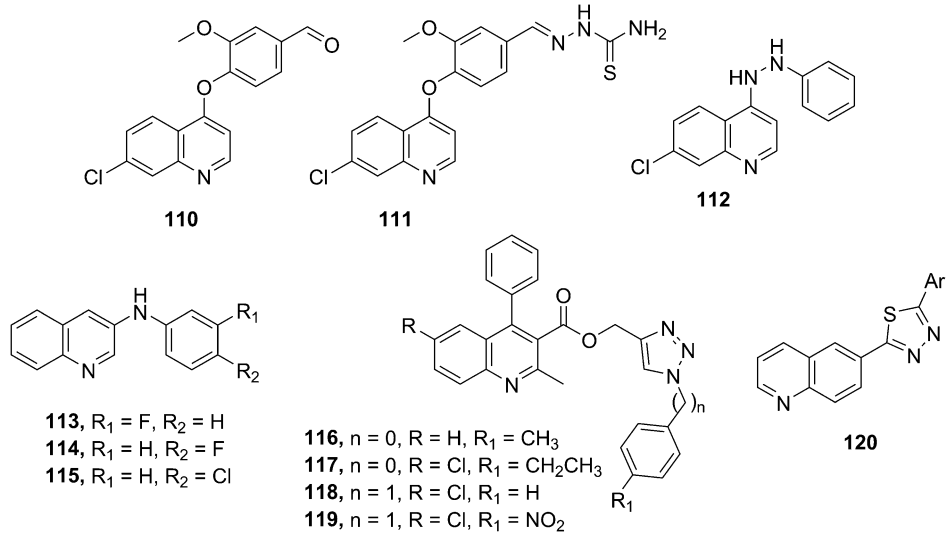


Fig. 16 Quinoline derivatives with antileishmanial activity

oxidative damage, which is not countered by the antioxidative defence system of the parasite.

Series of triazolyl esters of 2-methyl-4-phenylquinoline-3-carboxylic acid have been evaluated *in vitro* against *L. donovani* (Upadhyay et al. 2018). Most of the derivatives exhibited significant antileishmanial activity against promastigotes and intracellular amastigotes, with less cytotoxicity in comparison to the sodium stibo-gluconate. Four compounds **116–119** (Fig. 16) proved to be active derivatives, with lower toxicity and better selectivity index and were selected for *in vivo* evaluation. Compound **118** showed consistent activity up to day 28 post-treatment in a hamster model, which is promising finding as the hamster model of visceral leishmaniasis closely resembles the human condition.

Sixteen derivatives of 6-substituted quinoline analogues **120** (Fig. 16) showed high inhibitory potency against *L. major* promastigotes with IC_{50} = 0.04–5.60 μM, and all were more active in comparison with pentamidine (IC_{50} = 7.02 μM) (Almandil et al. 2019). Presence of either electron donating or electron withdrawing group on phenyl ring plays an important role in inhibition, but electron donating group showed better activity.

Leishmaniasis did not take much attention in R&D programs in comparison to other parasitic disease, like malaria. However, since the disease is continuously increasing in Eastern Mediterranean Region, with 5 times growth in the period 1998–2015 (World Health Organisation 2017), there is an urgent need for changing of such a treatment. Although many quinolines exhibited good antileishmanial activity, their targets are still unknown.

Identification of the targets is prerequisite to direct further structural modifications of quinoline scaffold in order to optimize their therapeutic activity.

6.3 Antitrypanosomal Activity

Trypanosoma is unicellular parasitic protozoa which causes infections in humans in the tropical and sub-tropical areas. Trypanosomes infect a variety of hosts and cause fatal disease, such as sleeping sickness in humans. More than 19 different *Trypanosoma* species were identified as causative agents of diseases that differ in symptoms, action and main host. *Trypanosoma cruzi* causes American trypanosomiasis, or Chagas disease, when it infects a wide variety of wild and domestic mammals, rodents, many species of bloodsucking triatomid insects – the usual vectors of disease, and humans. The other members of the genus *Trypanosoma* that cause disease in humans

are two subspecies of African trypanosomes, *T. brucei* subspecies *gambiense* and *T. brucei* subspecies *rhodesiense*. These organisms cause West African and East African trypanosomiases, respectively. The main limitation of the drugs that are currently in use against *Trypanosoma* is toxicity and requirement for intravenous dosing, thus novel drugs are needed. One approach is to develop compounds that show activity against molecular targets that are common for related pathogens. For example, *T. brucei* (human African trypanosomiasis), *T. cruzi* (Chagas' disease), *Leishmania* spp. (leishmaniases), and *Plasmodium* spp. (malaria) express kinases and phosphodiesterases which are involved in the process of cellular signalling (Gould and de Koning 2011; Parsons et al. 2005). Recently it was found that certain human kinase inhibitors could be also effective against *T. brucei* with EC_{50} in the low micromolar range (Katiyar et al. 2013), including tyrosine kinase inhibitors and new compounds developed on that basis (Patel et al. 2013).

Quinoline and quinoline-like compounds were tested for their antiprotozoan activity (Devine et al. 2015). From 4-aminoquinoline scaffold, derivatives **121–123** (Fig. 17), and derivatives **108** and **109** (Fig. 15) showed medium nanomolar activities against *Trypanosoma brucei* and *Trypanosoma cruzi*. In general, derivatives showed better activity against *T. brucei* (ED_{50} = 79–450 nM), than against *T. cruzi* (ED_{50} = 730–950 nM), with exception of derivative **122**, which is more active against *T. cruzi* (EC_{50} = 90 nM vs. EC_{50} = 350 nM).

The TbrPDEB1 enzyme, a member of cyclic nucleotide phosphodiesterases was used as a target for series of 3-carbamido-4-amino quinolines (Ochiana et al. 2015). Derivatives **124–127** (Fig. 18) exhibited the highest activity, inhibiting >80% of TbrPDEB1 and exhibiting IC_{50} in the range 3.5–6.4 μM.

Another derivatives of chalocone (**128;** general structure **A**, Fig. 18) and corresponding 4,5-dihydro-1H-pyrazole derivatives (**129**; general structure **B**, Fig. 18) were screened against intracellular amastigotes of *T. cruzi* and intracellular amastigotes of *Leishmania* (*Vianna*) *panamensis* (Ramirez-Prada et al. 2017). Compound **129** was highly active against *T. cruzi* (EC50 = 0.70 μg/ml), while the best antileishmanial activity exhibited compound **128**, which was active at 0.79 μg/ml.

Novel 7-phenyl-quinolines **130–133** (Fig. 18) were evaluated against bloodstream forms of *T. cruzi*. Nine quinolines were more effective against amastigotes than benznidazole (EC_{50} = 2.7 μM) and they showed EC_{50} values ranging from 0.6 to 0.1 μM (Nefertiti et al. 2018). All examined quinolines were highly active *in vitro* against African trypanosomes, showing EC_{50} values ≤0.25 μM. The most potent *in vitro* candidates **130** were tested in *in vivo* models of *T. b. rhodesiense* infection, and showed a more than 98% reduction of the parasitemia and curing a half number of infected mice, after 3 doses of 40 mg/kg intraperitoneal administration However, derivative **131** was even more efficacious, showed complete reduction of the parasitemia and cured a half of infected mice.

A series of sulfone derivatives were tested for their *in vitro* activity against *T. brucei*. From 22 examined compounds, derivatives obtained from 8-aminoquinoline **132** and **133** show the

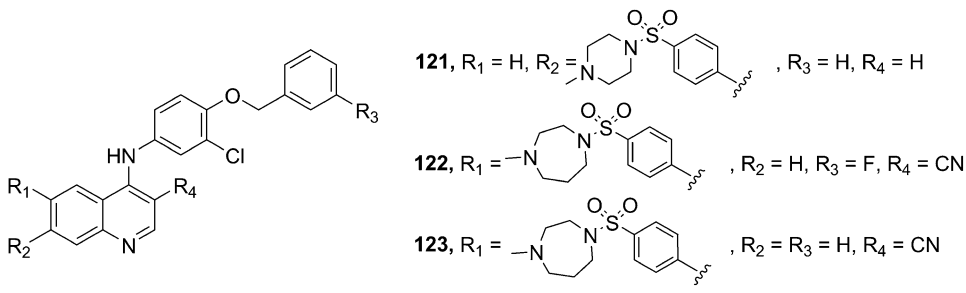


Fig. 17 4-Aminoquinolines with antitrypanosomal activity

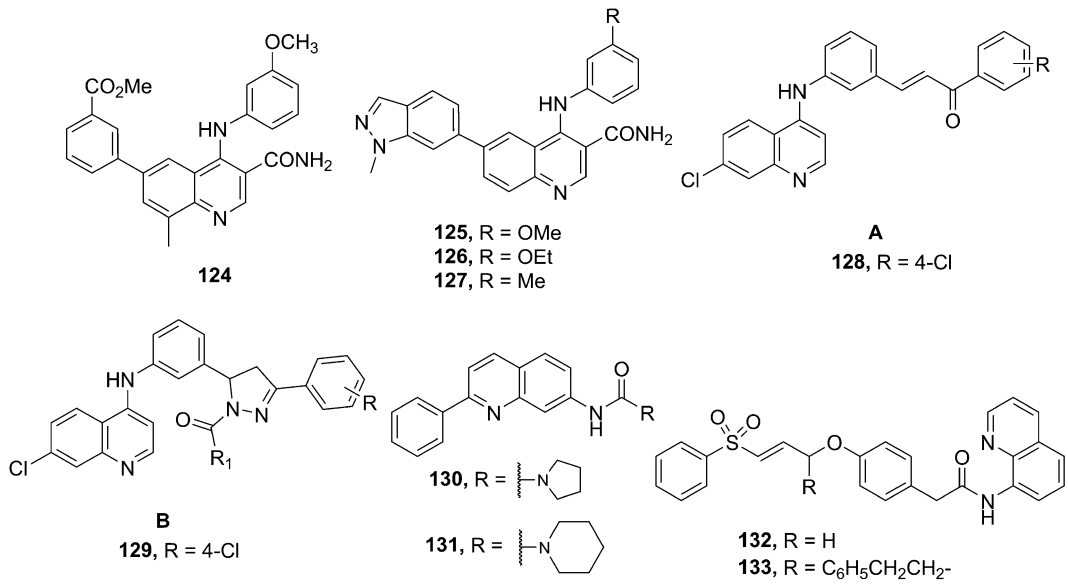


Fig. 18 Quinoline derivatives as antitrypanosomial agents

best inhibitory activity $IC_{50} = 0.76$ μM (Zhang et al. 2018). However, during in vivo evaluation against *T. brucei*, when infected mice were treated for four consecutive days intraperitoneally, derivative **132** did not shown activity, and the infected mice were positive for parasites 24 h post-treatment. However, second 8-quinoline derivative **133** was able to completely inactivate rhodesain, a cysteine protease essential for parasite survival and infectivity (Ettari et al. 2013), at 20 μM after 1 h of incubation with estimated IC_{50} value of 800 nM.

Pursuit for efficacious clinical therapy based on quinoline-based compounds still continues. Although there are increasing numbers of reports on trypanosoma growth inhibitors, and identification of potential targets, the mechanism of action (s) remain unknown.

7 Conclusion

As emergence and spread of the resistance to existing therapeutics are inevitable the novel advanced drugs and strategies for treatments of numerous infective diseases, affecting tens of millions of people worldwide, are continuously being demanded. Among large number of various pharmacophores used for development of effective antimicrobials, quinoline/quinolone scaffold can be considered a privileged structure since it displays wide range of bioactivities. The diversity of synthetized quinoline/quinolone derivatives provides high and selective activity through different modes of action and low cytotoxicity on human cells.

The drugs based on quinoline/quinolone structure are well-known and in use for over half a century in the treatment of bacterial, parasitic, and viral infections. Besides, in the last two decades quinoline/quinolone derivatives emerged as efficient anti-virulence agents targeting bacterial and fungal virulence factors, including formation of biofilms, characteristic of chronic infections. Of particular importance is the ability of certain quinoline-based compounds to target both bacteria and fungi, either their planktonic or biofilm life form, suggesting that these compounds are promising chemical matter for the development of therapeutics against mixed-species and/or biofilm-associated infections. Quinoline/quinolone derivatives exhibit strong activity by themselves and can increase efficacy of commonly used drugs when applied together. Recently, a

new promising approach was employed in the development of more successful quinoline-derived therapeutics based on the synthesis of hybrid compounds that contain two or more pharmacophores affecting different targets and/or employing distinct mechanisms of action. Hence, there are still plenty of possibilities for development of novel more effective antimicrobials.

The high potency of quinoline/quinolone derivatives is demonstrated by their activities in nanomolar and low micromolar concentrations against wide range of infective agents including resistant strains. The importance of quinoline/quinolone as a scaffold for drugs' development is emphasized by recently granted accelerated approval for clinical use to a new quinoline-based antibiotic developed to fight MDR mycobacteria.

Therefore, there are more than enough reasons to continue quest for efficacious clinical therapeutics based on quinoline/quinolone structures since they are still proving to be unprecedented source of much needed solutions for infections treatments.

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Current Research and New Perspectives in Antifungal Drug Development

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Abstract

In recent times, fungi are becoming more and more active as causal agents of human infections, which is primarily determined by the growing number of people with severe immunosuppression. Thus, the problems of elucidating the mechanisms of action of antifungal preparations, highlighting ways to obtain resistance to their action and research strategies aimed at discovering new compounds with antifungal properties remain the focus of contemporary biomedicine and pharmaceutics. This paper reviews the recent achievements in antifungal drug development and focuses on new natural antifungal remedies with a noticeable effect on pathogens with minimal adverse effects on the host organism.

Keywords

Drug resistance · Natural antifungals · Oxidative stress · Pathogenic fungi · Spirulina extracts

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1 Introduction

In recent times, fungi are becoming more and more active as causal agents of human infections, which is primarily determined by the growing number of people with severe immunosuppression. The list of this type of patients includes people suffering from haematological and autoimmune diseases, have undergone organ transplantation, or have a compromised immune status (Debourgogne et al. 2016). Even in the case of appropriate treatment, most invasive fungal infections are associated with high mortality rates of over 50% (Brown et al. 2012; Chowdhary et al. 2016). This explains the major interest towards the role of fungi in the development of various pathological conditions (Caggiano 2012). Although, much work is being done to develop more active and less toxic antifungal drugs and preventive measures are applied virtually worldwide, mycoses (especially deeply invasive ones) continue to be some of the most serious infectious complications that cause an unacceptable high mortality rate (Lehrnbecher et al. 2010). Fungal diseases may take the form of superficial (dermatophyte), invasive (systemic) and opportunistic infections. Skin fungal infections are caused by dermatophytes that live in the upper most keratin layer, the nail or the hair shaft. This concept is actually based on the specific location of affections and not on the systematic belonging of causal agents, although most dermatophytes belong to three genera *Microsporum*, *Trichophyton*

and *Epidermophyton*. It is worth mentioning that dermatomycoses are widespread among immunocompetent patients and are associated with a very high morbidity. According to some authors, approximately a quarter of the world's population is infected with these pathogens (Brown et al. 2012; Havlickova et al. 2008).

Invasive (systemic) fungal infections have a much lower incidence than superficial infections, but cause a greater concern due to an unacceptable high mortality rate. These infections annually take the life of about one and a half million people (Harrison et al. 2014). This type of infection occurs following the inhalation of spores that cause fungal pneumonia, which cannot be transmitted from person to person, but can also occur in healthy individuals. Many microorganisms that cause systemic fungal infections are characterized by specific geographic locations with favorable climate for their proliferation. Among the most common diseases of this type are coccidioidomycosis (*Coccidioides immitis*), histoplasmosis (*Histoplasma capsulatum*) and blastomycosis (*Blastomyces dermatitidis*). In Canada, for example, Ontario is an important region of endemicity for blastomycosis and histoplasmosis, and in USA- histoplasmosis and blastomycosis incidence is highest in the Midwest, but coccidioidomycosis incidence rate is highest in the West (Baddley et al. 2011; Brown et al. 2018). Nearly 90% of *fungal infections resulting in deaths are* caused by pathogenic fungi from the genera *Cryptococcus*, *Candida, Aspergillus* and *Pneumocystis* (Velayuthan et al. 2018).

Fungi causing opportunistic infections (*Candida albicans*, *Cryptococcus neoformans*, *Aspergillus* sp., *Murcor* sp., etc.) are not hazardous to healthy people. Usually, these fungi affect individuals with a weakened immune system, causing serious infections such as candidiasis, cryptococcal meningitis, aspergillosis, mucormycosis, etc. Patients *especially susceptible* to these infections include people with diabetes, leukemia, cancer, HIV and other types of immunodeficiencies (Cowen et al. 2014; Oltu and Rudic 2016). The *development* of medical practices with the introduction of new therapeutic procedures, such as the use of some aggressive chemotherapies or new immunosuppressive drugs, such as tumor necrosis factor (TNF) antagonists, anti-CD52 antibody (alemtuzumab) and interleukin-2 receptor antagonist (basiliximab), favored an increased incidence of invasive fungal infections (Debourgogne et al. 2016).

The number of patients susceptible to invasive infections caused by filamentous fungi has reached alarming proportions and continues to grow steadily. This group of microorganisms is ubiquitous in different natural habitats such as soil and various organic substrates. The most common etiologic agents of invasive mycoses are part of the genera *Aspergillus* and *Mucor*. In recent years, this list has been supplemented with less frequent filamentous fungi in the environment, such as *Fusarium spp.* and *Penicillium spp*. Nonetheless, the medical term for fungal invasion has also extended its application limits from invasive disease to less recognized entities previously, such as severe asthma with fungal sensitization, fungus-associated chronic cough, allergic bronchopulmonary mycosis and allergic fungal rhinosinusitis (Chowdhary et al. 2016; Ogawa et al. 2009; Singh et al. 2013).

Thus, the problems of elucidating the mechanisms of action of antifungal preparations, highlighting ways to obtain resistance to their action and research strategies aimed at discovering new compounds with antifungal properties remain the focus of contemporary biomedicine and pharmaceutics.

2 Antifungal Preparations and Their Mechanisms of Action

Appropriate antifungal therapy is prescribed in dependence on the patient's immune status, site of infection, the intrinsic properties of the pathogen and the pharmacokinetic characteristics of a drug. Antifungal preparations, which can be successfully applied in the treatment of mycoses in humans, should act on the basis of differences between the fungal cells and those of the human body. At the same time, fungi like mammals are eukaryotic organisms, and therefore, the

development of preparations that attack the fungal cells without causing damage to the human body is an extremely difficult task. One of the distinctions, which can be exploited in the pharmaceutical design of antifungal drugs, is the presence of a specific sterol found in the cell membrane of yeast – namely ergosterol (with many of the same functions that cholesterol serves in animal cells) (Parks and Casey 1996). Specific targets for the antifungal action are also the components of cellular wall – mannoproteins and β-glucans. Consequently, the vast majority of active drugs against pathogenic fungi are developed in particular on the basis of these essential differences.

Preparations that are currently used to treat fungal diseases may be divided by several criteria, the main of which is the mechanism of action. After this criterion, antifungal drugs are divided into 7 groups, according to Table 1.

The mechanisms of action of substances belonging to these seven groups are well known and fairly well studied and described. At the same time, there are many antifungal agents that cannot be attributed to any of these mechanisms. Ciclopirox – a topical antimycotic agent belonging to the chemical class of hydroxypyridones is not related to azoles or any other class of antifungal drugs. Its antifungal profile targets almost all clinically important dermatophytes, yeasts and molds, and is therefore wider than the profile of most antimycotics. It is active against certain azole-resistant strains of *Candida albicans* and against some bacteria. The mechanism of action is thought to be through the chelation of polyvalent metal cations, such as Fe^{3+} and Al^{3+}. These cations are cofactors of many enzymes, including cytochromes and their inhibition may lead to the disruption of the biosynthesis of ergosterol. Ciclopirox is also thought to act by modifying the fungal plasma membrane, resulting in the disorganization of internal structures. This unique mechanism provides a very low potential for the development of resistance to this drug (Subissi et al. 2010).

Organic acids such as caprylic acid, salicylic acid, undecylenic acid, propionic acid, and benzoic acid exhibit antifungal activity by interacting with non-specific components in the cell membrane. Reports over the years have indicated that short and medium-chain organic acids have antimicrobial properties. In addition, common aromatic acids such as benzoic acid, have found utility as preservatives in foods on the basis of their antifungal properties. Sorbic acid is an antimycotic agent which demonstrates broad spectrum activity against yeast and fungal molds. Most mentioned compounds act by targeting different stages of biotin biosynthesis, which is essential for fungal metabolism (Mazu et al. 2016).

Some natural preparations like haloprogin, the active part of which is allicin, appear to have a mode of action related to its ability to cross the cell membrane and combine with sulfur-containing groups in amino acids and proteins and interfering with cell metabolism. More often the effects of this preparation are associated with the fact that allicin blocks glutathione activity (Davis 2005).

Table 1 Classification of antifungal drugs

The groups, according to the mechanism of action	Examples
Inhibitors of fungal cell wall synthesis	Nikkomycins, Polyoxins, Caspofungin, Micafungin, Anidulafungin, Benanomicin, Pradimicin
Antifungals that operate by complexing directly with membrane ergosterol	Nystatin, Amphotericin B
Concomitant inhibitors of lanosterol and ergosterol biosynthesis	Terbinafine, Naftifine, Butenafine
Inhibitors of fungal ergosterol biosynthesis	Fluconazole, Itraconazole, Ketoconazole, Voriconazole, Clotrimazole
Inhibitors of nucleic acid synthesis	Flucytosine
Inhibitors of fungal mitosis	Griseofulvin
Antifungal drugs with other mechanisms of action	Ciclopirox, Haloprogin

Some of the mechanisms of antifungal activity relate not to the direct action on the fungi, as to the modeling of host-pathogen interaction. Very often it is noted that natural antifungal agents stimulate the cellular immunity of the affected macroorganism. These effects were shown in the process of study of tea tree oil, citronella oil, orange oil, palmarosa oil, lemon and myrtle oils, coconut oil – all exhibit pronounced antifungal activity. However, it is hypothesized that they might act by altering membrane properties and compromising membrane-associated functions. Olive leaf extract directly stimulates the process of phagocytosis of fungi in the body (Uniyal et al. 2012).

There are also a lot of preparations in this category, which act on membrane ATPases, on signal-transduction pathways, electron transport chains and other metabolic processes of vital importance. However, further research is required, as all these affect common elements of fungal and human metabolism, and therefore, do not meet the requirements for an ideal antifungal agent.

3 Antifungal Natural Products

The adverse antifungal therapy reactions, in association with the high rate of multi-resistant pathogens, require the development of new remedies for the treatment of fungal infections. Furthermore, the intensive use of fungicides in agriculture, food industry, etc. causes essential damage to both the environment and human health. For that reason, the natural compounds with antifungal activity have become an attractive alternative over the past two decades. There are several classes of phytochemicals with antifungal activity: terpenes, tannins, flavonoids, alkaloids, lecithin, various polypeptides and others (Castillo et al. 2012).

Plants produce a variety of medicinal components that can inhibit pathogen growth. A considerable number of studies of medicinal plants and alternative compounds, such as secondary metabolites, phenolic compounds, essential oils and extracts, have been performed (Negri et al. 2014). Most of the antifungal compounds of plant origin belong to the phenol category. This group is extremely heterogeneous, including over 10 thousand compounds, a large part of them present in leaf extracts, tree bark, wood, fruits and other tissues of fern, gymnosperm and angiosperm plants. Phenols perform important functions, like resistance to microorganisms (including fungi), insects and herbivorous animals that can affect them. In particular, polyphenols have strong antioxidant properties, making them active in the fight against stress and free radicals, maintaining plant integrity in the process of continuous exposure to ultraviolet radiation, relatively high temperatures and dehydration.

Plant phenolic compounds display high antifungal activity against various species (*Penicillium italicum, Penicillium expansum, Monilinia laxa, Aspergillus carbonarius, Botrytis cinerea, Monilinia fructicola* and others) (Gatto et al. 2013). Phenol action on yeasts differs from the mode of action on filamentous fungi (Ansari et al. 2013). Thus, *Candida* dimorphic species are affected by phenolic compounds in the process of transition from one form to another. For example, phenolic compounds of *Baseonema acuminatum* leaves, *Lycium chinense*, *Sida urens, and Curcuma longa* root bark have shown strong antifungal activity against various *Candida albicans* strains. Their effect is associated with an increase in the amount of reactive species of oxygen and nitrogen in fungal cells, with predominant location in membrane, which induces early apoptosis. It is very important that the effects are irreversible and cannot be removed by the action of antioxidants (Ansari et al. 2013; Zerbo et al. 2014).

The extract from *Curcuma longa* is extremely effective against *C. albicans* strains. The mechanism of antifungal effect is based on inhibition of desaturase expression (ERG3), leading to a significant reduction of ergosterol in fungal cells. Accumulation of biosynthetic precursors of ergosterol in cells leads to cell death by the generation of reactive oxygen species (ROSs). Thus, the curcumin, bisbibenzyl, carvacrol and thymol compounds have been successfully used in the candidiasis treatment, through their effects on dimorphic transformation, biofilm formation and ROS generation (Ansari et al. 2013).

Among the phenolic compounds, hydrolysable tannins are also recognized as antifungal agents. In particular, by their effect on filamentous fungi *Epidermophyton floccosum, Microsporum canis, Microsporum gypseum, Trichophyton mentagrophytes, Trichophyton rubrum, Trichophyton tonsurans, Trichophyton terrestre, Penicillium italicum, Aspergillus fumigatus, Mucor racemosus, Rhizopus nigricans,* as well as on pathogenic opportunistic yeasts *Candida albicans, Candida glabrata, Candidata krusei, Cryptococcus neoformans* (Latté and Kolodziej 2000). The biological action of these compounds on fungi is associated with the formation of stable bonds between tannins and fungal cell proteins, leading to inactivation of enzymes and degradation of structural proteins (Marichal et al. 1997). Condensed tannins (also called proanthocyanidins) are polymers of 3-flavanol and 3–4 flavandiol (catechin and leucoanthocyanidins). These low solubility substances exhibit biological properties, including antifungal action, with a particularly pronounced efficacy in disaggregation of biofilm matrix.

Terpenoids are another group of natural compounds that are known for multiple biological effects, including the antifungal one. They make up the largest class of plant secondary metabolites, most of which are insoluble in water. Different substances such as geraniol, nerol, citral, neral and geranial have been shown to be very active against dermatophytes and opportunistic pathogenic yeasts, the maximum activity level being expressed on *Trichophyton rubrum.* The mechanism of action of these substances is explained by their affinity for ergosterol, which leads to the modification of fungal membrane fluidity (Arif et al. 2009; Miron et al. 2014).

Many plant species produce alkaloids characterized by broad-spectrum antifungal activity. For example, the medicinal plant *Kopsia hainanensis* produces 15 indole alkaloids, which exhibit high antifungal activity against *Erwinia carotovora* and *Fusarium oxysporum* (Chandrasekar 2011). From the aerial parts of the *Waltheria* plant, 11 quinolone alkaloids with antifungal activity against *Candida albicans* (CMI <32 μg/ml) were obtained by extraction in dichloromethane. The microscopic examination of yeasts treated with these compounds revealed multiple deviations from the normal morphological structure (Cretton et al. 2016). Oxygen-containing alkaloids of microbial origin possess high antifungal activity against phytopathogenic fungi, making them attractive for development in agrochemistry (Wang et al. 2016).

Natural antimicrobial peptides (AMP) are probably one of the first-line forms in the chemical defense of eukaryotic cells against bacteria, protozoa, fungi and viruses (Silva et al. 2009). Antimicrobial peptides, including antifungal agents, are part of the innate immune system and practically all organisms – from bacteria to humans – produce these peptides that ensure the integrity of the body.

Defensins are the most common antimicrobial peptides present in all types of eukaryotic organisms. However, there are some practical challenges in new defensin-based drug development. First of all, it is about the amphiphilic cationic character and the protease lability, which leads to the rapid elimination of the drug from the circulating body fluids and limits its systemic administration. However, defensin combines antimicrobial, including antifungal capacity with the ability to positively modulate immune system activity, and has been found to be efficient during biological evolution, which make this peptide extremely attractive for anti-infective strategies (Silva et al. 2009).

4 Cyanobacteria as a Source of Antifungal Compounds

The research on the causative agents of mycoses, especially invasive ones, as well as effective preparations for treating mycoses and applied treatment strategies have marked a noticeable revival over the last two decades. The treatment of fungal infections has improved significantly in the few short years. New antifungal agents have been licensed for use, and indications for the use of various antifungal agents have changed considerably. The introduction of new antifungals from the echinocandin and azole class drugs and the

growing use of lipid formulations of amphotericin B have allowed safer and more effective therapy for severe fungal infections, especially in immunosuppressed patients (Baghi et al. 2016; Blyth 2012; Grover 2010; Kauffman et al. 2011; Perlin 2015; Rodríguez-Leguizamón et al. 2015). The scientific basis of antifungal therapy has been improved with new studies on the pharmacodynamics and pharmacokinetics of these drugs. However, these positive developments have been tempered by increasing resistance of causal agents to several classes of antifungal drugs. Recently, multiple forms of pathogenic fungi have been discovered, for which multidrug resistance to antifungal agents is an inherent capacity fixed at the genetic level (Sanglard et al. 2009).

Thus, the discovery of new substances with antifungal properties remains one of the most important tasks in this field.

Cyanobacteria, the most widely distributed group of photosynthetic prokaryotes, are a prolific source for bioactive compounds, which could be toxic or potentially new drug leads. Over 400 cyanobacterial strains were screened for the production of antifungal compounds, which belong to structural classes, such as peptides, polyketides and alkaloids. There are currently numerous studies that have highlighted the compounds extracted from biomass of various cyanobacterial strains responsible for antifungal effects. The macrolide scytophycin was identified from *Anabaena sp*. HAN21/1, *Anabaena cylindrica* PH133, *Nostoc sp*. HAN11/1 and *Scytonema sp*. HAN3/2, which exhibits antifungal activity against *Candida albicans* and *Aspergillus flavus*. *Anabaena spp*. BIR JV1, *Anabaena spp*. HAN 7/1 and *Nostoc spp*. CENA 219 produced glycolipopeptide hassallidins with pronounced antifungal activity (Kshetrimayum and Kant 2016; Shishido et al. 2015). Among the substances with antifungal effects detected in cyanobacterial extracts, the following compounds have been identified: fisherellin A, hapalindole, hassallidin/balticidins, *carazostatin,* phytoalexin, tolytoxin, scytophycin, toyocamycin, t*jipanazole*, nostocyclamide, nostodione and nostofungicidine (Abed et al. 2009; Burja et al. 2001; Vestola et al. 2014).

Aqueous extracts from biomass of several strains of *Nostoc commune* and *Spirulina platensis* are active against *Aspergillus flavus*, their activity being largely determined by the conditions under which biomass was obtained. The change in nitrogen content of a medium is a factor that determines the antifungal activity of the extracts from these two species (Shaieb et al. 2014). In *Nostoc insulare*, two metabolites were extracted and characterized: norharmane (9H-pyrido(3,4-b)indole) and 4,4-′-dihydroxybiphenyl, which exhibited antifungal activity against strain *Candida albicans ATCC 10231* in concentrations from 32 to 40 μg/ml (Volk and Franz 2006). Many cytotoxic metabolites of different *Nostoc* species possess antifungal activity. For instance, such cytotoxins include nostocyclophanes and borophycin, a boron-containing polyketide, which are associated with blue-green alga *Nostoc linckia* (Rawat and Bhargava 2011).

Different types of spirulina extracts exhibit a certain level of activity towards various strains of fungi. Thus, fractions of terpenoids and sterols extracted from *Spirulina platensis* biomass demonstrated antifungal activity against *Candida albicans* (Uyisenga et al. 2010). Spirulina extracts have also proved effective against fungi *Aspergillus fumigatus* and *Aspergillus niger*. In particular, methanol extracts from spirulina biomass are effective against species of filamentous fungi mentioned above, but also on pathogenic yeast species *Candida albicans* (Kumar et al. 2009).

Extracts in organic solvents from *Spirulina platensis* biomass, the major active components of which are phenolic compounds, possess antifungal action against *Aspergillus flavus* and *Aspergillus niger* (Moraes De Souza et al. 2011; Pugazhendhi et al. 2015). Purified and concentrated hydric extracts from spirulina showed antifungal activity against *Penicillium oxalicum* and *Fusarium solani* (Battah et al. 2014). It is considered that mechanisms of action of spirulina extracts on filamentous fungi are based on the inhibition of the synthesis of ergosterol, glucosamine and proteins.

Thus, there is now sufficient evidence, which confirms that the search for new compounds with

antifungal action must cover as many groups of substances – both chemical and natural synthesis. Such an approach allows the selection of new remedies with high activity and multiple mechanisms of action on fungal cells, which is a guarantee of success in contemporary research.

Effects of hydro-ethanol extracts (20 (Extr.20%), 50 (Extr.50%) and 70 (Extr.70% ethanol) from biomass of *Arthrospira platensis* and *Nostoc linckia* were tested. Hydro-ethanol extracts from spirulina biomass showed antifungal activity against *Trichophyton, Microsporum* and *Candida* (Fig. 1). The most significant antifungal activity was recorded for 20% hydro-ethanol extract, which in the case of *Candida albicans* ATCC®10231™ was more active than reference antifungal preparations included in the study, and in the case of *Microsporum gypseum* ATCC®24102™ was as active as ketoconazole. The 50% hydro-ethanol extract showed activity equivalent to that of ketoconazole against the reference strain *Trichophyton mentagrophytes* ATCC®9533™, and 70% extract demonstrated antifungal activity equal to that of itraconazole and naftifine hydrochloride (NHCh) against *Candida albicans* ATCC®10231™.

Hydro-ethanol extracts from *Nostoc linckia* CNM-CB-03 have shown high biological activity (Fig. 2). The most significant antifungal activity had 20% ethanolic extract, the obtained values being significantly higher than the activity of ketoconazole against *M. canis ATCC®36299™, M. gypseum ATCC®24102™* and *C. albicans ATCC®10231™;* itraconazole against *M. gypseum ATCC®24102™* and *C. albicans ATCC®10231™;* naftifine hydrochloride against *C. albicans ATCC®10231™. Both* 50% and 70% hydro-ethanol extracts also possess antifungal activity, but less pronounced. At the same time, 50% hydro-ethanol extract had antifungal activity equivalent to that of ketoconazole against *Trichophyton mentagrophytes ATCC®9533™,* and 70% hydro-ethanol extract – against *Trichophyton rubrum ATCC®28188™.*

Another series of extracts were obtained from spirulina biomass grown under biotechnological conditions of metal bioaccumulation (Zn(II), Fe (II), Co(II), Cr(II), Cd(II)). During the vital cycle, metals were accumulated in spirulina biomass, and some of them are incorporated into biomass components (proteins, oligopeptide *fraction*, polysaccharides, lipids). In the process of extraction with ethanol and purified water, the organically bound metals pass into respective extracts. *Hydric extracts* (HE_{Me}) and ethanol extracts (EE_{Me}) were standardized after dry mass, and then these were used in research. The most performing results were obtained in the case of applying the ethanolic extract from spirulina biomass with cobalt content. Thus, in three of tested strains, *Microsporum canis* ATCC®36,299™, *Microsporum gypseum* ATCC®24,102™ and *Trichophyton mentagrophytes* ATCC®9533™,

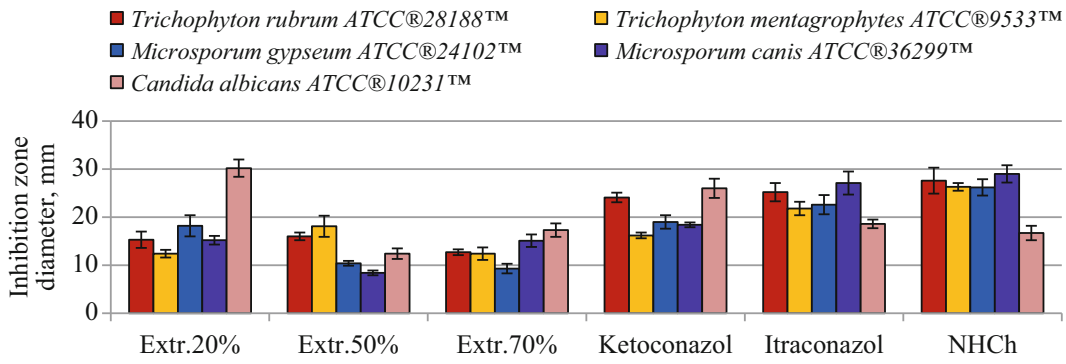

Fig. 1 Antifungal action of hydro-ethanol extracts from biomass of *Arthrospira plantesis.* Extr.20% – hidro-ethanol extract 20% of ethanol; Extr.50% – hidro-ethanol extract 50% of ethanol; Extr.70% – hidro-ethanol extract 70% of ethanol; NHCh – naftifine hydrochloride

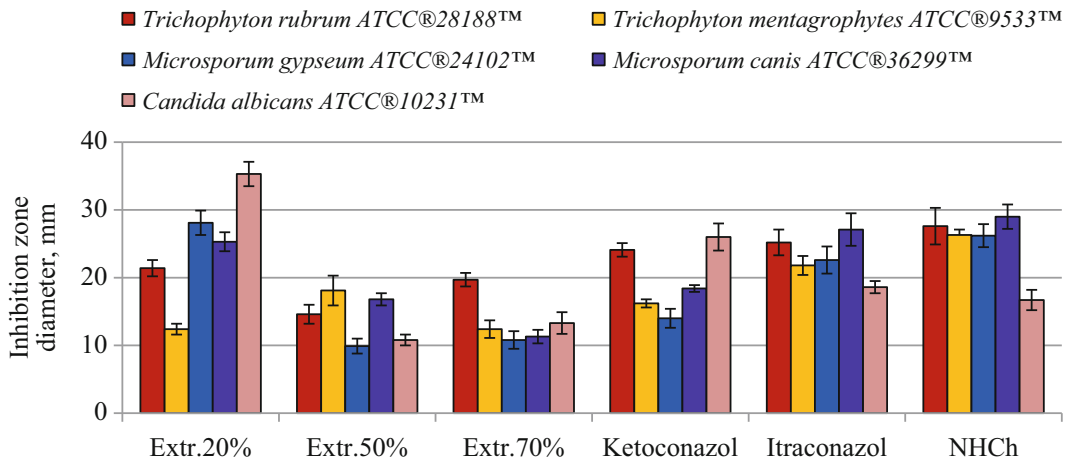

Fig. 2 Antifungal action of hydro-ethanol extracts from biomass of *Nostoc linckia*. Extr.20% – hidro-ethanol extract 20% of ethanol; Extr.50% – hidro-ethanol extract 50% of ethanol; Extr.70% – hidro-ethanol extract 70% of ethanol; NHCh – naftifine hydrochloride

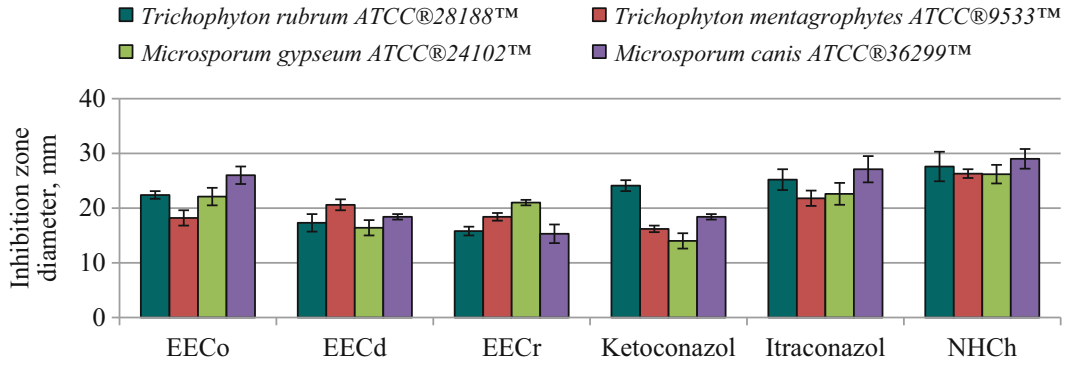

Fig. 3 Antifungal action of extracts from *Arthrospira platensis* biomass with metal content. EECo – ethanol extract from biomass with bioaccumulated cobalt, EECd – ethanol extract from biomass with bioaccumulated cadmium, EECr – ethanol extract from biomass with bioaccumulated chromium, NHCh – naftifine hydrochloride

the diameters of inhibition zones obtained under the influence of this extract were actually larger in comparison with those obtained under the influence of ketoconazole ($P < 0.001$) (Fig. 3).

For all substances with antifungal effect, minimum inhibitory concentration and minimum fungicidal concentration were *assessed by* standard method EUCAST 7.1. Table 2 contains a selection of the best results obtained for tested substances depending on the used strain.

Ethanolic extracts from spirulina biomass containing cobalt and cadmium exhibited high antifungal activity against dermatophyte strains *Trichophyton mentagrophytes* ATCC®9533™, *Trichophyton rubrum* ATCC®28188™ and *Microsporum canis* ATCC®36299™. The ethanol extract from chromium-containing biomass was active against strain *Microsporum gypseum* ATCC®24102™. In the case of filamentous fungi *Aspergillus fumigatus* CNM-FA-02, *Mucor vulgaris* CNMN-FD-07, *Penicillium expansum* CNMN-FD-05, low minimum inhibitory concentrations were obtained for aqueous and ethanol extracts from spirulina biomass

Table 2 Minimum inhibitory concentrations (MICs) and minimum fungicidal concentrations (MFCs) of selected substances against tested fungal strains

Substance	MICs	MFCs	Substance	MICs	MFCs
Trichophyton mentagrophytes ATCC®9533™			*Trichophyton rubrum* ATCC®28188™		
EE_{Co}	0.125 mg/ml	1 mg/ml	EE_{Co}	0.125 mg/ml	1 mg/ml
EE_{Cd}	0.065 mg/ml	0.5 mg/ml	EE_{Cd}	0.065 mg/ml	0.5 mg/ml
Intraconazole	0.25 μg/ml	1.0 μg/ml	Intraconazole	0.125 μg/ml	0.5 μg/ml
Microsporum canis ATCC®36,299™			*Microsporum gypseum* ATCC®24,102™		
EE_{Co}	0.125 mg/ml	1 mg/ml	EE_{Co}	0.125 mg/ml	1 mg/ml
EE_{Cd}	0.065 mg/ml	0.5 mg/ml	EE_{Cr}	0.065 mg/ml	0.5 mg/ml
Intraconazole	0.125 μg/ml	0.5 μg/ml	Intraconazole	0.065 μg/ml	0.5 μg/ml

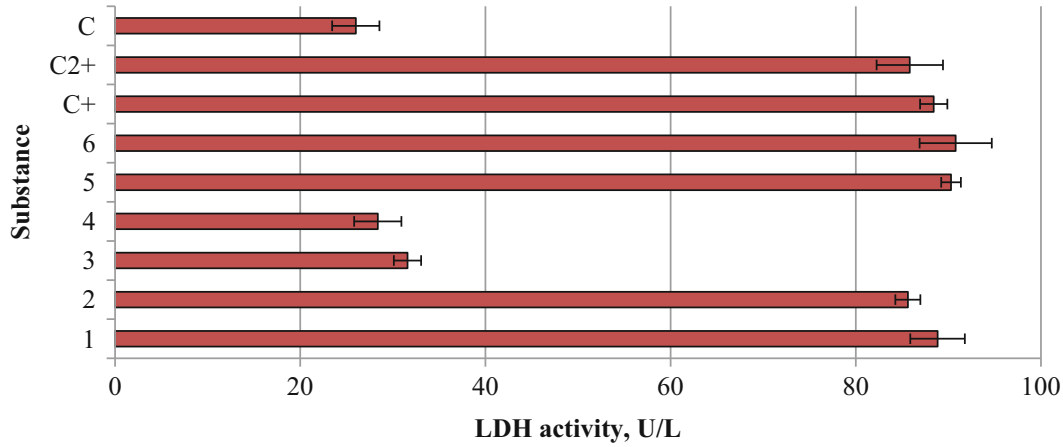

Fig. 4 *Lactate dehydrogenase activity released by Microsporum canis* ATCC®36,299™ under the action of tested substances. 1. *J. Regia* leaf extract (ethanol); 2. 70% hydro-ethanolic extract of *S. platensis* biomass; 3. 20% hydro-ethanolic extract of *N. linckia*; 4. 70% hydro-ethanolic extract of *N. linckia*; 5. EE_{Co}; 6. EE_{Cd}; C_+ – itraconazole; C_{2+} – naftifine hydrochloride; C – untreated control

containing cadmium and chromium. Ethanolic extracts from spirulina biomass containing cadmium and chromium also acted on both natural isolates of the genus *Fusarium.*

In order to identify the mechanisms of action of substances with antifungal effect on target cells, several biochemical tests were carried out. The first used test reffered to measuring the amount of released lactate dehydrogenase (LDH). Thus, increased lactate dehydrogenase activity in the extracellular environment was an indicator for toxic effects on cells and disruption of membrane permeability and structure. The effect of tested remedies was compared with that of the reference antifungal preparations.

In the case of dermatophyte strains *Trichophyton mentagrophytes* ATCC®9533™, *Trichophyton rubrum* ATCC®28188™, *Microsporum canis* ATCC®36299™ and *Microsporum gypseum* ATCC®24102™, the results are similar: substances with antifungal effect caused the loss of intracellular LDH and its release into the culture medium. Hydro-ethanol extracts from spirulina and nostoc biomass did not substantially increase the activity of released LDH. Figure 4 contains the results obtained for culture *Microsporum canis* ATCC®36299™.

The antioxidant status of fungal cultures was *assessed* on the basis of 2 parameters – the ability to reduce free radicals and the level of lipid peroxidation end products.

Both the used positive controls and the selected substances caused the *decrease of antiradical*

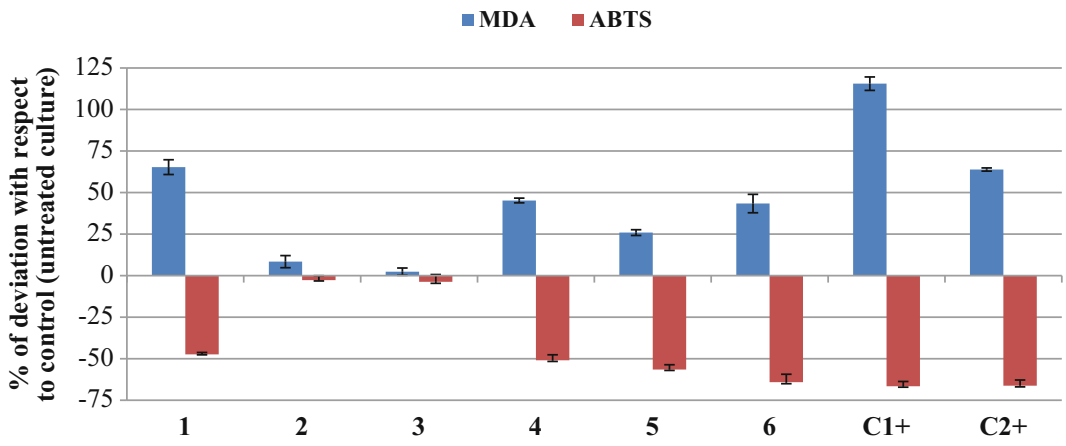

Fig. 5 Antioxidant activity (ABTS *radical* scavenging assay) and MDA levels at *Trichophyton mentagrophytes* ATCC®9533™ under the action of tested substances. 1. *J. Regia* leaf extract (ethyl acetate); 2. 50% hydro-ethanolic extract of *S. platensis* biomass; 3. 20% hydro-ethanolic extract of *N. linckia*; 4. EE_{Co}; 5. EE_{Cd}; 6. EE_{Cr}; $C+_1$ – itraconazole; $C+_2$ – naftifine hydrochloride

capacity and an overaccumulation of lipid peroxidation products. In Fig. 5, as an example, the results for the action of antifungal remedies on culture *Trichophyton mentagrophytes* ATCC®9533™ are presented. The concentration of malonic dialdehyde in control was 2.50 ± 0.12 nmol/g, and total antioxidant capacity was 35.05± % inhibition of $ABTS^{+\cdot}$ radical. The most pronounced effect of intensifying lipid peroxidation had itraconazole (MDA increase by 115.6% compared to untreated control). Close value effects showed naftifine hydrochloride and ethyl acetate extract of walnut leaves, which produced an increase in MDA levels by 62.6 and 65.3%, respectively. The ethanolic extracts from spirulina biomass containing cadmium, cobalt and chromium produced an increase of MDA levels with 25.9–45.2% compared to untreated control. Hydro-ethanol extracts from spirulina and nostoc biomass did not produce modification of MDA levels. Similarly, these extracts did not alter total antioxidant capacity of biomass of *Trichophyton mentagrophytes* ATCC®9533™. Both positive controls and ethanolic extract from chromium-containing spirulina biomass had similar effects on total antioxidant capacity of biomass, reducing it by 64.2–66.7% compared to positive control.

One of the fungal virulence factors that provide defence against the host immunity are the primary antioxidant enzymes – superoxidismutase (SOD), catalase (CT) and peroxidases, that remove reactive oxygen species generated by the host cells and ensure the spread of infection. Reduced activity of these enzymes leads to the inability of fungi to protect themselves from the consequences of oxidative stress and reduce their viability. Therefore, further research aimed at assessing the modification of SOD, CT and glutathione peroxidase (GPx) activity in the tested fungi cells under the influence of antifungal compounds. The activity of the three major antioxidant enzymes was evaluated in fungal cultures reaching the exponential growth phase after 24 h contact with the selected compounds in the concentrations corresponding to CMI. The obtained results showed substantial changes in the activity of the primary antioxidant enzymes in almost all experimental variants.

In the case of dermatophytes, the antifungals caused the decrease of the activity of the first line antioxidant enzymes, again, except the hydro-ethanolic extracts of the standard biomass of cyanobacteria, in which only a decrease in CT activity and an increase in SOD activity was observed. As example, Fig. 6 shows the results for the *Trichophyton mentagrophytes* ATCC®9533 ™ strain.

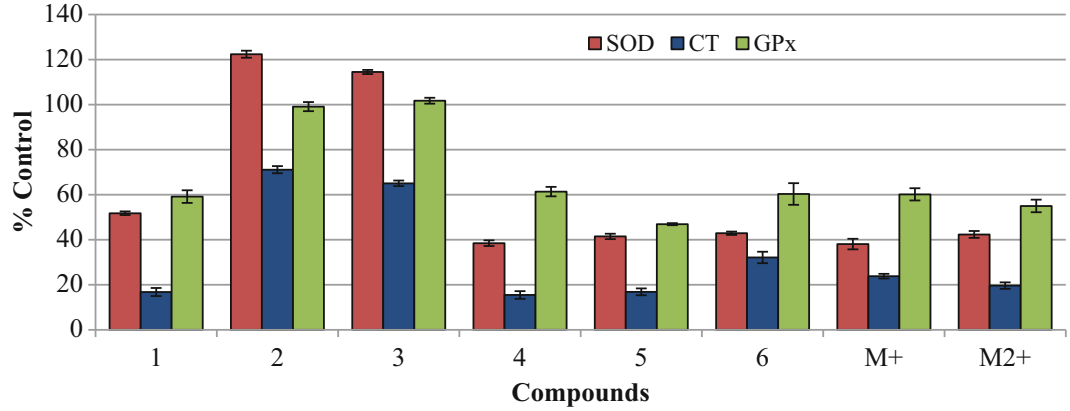

Fig. 6 The activity of antioxidant enzymes in *Trichophyton mentagrophytes* ATCC®9533 ™ under the influence of the studied compounds. 1. Leaf extract *J. regia* (ethyl acetat); 2. Hydro ethanolic etract 50% *S. platensis*; 3. Hydro ethanolic extract 20% *N. linckia*; 4. EE $_{Co}$; 5. EE $_{Cd}$; 6. EE $_{Cr}$; M_+ – Itraconazol; M_{2+} – Naftifine hydrochloride

Thus, the extracts from spirulina biomass obtained under biotechnological conditions of metal accumulation significantly reduced the activity of antioxidant enzymes. Hydro-ethanolic extracts from spirulina and nostoc biomass did not substantially influence GPx activity, decreased CT activity and increased SOD activity, which is explained abowe all by the lack of toxic potential in these cultures. At the same time, these extracts exerted an antifungal activity against studied dermatophytes. Cyanobacterial phycobiliproteins are considered as active elements of the water extracts. These compounds, in particular phycocyanin and phycoerythrin, are present in nostoc hydro ethanolic extracts, and phycocyanin and allophycocyanin – in spirulina extracts.

The tested in this study 50 and 70% hydro ethanolic cianobacterial extracts are also rich in such active components as phenols. Thus, it is certain that the extracts from the standard biomass of spirulina and nostoc, due to the safety for human consumption, but also due to the specific effects on some causative agents of the mycoses, deserve to be the subject of separate studies.

The results of our study suggest that one of the mechanisms of antifungal action of the tested natural extracts is the decrease of the primary antioxidant enzymes activity of the fungi, considered as a virulence factor of the fungal cells.

5 Final Remarks

Pathogenic fungi possess an impressive arsenal of intrinsic and acquired resistance mechanisms to the action of the most effective antifungal agents. Hence the need to develop and implement new remedies with a noticeable effect on pathogens with minimal adverse effects on the host organism. In the current concept there are several perspectives for the development of new antifungal agents, including the detection of natural antifungal compounds and the application of multivalent complex preparations acting on the various levels against multiresistant pathogens. The biomass of cyanobacteria *Arthrospira platensis* and *Nostoc linckia* is a suitable raw material for extracting compounds with potential for the development of new generation of antifungal drugs.

The hydro-ethanolic extracts from spirulina biomass obtained under biotechnological conditions of metal accumulation displayed an antifungal activity against all studied dermatophytes. The best results were recorded for water and ethanolic extracts from spirulina biomass containing cobalt, chromium and cadmium (Oltu and Rudic 2016). The following response reactions of fungicultures were observed at the application of these extracts: extracellular lactate dehydrogenase release; decreased activity

of first line defense antioxidant enzymes – superoxide dismutase, catalase and glutation peroxidase; decreased total antioxidant activity of the fungal biomass and increased quantity of the lipid peroxidation products. These reactions suggest the possible mechanism of antifungal action of tested extracts, based on the generation of oxidative stress, which coincides with the intense degradation of cellular components, primarily membrane ones. As a result, the normal permeability and physical integrity of the fungal wall and cell membrane is compromised (Oltu and Rudic 2016). Reduced activity of primary antioxidant enzymes, considered as virulence factor, and of total antioxidant capacity of causative agents of mycoses under the influence of studied extracts, leads to the inability of fungi to protect themselves from the consequences of oxidative stress and reduce their viability.

Compliance with Ethical Standards

Conflict of Interest: Author Iulian Oltu declares that he has no conflict of interest. Author Liliana Cepoi declares that she has no conflict of interest. Author Valeriu Rudic declares that he has no conflict of interest. Author Ludmila Rudi declares that she has no conflict of interest. Author Tatiana Chiriac declares that she has no conflict of interest. Author Ana Valuta declares that she has no conflict of interest. Author Svetlana Codreanu declares that she has no conflict of interest.

Ethical Approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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HCV Eradication: A Duty of the State, an Option for the Individual

Lucia Craxì

Abstract

In recent years, the debate on ethical issues related to hepatitis C virus therapies has been focused on the problem of drug prices and access to therapies. Nonetheless, the goal of hepatitis C virus eradication set by the World Health Organization in 2016 is raising new ethical issues, since governments are faced with a new challenge: reaching through screening, diagnosis and treatment a large amount of subjects with undiagnosed hepatitis C infection. National governments, especially high-income countries with a Welfare State, are compelled to provide access to therapies, but also to involve those who are still unaware of their disease status.

Since people cannot be forced but should be guided towards the choice of screening, diagnosis and treatment, three concepts will be instrumental in the success of any HCV elimination policy: involvement, communication and protection of vulnerable individuals.

Given the importance of diagnosis and treatment both in terms of individual benefit and social benefit, while respecting individual freedom and autonomy, the government has a moral obligation to try to drive individuals on the path of therapy. Even if it fails to get a complete success, the hepatitis C virus eradication campaign will lead to a significant reduction in the incidence of the disease and it will convey a very important message: today more than ever public health interventions must be thought in a global perspective, far beyond the borders of National States.

Keywords

Autonomy · Cost-effectiveness · Eradication · Ethics · HCV

Abbreviations

HCV	Hepatitis C Virus
WHO	World Health Organization

1 Background

Over recent years a revolution took place in the treatment of hepatitis C virus (HCV). Direct-acting antivirals, the first of which was approved in 2013, can presently cure more than 95% of patients in a matter of weeks with all-oral and well tolerated regimens (European Association for the Study of the Liver 2017). In 2016, the

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194 Member States of the World Health Organization (WHO) agreed to eliminate all forms of viral hepatitis as a public health threat by 2030. WHO's global strategy targets reductions of 65% in HCV-related mortality and 80% in new infections, relative to 2015 levels (World Health Organization 2016). In March 2018 the progress report published by the WHO itself showed that, despite an increasing availability of access and several success stories for singular nations, the bulk of the 71 million people infected with HCV in the world remained untreated, with most of the progress made only in a small number of high-income countries with a high disease burden levels (World Health Organization 2018). According to the report, in 2016 about one out of 5 infected people worldwide had a diagnosis, with a significant difference between low-income countries, where the ratio was only 8%, and high-income countries, where it went up to 43%. As data show, the major obstacles to the ambitious goal of eradication are economic and organizational restrictions, that affect especially low and middle-income countries. Nonetheless, if the goal is eradicating the infection worldwide, the obstacles to overcome will not only be economic and organizational. Governments are indeed facing a further challenge and a new public health issue: reaching through screening, diagnosis and treatment a large amount of subjects with undiagnosed HCV infection.

An overall strategy to fight against HCV must in fact aim to protect individuals and the community through the treatment of infected individuals (already diagnosed or to be diagnosed) and the prevention of new infections. To achieve these objectives, an effective plan for diagnosis and prevention is needed and new ethical issues need to be carefully analyzed.

In recent years, the debate on ethical issues related to HCV therapies has been focused on the problem of drug prices and access to therapies. Today a sound ethical analysis on these topics is still necessary in many countries, but meanwhile, the goal of eradication is raising new ethical issues.

2 What Obligations for the State?

If effective therapeutic options with sustainable costs are available, National governments, especially high-income countries with a Welfare State, are compelled to provide access to therapies, but also to involve undiagnosed subjects, in order to promote health as a good and as a right both for the individual and for the society.

As well as in legal terms, the effort to eliminate HCV infection is also justified from an ethical point of view, as consistent with the principle of beneficence.

The principle of beneficence underlies the obligation for the State to meet the essential needs of the individual, particularly the right to health. In a policy aimed at eliminating HCV the benefit is twofold: there is an individual benefit for the treated subject and a benefit for the society as a whole, because the removal or reduction of the threat of infection is something the entire community benefits from. If we consider how benefits and risks are distributed between the patient and the population as a whole, the HCV eradication plan enjoys additional specificity compared to other eradication plans based on vaccination campaigns. In the case of a standard medical treatment, the recipient of the therapy is expected to be the main beneficiary, consistently with the principle of beneficence that rules medical ethics. In eradication plans based on vaccination campaigns, the single person vaccinated is expected to be the main beneficiary, but at the same time the goal is to create cascading benefits for the other subjects through herd immunity. As the vaccination campaign proceeds, the potential benefit for the individual is reduced as the risk of infection decreases, by virtue of the fact that the individual already enjoys herd immunity. In HCV eradication campaign, the benefit for the individual resulting from the therapy remains unchanged and two opposite cases may arise. In the first case both individual and social benefit are reduced, e.g. very old patients or patients with other severe illnesses, who presumably will have little benefit

from clearing the infection, and are unlikely to infect others since they are not prone to risk behaviors. On the other hand, individual benefit and collective benefit can be both high, as in the case of subjects who use injectable drugs which, if cared for, will benefit greatly and at the same time stop being a potential source of contagion.

HCV eradication campaign should be also analyzed according to the principle of Equity. The concept of equity refers to fair distribution of resources, opportunities and results. In this respect, further considerations about the cost-effectiveness should be done in an eradication policy, because the eradication campaign has to be cost-effective to be ethical. To achieve this goal, both at global and national level, the campaign will have to proceed quickly and effectively, because the more it slows down the less it will be cost-effective, due to the persisting costs arising from the progression of the disease in untreated subjects, the costs of surveillance, the decreasing economies of scale when small population groups are to be reached and, last but not least, the chance of new infections and reinfections in the subjects treated (Omer et al. 2013; Barrett 2013).

In terms of cost-effectiveness, another important element should be evaluated yet. If we compare the results obtained with the global eradication of HCV with those obtained with a control and elimination campaign that suppresses the incidence of infection to very low levels, we might think that the additional benefit obtained with the eradication intervention is quite low. However, if we evaluate the results over a very long period of time we realize that the percentage of residual incidence acquires a much greater weight in terms of DALY (Disability-Adjusted Life Years). In health care economics there is a tendency to give greater weight to the short- and medium-term benefits and risks than to long-term ones, due to the uncertainty of predictions about the future and due to the natural tendency of the human being to give more importance to what is closer both in time and in space. In the last few decades, however, the idea of collective responsibility for global health promotion has grown stronger, together with an increased sense of responsibility towards the future repercussions of our current choices in terms of health for the individual and for the society. Accordingly, it is necessary to carefully evaluate the weight of a result different from eradication, which will bring with it long-term and indefinite consequences in terms of morbidity and mortality related to HCV.

The elimination plan is consistent with two other important principles: utility and solidarity. The principle of utility binds us to pursue actions aimed at obtaining the maximum benefit for as many people as possible. The principle of solidarity invokes the commitment of the individual in undertaking actions aimed at promoting the well-being of others. In this respect, the choice to take care of ourselves also has an implication in terms of individual participation in the well-being of the community.

3 What Rights for the Individual?

In moving towards HCV eradication another fundamental right plays a key role: the individual right to freedom. The right to freedom overrides the right to health, justifying the exercise of treatment refusal. Imposing a treatment is acceptable only in cases laid down by law (e.g. compulsory vaccination); this kind of limitation of personal freedom is justified by the fact that the treatment is aimed at safeguarding not only the health of the individual, but also of the entire community. This kind of restriction, however, is justified only if it is strictly necessary, in the presence of an extremely high risk for public health and in the absence of any other possible alternative; it is expected to be rigorously evaluated in order to justify the derogation to the respect of a fundamental human right recognized internationally, especially in the case of sudden outbreaks of infectious epidemics with high contagiousness and high risk (American Association for the International Commission of Jurists 1985).

In addition to the legal aspect, a strong ethical issue arises, namely the observance of one of the

pivotal principles of medical ethics: the principle of autonomy. Based on the recognition of freedom as a fundamental human right, the principle of autonomy requires compliance with the decisions that each individual takes in the area of his health, according to his own values and preferences.

Despite having an important symbolic value and being a good for the community at a global level, eradication policies, as public health policies, do not therefore enjoy by themselves a status of exceptionality that justifies coercive interventions and do not imply additional obligations for the citizen (Wilson 2014).

In the setting of hepatitis C, where transmissibility of the virus is relatively modest and infection can be mostly prevented in the absence of a vaccine through modification of risk behaviors, imposing screening or treatment is neither ethically nor legally sustainable. This intervention is not justifiable even for prisoners, whose personal freedom has already been restricted. This restriction is in fact related to the crime committed and justified by security reasons, but cannot be unnecessarily extended to anything else.

Finally, even in cases where these kind of coercive interventions are legitimate (e.g. mandatory vaccinations), the mere imposition of the obligation does not always give optimal results and shows a failure in trying to educate public about health culture.

4 Involvement, Communication and Protection of Vulnerable Subjects

Three basic pillars will contribute to the success of the eradication plan:

1. Targeted screening campaigns with quick and simple tests for population groups at higher risk
2. Access to therapies, which means effective linkage to care and the widest possible involvement of subjects through information campaigns
3. Simplicity in treatment

Continuous innovation in the field of therapy and diagnosis, a considerable organizational effort and a solid long-term commitment will be necessary. But above all, as people cannot be forced but must be guided towards the choice of screening, diagnosis and treatment, three concepts will be instrumental in the success of any HCV elimination policy: involvement, communication and protection of vulnerable individuals. These concepts are consistent with the principle of procedural justice. Procedural justice refers to the idea of a fair decision making process. Its essential elements are: participation, transparency, involvement of stakeholders in the decision-making process. According to the principle of justice, resources should be distributed fairly, providing access to all sick people, protecting the most vulnerable groups, avoiding discrimination and involving all those concerned in the decision-making process.

In order to reach the elimination goal, high-income countries with a high prevalence of HCV, are now paying special attention to specific high risk population groups, such as injectable drug users and prisoners, that are key drivers of HCV transmission (Wiessing et al. 2014). Identification and treatment of high-risk individuals will allow to hit the epidemic in those who are maintaining it, realizing the concept of treatment as prevention.

These classes of subjects still have specific features that must be understood and addressed in order to reach the goal: they often have very low health-related values and low trust in institutions; they also may not be aware of their condition, do not want to know it or do not want to be cured, have difficulty accessing treatment, have poor compliance.

How to promote diagnosis and linkage to care for this kind of groups? The traditional model of health system doesn't work for these population groups: they will not refer to the health system, it is the health system that should try to approach them.

To achieve the goals set, an active collaboration of multiple different subjects (political decision makers, health system, medical class, patient associations, volunteer groups, etc.) will be

necessary, all of whom share responsibility for the success of the plan itself. The primary responsibility for organizing the program lies with the State, which must work to promote health as an individual's right and a community's good, but this effort will be ineffective without involving the other parties in a network of responsibility. In order to obtain good results, an early and continuous involvement of the population groups targeted will be necessary, to give voice to their needs. To reach this goal active social networks between the representatives of these groups and the decision makers should be developed. In the process of community involvement, the decision makers should be ready to review and question the policies adopted for the recruitment of subjects at risk in screening and therapy protocols: this can strengthen trust and make the communities feel involved in a real dialogue.

Communication plays a vital role to ensure effective involvement: therefore, communication tools and platforms that are culturally and linguistically adequate for the targets to be achieved should be created. This will require the commitment of the State to create information and awareness campaigns, to determine an increase in voluntary participation by citizens, convinced of the value of what is being achieved.

Media will play an essential role in communication and therefore it will be necessary to make sure that they have access to accurate and up-to-date information about the disease and therapy.

Special regard should be given to vulnerable subjects, who are faced with situations of discrimination and exploitation. The vulnerability is defined as an increased susceptibility to harm or injustice. Many of the features that make a subject vulnerable in fact make it difficult for him to access screening and therapy (e.g. the linguistic obstacle for immigrant foreigners, or illiteracy, or the inability to access the internet or other media). To overcome these barriers, the message should be conveyed through multiple communication tools. The health authority cannot assume that the public will actively seek information but instead must strive to reach the potentially interested public in every possible way.

Stigma and discrimination represent a risk in the HCV eradication process: they affect the ability and willingness of a person to have access to treatment or screening. Policy makers will have to develop specific plans to try to meet the needs of these individuals, for example through community education regarding HCV.

As has been done for the tuberculosis control plan, the work should be based on the concept elaborated by the WHO of " person-centered care" (World Health Organization 2006), that implies the idea of seeing health from the patient's point of view and adapting the care to better meet the needs and expectations of individuals (Macq 2007; World Health Organization & Stop TB Partnership 2010; World Health Organization 2010). A person-centered approach recognizes that the beneficiary of the therapy is the sick individual and that therefore the strategies must be outlined focusing on the individual's rights and well-being.

Given the importance of diagnosis and treatment both in terms of individual benefit and social benefit, while respecting individual freedom, the government has a moral obligation to try to drive individuals on the path of screening and therapy.

Patients who refuse screening or treatment should receive counseling about the risks they run for themselves and for the others. Operators should try to understand the reasons for the refusal and try to work together to identify ways to meet the subject's concerns and fears.

Another obligation for health care is to assist patients who have problems with adherence to therapy. The efforts to contact patients who do not show up for treatment, however, create the risk of an intrusion into the privacy and autonomy of the individual. Therefore, consistently with a person-centered approach, any attempt to contact must be carried out in such a way as to minimize intrusions. Patients should be informed at the beginning of the treatment that they will be contacted if they do not show up and, as far as possible, should be given the opportunity to choose how to be contacted.

5 Conclusions

Although eradication is an unquestionable success in terms of public health, it may not always be reasonable or necessary to pursue it for any widely spread infectious disease. There are in fact numerous types of assessments, in terms of cost-effectiveness of the intervention and of allocation of finite resources, that lead us to believe that elimination or control could be not only more achievable goals, but also somehow more ethically appropriate goals in terms of resource allocation and in terms of respect for the autonomy of the individuals (Caplan 2009). To date, smallpox remains the only successful case of global infectious disease eradication, while many others eradication campaigns, which have entailed a considerable economic effort, have never been able to reach a complete success (Stepan 2011).

With regard to HCV, is the eradication plan ethically suitable? The answer is yes, as long as it meets certain conditions: considering the principle of equity, the plan must be cost-effective to be ethical, moreover, with respect for the principle of autonomy it always has to be a choice for the individual.

Is HCV eradication feasible? We don't know it for sure, but the most suitable way to achieve overall global eradication is to progressively eliminate the disease in different areas, with methodology and timing specific for the considered area. To reach the target, HCV must be tackled down not only in the well-defined setting of developed countries but also in the more intricate situation of developing countries, where the disease is generally not confined within restricted population groups and economical resources are limited. Nonetheless, we have to pay attention not only to economical and organizational constraints in low and middle-income countries, but also to proper ways to eliminate HCV infection in high-income countries, especially those with a high prevalence of the infection, because the goal of elimination in each single country will be a fundamental brick in the achievement of global eradication.

Even if it fails to get a complete success (Lombardi et al. 2019), the HCV eradication campaign will lead to a significant reduction in the incidence of the disease and it will convey a very important message. The boost by the WHO towards ambitious goals such as HCV eradication for 2030 must in fact continue to exist and must be above all a stimulus and a message: today more than ever public health interventions should be thought in a global perspective, beyond the borders of national states. Not only because in epidemiological terms it is the most effective type of intervention, especially in a world with a level of mobility never known before in history, but also because the reflection developed from the end of the Second World War to today on universal human rights requires us to promote the right to health as a universal right for every individual, a good to be guarded and promoted far beyond the borders of our States.

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Ten-Year Retrospective Analysis of *Legionella* Diffusion in Hospital Water Systems and Its Serogroup Seasonal Variation

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Abstract

Introduction

Legionella spp. are ubiquitous aquatic organisms found to be associated with community-acquired pneumoniae (CAP) as well as hospital-acquired pneumonia (HAP). Direct inhalation of aerosols from environmental colonisation is typically the source of infection. The aim of this study was to determine the level of colonisation in hospital water supply systems in order to assess the criticality of the water distribution network and strengthen preventive measures.

Methods

From 2009 to 2018, 769 water samples were collected and then analysed according to the standard methods indicated in ISO11731-2:2004 and ISO11731:2017 for *Legionella* detection.

Results

The samples were positive in 37.1% cases (n. 285) and negative in 62.9% cases (n. 484).

The threshold of 10,000 CFU/L was exceeded in 15.1% cases and led to decolonisation as indicated by Italian and European ECDC guidelines. In the autumn-winter period SG1 showed a positivity of 41.2% (n. 40) with a decrease in the spring-summer period with 9.6% (n. 18) of positivity. In contrast, SG2-15 showed a positivity of 30.9% (n. 30) in autumn-winter, which tends to increase to 56.9% (n. 112) in spring-summer ($p < 0.001$).

Conclusion

Surprisingly, besides showing a seasonal trend already described previously in the literature, the positivity of our sample was not balanced even for serogroups in the two periods. This could be due to genetic differences and ecological niches to be further investigated that could also have links with the greater pathogenicity of SG1. Environmental microbiological surveillance and risk assessment should be

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performed more frequently and disinfection must be carried out, especially in health facilities where people are more susceptible to infections.

Keywords
Environmental surveillance · Hospital water systems · *Legionella* spp. · Seasonal serogroups · Serotyping of *Legionella* spp.

1 Introduction

Legionella spp.is a Gram-negative microorganism that can develop at a temperature between 25 °C and 42 °C, especially in collection systems. Microorganisms of the *Legionella* genus are ubiquitous and can be found in natural and artificial aquatic environments, such as spring waters, including thermal ones, rivers, lakes, mud, etc. From these environments *Legionella* reaches city pipelines and building water systems, such as reservoirs, pipes, fountains and swimming pools, where it can be a potential risk for human health (Declerck 2010; Fliermans et al. 1981).

Sediments and soils contain *Legionella* as a free form, but it is mostly found in colonising amoeba or within biofilms (Fields 1996). *Legionella* colonise man-made water systems where the temperature conditions are suitable for their optimal growth, its hosts include amoebae like Acanthamoeba, Naegleria, Balmuthia, Dictyostelium and ciliates such as Tetrahymena (Boamah et al. 2017).

Legionella can be isolated for example in hot and/or cold-water systems, wet soil, air conditioning systems, cooling towers, spas, swimming pools and fountains. Biofilm formation is also an important protection and survival mechanism that allows this bacterium to remain as a coloniser in water systems (Declerck 2010). *Legionella* spp. can cause Legionnaires' disease (LD) and the flu-like Pontiac fever in humans (McDade 2009; Spagnolo et al. 2013).

Risk-minimisation of *Legionella* colonisation in hot water systems can be guaranteed by maintaining the temperatures at the safety levels indicated in the technical guideline standards which set this threshold at 50 °C for hot water and <20 °C for cold water (ESCMID 2017; Graells et al. 2018; Richards et al. 2013). The most isolated species in the *Legionella* spp. *genus* is *L. pneumophila* which is divided into 16 serogroups, of which serogroup 1 (SG1) is the most virulent and associated with high morbidity and mortality (McDade 2009).

There was a high incidence of LD in Italy in the summer of 2018, which led to 5 deaths. This happening created media alarmism in the population, but also increased awareness of how legionellosis is contracted, thereby highlighting the importance of recognising symptoms and improving prevention.

Water distribution systems are the major reservoirs of *Legionella* spp. which commonly colonise the water systems in large public buildings, households and industrial systems. The most dangerous kind of colonisation is when *Legionella* occurs in the water distribution systems in hospitals, medical equipment (e.g. respirators, dialysers, inhalers, humidifiers, water massage equipment used in balneotherapy) and the dental turbines used in dental offices. This poses a risk for human infection with these bacteria, particularly for patients in health centres (Sikora et al. 2015). This microorganism can multiply and lead to the acquisition of infections by patients through the direct inhalation of aerosols. In fact, the most common route of exposure is represented by the inhalation of aerosol drops containing *Legionella*, and the risk of transmission increases if one considers the complexity of hospital water systems, along with the vulnerability of hospitalised patients. Hospital legionellosis is a preventable disease through specific measures applied to the water systems within

buildings (O'Neill and Humphreys 2005; Spagnolo et al. 2013). Environmental surveillance for *L. pneumophila* in hospitals is a useful strategy to prevent nosocomial LD.

In Italy, the latest available ECDC report describes an annual incidence rate of 2.8 cases/100,000 people, which is among the highest in Europe (European Centre for Disease Prevention and Control 2015). In our country, according to a 2015 report by the "Istituto Superiore di Sanità" (Chochlakis et al. 2017; Italian Ministry of Health 2016), nosocomial LD represents 5.3% of the total number of cases, although the Sardinian Region reports zero incidence of nosocomial infection (ISS 2018).

We addressed three specific aims: (1) to determine the prevalence of *L. pneumophila* in the water supply systems of eleven hospitals in southern Sardinia in order to assess the criticality of the water distribution network and, if necessary, to strengthen environmental preventive measures; (2) to identify potential risk factors for a positive sampling of *Legionella* spp.; (3) and to define whether the season can influence SG1 distribution.

2 Methods

2.1 Study Design and Sample Collection

We retrospectively reviewed 769 samples tested for *Legionella* spp. collected from the hospital distribution network, during the study period between January 2009 and December 2018. The following data were recorded for each sample in the database: collection date, hospital, ward, sampling point, temperature at the time of collection, free chlorine, possible positivity for *Legionella* spp., serogroup and count. Samples were divided *a posteriori* according to the season (autumn-winter and spring-summer) to assess whether climatic changes could play a role on positivity and serogroups.

2.2 Hospitals Enrolled

Eleven hospitals in Southern Sardinia were enrolled in this study period. They were divided by date of construction, that is before 1950 or more recently. We also divided the structures according to the number of beds (more/less than or equal to 150 beds) and type of construction (monoblock or pavilion) in order to find any possible link between colonisation and these variables (D'Alessandro et al. 2015; Danila et al. 2018; Montagna et al. 2018).

Wards or sample points were identified and then, in order to dichotomise the variable, we also divided the wards into two groups, high risk wards (with high risk patients: Haematology and ICUs) and low/middle risk wards (Medicine, Surgery and others) (Italian Ministry of Health 2016; Montagna et al. 2018).

2.3 Microbiological Analysis

Microbiological analyses for *Legionella* spp. were carried out by the Hygiene Laboratory, Department of Medical Science and Public Health, University of Cagliari. Collection bottles, containing 1% sodium thiosulfate to neutralise the presence of chlorine, were returned to the laboratory immediately after sampling for bacteriological and chemical-physical examination. After collection, the samples were kept at room temperature and protected from direct light during transport to the Food Hygiene Laboratory which operates with UNI standard *ISO 17025:2005*. If analyses could not be carried out immediately, samples were kept at +5 °C ± 3 °C and processed within 24 h of collection. At the time of sampling, the temperature and free residual chlorine in the water were measured (Belladonna and Ottaviani 2007).

The samples were analysed according to the standard methods indicated in ISO 11731-2:2004 and in *ISO* 11731:2017 for the detection of *Legionella* spp. with a quantitative evaluation,

expressed in CFU/L. As regards the first method, we analysed 1- L of the water sample. The sample was treated, directly on the filter, with 30 ± 5 mL of acid solution (HCl-KCl) for 5 min, the membrane was connected and washed with 20 ± 5 mL of PAGE solution and placed directly on BCYE culture medium containing L-cysteine, needful for microorganism growth. The samples were incubated in a humid microaerophilic environment at 36 ± 2 ° C for a period of 10 days and then identified.

Instead, the following procedure was adopted for the second method: 1-L water samples were concentrated (through the use of a Millipore filtering ramp) to 10 mL through 0.2 μm porous membrane filters (in polycarbonate or polyethersulfone – Microbiol Diagnostici s.n.c.). After sample concentration, the membrane was deposited with sterile tweezers in a sterile container containing 10 ml of Page's saline. The deposit collected on the membrane was suspended by vortex shaking (2 min minimum). This concentrated suspension was the untreated sample.

After this procedure the untreated sample was divided into three parts: (1) the untreated sample itself; (2) the acid treatment sample (1 mL of the untreated sample was added to 9 mL of HCl-KCl buffered solution for 5 ± 0.5 min); (3) the heat treatment sample (3 mL of the untreated sample were placed in a sterile tube and then in a thermostatic bath at 50 ° C ± 1C ° for 30 ± 2 min). 0.1 ml of each part (untreated, after acid treatment, and after heat treatment) was subsequently inoculated and distributed on BCYE agar medium and on BCYE agar + AB selective medium or alternatively on GVPC (Microbiol Diagnostici s.n.c.).

Plates were incubated at 36 ± 2C ° for 7–10 days in a microaerophilic environment. Plates with the highest count of presumed *Legionella* CFU/L were subcultured both on BCYE agar and blood agar (Microbiol Diagnostici s.n.c.). The growth of *Legionella* colonies can only occur on BCYE agar due to *Legionella*'s inability to multiply in the absence of L-cysteine; there can be no growth on blood agar, where the latter is not available. Any colonies that grew on BCYE agar but showed no growth on blood agar were incubated at 36 ± 2 °C for 2–5 days and recorded as *Legionella*.

The samples were divided into positives with a high level of colonisation (≥ 1000 CFU/L), those with a low level of colonisation (100 < x < 1000 CFU/L) and negatives (<100 CFU/L) (Montagna et al. 2018). The final confirmation was serum agglutination: colonies of the *Legionella* genus were serologically identified with a special kit which allows the identification of Serogroup 1 (SG1), Serogroup 2–15 (SG2–15) or *Legionella* spp. (Oxoid Microbiology Products®).

2.4 Statistical Analysis

Descriptive analysis of the data was carried out using median values and the interquartile range for the quantitative variables and percentage values for the qualitative ones. Normality of the variables was assessed with the Shapiro-Wilk test and values of asymmetry and kurtosis related to their standard error.

The association between endpoint variable and explicative variables was verified with a non-parametric approach using the χ^2 or Fisher exact test and the Mann-Whitney test.

We computed univariate logistic regression to identify predictors of positive samples of *Legionella* spp. For covariates that showed a p value <0.10 in the univariate regression, independent predictors were identified by a multivariate logistic regression. A p value of <0.05 was considered statistically significant (Borella et al. 2005; Gavaldà et al. 2019; Hosmer and Lemeshow 1989). All analyses were conducted

using the SPSS statistical package (version 23 for Windows. SPSS, Inc. Chicago, Ill).

3 Results

3.1 Descriptive Data

As summarised in Table 1, out of a total of 769 samples, 62.9% (n. 484) were negative and 37.1% positive for *Legionella* spp. (n. 285).

The following results emerged from the analysis of only the positive samples: older hospitals (built before 1950) had 44.9% (n. 128) of positive samples for *Legionella* spp. while other hospitals had 55.1% (n. 157).

Hospitals with more than 150 beds had 50.2% (n. 143) positivity, whereas the others had 49.8% (n. 142) samples positive for *Legionella* spp. As regards the subdivision of the hospitals into "monoblock" and "pavilion", the former showed 75.8% (n. 216) of positivity and the latter 24.2% (n. 69).

Medicine wards recorded the highest number of positive samples in the study period with 30.5% (n. 87), followed by the Surgery 28.8% (n. 82) and Oncology-Haematology departments with 19.6% (n. 56) followed by ICUs with 8.4% (n. 24) and then the other wards. The subdivision into "low-middle risk" and "high risk" wards produced a significant difference ($p = 0.008$) with a positivity for *Legionella* spp. of 71.9% (n. 205) for the "low-middle risk" group and 28.1% (n. 80) for the "high risk" one.

The sample analysis based on temperature at the time of collection showed that 97.5% (n. 278) of positive samples had a temperature in the range of 20–50 °C while the remaining 2.5% (n. 7) were outside this range ($p = 0.069$).

As regards the positive samples, 20.4% (n. 58) were found to be SG1, 49.8% (n. 142) were SG2–15, 15.4% (n. 44) were *Legionella* spp. while 14.4% (n. 41) showed the simultaneous presence of SG1, SG2-15 and *Legionella* spp.

Our analysis of the positive samples based on the level of colonisation allowed us to observe that 38.2% (n. 109) showed a value of CFU/L < 1000; 46.7% (n. 133) a value of CFU/L between 1000–10,000 and 15.1% (n. 43) >10,000 (For more details about colonization levels see supplementary material – Figures 1 and 2). Several guidelines including the Italian ones for microbiological evidence of these colonisation thresholds recommend disinfection, even in the absence of illness (Italian Ministry of Health 2016).

Table 2 shows the concentration of residual chlorine (mg/L). The lower interquartile range value is associated with a lower probability of having a positive sample ($p = 0.008$). The total count of positive samples showed a median of 1000 CFU/L.

3.2 Examination of Risk Factors

Table 3 illustrates the Odds Ratio, the 95% confidence interval and the relative p-value in univariate and multivariate models in order to evaluate the strength of association regarding the probability of having a positive sample.

A sample taken in spring-summer had a 1473-fold increased probability of being positive compared to a sample taken in autumn-winter ($p = 0.013$); this result was not confirmed in multivariate analysis but with a p value borderline ($p = 0.089$).

Instead, a positive sample is less likely to be isolated in a high-risk ward (OR 0.653, $p = 0.009$), confirmed by multivariate analysis ($p = 0.016$). Moreover, the probability of isolating a positive sample according to the levels of residual chlorine is lower, and the probability decreases by 2% with each mcg/L increase in water (OR 0.981, $p = 0.027$), a result confirmed by a multivariate model ($p = 0.023$). As regards temperature, the p value is borderline and showed

Table 1 Summary table of qualitative variables examined ∗chi square test or Fisher's test

Variables		Samples				p value∗
		Negative		Positive		
		N	%	N	%	
Old Hospitals (<1950)	No	262	54.1%	157	55.1%	0.797
	Yes	222	45.9%	128	44.9%	
Hospital >150beds	No	240	49.6%	142	49.8%	0.949
	Yes	244	50.4%	143	50.2%	
Hospital type	Monoblock	349	72.1%	216	75.8%	0.264
	Pavilion	135	27.9%	69	24.2%	
Sample origin	Medicine	132	27.3%	87	30.5%	**< 0.001**
	Surgery	109	22.5%	82	28.8%	
	ICU	23	4.8%	24	8.4%	
	Oncology–Haematology	158	32.6%	56	19.6%	
	Tank – Pool	21	4.3%	5	1.8%	
	Others	41	8.5%	31	10.9%	
Low/middle risk wards High risk wards	Medicine, Surgery, Others	303	62.6%	205	71.9%	**0.008**
	ICUs and Haematology	181	37.4%	80	28.1%	
Temperature sample range 20°-50 °C	No	25	5.2%	7	2.5%	0.069
	Yes	459	94.8%	278	97.5%	
Serogroup	Negative	484	100.0%	0	0.0%	–
	SG 1	0	0.0%	58	20.4%	
	SG 2–15	0	0.0%	142	49.8%	
	Mixed SG	0	0.0%	41	14.4%	
	Legionella spp.	0	0.0%	44	15.4%	
Count	Negative	484	100.0%	0	0.0%	–
	< 1000 CFU/L	0	0.0%	109	38.2%	
	1000–10,000 CFU/L	0	0.0%	133	46.7%	
	> 10,000 CFU/L	0	0.0%	43	15.1%	
Total		484	100.0%	285	100.0%	

Table 2 Summary table of quantitative variables ∗Mann-Whitney's test

Variables	Samples						p value∗
	Negative			Positive			
	Median	Percentile 25	Percentile 75	Median	Percentile 25	Percentile 75	
Chlorine (mg/L)	0.020	0.020	0.020	0.020	0.010	0.020	**0.008**
Count (CFU)	0.0	0.0	0.0	1000.0	100.0	5000.0	–

an increased risk in the 20–50 °C temperature range in both univariate and multivariate analysis.

Finally, Table 4 regards the frequency of the different serogroups isolated by season. In the autumn-winter period SG1 showed a positivity of 41.2% (n. 40) followed by a decrease in the spring-summer period with 9.6% (n. 18) of positivity. In contrast, SG2-15 showed a positivity of 30.9% (n. 30) in autumn-winter, which tended to increase to 56.9% (n. 112) in spring-summer (p value = <0.001).

Moreover, from the analysis of the data it emerged that the samples taken in the autumn-winter period were positive for *Legionella* in 34% of cases (n. 97) and a significant increase was recorded (p = 0.012) in the spring-summer period with 66% (n. 188) of positivity.

Table 3 Positive samples for Legionella spp. univariate and multivariate analysis. OR – Odds Ratio, bold values are for variables with a p value ≤0.05

	Univariate analysis			Multivariate analysis		
Variables	*OR*	*CI 95%*	*p value*	*OR*	*CI 95%*	*p value*
Spring – Summer	1.473	1.087–1.996	**0.013**	1.324	0.958–1.832	0.089
ICUs and Haematology	0.653	0.476–0.897	**0.009**	0.659	0.469–0.925	**0.016**
Temperature range 20°-50 °C	2.163	0.923–5.067	0.076	2.227	0.934–5.310	0.071
Chlorine (mcg/L)	0.981	0.965–0.998	**0.027**	0.979	0.962–0.997	**0.023**
Pavilion Hospital	0.826	0.590–1.156	0.264	–	–	–
Hospital (N. beds >150)	0.991	0.739–1.327	0.949	–	–	–
Old Hospital (<1950)	0.962	0.717–1.291	0.797	–	–	–

Table 4 Variability per season $*\chi^2$ test or Fisher's test

		Season				
		Autumn – Winter		Spring – Summer		
Variables		N	%	N	%	p value*
Serogroup	SG 1	40	41.2%	18	9.6%	**< 0.001**
	SG 2–15	30	30.9%	112	59.6%	
	Mixed SG	9	9.3%	32	17.0%	
	Legionella spp.	18	18.6%	26	13.8%	
Sample	Negative	209	68.3%	275	59.4%	**0.012**
	Positive	97	31.7%	188	40.6%	

4 Discussion

Our data indicate that at the time of this study, 37.1% of the water distribution system samples in southern Sardinian hospitals were positive for *Legionella* spp.

A study in Taiwan reported an *L. pneumophila* positive rate greater than 30% (Yu et al. 2008); four studies in Greece have shown that 27.3–75% of hospital water systems are colonised with *Legionella* spp. (Fragou et al. 2012; Mavridou et al. 2008; Mouchtouri and Rudge 2015; Velonakis et al. 2012) and a Polish paper reported a positive rate of isolation of 78.57% (Sikora et al. 2015); the overall positivity of our samples was also similar to Tesauro et al.'s findings (Tesauro et al. 2010) but lower than those found in another recent Italian study (Laganà et al. 2019).

The 10,000 CFU/L threshold has been exceeded in a significant number of cases as previously reported which has led to remediation consisting of thermal shock and hyperchlorination treatment, as indicated by Italian and European ECDC guidelines (ESCMID 2017; Italian Ministry of Health 2016).

We did not find any association between older hospitals (built before 1950) and positive samples of *Legionella* spp. This finding is in contrast with Montagna et al. (2018) and Marchesi et al. (2011).

Our results for small or large nosocomial buildings is also in contrast with the current literature, since the territorial extension of the network in our setting did not seem to influence the presence or absence of *Legionella* spp. (Montagna et al. 2018)

Wherever we find water systems from water reserves with a temperature between 20 °C and 50 °C, the bacterium can easily multiply itself (as is well-known) and hence it is transmitted by inhalation of aerosol droplets.

Hot water samples were often at a non-optimal temperature of around 40 °C (Table 1), which was too low and could represent an issue for *Legionella* spp. colonisation, as reported by Marchesi et al. (2011). Unfortunately, the clear limitation of this variable is the unbalanced set of

samples, with most of them resulting in the critical temperature range. Despite this limitation, we report the variable here for the sake of completeness. A feasible corrective manoeuvre could be to install a boiler and decrease the distance between source and tap.

SG2-15 were the most frequently isolated representing almost half the number of isolations as described by Montagna et al. in a recent Italian survey (Montagna et al. 2018) and by other authors (Laganà et al. 2019; Tesauro et al. 2010). Moreover, the number of cases of colonisation was similar to another study carried out in Italy (Iatta et al. 2013). Although the literature states that *L. pneumophila* SG1 is more frequently isolated in humans, an increasing number of cases are attributed to other species and serogroups. Moreover, a large European study, isolated from clinical samples, showed that 33.9% of hospital acquired infections were caused by *Legionella* spp., which is why *Legionella* colonisation of the environment should not be underestimated. In our experience it represented 15.4% of the samples (Yu et al. 2003).

As regards risk factors, multivariate analysis showed that "High risk" wards proved to have a lower risk as regards the isolation of positive samples of *Legionella* spp. than other wards. This is a desirable result in contrast with other Italian surveys published in 2013 (Iatta et al. 2013) and in 2019 (Laganà et al. 2019). Furthermore, the fatality rate of hospital-acquired legionellosis in patients affected by chronic degenerative diseases, tumours, immunocompromised patients, or those who have undergone organ transplantation, is much higher than the one observed in community-acquired legionellosis (33.3% vs 7.5%, respectively) (Herwaldt and Marra 2018; Rota et al. 2009).

As O'Neill et al. stated in their review, the incidence of hospital-acquired legionellosis is underestimated for a variety of reasons, including a lack of clinical awareness or a missed diagnosis, *e.g.* non-classical presentation especially in very ill or immunocompromised patients, infection at sites other than the respiratory tract, including soft tissue infections and endocarditis, delayed seroconversion, or lack of specialised culture facilities or urinary antigen detection tests in diagnostic microbiology laboratories (O'Neill and Humphreys 2005).

A temperature range between 20 °C and 50 °C doubles the risk of finding *Legionella* spp. with a p value borderline near to significance value in multivariate analysis ($p = 0.071$); free chlorine is shown to be a protective factor (OR 0.979, $p = 0.023$) which is similar to the results found in other previous studies. (Darelid et al. 2002; Orsi et al. 2014; Rogers et al. 1994).

Our study showed that the residual chlorine varies significantly in positive samples, while the temperature undergoes no significant variations, unlike the findings of Vincenti et al. 2019).

This retrospective ten-year sampling study allowed the determination of the risk of colonisation by *L. pneumophila* associated with water in the water systems of a number of hospitals in southern Sardinia. The study showed the presence of *L. pneumophila* in all the structures tested, but with different levels of colonisation (data not shown).

Seasonality is confirmed by the literature and this finding is in accordance with the ECDC report for the 2012–2016 period (European Centre for Disease Prevention and Control 2015). Moreover, D'Alessandro et al. reported an increase in positive samples in the spring-summer period and also found an association between low levels of free chlorine and an increase in positive samples. However, they did not calculate the strength of this association and the relative odds ratio (D'Alessandro et al. 2015).

The present study highlights the different seasonality of SG1, which appears to be isolated with a significantly higher frequency in the autumn-winter period compared to the spring-summer period. This result is opposite for SG2-15, since these serogroups are more present in the spring-summer period than in the autumn-winter period. Another ten-year analysis by Marchesi et al. did not find any difference in serogroups and season (Marchesi et al. 2011).

Borella et al.*'s* analysis of hot water found that *Legionella* SG1 was more present in water at a

low temperature (Borella et al. 2004). Our findings suggest that different serogroups may have different ecological niches and that SG1 needs further examination on account of its level of risk.

The prevention of LD is based on the correct design and construction of technological systems, which involve water heating and/or nebulisation systems, such as in hydro-sanitary systems, air conditioning systems with water humidification, cooling systems with evaporative towers or evaporative condensers, systems that distribute and supply thermal waters, pools and hot tubs. Furthermore, it is essential to adopt preventive measures such as maintenance and occasional disinfection, aimed at counteracting the multiplication and spread of *Legionella* spp. in waterworks at risk. Although these measures do not guarantee that a system or one of its components is *Legionella* free, they do contribute to reducing the probability of serious bacterial colonisation (Italian Ministry of Health 2016).

5 Conclusion

This study highlights the relationship between seasonality and frequency of isolation of Legionella serogroups, an aspect not yet reported in the literature.

The variation of frequency in the isolation of *Legionella* serogroups according to seasonality could be an interesting new factor that deserves further investigation.

In vitro studies are required to evaluate the different serogroup behaviours that are probably due to temperature. This is because SG1, apart from its greater incidence, also seems to have the greatest virulence and lethality (Herwaldt and Marra 2018; McDade 2009; Yu et al. 2003).

Therefore, the fact that we know that in our setting it is mainly present in autumn-winter makes us suppose that it is better suited to a lower average temperature. This may also partly explain the few cases of legionellosis reported in our region (European Centre for Disease Prevention and Control 2015). However, it cannot be excluded that they are the result of an underreporting at hospital level.

Environmental microbiological surveillance and risk assessment should be performed more frequently to control the environmental spread of *Legionella*. Since the emission of *Legionella* from water systems is not necessarily constant over time, disinfection must be carried out, even in the presence of low levels of colonisation especially in health facilities, where people are more susceptible to infections. This suggests the need for health authorities to pay greater attention to the problem and to effective risk containment measures.

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Ability of Three Lactic Acid Bacteria to Grow in Sessile Mode and to Inhibit Biofilm Formation of Pathogenic Bacteria

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Abstract

In this study, we explored the effect of three lactic acid bacteria (LAB), i.e. *Enterococcus sp* CM9, *Enterococcus sp* CM18 and *Enterococcus faecium* H3, and their supernatants, on seven biofilm-forming pathogenic strains isolated from human urinary tract or nose infections. By quantitative biofilm production assay, a strong adherence ability of *Enterococcus sp* CM9 and *Enterococcus sp* CM18 was revealed while *E. faecium* H3 resulted to be moderately adherent. Inhibition tests demonstrated an antimicrobial activity of LAB against pathogens.

The presence of cell free supernatant (CFS) of CM9 and CM18 strains significantly decreased the adhesion of *S. aureus* 10,850, *S. epidermidis* 4,296 and *E. coli* FSL24. The CFS of H3 strain was effective against *S. epidermidis* 4,296 and *P. aeruginosa* PA1FSL biofilms only. Biofilm formation of *K. pneumoniae* Kp20FSL, *A. baumannii* AB8FSL and ESBL+ *E. coli* FS101570 have not been affected by any CSF while *P. aeruginosa* PA1FSL biofilm increase in presence of CM9 and CM18 CFS.

Confocal Laser Scanning Microscopy revealed that *K. pneumoniae* Kp20FSL biofilm was inhibited by *Enterococcus sp* CM9, when grown together.

Our results suggest that the LAB strains and/or their bacteriocins can be considered as potential tools to control biofilm formation of some bacterial pathogens.

Keywords

Bacteriocins · Biofilm inhibition · Lactic acid bacteria · Pathogenic bacteria

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1 Introduction

Biofilm is defined as a complex community of microorganisms growing attached to a surface, producing extracellular polymeric substances and exhibiting an altered phenotype compared with planktonic cells (Lindsay and von Holy 2006). Biofilms are often constituted by different species that interact with each other in a synergistic or antagonistic manner (Madsen et al. 2016).

Control of microbial biofilms is an important goal in medical research and clinics, since pathogenic microorganisms growing in a sessile mode on biotic or medical devices surfaces commonly cause biofilm-associated infections difficult to treat (Okuda et al. 2013). Microbial biofilm is also a relevant issue in the field of food safety; in fact, intensive research efforts have been directed towards controlling the occurrence and survival of *Listeria monocytogenes,* for example in the model cultured milk "Lben" (Benkerroum et al. 2002).

Considering the increasing spread of multidrug resistant pathogens, it is becoming necessary to find alternative ways to counteract infections caused by these microorganisms. Bacteriotherapy, consisting in the use of lactic acid bacteria (LAB) to displace pathogens, can be considered as a promising tool to fight infectious diseases (Borody et al. 2004).

In fact, the LAB are known to produce a variety of antimicrobial compounds, as metabolic by-products or natural antimicrobials, that allow to establish themselves in the competitive niches shared with closely-related microorganisms (Sharma and Srivastava 2014).

In addition, the largely reported biofilm-growing ability of some species of LAB, such as *Lactobacillus*, *Enterococcus* and *Pediococcus* (Izquierdo et al. 2009; Belgacem et al. 2010; Borges et al. 2012; Ait Ouali et al. 2014) can be considered as an added value to compete for adhesion sites with other microbial pathogens.

A number of papers reported the effect of antibacterial agents and antimicrobial peptides produced by LAB also on pathogens growing as biofilm (Okuda et al. 2013; Ait Ouali et al. 2014; Jalilsood et al. 2015; Bolocan et al. 2017; Mathur et al. 2018).

Therefore, the aim of this study was to determine the effects of *Enterococcus* sp. CM9, *Enterococcus* sp. CM18 and *Enterococcus faecium* H3 (collected from fermented wheat) and their supernatants on the following biofilm-forming pathogenic bacterial strains: *Klebsiella pneumoniae* Kp20FSL, *Acinetobacter baumannii* AB8FSL, *Escherichia coli* FSL24, *Pseudomonas aeruginosa* PA1FSL, extended-spectrum beta-lactamases (ESBL)-producing *E. Coli* FS101570, *Staphylococcus aureus* 10,850 and *Staphylococcus epidermidis* 4296.

2 Materials and Methods

2.1 Bacterial Strains and Culture Conditions

2.1.1 LAB Isolates

The *Enterococcus* sp. CM9 and *Enterococcus* sp. CM18 strains have been isolated from Camel milk by the Laboratory of Micro-organisms Biology and Biotechnology at the University of Oran. Serial dilutions were prepared (from 10^{-1} to 10^{-6}) from 100 ml of camel milk, and 100 µL of each dilution were spread on M17 agar surface in duplicate (Fluka, St. Louis, MO). After 24 h incubation at 30 °C, distinct colonies were selected randomly and purified by re-streaking on M17 agar plates until only a single type of colonies was observed. The LAB strains were selected by Gram staining, catalase production, and cell morphology, and the *Enterococcus* sp. CM9 and CM18 strains were selected for their interesting bacteriocinogenic properties (Benmouna et al. 2018). *E. faecium* H3 has been isolated and identified by Lazreg et al. (2015).

These LAB strains were cultivated in buffered MRS (De Man, Rogosa and Sharpe) broth (De Man et al. 1960) or agar (Fluka, Switzerland) and incubated at 30 °C for 20–22 h.

2.1.2 Pathogenic Strains

As target bacteria for the inhibitory effects of LAB, 5 clinical isolates collected from urines of patients affected by urinary tract infections (*K. pneumoniae* Kp20FSL, *E. coli* FSL24,

P. aeruginosa PA1FSL, ESBL$^+$ *E. coli* FS101570 and *A. baumannii* AB8FSL) and 2 clinical isolates collected from nose infections (*S. aureus* 10,850 and *S. epidermidis* 4,296), provided by the Microbial Biofilm Laboratory (MBL) in Rome, were cultured in Luria Bertani (LB) or Tryptic Soy (TS) broth or agar (Oxoid, Basingstoke, United Kingdom), depending on the isolate. For the quantitative biofilm production assay and confocal laser scanning microscopy (CLSM) analysis, strains were grown in broth supplemented with 1% (w/v) glucose.

2.2 Preparation of Cell Free Supernatant (CFS) of LAB

Each LAB strain was inoculated twice in modified buffered MRS medium (Benmouna et al. 2018). After incubation at 30 °C for 18 h, the CFS was collected after centrifugation at 6000 rpm for 20 min, at 4 °C. The CFS was stored at −20 °C until use.

2.3 Antimicrobial Activity of LAB Against Pathogenic Bacteria

2.3.1 Bacteriocin Activity Assay

The LAB strains were inoculated in buffered MRS broth, at 5% of inoculum, and incubated for 20 h at 30 °C. The CFS of the LAB were treated with catalase and proteolytic enzymes: trypsin, pepsin and pronase E at 2 mg/mL for 2 h at 37 °C. Then, the samples were boiled for 5 min in order to inactivate the enzymes. The treated and untreated (without enzymes) CFS were tested by the below described well diffusion assays. Briefly, treated and untreated CFS collected from CM9 and CM18 strains were tested against H3 (used as indicator strain), and the treated and untreated CSF collected from H3 strain were tested against CM9 (used as indicator strain).

2.3.2 Direct Inhibition

The inhibition of the pathogenic bacteria by the LAB was realized by the method described by Fleming et al. (1975). Briefly, LAB dropped on the MRS plate were incubated for 18 h at 30 °C. After that, 10 mL of soft agar medium were seeded with the target bacteria (OD = 1.0) and poured on the surface of the MRS plate on which LAB were grown. After incubation at 37 °C from 24 h to 48 h, the diameter of inhibition zone was measured.

2.3.3 Indirect Inhibition – Well Diffusion Assay

The effect of the CFS of the LAB on the target bacteria was tested by the well diffusion assay described by Schillinger and Lücke (1989). Briefly, 50 µL of the CFS was put into a well created by punching the soft agar seeded with pathogenic strains. After an overnight diffusion at 4 °C, the plates were incubated at 37 °C for 24 h. The presence of a clear halo around the well indicated the presence of an inhibiting activity of LAB against the target bacteria.

2.4 Quantitative Biofilm Formation Assay

According to the protocol described by Donelli et al. (2012), 20 µL of LAB overnight culture adjusted at OD_{600} = 0.1 was put into each well of a 96-wells plate already filled in with 180 µL of sterile MRS broth supplemented with 1% (w/v) glucose, with OD_{600} = 0.01 as final bacterial concentration. As control, 20 µL of sterile broth medium plus 180 µL of MRS broth supplemented with 1% (w/v) glucose were used. Each sample was performed in triplicate. After incubation in aerobic conditions at 30 °C for 48 h, the content of the wells was removed and each well was gently washed three times with 200 µL of phosphate buffer saline (PBS). After drying the plate at 60 °C for 1 h, the content of the wells was stained with 180 µL of 2% Hucker's crystal violet for 15 min. Then, the plate was rinsed with distilled water in order to remove the excess of stain, and dried. The dye bound to the adherent cells in each well was solubilized with 180 µL of 33% glacial acetic acid. The OD of each well was measured at 570 nm using microplate photometer

(Multiscan FC, Thermo Scientific). All the strains were classified on the basis of the adherence abilities into the following categories: non-adherent (OD $\leq$ ODc); weakly adherent (ODc < OD $\leq$ 2 $\times$ ODc); moderately adherent (2 $\times$ ODc < OD $\leq$ 4 $\times$ ODc); and strongly adherent (4 $\times$ ODc < OD); where cut off OD (ODc) is defined as three standard deviations (SD) above the mean OD of the negative control.

2.5 Effect of CFS on Biofilm Formation by Pathogenic Bacteria

The wells of the 96-wells plate were filled with 200 µL of overnight culture of pathogenic bacteria in broth supplemented with 1% of glucose, adjusted at $OD_{600} = 0.125$ mixed with 50 µL of the CFS of LAB, in order to obtain a final $OD_{600} = 0.01$. As positive control, wells were filled in with 250 µL of broth culture of the pathogenic bacteria at the same OD. As negative control, wells were filled in with 250 µL of fresh medium supplemented with 1% (w/v) of glucose. Each sample was performed in triplicate. After 24 h and 96 h incubation, biofilm formation was quantified as above described.

2.6 CLSM Analysis of *Klebsiella pneumoniae* Biofilm Inhibition by LAB

Each well of a 24-wells plate containing glass coverslips on the bottom was filled in with 1.6 mL of broth supplemented with 1% (w/v) glucose and 400 µL of broth culture of *K. pneumoniae* Kp20FSL and LAB (200 µL, with OD of the strains adjusted at 0.1). As control, each strain was inoculated alone by using 1.8 mL of broth supplemented with 1% glucose and 200 µL of broth culture at $OD_{600} = 0.1$. After incubation at 37 °C for 24 h, each well was washed three times with 1 mL of PBS and the biofilms formed on the glass coverslips were fixed with 3.7% of paraformaldehyde at room temperature for 30 min. Then, each well was washed three times with 1 mL of sterile distilled water. Biofilms were stained with the Live BacLight Bacterial Gram stain kit (ThermoFisher Scientific) for 15 min at room temperature in the dark. The dye was aspirated and the coverslips were washed gently twice with distilled water.

The Hexidium iodide penetrates in all of bacteria while the Syto 9 penetrates only in Gram-negative bacteria. Thus, LAB were stained with Hexidium Iodide (red), while *K. pneumoniae* Kp20FSL was stained with Syto 9 (green). Fluorescence from stained biofilms was observed by using a CLSM Nikon mod. C1si.

2.7 Statistical Analysis

All analyses were carried out in triplicates. Data were presented as mean $\pm$ SD. Data were analyzed by one-way analysis of variance in an ANOVA using the Scheffe post hoc test (León-Romero et al. 2016) for multiple comparisons ($P < 0.05$; SPSS V20 software).

3 Results and Discussion

Biofilm formation can be benefic or harmful, depending on the bacteria that adhere to the surface. Thus, the control of biofilm formation by pathogenic bacteria can be achieved by the introduction of beneficial sessile-growing bacteria which can compete by forming biofilm at the expense of pathogenic bacteria (Tait and Sutherland 1998; Simoes et al. 2010; Vuotto et al. 2013). The LAB strains are known for their competition with other bacteria, possibly due to the production of lactic acid, hydrogen peroxide or bacteriocins (Fleming et al. 1975; Ammor et al. 2006). Interestingly, several studies mention the anti-biofilm effect of lactic acid bacteria and of their bacteriocins (Okuba et al. 2013; Lin et al. 2015).

3.1 Bacteriocins Production by LAB

According to our results (Table 1), the antimicrobial activity of the CFS was affected mostly by trypsin and pronase E. Since bacteriocins are proteinaceous compounds, it can be presumed that the antimicrobial effect of LAB is due to bacteriocins. In agreement with our hypothesis, several other researchers suggested that the reduction of the antimicrobial activity of the CSF of LAB after treatment with proteolytic enzymes was due to bacteriocins (Kim 1996; Yang et al. 2012).

3.2 Screening for Antimicrobial Activity of LAB

The three selected LAB strains, analysed by the method described by Fleming et al. (1975), resulted to inhibit all the tested pathogenic bacteria (Table 2), with *P. aeruginosa* PA1FSL not being tested. On the contrary, while nisin at 10 and 100 mg/mL inhibited some strains (Table 2), the CFS of all the tested LAB did not exhibit any inhibiting activity against the pathogenic bacteria, as already reported by other authors (Arena et al. 2016). Presumably, the absence of inhibition might be due to a bacteriocin concentration produced by CM9, CM18 and H3 not enough to affect these strains and/or their resistance to the bacteriocins produced by LAB strains.

3.3 Biofilm Formation by LAB

The three LAB strains showed the capacity to adhere to the bottom of a 96-wells plate (Fig. 1). The CM9 and CM18 strains were classified as strongly adherents ($4ODc < 2 \pm 0.3$ and $4ODc < 1.98 \pm 0.47$, respectively), while the H3 strain was moderately adherent ($2ODc < 1.04 \pm 0.19 \leq 4ODc$). These results showed the ability of the three strains to form biofilms, even if to a different extent.

Consistent results have been obtained from several researchers by studying the biofilm formation of LAB in different application fields. Kubota et al. (2008) have examined the formation of biofilms by forty-six strains belonging to different genera of LAB, with all of the bacteria showing the ability to form biofilm. Lortal et al. (2009) have isolated LAB from Tina wooden vat biofilm as predominant bacteria, such as *S. thermophilus*, thermophilic lactobacilli, mesophilic lactobacilli, lactococci and enterococci. On the contrary, Ait Ouali et al. (2014) have studied the ability of 130 LAB isolated from milk tanks and milking machine surfaces, and only five of them were able to form biofilm.

3.4 Effect of CFS of LAB on Biofilm Formation of Pathogenic Bacteria

According to the results reported in Fig. 2, all the pathogenic bacteria tested were strongly adherent, with the only exception of ESBL-producing *E. coli* FS101570 strain which was moderately adherent. The presence of CFS collected from CM9 and CM18 strains significantly decreased the adherence ability of *S. aureus* 10,850 and *S. epidermidis* 4296, from strongly adherent to moderately adherent bacteria. In terms of percentage, a reduction of *S. aureus* 10,850 biomass of

Table 1 The effect of the catalase and the proteolytic enzymes on the antimicrobial activity of the CFS

	CFS of CM9 on H3	CFS of CM18 on H3	CFS of H3 on CM19
Control	10	10	9
+ trypsine	–	–	–
+ pepsine	9	9	nd∗
+ pronase E	–	–	–
+ catalase	10	10	nd

The diameter of zone inhibition was measured in mm. ∗nd not determined; – absence of inhibition

Table 2 Screening of antimicrobial activity of the target bacteria by the LAB

Strains		Origin	Fleming et al. 1975			Schillinger and Lücke 1989				
			CM9	CM18	H3	Nisin[1]	Nisin[2]	CM9	CM18	H3
Gram negative	*K. pneumoniae* Kp20FSL	Urinary tract infection	10	16	10	–	–	–	–	–
	E. coli FSL24		20	18	10	16	30	–	–	–
	P. aeruginosa PA1FSL		nd∗	nd	nd	–	–	–	–	–
	ESBL *E. Coli* FS101570		20	22	10	–	–	–	–	–
	A. baumannii AB8FSL		16	10	5	14	34	–	–	–
Gram positive	*Staphylococcus aureus* 10,850	Nose infection	10	10	8	12	16	–	–	–
	S. epidermidis 4296		4	5	4	8	10	–	–	–

The diameter of zone inhibition was measured in mm. Nisin[1]: 10 mg/mL; Nisin[2]: 100 mg/mL ∗nd: not determined; −: absence of inhibition,;+ and ++: presence of inhibition

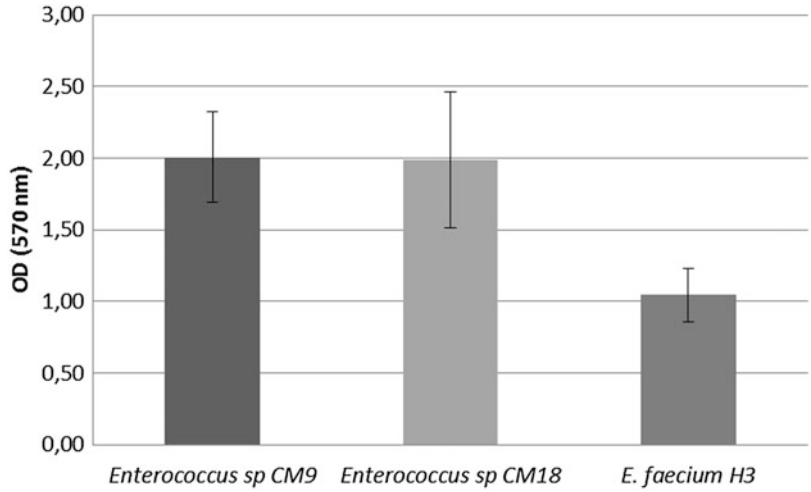

Fig. 1 Adhesion values (OD_{570}) of the three LAB strains obtained by the quantitative biofilm formation assay. Data are mean ± standard deviations

62.4% (4.2 times reduction in presence of CM9 CFS) and 76.2% (6.1 times reduction in presence of CM18 CFS) was detected. Regarding *S. epidermidis* 4296, 74% (corresponding to 5.7 times reduction) and 67.9% (corresponding to 4.8 times reduction) of biomass reduction were revealed when CM9 CFS and CM18 CFS, respectively, were added.

Comparable results have been obtained by several authors. Ait Ouali et al. (2014) have noted that the CFS from LAB isolates were able to counteract the adhesion and subsequently the biofilm formation of *S. aureus* SA3, so considering LAB as good candidates for applications as natural barriers or competitive-exclusion microorganisms to control staphylococcal biofilm formation. Pimentel-Filho et al. (2014) have indicated that bovicin HC5 and nisin can inhibit *S. aureus* adhesion and consequent biofilm development.

On the contrary, conflicting results have been achieved with the *S. aureus* 10,850 and *S. epidermidis* 4296 strains in presence of the CFS of H3 strain. In fact, this supernatant decreased significantly the adherence ability of *S. epidermidis* 4296 (Fig. 2), with a percentage

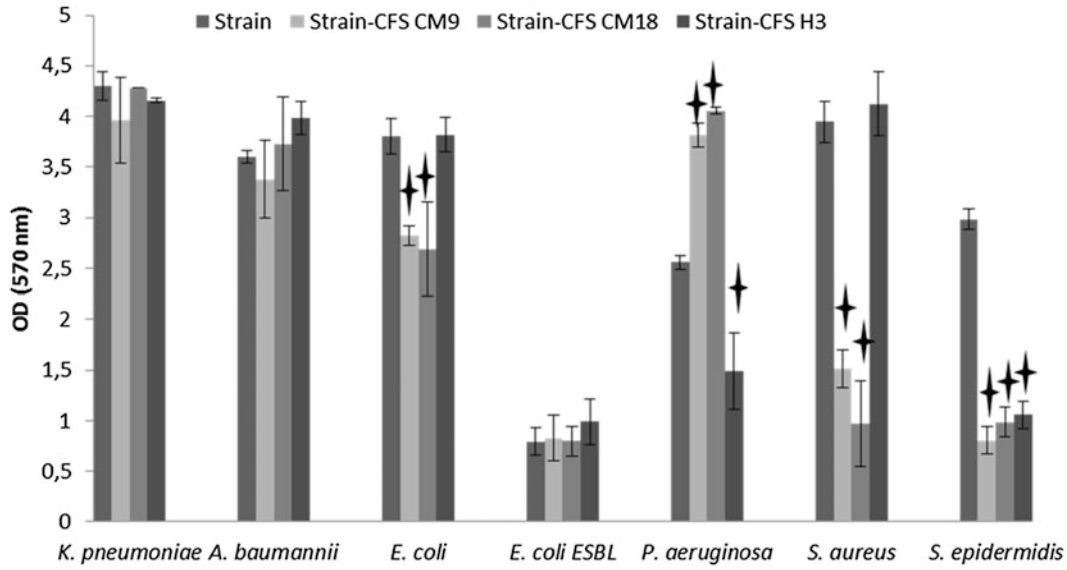

Fig. 2 Effect of LAB supernatants on biofilm formation of the pathogenic bacteria. Results are expressed as the mean ± SD of triplicate measurements. Samples statistically different at $p < 0.05$ are signed by ✦

of reduction of 65.4%, while did not affect the adherence of *S. aureus* 10,850 (OD of 3.9 in the absence of CFS of H3 and OD of 4.1 in the presence of CFS of H3). This result indicated that the antimicrobial agent present in the CFS collected from H3 does not affect the adherence of this strain.

Otherwise, the presence of CFS collected from CM9 and CM18 culture increased significantly the biofilm formation of *P. aeruginosa* PA1FSL (Fig. 2). At our knowledge, this result has never been mentioned in the literature, and it may be due to the presence of substance in CFS that increase the biofilm formation, or that encourage cell growth. On the contrary, a significant decrease was observed for *P. aeruginosa* PA1FSL biofilm in presence of CFS of H3, corresponding to 41.8% of reduction (reduction of 2.4 times).

Biofilm formation of *K. pneumoniae* Kp20FSL, *A. baumannii* AB8FSL and ESBL-producing *E. coli* FS101570 strains have not been affected by the CSF of the three LAB strains, while the biofilm formation of *E. coli* FSL24 significantly decreased of 26% and 29.4% in presence of CFS collected from CM9 and CM18, respectively (Fig. 2).

3.5 Analysis of LAB Biofilm Formation by CLSM

Results obtained by quantitative biofilm formation assay on the three LAB strains have been confirmed by CLSM analysis, with H3 strain producing less biofilm compared to CM9 and CM18 strains (Fig. 3a, b, c). According to Andrade et al. (1998), the attachment of *E. faecium* cells to the surface depends on various factors such as pH, culture media and microorganisms.

The LAB biofilm formation has been studied mostly for probiotics *Lactobacillus* genus (Somers et al. 2001; Kubota et al. 2008; Ait Ouali et al. 2014; Gómez et al. 2016), while there are few studies on biofilm formation by *Enterococcus* strains (Andrade et al. 1998; Minei et al. 2008; Pieniz et al. 2015).

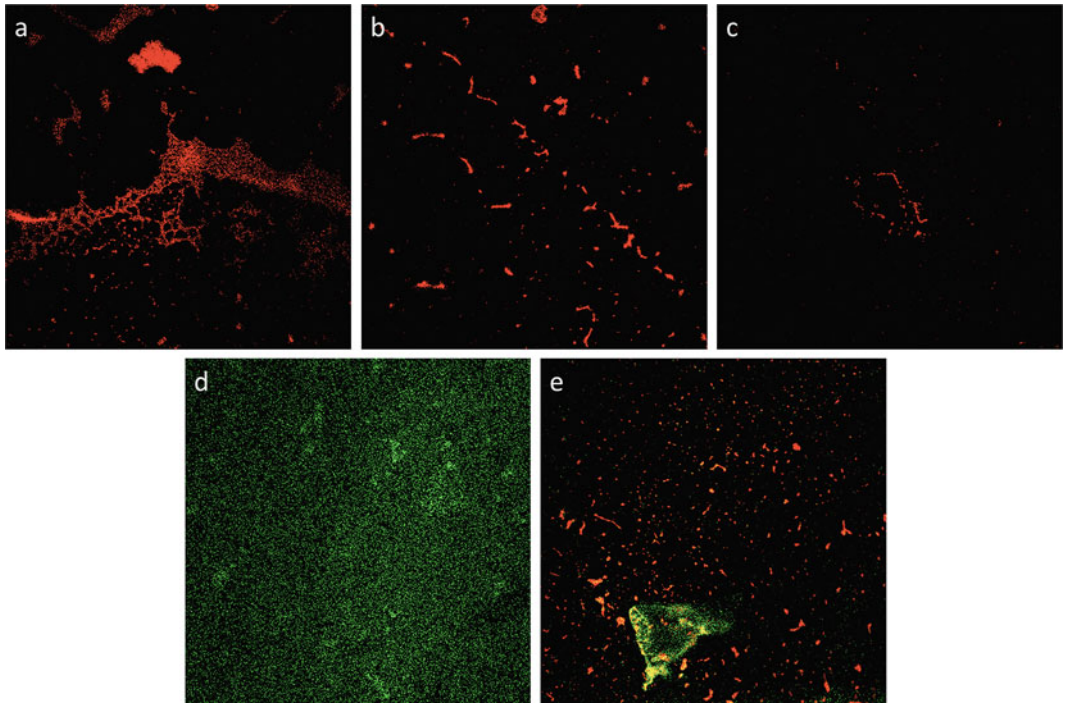

Fig. 3 CLSM images of biofilms formed by (**a**) CM9 strain, (**b**) CM18 strain, and (**c**) H3 strain, *K. pneumoniae* Kp20FSL (**d**), and a dual species biofilm formed by CM9 strain and *K. pneumoniae* Kp20FSL (**e**)

3.6 Analysis of the Competition Between CM9 Strain and *K. pneumoniae* Kp20FSL

To determine the effect of LAB cells on pathogens against which LAB supernatant alone did not show any anti-biofilm effect, we inoculated CM9 and *K. pneumoniae* Kp20FSL strains into the same well and we analyzed the resulting biofilm by CLSM (Fig. 3d, e). It was observed that biofilm formation of *Kp20FSL* (Fig. 3d) was inhibited when inoculated with the CM9 strain (Fig. 3e), with the Kp20FSL strain covering a small portion of the total area. The inhibition of biofilm formation of *K. pneumoniae* may be due to the ability of *Enterococcus* sp. CM9 to compete with *K. pneumoniae* for adhesion site, or to some antimicrobial compounds, produced by the LAB, which are known to inhibit other bacteria in the ecosystem. Obviously, more in-depth studies have to be planned in order to confirm the usefulness of the CM9 strain in the food industry, with the aim to prevent the contamination of food by *K. pneumoniae*.

4 Conclusions

The formation of biofilm by the LAB could represent the next major scientific challenge to microbiologists and food processors for controlling biofilm formation by pathogenic bacteria (Gómez et al. 2016). Based on, a growing number of studies in the last years have pointing out the competitive nature of LABs towards biofilm-growing pathogenic or spoilage bacteria (Zhao et al. 2013; Vuotto et al. 2014; Pérez-Ibarreche et al. 2014; Gómez et al. 2016).

According to our study, the supernatants of the CM9 and CM18 strains have resulted effective against biofilm formation by *Staphylococcus* strains but not by Enterobacteriaceae. The lack of inhibition of biofilm formation in the latter strains may suggest that a higher concentration of bacteriocins is required.

However, by observing the antagonistic effect of CM9 strain on the biofilm formation by *K. pneumoniae*, we have demonstrated that, even if CM9 supernatant alone is not able to counteract Kp20FSL biofilm, the CM9 whole cells are instead able to overgrow *K. pneumoniae,* thus neutralising pathogenic biofilm.

However, to confirm the use of LAB or their bacteriocins for the control of sessile-growing harmful bacteria, further in depth investigations are needed.

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Conflicts of Interest The authors declare no conflict of interest.

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Intracellular Survival and Translocation Ability of Human and Avian *Campylobacter jejuni* and *Campylobacter coli* Strains

Raffaella Campana and Wally Baffone

Abstract

Campylobacter acts using complex strategies to establish and promote intestinal infections. After ingestion via contaminated foods, this bacterium invades and can survive within the intestinal cells, also inducing epithelial translocation of non-invasive intestinal bacteria. In this investigation, the ability of human and avian *C. jejuni* and *C. coli* isolates to survive within two different intestinal epithelial cells lines, Caco-2 and INT 407, as well as the intestinal translocation phenomenon, was assessed. Our data demonstrated that both *C. jejuni* and *C. coli* strains survived in Caco-2 (81.8% and 100% respectively) and INT 407 monolayers (72.7% and 100% respectively) within the first 24 h post-infection period, with a progressive reduction in the prolonged period of 48 h and 72 h post-infection. The translocation of the non-invasive *E. coli* 60/06 FB was remarkably increased in *C. jejuni* treated Caco-2 monolayers (2.36 ± 0.42 log cfu/mL) ($P < 0.01$) and less in those treated with *C. coli* (1.2 ± 0.34 log cfu/mL), compared to *E. coli* 60/06 FB alone (0.37 ± 0.14 log cfu/mL). Our results evidenced the ability of both human and avian strains of *C. jejuni* and *C. coli* to efficiently survive within intestinal cells and induce the translocation of a non-invasive pathogen. Overall, these findings stress how this pathogen can interact with host cells and support the hypothesis that defects in the intestinal barrier function induced by *Campylobacter* spp. could have potentially negative implications for human health.

Keywords

Campylobacter spp. · Virulence · Gut health · Intracellular survival · Epithelial translocation

Abbreviations

CJHS	*Campylobacter jejuni* human sample
CJAS	*Campylobacter jejuni* avian sample
CCHS	*Campylobacter coli* human sample
CCAS	*Campylobacter coli* avian sample

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1 Introduction

Campylobacter is considered the most common and important cause of bacterial gastroenteritis in humans in the developed world (Kaakoush et al. 2015; Cody et al. 2019). In the European Union (EU) the number of confirmed cases has continued to escalate over the years (214,000 in 2013 to 246,000 in 2016 and 2017) (European Food Safety Authority 2018), and in the United States, more than 800,000 cases are annually estimated (Scallan et al. 2011). Poultry is considered to be one of the most important reservoirs of human infections (Wilson et al. 2008; Friesema et al. 2012) and consumption, as well as handling of poultry meat, have been identified as important risk factors (Humphrey et al. 2007; Krutkiewicz and Klimuszko 2010; Wysok and Wojtacka 2018).

The molecular genetics of *Campylobacter* pathogenesis has been extensively studied (Krutkiewicz and Klimuszko 2010) and it is well acknowledged that important virulence factors are involved in the pathogenesis of *Campylobacter* infections, such as the flagella-mediated motility, adherence to intestinal epithelial cells, invasion and survival in the host cells, as well as the ability to produce toxins (Bang et al. 2003; Fouts et al. 2005). During the infection process, *Campylobacter* spp. can pass through the mucous layer of the epithelial cells, attach and then penetrate them (Bolton 2015; Igwaran and Okoh 2019). This interaction is possible to the motility of the bacteria, reached by flagellin, a protein of flagella encoded by *fla*A and *fla*B genes (Lertsethtakarn et al. 2011). Among these, *fla*A is the gene expressed at a higher level, thus considered crucial for bacterial motility. The other genes, *cad*F and *rac*R, control *Campylobacter* spp. colonization (de Oliveira et al. 2019), while invasion ability is supported by the products of *virB*11 and *pld*A genes (Talukder et al. 2008; Wysok and Wojtacka 2018). Other important virulence genes and proteins synthesized by *Campylobacter* species including the invasion antigen C protein, involved in the full invasion of INT 407 cells, and the invasion associated protein gene (*iam*A) (Bolton 2015). Once inside the host cell, *Campylobacter* is able to survive intracellularly for a relatively long period of time, both in phagocytes and intestinal epithelial cells (Naikare et al. 2006; Šikić Pogačar et al. 2009; Buelow et al. 2011). Indeed, it is assumed that the invasion factors activate host cell plasma membrane invaginations (Cróinín and Backert 2012), forming an endosomal compartment that is transported from the apical to the basolateral surface, enabling *Campylobacter* translocation across the intestinal epithelial barrier (Konkel et al. 1992; Hu et al. 2008; Louwen et al. 2012; Backert and Hofreuter 2013). The *Campylobacter* translocation phenomenon may also translocate across the intestinal epithelium other commensal pathogens (Kalischuk et al. 2009), leading to important implications in human mucosal inflammatory responses towards intestinal bacteria.

In this research, the intracellular survival ability of different human and avian *C. jejuni* and *C. coli* strains, as well as the *Campylobacter* induced translocation phenomenon of a non-invasive pathogen was investigated.

2 Materials and Methods

2.1 Bacterial Strains and Growth Conditions

A total of 20 *Campylobacter* spp. belonging to our laboratory collection of strains isolated in Central Italy from human and animal were considered in this study. Samples included 11 *C. jejuni* strains, isolated from patients with gastroenteritis (n = 6) and from avian collected from slaughterhouse environment (n = 5), and 9 *C. coli* from human patients with gastrointestinal diseases (n = 5) and from avian collected from slaughterhouse environment (n = 4). All isolates were previously characterized for adhesion and invasion properties, as well as for the presence of several virulence genes (*cad F*, *flaA*, *dnaJ*, *ciaB*, *pldA*, *cdtA*, *cdtB* and *cdtC*) (unpublished data). The strains were grown on Columbia agar base (VWR, Milan, Italy) plates

supplemented with 5% of Laked Horse Blood (Oxoid, Milan, Italy) and Preston Campylobacter Selective Supplement (VWR) h at 37 °C for 48 h under microaerophilic conditions (5% O_2; 10% CO_2; 85% N_2).

2.2 Epithelial Cells

Caco-2 (human colon adenocarcinoma) cells were propagated as monolayers in Dulbecco's Modified Eagle's Medium (D-MEM) (Sigma, Milan, Italy) supplemented with 10% foetal bovine serum (FBS) (Sigma), 1% non-essential amino acids (Sigma) and 1% antibiotics solution (5000 U of streptomycin-penicillin; Sigma). Intestine 407 (INT 407) cells were cultured in Minimal Essential Media (MEM) (Sigma) supplemented with 10% foetal bovine serum (FBS) (Sigma) and 1 mM sodium pyruvate (Sigma). All the cells were maintained at 37 °C in a 5% CO_2 humidified atmosphere. For internalization and survival assays, Caco-2 and INT 407 cells were seeded at 2×10^4 cells per well in 6-well plastic plates (VWR) and incubated for 7 days at 37 ° C in a 5% CO_2 humidified atmosphere. Before the assays, the cell monolayers were washed twice with phosphate-buffered saline (PBS) pH 7.2.

2.3 Intracellular Survival of *C. jejuni* and *C. coli* Strains in Caco-2 and INT 407 Cells

C. jejuni and *C. coli* strains were cultured for 48 h in Mueller Hinton broth (VWR) with 5% of FCS at 37 °C in a shaking incubator under the above-mentioned microaerophilic conditions. Each bacterial suspension was centrifuged at 1.500 × g for 15 min, the pellet was resuspended in D-MEM containing 1% FBS and adjusted spectrophotometrically to approximately 1×10^8 cell/mL (OD_{660}). One mL of this suspension was inoculated in 6-wells plates containing semi-confluent Caco-2 and INT 407 cells monolayers. Infected cells were then incubated at 37 °C for 3 h in an atmosphere of 5% CO_2. After incubation, the infected Caco-2 and INT 407 cells were washed twice in PBS after 250 μg/mL gentamicin killing and further cultivated in D-MEM containing a sub-lethal dose of gentamicin (10 μg/mL) to suppress eventual growth of viable bacteria from cells. Quantification of intracellular bacteria after until 72 h was performed by twice washings in PBS and subsequent lysing with 1% Triton X-100. The number of surviving bacteria was determined by plating serial dilution of the lysates in sterile physiological solution on Columbia Agar base (VWR); the plates were incubated for 48 h at 37 °C under the described microaerophilic conditions for the quantification of the colonies forming units (cfu). Data were expressed as mean ± sd (cfu/mL) of three independent experiments performed in duplicate.

2.4 Selection of Non-invasive *E. coli* Strain

Four human strains of *E. coli* (*E. coli* 21/01, *E. coli* 126/18 FB, *E. coli* 85/27 AP, and *E. coli* 60/06 FB), kindly furnished by Gamma Laboratory (Fano, Pesaro, Italy), were used in this study. The identification, as well as the antibiotic susceptibility, was performed using VITEK 2 Compact (bioMérieux, Vercieux, France). All the strains were maintained in Tryptic Soya Agar (TSA) (VWR) at 37 °C, while stock cultures were kept at −80 °C in Nutrient Broth n°2 (Oxoid) with 15% glycerol.

To select one non-invasive *E. coli*, invasion assay on Caco-2 cell monolayers was carried out. Briefly, *E. coli* strains were grown in 20 mL of Tryptic Soya Broth (TSB) (VWR) at 37 °C for 18 h. At the end of the incubation period, the suspensions were centrifuged at 3500 rpm for 15 min and bacteria were harvested and re-suspended in 10 mL of DMEM at 1% FBS; for each strain, the optical density was spectrophotometrically adjusted to approximately 1×10^8 cell/mL ($OD_{610\ nm}$) and 1 mL was added to 24 wells plate containing Caco-2 cells prepared as described above. After 6 h of incubation at 37 °C in an atmosphere of 5% CO_2, infected cells were washed twice in PBS and

incubated for another 2 h in DMEM containing 250 μg/mL of gentamicin to kill remaining viable extracellular bacteria. At this point, monolayers were lysed with 1% Triton X-100 (Sigma), serially diluted in physiological saline solution and plated onto TSA (VWR) for the enumeration of invasive *E. coli* (cfu/mL) as described above. Data are expressed as the mean values obtained in three independent experiments performed in duplicate (mean ± sd of cfu/mL). The invasion index, in percentage, was then calculated as a mean of cfu invasive bacteria/cfu inoculated bacteria. The *E. coli* strain with the lowest invasion index was identified as non-invasive and then used for the following experiments.

2.5 Caco-2 M-Like Cell Model

Caco-2 cells were grown in DMEM supplemented with 10% (v/v) fetal bovine serum, 200 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and incubated at 37 °C and 5% CO_2. For *E. coli* translocation assay, cells were seeded onto Transwell filters (3 μm pore size, 1.13 cm^2; Costar Corning Inc., Corning, NY) at 1.5 × 10^5 cells per filter and grown for 21 days, with a change of medium every 2 days.

2.6 *E. coli* Translocation Assays

Briefly, transwell-grown monolayers were washed with PBS and antibiotic-free DMEM was added to the apical and basal compartments. *E. coli* inoculum was added to the apical compartment of all monolayers to achieve a multiplicity of infection (MOI) of 100 cfu per enterocyte. Monolayers were then divided into two groups and half were inoculated with the selected strains of *C. jejuni* or *C. coli* at MOI of 100, whereas the other half received an equivalent volume of sterile broth (control treatment). Following incubation, *E. coli* recovered in the basal compartment (indicating translocation) were enumerated by spreading serial dilutions onto McConkey agar (VWR), incubating the cultures aerobically at 37 °C for 24 h, and enumerating at the dilution yielding 30–300 colony forming units (cfu) per culture. Preliminary experiments were performed to verify that 6 h was the optimal incubation time for *E. coli* internalization and/or translocation (data not shown).

2.7 Statistical Analysis

Statistical analysis was performed using Prism 5.0 (GraphPad Software, Inc., La Jolla, USA). All the data are expressed as the mean values obtained in three independent experiments performed in duplicate. The conditions necessary to perform the parametric tests (one-way Anova, Student t-test) were checked before conducting the analysis, otherwise, non-parametric tests (such as Kruskall-Wallis or Mann-Whitney U test) were utilized. The level of significance was always considered $\alpha = 0.05$.

3 Results

3.1 Survival Ability of *Campylobacter* Spp.

A total of 20 *Campylobacter* spp. were examined for their ability to intracellularly survive in Caco-2 and INT 407 cells up to 72 h post-infection. As shown, most of the strains were able to survive within Caco-2 (90.0%) (Fig. 1a) and INT 407 (85.0%) (Fig. 1b) 24 h post-infection period. A progressive reduction in the survival ability of *Campylobacter* spp. was observed in the following 48 h post-infection period (55% and 40% within Caco-2 and INT 407 respectively). Finally, in the last 72 h post-infection period, only 15% of the strains survived within Caco-2 cells and 20.0% within INT 407 (Fig. 1a, b).

3.1.1 *Campylobacter* Spp. Survival Within Caco-2

The survival of *C. jejuni* and *C. coli* strains within cultured Caco-2 cells was analysed over a 72 h period of incubation (Fig. 2). Quite similar survival characteristics were observed for *C. jejuni*

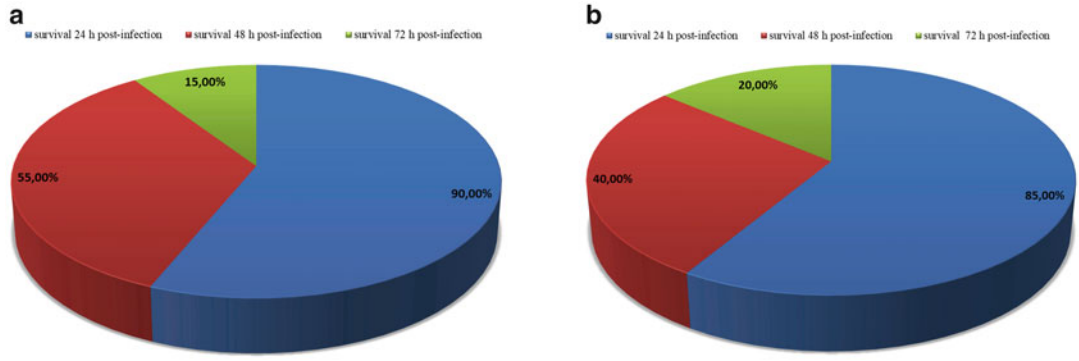

Fig. 1 Percentage of *Campylobacter* spp. survival ability within Caco-2 (**a**) and INT 407 cells (**b**)

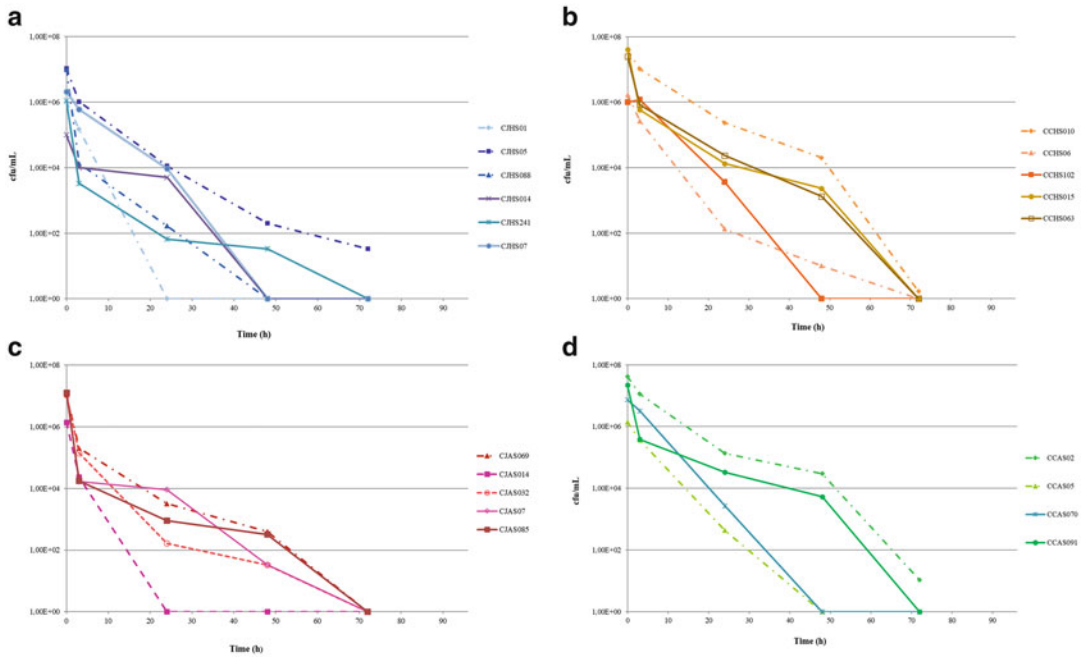

Fig. 2 Survival ability of *Campylobacter* spp. within Caco-2 monolayers up to 72 h post-infection period: (**a, b**) *C. jejuni* and *C. coli* human strains; **(c, d)** *C. jejuni* and *C. coli* avian strains. Data represent mean values of three independent experiments performed in duplicate

and *C. coli* human isolates, though with different cfu/mL values, with a progressive decrease in the number of intracellular bacteria after 24 and 72 h post-infection. In detail, in the case of CJHS05, 1.3×10^4 cfu/mL were recovered 24 h post-infection period higher than the values observed for the other strains (ranging from 6.67×10^1 to 9.33×10^3 cfu/mL); the only strain CJHS01 resulted unable to survive for 24 h within Caco-2 cell. In the following 48 h post-infection, the bacterial survival within Caco-2 cells was remarkably reduced, as showed by the low cfu/mL values recovered for only two *C. jejuni* strains (2×10^2 and 3.3×10^1 cfu/mL for CJHS241 and CJHS05 respectively); moreover, most of the strains did not survive 72 h post-infection (Fig. 2a). Considering *C. coli* human strains, the obtained cfu/mL values after 24 h of incubation were slightly higher compared to *C. jejuni* human isolates, reaching

2.37×10^5 cfu/mL in the case of CCHS010 and ranging from 1.33×10^2 to 2.33×10^4 cfu/mL for the other strains. After 48 h of incubation, CCHS010, CCHS015 and CCHS063 still survived within Caco-2 cells, showing 2×10^4, 2.33×10^3 and 1.33×10^3 cfu/mL respectively. Furthermore, in this case, no strain was able to survive up to 72 h (Fig. 2b).

As regards the survival of *C. jejuni* avian isolates within Caco-2 monolayers, 9×10^3 and 3.3×10^3 cfu/mL were recovered for CJAS07 and CJAS069 respectively 24 h post-infection, while for the other strains about 10^2 cfu/mL were determined; the only strain CJAS014 did not survive. After 48 h of incubation, most of the strains showed low survival ability (about 10^1 cfu/mL) with the only exception of CJAS069 and CJAS1085 that resulted still present within Caco-2 cells (4×10^2 and 3.2×10^2 cfu/mL respectively). No strain survived up to 72 h (Fig. 2c). In the case of *C. coli* avian strains, a higher survival ability compared to *C. jejuni* human isolates was evidenced after 24 h of incubation, with values ranging from 4.3×10^2 to 1.37×10^5 cfu/mL; in the prolonged 48 h post-infection period, only CCAS02 and CCAS091 still survived within Caco-2 cells, showing 5.3×10^3 and 3×10^4 cfu/mL respectively, while no strain was able to survive up to 72 h (Fig. 2d).

3.1.2 *Campylobacter* Spp. Survival Within INT 407

As regards the survival ability of *C. jejuni* and *C. coli* strains within INT 407 cells, data are presented in Fig. 3. Specifically, 24 h post-infection period, most of the human *C. jejuni* strains showed very low survival ability with values ranging from 6.7×10^1 to 3.0×10^2 cfu/mL and, in some cases, no survival was observed (CJHS01, CHJS088 and CJHS24). In the following 48 h post-infection, only the strain CJHS07 still survived with 2.3×10^2 cfu/mL and, as expected, no survival was evidenced 72 h post-infection period (Fig. 3a). Considering the human *C. coli* strains, after 24 h of incubation the

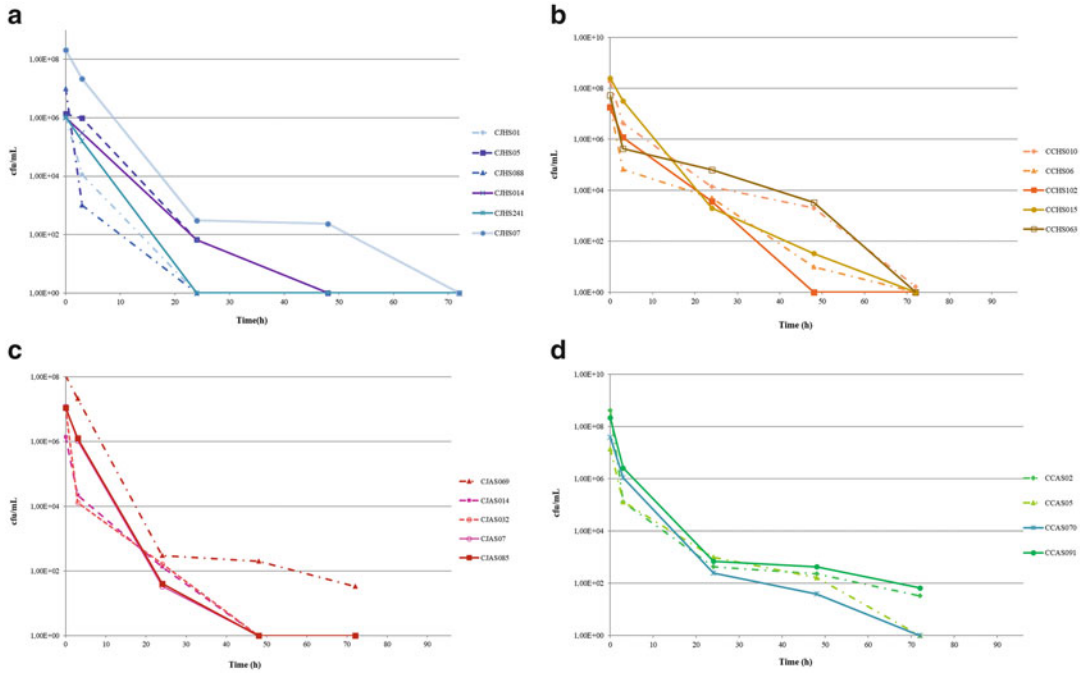

Fig. 3 Survival ability of *Campylobacter* spp. within INT 407 monolayers up to 72 h post-infection period: (**a, b**) *C. jejuni* and *C. coli* human strains; (**c, d**) *C. jejuni* and *C. coli* avian strains. Data represent mean values of three independent experiments performed in duplicate

survival ability resulted higher compared to *C. jejuni* human isolates, with values ranging from to 2×10^3 cfu/mL (CCHS015) to 6.3×10^4 cfu/mL (CCHS063). Most of the strains (CCHS010, CCHS015, CCHS063) were also able to survive 48 h post-infection (values ranging from 1.0×10^1 to 3.3×10^3 cfu/mL) but not up to the prolonged 72 h post-infection period (Fig. 3b).

In the case of the avian *C. jejuni* isolates, all the strains survived only after 24 h post-infection with values ranging from 3.3×10^1 to 1.6×10^2 cfu/mL, while no strains resulted able to survive up to 48 or 72 h post-infection (Fig. 3c). As already noted, all the *C. coli* avian strains showed higher ability to survive after 24 h post-infection in comparison to *C. jejuni* avian isolates, with values ranging from 7.0×10^2 cfu/mL (CCAS091) to 1.1×10^3 cfu/mL (CCAS02). In the following 48 h post-infection period, all the strains resulted still alive within INT 407 (from 3.93×10^1 cfu/mL to 4.33×10^2 cfu/mL), while in the prolonged 72 h post-infection period, only two strains (CCAS02 and CCAS091) still survived within INT 407 cells with low values (about 10^1 cfu/mL) (Fig. 3d).

3.2 Comparison of *C. jejuni* and *C. coli* Survival Ability

In Table 1 are summarized the different survival ability of *C. jejuni* and *C. coli* strains (human and avian source) in both the used cell lines. As shown, in the first 24 h post-infection period, most of *C. jejuni* strains (81.1%) were able to survive within Caco-2 cells regardless of the source (human and avian), as evident by the similar obtained percentages of survival (83.3 and 80% respectively), whilst in the case of *C. coli* 100% of the strains (both for human and avian) resulted surviving. In the second 48 h post-infection period, among the *C. jejuni* isolates, survived only 33.3% of the human strains in comparison to 80% of the avian ones. Similarly, 60% of human and 50% of avian *C. coli* strains were survived within Caco-2 cells. In the last 72 h post-infection period, only a survival percentage of 9.09% was determined for the *C. jejuni* strains, remarkable lower compared to 22.2% of the *C. coli* isolates (20% human strains and 25% avian strains).

As regards the survival ability within INT 407 cells, as already observed for Caco-2 cells, in the first 24 h post-infection period, 72.7% of

Table 1 Survival of *C. jejuni* and *C. coli* strains of different sources (human and avian) within Caco-2 and INT 407 cells after 24 h, 48 h and 72 h post-infection period

	C. jejuni			*C. coli*		
	Human (n = 6)	Avian (n = 5)	Total (n = 11)	Human (n = 5)	Avian (n = 4)	Total (n = 9)
Survival within Caco-2 cells						
24 h post-infection	5 (83.3%)	4 (80%)	**9 (81.8%)**	5 (100%)	4 (100%)	**9 (100%)**
48 h post-infection	2 (33.3%)	4 (80%)	**6 (54.5%)**	3 (60%)	2 (50%)	**5 (55.5%)**
72 h post-infection	1 (16.7%)	0 (0%)	**1 (9.09%)**	1 (20%)	1 (25%)	**2 (22.2%)**
Survival within INT 407 cells						
24 h post-infection	3 (50%)	5 (100%)	**8 (72.7%)**	5 (100%)	4 (100%)	**9 (100%)**
48 h post-infection	1 (16.7%)	1 (20%)	**2 (18.2%)**	4 (80%)	2 (50%)	**6 (66.7%)**
72 h post-infection	0 (0%)	1 (20%)	**1 (9.09%)**	1 (20%)	2 (50%)	**3 (33.3%)**

Data are expressed as number of surviving bacteria (%) recovered after gentamicin protection assay

C. jejuni strains were able to survive (50% human and 100% avian), as well as all the *C. coli* strains (100% human source and 100% avian source). In the second 48 h post-infection period, only 18.2% of *C. jejuni* isolates survived (16.7% human strains and 20% avian strains), in comparison to 66.7% of *C. coli* (80% human strains and 50% avian strains). In the last 72 h post-infection period, only 9.09% of the *C. jejuni* strains survived in opposition to 33.3% of the *C. coli* isolates (20% human strains and 50% avian strains).

3.3 *E. coli* Translocation Induced by *Campylobacter* Spp.

In the last part of this investigation, the *E. coli* translocation induced by *Campylobacter* spp. was examined. As first, we have selected two strains of *Campylobacter*, specifically *C. jejuni* CJHS05 and *C. coli* CCHS06, possessing broad invasive abilities (ID 38.2% and 25.7% respectively, unpublished data), as well as the non-invasive E. coli 60/06 FB resulted to possess the lowest invasive index (ID 0.67%) among the four examined strains (Table 2). In the following experiments, the translocation of *E. coli* 60/06 FB was remarkably increased in CJHS05 treated Caco-2 monolayers (2.36 ± 0.42 log cfu/mL) ($P < 0.01$) and less in CCHS06 treated Caco-2 monolayers (1.2 ± 0.34 log cfu/mL), compared to related control (0.37 ± 0.14 log cfu/mL) (Fig. 4).

4 Discussion

Campylobacteriosis is a significant public health concern worldwide with most human infections caused by *C. jejuni* and *C. coli* (Thakur et al. 2010) through the ingestion of poultry and retail meat products (Igwaran and Okoh 2019). Indeed, these microorganisms can colonise the intestine caecum in extremely high numbers causing severe watery or bloody diarrhoea depending on the individual itself (Scallan et al. 2011). *In vitro* culture assays and molecular methods (Friis et al. 2005; Zheng et al. 2006; Casabonne et al. 2016) have stressed the importance in determining the pathogenicity of *Campylobacter* spp. In addition, the survival of these microorganisms inside various cells has been proposed as a fundamental key in their pathogenesis and infections persistence (Klančnik et al. 2006; Buelow et al. 2011).

Table 2 Invasive index (ID) on Caco-2 cells of the examined *E. coli* strains

Strains	ID
E. coli 21/01	5.67%
E. coli 126/18 FB	3.33%
E. coli 85/27 AP	0.70%
E. coli 60/06 FB	0.67%

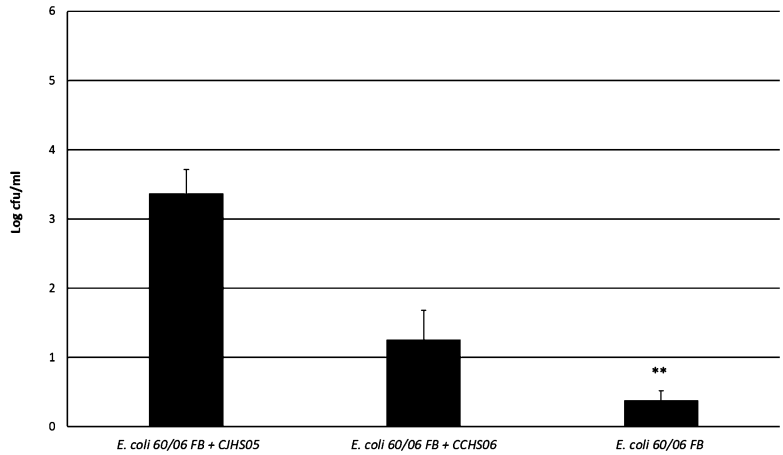

Fig. 4 *E. coli* translocation in Caco-2 monolayers inoculated with *E. coli* 60/06 FB and *C. jejuni* CCHS05 or *C. coli* CCHS06 versus monolayers treated with *E. coli* 60/06 FB alone (control). Data represent mean values of three independent experiments performed in duplicate and asterisks denote values statistically significant compared to the related control (**$P < 0.01$; Student t-test)

In this context, we have examined the survival ability of 20 human and avian *Campylobacter* strains (11 *C. jejuni* and 9 *C. coli*) within two different cell lines, specifically Caco-2 and INT 407, widely used to study *Campylobacter* virulence (Naikare et al. 2006; Buelow et al. 2011; Negretti et al. 2019). The *in vitro* cellular model herein applied was utilized to understand the pathogenic behavior of *C. jejuni* and *C. coli* in a different type of epithelial cell. Indeed, INT 407 cells are considered HeLa derivative because were originally derived from the jejunum and ileum of a 2-month-old Caucasian embryo, later contaminated with HeLa cells, while Caco-2 cells were originally derived from human colon carcinoma. In term of performance, INT 407 cells have the advantage to grow rapidly, we're easy to maintain and manipulate, while Caco-2 cells have a slower grown and are less applicable for some experimental design. Our results showed that both *Campylobacter* spp. were efficiently internalized in Caco-2 and INT 407 monolayers within the first 24 h post-infection period (90% and 85% respectively). Interestingly, 48 h post-infection period, the number of survived bacteria was reduced almost twice within Caco-2 cells (55.5%) and INT 407 (40%), while after 72 h post-infection period, no substantial difference in the survival ability were visible in both the used cell lines (15% and 20% respectively). As regards the relation between *Campylobacter* isolation source (human and avian) and the observed survival ability, was evident that, after 24 and 48 h post-infection periods within Caco-2, the avian strains showed survival percentages higher (from 50% to 100%) compared to those of the human isolates (from 33.3% to 100%). On the contrary, in the case of INT 407 monolayers, in the same post-infection periods, the avian strains showed survival percentages ranging from 20% to 100%, quite similar to those of the human *Campylobacter* isolates (from 16.7% to 100%). All these observations are in line with data of similar researches that reported the ability of *Campylobacter* to survive within various types of non-phagocytic cells for at least 24 h (Backert and Hofreuter 2013) or more prolonged time (Naikare et al. 2006). Nevertheless, while the entry process of *C. jejuni* or *C. coli* into eukaryotic cells has been focused by many studies (Corcionivoschi et al. 2015; Rawat et al. 2018), only a few reports have examined the intracellular fate of these pathogens within eukaryotic cells (Naikare et al. 2006; Watson and Galán 2008; Buelow et al. 2011). Moreover, in the case of *C. coli*, to our knowledge, no reports are available on its ability to survive within intestinal cells, making difficult a comparison of the presented data. In this direction, our data give novel and additional information on the survival ability of *Campylobacter* species, with particular attention to *C. coli* strains that resulted able to highly survive in both the used cell lines.

The functionality of the intact gut barrier is fundamental to gut health, and any impairment of this barrier, including pathogens, toxins or drugs, can increase host susceptibility to various infectious and inflammatory diseases. As a result, certain bacteria could utilize this "active passage" to translocate across the gut epithelium, leading to a different typology of gut-associated diseases (Nagpal and Yadav 2017). This mechanism, defined as bacterial translocation, has been recognized for *Campylobacter* as a phenomenon that may facilitate the translocation of different non-invasive commensal bacteria. In literature Lamb-Rosteski and collaborators (2008) have hypothesized that *C. jejuni* can induce tight junctional disruption, allowing basolateral receptors to migrate to the apical cell surface and promote the following translocation of *E. coli*. In the last part of our research, a Caco-2 M-like cell model, as described by Kalischuk et al. (2010), was developed on transwell filters to study the induced translocation by *C. jejuni* or *C. coli* of a selected non-invasive *E. coli*. In the literature is well documented the translocation of *E. coli* induced by *C. jejuni* 81–176 strain (Kalischuk et al. 2009; Kalischuk et al. 2010) and, to reinforce this observation, here we presented evidence that not only *C. jejuni* but also *C. coli* can cross the intestinal epithelial barrier allowing the translocation of *E. coli*. Therefore, our data support the hypothesis that defects in the intestinal function and/or the disruption of intestinal barrier induced by pathogens, such as *C. jejuni* and

C. coli, can increase the translocation of other bacteria across the intestinal barrier, with possible negative effects on gut and human health. Indeed, this phenomenon can lead to the loss of immunological tolerance (Shen and Turner 2006) and, the failure to downregulate these inflammatory responses in particularly susceptible individuals, can promote the insurgence of chronic inflammations (Resta-Lenert et al. 2005; Kalischuk and Buret 2010).

In conclusion, our investigation showed that both human and avian strains of *C. jejuni* and *C. coli* were efficiently internalized and survived within Caco-2 and INT 407 monolayers for 24–48 h after infection, but not for the prolonged period of 72 h. In addition, we showed that *Campylobacter* spp. can cross the intestinal epithelium thus promoting the translocation of other microorganisms, such as non-invasive E. coli, to extra-intestinal organs. In view of this, further investigations can be addressed to verify whether *Campylobacter* species may favor the translocation of other non-invasive intestinal bacteria, as well as to delineate the consequences of host responses in order to obtain more information on the gut ecosystem equilibrium in human health and diseases.

Conflict of Interest The authors declare that they have no competing interests.

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Evaluation of Bacterial Biofilm Removal Properties of MEDSTER 2000 Cold Sterilant on Different Materials

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Abstract

We studied the antibacterial and anti-biofilm properties of MEDSTER 2000, a pH neutral biodegradable mixed acidic peroxide disinfectant belonging to the class IIb medical device which has been designed for decontamination and cold sterilization of hospital instruments. The broth microdilution method was used to define the antibacterial activity against planktonic form of both classified bacteria and antibiotic resistant strains of clinical source, whereas effectiveness toward their biofilm was determined on mature biofilm, grown both on plastic and stainless steel surfaces. The results showed that for the planktonic form the antibacterial activity of MEDSTER 2000 was already observed after 10 min at the lowest concentration (0.1%), and this effect was not exposure-and/or concentration-dependent. After the same time of exposure at the concentration of 2% the disinfectant was able to completely eradicate all tested bacteria grown in sessile form on both surfaces, with a greater than 6 log CFU/cm^2 reduction in viable cells. This result is supported by the microscope observation by crystal violet and live/dead assays. For the high antibacterial and anti-biofilm ability emerged, MEDSTER 2000 could represent a new and more effective approach for semicritical devices that need a high-level disinfection and could not sustain the process of heat sterilization.

Keywords

Antimicrobial activity · Biocide · Biofilm · High-level disinfection · Medical device · Pathogens

1 Introduction

Biofilm is a structured community of microorganisms, enclosed in a self-produced polymeric matrix (mainly polysaccharides), variously stratified and containing bacterial cells of different living and dormant species inside. Biofilm, in fact, has been defined by some as "a city for microbes", while by others it is equated with an analogue of a multicellular organism. The formation of biofilms offers ecological advantages to the resident microorganisms, including protection from the environment (eg temperature, pH and osmotic extremes, exposure UV light, drying),

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increased availability of nutrients, metabolic enhancement and facilitation of genetic material transfers (Davey and O'Toole 2000; Watnick and Kolter 2000; Assere et al. 2008; Messi 2013).

Unlike the more commonly studied and well-understood planktonic (fluctuating) form, biofilms represent the predominant form of bacterial growth, and it is estimated that 80% of all human infections are of biofilm origin (Hu et al. 2015; Percival et al. 2015; Costa et al. 2019). Various inert substrates such as Teflon ™, stainless steel, rubber and polyurethane can even support the adherence and growth of biofilm, which is regulated by various environmental conditions such as pH, temperature and concentration of dissolved mineral salts. This microbial consortium is a complex system that protects microbes from environmental stress and allows its "inhabitants" to better resist, providing a real physical barrier against antimicrobial substances such as antibiotics, disinfectants and bacteriocins produced by competing microorganisms.

This increased resistance to antibiotics and disinfectants can be estimated from 10 to 1000 times, depending on the studies (Davies 2003; Otter et al. 2015). This represents a real problem and a potential health hazard, especially in health facilities and hospitals where these wild strains can establish themselves and spread as resident surface environmental flora on surgical and diagnostic equipment.

Most of the products used for the disinfection of hospital's surfaces and instruments include quaternary ammonium salts (QACs), aldehydes, chlorine-based products, hydrogen peroxide and peracetic acid. Many of these, however, show serious limits when tested against biofilms. In fact, in order to be registered, all disinfectants must be tested on bacteria in suspension tests, but only few compounds have been tested on consolidated biofilms. QACs have long been shown to be ineffective on *Staphylococcus aureus* and *Pseudomonas aeruginosa* hospital isolated strains (Guerin-Mechin et al. 1999; Méchin et al. 1999). The limited penetration of chlorine-based products into the biofilm matrix can explain the reduced killing action towards microorganisms living inside (de Beer et al. 1994; Jang et al. 2006). More interesting data have been reported on oxidizing agents, especially on peracetic acid which has been demonstrated to have a better performance on consolidated biofilm on different materials and surgical instruments than aldehydes (Neves et al. 2016), chlorine-based products, alcohol and others (Tote et al. 2010; Ledwoch and Maillard 2018; Skowron et al. 2018; Chowdhury et al. 2019).

Given all these data from literature, we tested the anti-biofilm property of MEDSTER 2000 – cold sterilant (Euro Medical Center srl – Firenze – Italy), a class IIb medical device in a sophisticated powder form used to reprocess medical and odontoiatric instruments. Once dissolved into water, this compound shows a synergistic activity of peracetic acid (1500 ppm a 1% concentration) with hydrogen peroxide and organic acids (acetic acid and citric acid), thus improving its biocidal action at a balanced neutral pH environment (around 7). Unlike other similar products, technical data confirm that MEDSTER 2000 solutions have a more stable peracetic acid titration even several days after its preparation. Its bactericidal, virucidal, mycobactericidal, fungicidal and sporicidal activity has been largely demonstrated in standard UNI EN protocols since 0,1% dilution and 5 min contact time. The peracetyl ions released in such a neutral pH environment have been recently described as a crucial and as a further advantage in the reduction of viable count of biofilms over conventional peracetic acid acting at pH value lower than 4 (Meyer et al. 2019).

2 Materials and Methods

2.1 Bacteria, Culture Conditions and Biocide

Reference strains (ATCC-American Type Culture Collection) and ntibiotic resistant clinical isolates were used. Gram positive *Staphylococcus aureus* ATCC 6538 and methicillin-resistant *Staphylococ-*

cus aureus (MRSA), Gram negative *Escherichia coli* ATCC 25922 and extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli*, *Pseudomonas aeruginosa* ATCC 9027 and *Clostridium difficile* ATCC 9689 were grown in Tryptic Soy Broth (TSB, Difco Laboratories, Detroit, MI) supplemented with 0.6% yeast extract (TSB-YE) (Difco), and kept at 30 °C for 18 h. All cultures were centrifuged at 2000g for 20 min. After discarding the supernatant fluid, the pellets were re-suspended in 5 ml sterile deionized water. Centrifugation, supernatant discard and re-suspension were repeated three times. The density of the final suspensions was measured on selective media (Mannitol Salt Agar for Gram-positive bacteria, MacConkey Agar for Gram-negative strains and Clostridium Difficile Agar with 7% Sheep Blood for *Clostridium difficile*), all from Difco Laboratories, Detroit, MI) by the plate count method.

The obtained suspensions were stored until required in phosphate-buffered saline (PBS; 8 g NaCl, 0.2 g KCl, 2.9 g $Na_2HPO_4 \cdot 12H_2O$, 0.2 g KH_2PO_4 with 1 l of distilled water) supplemented with 30% (vol./vol.) glycerine at −80 °C.

MEDSTER 2000 (Euro Medical Center srl, Firenze, Italy) is a class IIb medical device which has been designed for decontamination and cold sterilization of invasive and non-invasive odontoiatric, surgical, hospital and laboratory surfaces and instruments which cannot be processed through heat sterilization or autoclaving.

MEDSTER 2000 can be defined as a pH neutral biodegradable mixed acidic peroxide disinfectant in powder form. Once activated into water it can release active oxygen in a neutral pH solution. The formulation is enhanced by the presence of surfactant, corrosion inhibitors and a mixture of organic acids with their own specific biocidal properties offering a rapid killing action against microbial contaminants within a few minutes of contact.

Its cold sterilizing profile has been well documented with all European Standard tests to obtain the CE mark registration as a medical device but no tests against biofilms have been done before. The aim of this study is to verify the efficacy profile of this product against a mature biofilm on different materials.

2.2 Antibacterial Activity of Medster 2000

The study was divided into three set of experiments: treatment of bacteria in planktonic form, eradication of preformed (mature) biofilm on plastic surface, eradication of preformed (mature) biofilm on stainless steel surface.

All the experiments were carried out in triplicate and the bacterial count was performed on three plates. The arithmetic means of the three determinations, expressed as log bacterial count, was plotted against biocide concentrations. The results were analysed statistically with the Student's *t*-test and differences were considered significant when $p < 0.05$.

2.2.1 Treatment of Bacteria in Planktonic Form

The antibacterial activity of MEDSTER 2000 was assessed by the broth microdilution method. The washed suspensions were diluted up to 10^8 CFU/ml (colony forming units) and 100 µL inoculated into on 96-well polystyrene microtiter plates and the biocide solution (100 µL) was added to the suspensions at various concentrations: 1%, 0.5%, 0.25% and 0.1%. After 10- and 20-min incubation at room temperature, the viable cells were measured by the plate count method, performed spreading 100 µL of samples on plates added with selective media, incubated at 37 °C for 24 h.

2.2.2 Eradication of Preformed (Mature) Biofilm on Plastic Surface

The effectiveness of biocidal treatments was tested on '2 day-old' pre-formed biofilm using Gram-positive *Staphylococcus aureus* ATCC 6538 and MRSA *Staphylococcus aureus*, Gram-negative *Escherichia coli* ATCC 25922 and ESBL *Escherichia coli*, *Pseudomonas aeruginosa* ATCC 9027 as single-inoculated cultures. The assay was performed using a modified 96-well microtiter-plates method (Stepanović et al. 2007; Condò et al. 2020) under static conditions. The mature biofilm was obtained adding 180 µl of TSB and 20 µl of washed suspensions (10^5 CFU/ml) to each wells

of a 96-well polystyrene microtiter plates. After incubation for 48 h at 30 °C, to allow for cell attachment, the bottoms of the 96 well-plates were washed three times with sterile *PBS* in order to remove planktonic cells and 100 µL of MEDSTER 2000 at 2% concentration was added. Following a contact time of 10 min at room temperature, the possible residual biofilm was determined removing biofilm by scraping the entire surface of each well bottom with a sterile plastic loop. Serial tenfold dilutions of the obtained re-suspensions were spreaded onto appropriate agar plates for the viable cell count (CFU cm^{-2}). The colonies were counted following incubation at 37 °C for 24 h. Controls were performed by plate count method, adding in the wells bacterial culture only.

2.2.3 Eradication of Preformed (Mature) Biofilm on Stainless Steel Surface

Biofilms produced by all the species as above were grown on stainless steel AISI 316 coupons (25 cm^2), previously treated with HCl 5 N for 10 min and washed in a detergent solution (ethanol 70%) with sonication (model Elma Transsonic T570. Elma GmbH & Co KG, Elma GmbH & Co KG Kolpingstr. 1-7 D-78224 Singen/Germany) for 20 min to detach debris, rinsed in distilled water, and sterilized by autoclaving at 121 °C for 15 min. The mature biofilm was obtained introducing the coupons in a 50-ml tube containing TSB broth added with 0.1 ml of washed bacterial suspensions (10^5 CFU/ml). After incubation at 25 °C for 48 h, to allow for cell attachment, each coupon was rinsed in 500 ml of sterile PBS and the anti-biofilm activity determined after 10 min contact, both immerging the coupon in a 2% solution or sprinkling the surfaces with the same solution. After this time, the coupons were washed three times with sterile saline solution to remove unattached cells, sonicated for 15 min, and vortexed. Serial tenfold dilutions of the obtained re-suspensions were spreaded onto appropriate agar plates for the viable cell count (CFU cm^{-2}). The colonies were counted following incubation at 37 °C for 24 h. Controls were performed by plate count method, adding in the wells bacterial culture only.

2.3 Evaluation of Anti-biofilm Activity by Light Microscopy

The effectiveness of biocidal treatments on pre-formed biofilm on plastic and stainless steel surfaces was also evaluated with a morphological study using a Light Microscope. Both the mature biofilms formation and the anti-biofilm activity of MEDSTER 2000 at 2% were tested as described above. Concerning biofilm on plastic surface the assay was performed using 6-well polystyrene microtiter plates, to have a larger optical field to observe under microscope.

2.3.1 Anti-biofilm Evaluation by Crystal Violet (CV) Assay

Biofilms grown on polystyrene wells, both treated with MEDSTER 2000 at 2% and untreated control, were washed three times with sterile PBS and fixed with 150 µL of methanol for 5 min. Then, the supernatant was removed again and 150 µL of CV solution at 0.1% was added to each well. After incubation at room temperature for 30 min, the excess of CV was removed by washing three times with sterile PBS. The biofilm on the stainless steel coupons treated and untreated with biocide solution at 2% was washed three times with sterile PBS and the attached cells were fixed with paraformaldehyde for 1 h at 4 °C. Subsequently, the stainless steel surfaces were washed twice with sterile PBS and 150 µL of CV solution at 0.1% was added. After incubation for 30 min at room temperature, the unbound dye was removed by washing three times with sterile PBS and a Scotch tape was sticked by the adhesive side to the stainless steel coupon for 15 min at room temperature. Then, the tape with the adherent CV stained biofilms was removed and put on a glass slide.

Both the glass slides and the polystyrene wells were analyzed by Light Microscope Nikon Eclipse 90i imaging system equipped with Nomarski DIC optics (Nikon Instruments, Melville, NY, USA). DS-2Mv Nikon digital camera was employed to obtained images.

2.3.2 Anti-biofilm Evaluation by Live/Dead Assay

Both biofilm formation (used as control) and biocidal activity evaluation of MEDSTER 2000 at 2% was performed as described above. Biofilms treated with MEDSTER 2000 at 2% and untreated control were washed twice with sterile PBS and stained by the "live/dead cells stain kit" (Thermo Fisher Scientific, Waltham, MA, USA), according to manufacturer's instructions. The method is based on the use of propidium iodide (PI) as marker of dead cells and 5(6)- carboxyfluorescein diacetate (cFDA) to detect alive cells. After incubation in the dark at room temperature for 30 min, the samples were analyzed using the same light instrument as above.

3 Results

3.1 Antibacterial Activity of Medster 2000

3.1.1 Treatment of Bacteria in Planktonic Form

The activity of MEDSTER 2000 against planktonic bacteria was determined by viable count following 10 and 20 min of exposure to 1%, 0.5%, 0.25% and 0.1% concentrations of the biocide. MEDSTER 2000 inhibited the growth of all the strains and this effect was not exposure-and/or concentration-dependent. In fact, already after treatment for 10 and at all the biocide concentrations MEDSTER 2000 was able to eliminate the viable cells of all tested bacteria (range of p-value from 0.00014 to 0.0011) as no cells grew, as revealed by plate count method.

3.1.2 Eradication of Preformed (Mature) Biofilm on Plastic Surface

Biofilm eradication is considered the strictest measure of the efficacy of a biocide rather than bacterial viable count reduction. Biofilm is a structured community of microorganisms that offers ecological advantages, including protection from the antibiotics and disinfectants. After10 min of exposure at the concentration of 2% MEDSTER 2000 was able to eradicate all tested bacteria grown on plastic surface in sessile form, with a greater than 6 log CFU/cm^2 reduction in viable cells of biofilm (range of p-value from 0.0004 to 0.0009) (Fig. 1).

3.1.3 Eradication of Preformed (Mature) Biofilm on Stainless Steel Surface

The anti-biofilm activity of MEDSTER 2000 at 2% was also determined on the stainless steel AISI 316, a surface usually employed for devices

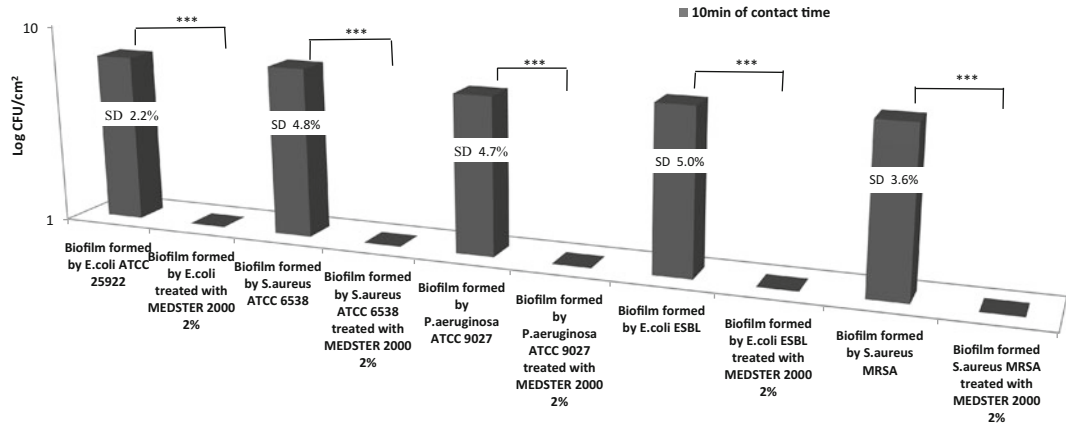

Fig. 1 The anti-biofilm activity of MEDSTER 2000 at 2% on the plastic surface after 10 min of exposure. Results were expressed in $\log_{10}$ CFU/cm^2 as the arithmetic mean of the three determinations. The standard deviation (SD) presented a range from 2,2% to 5% for the controls and 0% for the samples. p- value of <0.05 (∗), $p < 0.01$ (∗∗), $p < 0.001$ (∗∗∗) and $p < 0.0001$ (∗∗∗∗) were considered significant

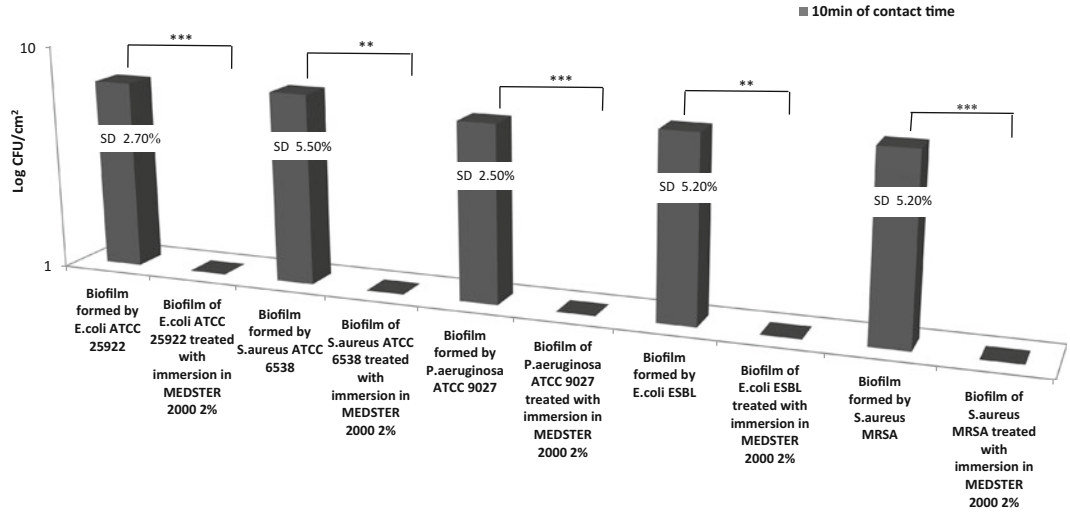

Fig. 2 The anti-biofilm activity of MEDSTER 2000 at 2% on the stainless steel AISI 316 surface after 10 min of exposure by immerging the coupon in a 2% solution. Results were expressed in $\log_{10}$ CFU/cm^2 as the arithmetic mean of the three determinations. The standard deviation (SD) presented a range from 2,7% to 5,5% for the controls and 0% for the samples. *p*- value of <0.05 (∗), $p < 0.01$ (∗∗), $p < 0.001$ (∗∗∗) and $p < 0.0001$ (∗∗∗∗) were considered significant

and implant materials in the hospital setting. The '2 day-old' biofilms on stainless steel were treated with the biocide solution for 10 min both immerging the coupon in a 2% solution and sprinkling the surfaces with the same solution. Even on this surface MEDSTER 2000 was able to eradicate biofilms formed by all strains examined, with cell counts reduction greater than 6 log CFU/cm^2. Furthermore, no differences were found between the two methods used (range of p-value from 0.00012 to 0.0014). The Fig. 2 shows, as example, of the data obtained with the method of immersion. Lastly, the anti-biofilm activity of MEDSTER 2000 at 2% was equal both on plastic and on stainless steel surfaces, so its effect was not typology surface-dependent.

3.2 Evaluation of Anti-biofilm Activity by Light Microscopy and by Live/Dead Assay

The anti-biofilm activity of MEDSTER 2000 at 2% on pre-formed biofilm on plastic and stainless steel surface was evaluated using a Light Microscope.

3.2.1 Anti-biofilm Evaluation by Crystal Violet (CV) Assay

The microscopic observations showed a meaningful eradication in the structures of biofilm and an evident decrease of the number and the adherent cells on both surfaces (Figs. 3 and 4).

3.2.2 Anti-Biofilm Evaluation by Live/Dead Assay

To verify the viability of bacteria observed with the Light Microscope, after the treatment with MEDSTER 2000 at 2% on the different surfaces, the cells were stained with PI and CFDA to discriminate live from dead cells. As shown in Figs. 5 and 6, the treatment with the biocide solution for 10 min led to the decrease in the amount of cells embedded in the polymer matrix of biofilm. Moreover, the few remaining cells were red colored, which makes them definable as dead cells.

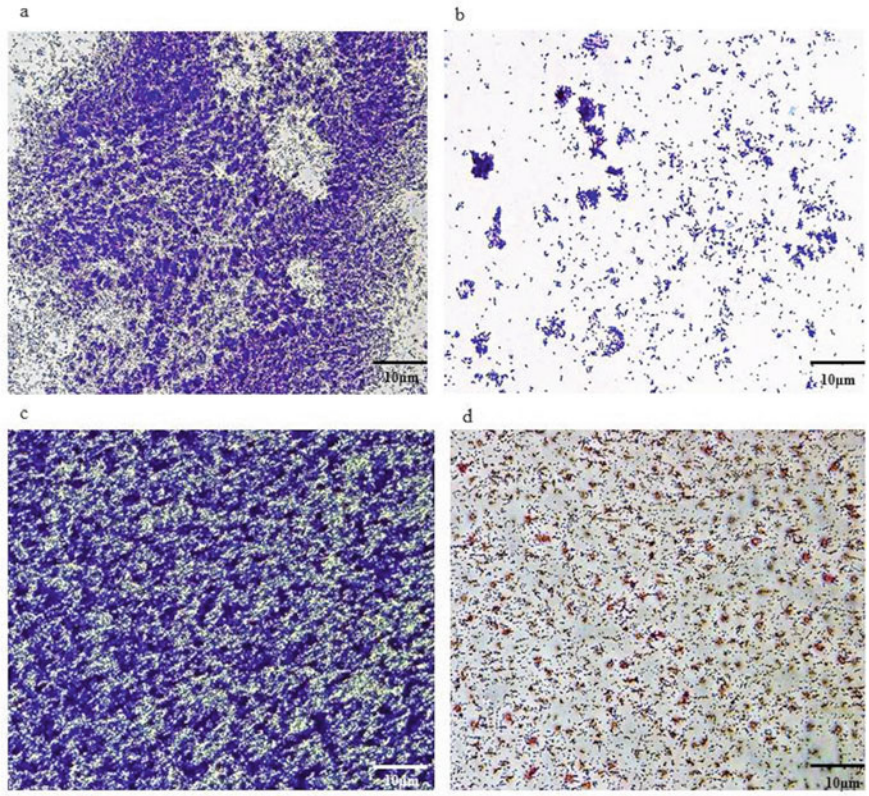

Fig. 3 *Escherichia coli* ATCC 25922 (**a** and **b**) and *Staphylococcus aureus* ATCC 6538 (**c** and **d**) '2 day-old' mature biofilm on plastic surface. Images of light microscopy obtained by using Crystal Violet (CV) Assay before (**a**) (**c**) and after (**b**) (**d**) 10 min disinfection with MEDSTER 2000 at 2%. The scale bars indicate 10 μm

4 Discussion

The constant increase of hospital acquired infections (HAI) is a cause of concern, particularly when they are due to multidrug-resistant (MDR) bacteria. The risk of acquiring methicillin resistant *Staphylococcus aureus* (MRSA), vancomycin resistant enterococci (VRE), extended spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae*, *Clostridium difficile* infections is increased over time (ECDC 2019) and it contributes to morbidity and is considered a major risk factor for mortality. The environment has long been recognized for having an important role in dissemination of microbial pathogens, and biofilm in particular represents an important reservoir of the involved bacteria. Biofilm represents a functional ecological niche for pathogenic and opportunistic strains, and there is now proof that some HAI outbreaks are related to the presence of biofilms (Hall-Stoodley and Stoodley 2005; Percival et al. 2015). The ability to form biofilm, is a critical feature already reported in a lot of studies and represents an evolutionary advantage for the microorganisms living within because this microbial consortium offers protection against different adverse conditions, including disinfection treatments.

In these scenarios the best studied biofilm infections are those related to the colonization of central venous catheters which lead to a mortality ranging from 12% to 25% with an additional cost for the healthcare facility estimated at $ 33,000–35,000 per event (El-Azizi et al. 2016).

In instruments with a complex structure, such as gastroscopes and endoscopic fibers in general,

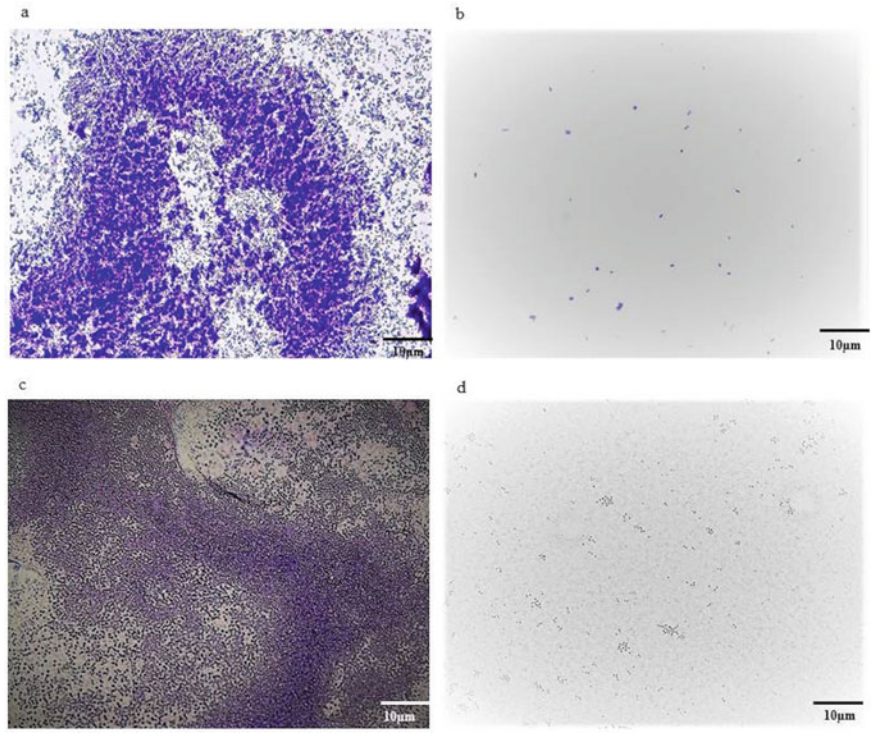

Fig. 4 *Escherichia coli* ATCC 25922 (**a** and **b**) and *Staphylococcus aureus* ATCC 6538 (**c** and **d**) '2 day-old' mature biofilm on stainless steel. Images of light microscopy obtained by using Crystal Violet (CV) Assay before (**a**) (**c**) and after (**b**) (**d**) 10 min disinfection with MEDSTER 2000 at 2%. The scale bars indicate 10 µm

the operating channels are the perfect site for the colonization by a resistant biofilm (Alfa and Singh 2020). The presence of stagnant water, contamination from organic dirt and high initial microbial load are all factors favoring the biofilm formation. In simulation studies of contamination of the working canals with high microbial charges it was shown how decontamination and prewashing procedures are fundamental in the reduction of the initial microbial load and how the use of common disinfectants does not totally eliminate the microbial risk (about 5–18% of endoscopes remained contaminated). With regard the time factor, the fast anti-biofilm activity shown by MEDSTER 2000 allows to speed up the disinfection practices and the ability to completely eliminate any viable cells observed in the present investigation represents an important feature for an optimal infective risk management because the bacterial growth in sessile form (biofilm) begins immediately after the high disinfection procedure, when the instrument is stored (Neves et al. 2016). MEDSTER 2000 inhibited the growth of all the strains, both in planktonic form and organized in biofilm. For the planktonic form the antibacterial activity of MEDSTER 2000 was already observed after 10 min at the lowest concentration (0.1%), and after the same time of exposure at the concentration of 2% the disinfectant was able to totally eradicate all tested bacteria grown in sessile form on both plastic and stainless steel surfaces, with a greater than 6 log CFU/cm^2 reduction in viable cells. This result is supported by the light microscopy observation of the dead cells marked in red with the live/dead assay. Furthermore, the resistance to sterilization processes of dry surface biofilms is not only to chemical disinfectants but has also been described to 121C° autoclaving for 30 min (Almatroudi et al. 2018). Acid peroxidic systems are more effective than glutaraldehyde and orthophtaldehyde (Chino et al. 2017), and in

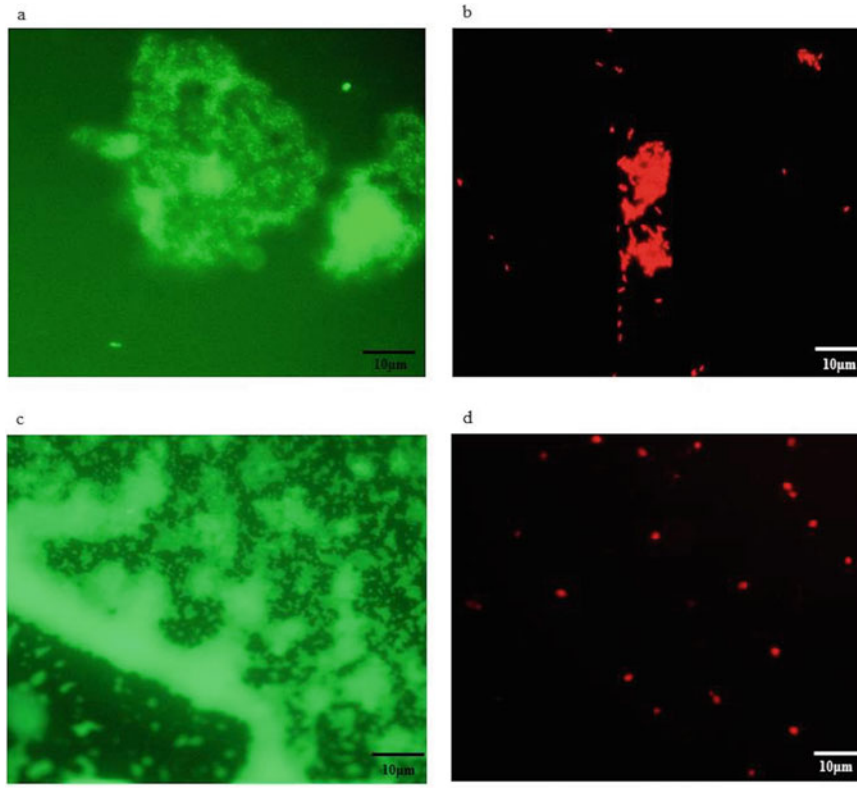

Fig. 5 *Escherichia coli* ATCC 25922 (**a** and **b**) and *Staphylococcus aureus* ATCC 6538 (**c** and **d**) '2 day-old' mature biofilm on plastic surface. Images of light microscopy obtained by using "live/dead cells stain kit" before (**a**) (**c**) and after (**b**) (**d**) 10 min disinfection with MEDSTER 2000 at 2%. Green fluorescence labels live cells, whereas red fluorescence labels dead cells. The scale bars indicate 10 μm

conditions of dry surface *Candida auris* biofilm they showed again a significant higher efficacy then other chlorine based products, such as chlorine dioxide (Ledwoch and Maillard 2018). This higher activity has also been demonstrated in presence of organic load and for relatively short exposure times (Chowdhury et al. 2019). Moreover, for some chemical compounds the removal of the biofilm can be achieved with the increase of the temperature only, but the biofilm reduction obtained in this way is detrimental to the integrity of the materials (just think of the aggressive action on the chlorine materials). On the other hand, a direct or catalyzing action of corrosion of metals and degradation of prosthesis and medical instrumentation materials has been demonstrated precisely for some types of biofilms (Beech et al. 2006; Procópio 2019). Under our study conditions we demonstrated that a complex mixture of precursors of reactive oxygen and peracids, working in a neutral pH solution and at room temperature, is able to remove biofilm from different materials such as plastic or surgical stainless steel AISI 316 both through spraying or immersion test. These results are consistent with other recent studies confirming a higher activity in biofilm removal for peroxyacetic acid under neutral buffered pH than in acid conditions (Meyer et al. 2019).

In conclusion, instead of conventional instrument processing through decontamination, precleaning and final high level disinfection with 3 different products that could interfere with each other, in the present study an "overkill approach" based on a unique antibacterial compound MEDSTER 2000 is proposed. Based on

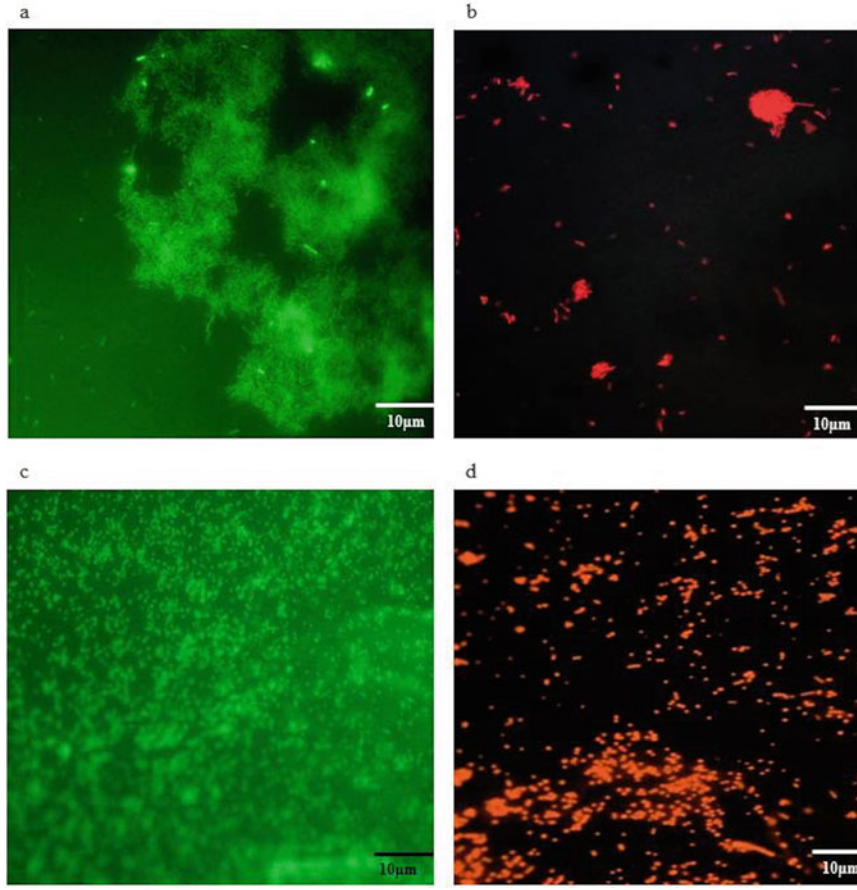

Fig. 6 *Escherichia coli* ATCC 25922 (**a** and **b**) and *Staphylococcus aureus* ATCC 6538 (**c** and **d**) '2 day-old' mature biofilm on stainless steel. Images of light microscopy obtained by using "live/dead cells stain kit" before (**a**) (**c**) and after (**b**) (**d**) 10 min disinfection with MEDSTER 2000 at 2%. Green fluorescence labels live cells, whereas red fluorescence labels dead cells. The scale bars indicate 10 µm

the results obtained, this disinfectant highly effective against biofilm and endowed with biosafety margins can be employed at different concentrations to perform the 3 conventional steps (decontamination, precleaning, disinfection). This treatment could be a new a more effective approach for semicritical devices that need of a high-level disinfection than any other reusable medical device.

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The Struggle Against Infant Scrofula in Siena Between the Nineteenth and Twentieth Centuries

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Abstract

Poverty, high population density and unhealthy dwellings in Siena's historic district accounted for the spread of tuberculosis in its various forms between the mid-nineteenth century and the first three decades of the twentieth century. In this paper, the author relies on statistical data relating to a time span between 1898 and 1935 to discuss the high incidence of scrofula, or tuberculosis of the lymphatic glands, among Siena's infant population.

The result is a description of the most important actions implemented at city level to prevent tuberculosis and to assist and treat sick children: stays in seaside hospices promoted by Carlo Livi in order prevent and treat poor and malnourished children, the establishment of a Preventorium to accommodate children from families that included members infected with pulmonary tuberculosis, the realization of activities in the green areas of the Fortress on advice of Achille Sclavo. Furthermore, the author recalls the work of great hygienist Sclavo to raise awareness of the hygienic practices among Siena's population, which was fundamental in the prevention of epidemic diseases such as tuberculosis.

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Keywords

Achille Sclavo · Carlo Livi · Scrofula · Seaside hospices · Tuberculosis and childhood

1 Introduction

In the Unification years, Italy's sanitary and hygienic conditions were extremely precarious and challenging, as described by Carlo Maggiorani (1800–1885), a doctor and Senator of the Kingdom of Italy, in the speech he delivered in the Senate on March 12, 1873: "Phthisis, scrofula and rickets are more rampant than ever before; pellagra is becoming more widespread; and malaria, with its sad effects, afflicts much of the peninsula. [. . .] Syphilis unruly meanders among citizens and in particular among militias" (Atti parlamentari 1873).

Together with poverty, ignorance and hunger, scarce knowledge of the etiology of the most widespread epidemic diseases, the danger of contagion and insufficient notions of hygiene yielded a picture of alarming severity.

Among all contemporary endemic diseases, tuberculosis, in its many forms, was the most pernicious and responsible for a very high number of deaths, especially among the poor and children.

And precisely because it mostly affected certain age groups, the tuberculosis epidemic produced serious effects also from a social point of view.

In Siena, between the end of the nineteenth century and the first decades of the twentieth

century this slow and silent disease had a very high morbidity rate due to the poor hygienic-sanitary conditions of the entire residential area within the ancient city walls, as well as the high population density of some districts and unhealthy dwellings inhabited by the poorest classes. It is easy to grasp the severity of the situation if one takes into account that a modern aqueduct was built in the city only in 1914, and that the sewerage system would not be ready until the end of WWI. The ease of contagion dissemination was matched by a very high mortality rate, which, at some junctures, reached levels recorded in Italy's largest cities, to the point that in 1929 Giorgio Alberto Chiurco (1895–1975), politician and director of the Institute of Surgical Pathology of the University of Siena, "referring to the serious disease that afflicts the land of Siena in an impressive manner" lamented that the city "according to statistics, ranks second for tuberculosis-induced mortality" (Chiurco 1928).

And if the lung form was the main cause of deaths in the age groups between 15 and 30 years the infant population was mostly affected by scrofula, which caused swollen suppurating lymph glands in the neck.

2 Etiology and Clinical Characteristics of Scrofula

The origins of scrofula represented a much-debated topic in the second half of the nineteenth century.

For some "it is a disease in the blood, dyscrasia, in which broken matter, through blood circulation, eventually deposited itself in some parts of the body, triggering its rotting". According to others, "it is a group of diseases that affect weak people and because of such weakness, the diseases become more malignant and more difficult to cure". Yet, for others, "it is vicious overnutrition of parts of the body: skin, mucous membranes, bones and especially lymphatic glands" (Livi 1873).

In reality, scrofula, or tuberculous lymphadenitis, is an infection of lymph nodes of the neck generated by mycobacteria.

Although it was originally considered a childhood disease, it is widely represented in the 20–40 age group. In adult population, it is caused by Mycobacterium tuberculosis, responsible for pulmonary tuberculosis, which penetrates the lymphatic circulation and affects some lymph nodes, and, in particular, those below the jaw. In children – topic of this research paper – this disease is caused by other "atypical or non-tuberculous" mycobacteria, such as the Non-tuberculous mycobacteria.

Scrofula presents disproportionate enlargement of the glands in the neck, especially those at the base of the jaw. It is characterized by the absence of phenomena typical of the inflammatory process, such as redness and increased skin temperature, resulting in characteristic swelling called "cold abscess". It is often accompanied by fever, chills and malaise.

The impairment of the lymph nodes does not cause pain but, if left untreated, makes lymph nodes hard-elastic, with swelling of or exceeding 2 cm in diameter (lymphadenomegaly).

The affected lymph nodes, in particular those that merge into masses of considerable size, sometimes cause the rupture of overlying skin and the explosion of the abscess (fistula) with abundant pus leakage and subsequent formation of retractile, deforming and permanent scars.

In addition to damage to glands in the neck, there are lesions of ocular, nasal and lip (peribuccal) mucosa, eczema of the face and scalp, and swelling of nose and upper lip. These deformations, associated with the formation of enlarged lymph node aggregates, gives the sick (especially children) the typical appearance of a pig (facies scrofola), hence the name of the disease.

3 Siena's Poor Scrofulous Children

Particularly significant is the description by Carlo Livi (1823–1877) in 1873, at that time the director of Siena's San Niccolò asylum, who was very committed to assisting scrofulous children: "Poor children with twisted and deformed legs; with

bloody eyes and a neck gnarled with swollen glands and disfigured by sores and scars. [. . .] But the spectacle will be even sadder if you venture through the filthy, damp and dark alleys of the Districts of the Wave, the Tower and the Caterpillar. There, scrofula and rickets have long taken residence, and are inflicting miserable torment upon innocent flesh" (Livi 1873).

We can obtain precise data on tuberculosis mortality in children aged between birth and 15 years from three different studies carried out at the time by Filippo Neri, Health Officer of the Municipality of Siena and assistant of Achille Sclavo (1861–1930) at the Institute of Hygiene of the Siena University, and Aristide Londini, Neri's successor in the municipal office.

In the first period into consideration, namely, from the end of the nineteenth century to 1913, mortality from tuberculosis in Siena in the 1–5 year age group reached 64.7 deaths per 10,000 inhabitants; it dropped to 44.1 in the 6–10 year range to rise to 61.4 for the 11 to 15 year range (Neri 1915).

In the subsequent period, 1914–1920, the subject of the second study, tuberculosis mortality dropped significantly in response to the implementation of preventive actions, amounting to 42.96 deaths for every 10,000 inhabitants in the 1–5 year age group; 35.80 in the 6–10 year group; and 70.11 in the 11–15 year group (Neri 1921).

In the last period taken into consideration, from 1921 to 1935, it is notable that tuberculosis-induced mortality among Siena's infant population decreased still: 39.53 deaths per 10,000 inhabitants in the 1–5 year age group; 19.78 in the 6–10 year age group; and 41.07 in the 11–15 year group (Londini 1937).

These values, though lower than those of the 15–30 year age group, were high nonetheless and an indication of very challenging sanitary conditions.

4 Sun and Sea: "Great Drugs" for Scrofulous Children

As early as the mid-nineteenth century, given the lack of drugs capable of treating tuberculosis, actions aimed at children and young people looked, in particular, to thalassotherapy, both for preventive and curative purposes.

Among the proponents of seaside hospices there was Giuseppe Barellai (1813–1884), a doctor at the Hospital of Santa Maria Novella in Florence and scholar of tuberculosis prophylaxis (Carnevale and Diana 2014), who, in 1853, claimed that there was no "medicine better than air and sea water" (Barellai 1853) to cure childhood scrofula and rickets. Three years after the publication of his book, a first seaside hospice was built in Viareggio and Barellai, spurred by this positive experience, urged the establishment of other similar structures on the Tuscan coast.

In Siena his plea was heeded by Carlo Livi, who in 1864 founded the Popular Association for Scrofulous Children.

His experience in this field led him to define the causes of tuberculosis: among these, "hereditariness", that is, hereditary disposition to the disease; unhealthy dwellings; poor hygiene, drinking water "contaminated with putrid materials" and unhealthy air. Indeed, he understood the importance of studying the relationships between illness and social status, imagining an evolution of medical science towards a form of preventive social medicine addressed to the community as a whole. And in support of his ideas, he exhorted taking a look at medical texts: "You will not find one that does not tell you that, aside from hereditary disposition, unhealthy, damp, cold and poorly illuminated dwellings, which can hardly be aired out and are supplied with dirty and infected water are the most powerful causes of two fatal diseases: scrofula and rickets" (Livi 1873).

For this reason, Carlo Livi endeavored to keep children away from environments where diseases thrived, offering them better nutrition and life outdoors, in the sun and pure air. It was essential for Livi to remove children from environments where contagion lurked, and place them in healthy environments, and especially seaside, "plunging them into air, light and water baths, which give children their color back, replenishing and regenerating them" (Livi 1873).

In the five-year period from 1867 to 1871, Siena's scrofulous children were sent to seaside hospices in Viareggio: Paolo Funaioli

(1848–1911), a student of Livi's, published a medical report with statistical data. Of 75 children, between 8 and 14 years, who spent a month seaside, 16 were healed, 36 improved their general conditions, 36 improved their general conditions and local disease manifestations, and 7 showed no improvement (Funaioli 1874). On the basis of such positive results, Funaioli asserted that: "Medicine has no other remedy to fight scrofula than the sea", though, arguing, at the same time, that even better results could be achieved and more children could be healed if only they could stay by the sea for longer stretches of time.

The seaside hospice of Porto Santo Stefano was inaugurated in 1872; upon Livi's suggestion became the reference point for thalassotherapy administered to Siena's children. The facility was built on the Argentario coast. In a letter sent by Barellai to Livi in 1869, we can read as follows: "The air surrounding Monte Argentario is so oxygenated and pure that when you breathe it, especially as you face the gulf, you feel that your lungs become more elastic and let in vigor and health [...]. Seaside hospices and the resulting natural baths offered by these facilities would find therefore in Porto Santo Stefano the most suitable, rewarding, beautiful and economical solution" (Carnevale and Diana 2014).

Over the years, results became more and more evident. Indeed, the Health Report on Siena's scrofulous children sent to the Porto Santo Stefano seaside hospice, published in 1878 by Flaminio Tassi, indicated that of about 55 children (32 girls and 23 boys) between the age of 5 and 14 years, who stayed at the hospice in the summer of 1877, most of the children, except for 6 of them, showed significant improvement or were cured of scrofula. "In conclusion – stated Tassi – the results achieved after seaside care, as the statistical reports show, are of indisputable utility, and in some cases the successes have been so brilliant as to appear almost prodigious. The sea is responsible for such wonders. [...] Now, faced with these facts, is it still possible to question the beneficial action of the sea on scrofula?". "The sea is therefore a great drug for scrofulous children" (Tassi 1878).

The institution of seaside hospices can therefore be considered works of civilization and progress; they led to the involvement of Public Administrations and generous citizens alike. The latter's donations, in particular, afforded needy children the benefit of seaside stays.

Crediting Barellai the successful intuition of seaside hospices for scrofulous childhood, Livi remembered his colleague with these words: "Heart can be highly inspired the same way brilliance is; and it was a great heart that propelled the modest Florentine physician to pursue the hygienic, civil and Christian idea of opening free seaside hospices for the craftsman's children stricken with scrofula. [...] Every year the beneficial [...] sea opens its great arms to welcome infirm, emaciated, hunched children and youngsters, seemingly blinded by scrofula, sending them back to their homes healthy, energetic, robust and cheerful" (Livi 1873).

Despite these important results, a sick childhood in the late nineteenth and early twentieth century Siena was still a serious problem until very strong choices were implemented in the sanitary field, thus finding solutions for what for decades had been singled out as the causes of the high mortality rate in the center of Siena: population density and unhealthy dwellings. In 1928, work began to demolish the district of Salicotto, one of the most affected by tuberculosis. In this area, in the heart of Siena, as late as the first half of the twentieth century, poor families often cohabited with tuberculosis patients in dwellings located below street level, consisting of one or, at most, two dark rooms that received air from the stairs. "These poor children are held by coughing old men, made to sleep together with TB patients, sit in front of the fireplace alongside sick individuals who, without any medical guidance, are allowed to sow bacilli left and right, infecting infants" (Chiurco 1928).

Over the years, other preventive actions were also implemented: starting in 1919, a summer camp was held inside the Fortress, welcoming about 100 children, 3–12 years of age, who were placed under the care of health personnel also during the year; it was also created an outpatient heliotherapy center – with 14 beds – inside

the garden of the Hospital *Santa Maria della Scala.* This center followed the guidelines set by Dr. Auguste Rollier (Corsini 1942). Therefore, for decades, before antibiotics became available, sun exposure played a fundamental role in the prevention and treatment of tuberculosis.

Furthermore, at the end of the 1920s, the *Podestà* of Siena, Fabio Bargagli Petrucci (1875–1939), supported by the positive opinion of Achille Sclavo, though not without challenges, arranged for the construction of a Preventorium on the premises of the monastery of Santa Maria Maddalena, designed to accommodate up to 60 children removed from families exposed to the danger of contagion by infected people within the family (Comune di Siena 1934).

In the words of Antonio Cammarata, in order to avoid exposure to tuberculosis, it was fundamental "to remove healthy children from an infected family environment, and to place them in a healthy environment, either by dispatching separately to other families (family placement) or collectively in specialized institutions (collective placement)" (Cammarata 1925).

Removal from an environment that led to contagion, better nutrition, outdoor living in the sun and pure air were fundamental remedies at a time when no drugs were available to treat TB.

5 "Hygiene and How to Implement It"

To be sure, Achille Sclavo, who endeavored to raise awareness of the culture of hygiene by every means at his disposal, surely gave a fundamental contribution to the prevention of epidemic diseases, such as tuberculosis.

In 1904 Sclavo, Professor of Hygiene at the University of Siena, founded in the city the *Istituto Sieroterapico Vaccinogeno Toscano*, after closely studying and understanding epidemic diseases. In so doing he realized that, in many cases, these diseases could be avoided by following simple hygiene rules that were useful to reduce contagion.

He became persuaded of the need for hygienic propaganda to engender a true "inner revolution" among people, in order to promote hygiene and a healthy respect for human life.

For this reason Sclavo, who was one of the first to held university courses in Hygiene, decided to turn to attentive audiences: soldiers, priests, doctors, people working within the public services, and even ordinary people.

However, he soon realized that the sound hygienic awareness could only arise out of a slow learning process that could certainly not be harbored within the domestic environment, due to the misery, ignorance and prejudices of parents. It was therefore necessary to turn to schools, teachers and above all, young people, who were free from the mistaken beliefs of adults and, thus, more open to change.

To be effective, teaching hygiene had to be devoid of its purely theoretical character, to be delivered hands-on.

Hence, Sclavo's writings dedicated to challenges in the educational environments: *Igiene ed edilizia scolastica* (1914), in which he denounced the disconcerting state of school buildings, proposing solutions to the most widespread health problems. Noteworthy are also writings in which he prescribed proper nutrition, outdoor education and physical activity for the healthy development of young people: *Per l'educazione fisica* (1914); *Diamo aria ai nostri polmoni* (1915) a manifesto of the anti-tubercular struggle in favor of sun and pure air; *Sull'alimentazione umana. 5 lezioni tenute in Firenze alle infermiere della Croce Rossa italiana* (1917); and *Per l'igiene sociale* (1918).

In this way, Sclavo placed great hope in the so-called "ascending education", resting on the axiom that young people were to become spokespersons for good practices among adults (parents and relatives) who, in turn, would end up appreciating their importance and recognizing their usefulness.

In his *Il Decalogo dell'Igiene*, we read: "Love sunlight, which gives you everything together with health to your body. Love fresh air and store plenty of it in your home, keeping windows open as long as you can" (Sclavo 1924).

This precept, fundamental in the prevention and treatment of Siena's children affected by

forms of tuberculosis, was consistently reaffirmed by Sclavo during his life. It is within this frames that we must understand his tirade against unhealthy dwellings in the historic center of Siena: "it has not yet been possible to build dwellings in which, in addition to protection from harmful external influences, the enjoyment of pure air and sunlight is guaranteed" (Sclavo 1924). And again, when he described schools: classrooms, even when they are in old buildings, must be renewed by "flooding them with air and light, without forgetting that, weather permitting, lectures should be delivered outdoors, under trees" (Sclavo 1924). Indeed, he promoted outdoor schools, building one on the bastions of the Fortress of Siena, as well as heliotherapy camps, with the intent of removing children from the unhealthy conditions of so many city districts, albeit for part of the day.

Let the sun flood homes became one of the topics discussed at school: "The healthiness of a house greatly depends on the amount of sunlight reaches into it. Let us take a brick to represent a house and orient it so that one of the major façades faces north, the opposite face will face south and the two warheads will face east and west, respectively. Children must be shown that in such conditions the brick will never receive the sun on the north façade; that the eastern head will be illuminated throughout the morning, until midday; that the west end will be exposed to sun from midday until sunset; and that throughout the day the sun will be on the south façade" (Sclavo 1924).

Sclavo did not lecture but conversed with pupils to make them reflect and reason, through concrete scientific examples, and sometimes even complex ones.

With the same approach, with simple language and lots of examples, Sclavo also discussed tuberculosis, "one of the most serious diseases afflicting humanity" (Sclavo 1924). In his book *Per la propaganda igienica. Scuola ed igiene*, he concisely summarized useful information "that is most necessary to disseminate among the public through school" (Sclavo 1924). He explained causes and forms of tuberculosis, putting to rest false beliefs, such as whether tuberculosis was hereditary, or that it did not affect the elderly. And he gave a series of important hygienic precepts: avoid spitting because tuberculosis bacilli were in saliva, and cover your mouth with a handkerchief when sneezing. He explained that it is very likely to find tuberculosis bacilli in cow's milk which could infect humans, hence he recommended boiling milk before drinking it.

"Crockery, cutlery and glasses used at home by tuberculosis patients must be disinfected and, to this end, be kept for fifteen minutes in water and ash, at boiling temperature". Clothes and linen used by the sick must be aired out for a long time, often under the sun, since "direct sunlight kills the Koch's bacilli". "Tuberculosis patients must refrain from kissing other people, and especially children, and it is very important that they sleep alone in a separate room" (Sclavo 1924).

He ended with a recommendation to all children and teenagers: "To make the human body strong, to neutralize the tuberculosis infection before it becomes manifest, and to recover from the disease it is necessary to live according to hygienic rules, which among other things, prescribe to stay long outdoors; keep the house clean and ventilate it often by opening windows; eat in a suitable and sufficient manner; and ensure thorough cleaning of the body and adequate physical activity" (Sclavo 1924).

6 Conclusion

Between the second half of the nineteenth century and the first three decades of the twentieth century, tuberculosis, mainly in its non-pulmonary form, as scrofula, was one of the most pernicious disease in Siena due to its high rate of morbidity in children.

Despite the lack of drugs available to treat this disease, significant results were achieved in the preventive and curative fields through seaside stays and exposure to sun and fresh air, which, as great hygienist Achilles Sclavo recommended, should always occur in conjunction with the application of the most basic hygiene standards.

It is interesting to highlight that at the time the interaction between sun and vitamin D and the treatment of tuberculosis was still unknown. For decades, before the discovery of antibiotics, TB patients were sent to sunny places to undergo heliotherapy treatments, without knowing the reason of the recovery.

In recent years, a study of the Queen Mary University in London brought to light that high-dose of vitamin D, given in addition to antibiotic therapy, may result in a quicker recovery of patients thanks to a better immune response.

At the Queen Mary University, 95 patients affected by tuberculosis underwent a standard antibiotic therapy. Forty-four of them were also treated with high-dose vitamin D, while the remaining 51 were given a placebo. The TB bacterium disappeared much quicker in the patients treated with vitamin D than the others: 23 days the first ones, 36 days the others.

This "study represents the most detailed characterization of the effects of antituberculous therapy on the immune response conducted to date, and is unique in being a clinical investigation in to the immunomodulatory actions of in vivo vitamin D supplementation during treatment of an infectious disease" (Coussens et al. 2012).

This is further supported by a recent study carried out by Danilo Buonsenso and some colleagues of his: "Fifty-seven children were included: 24.6% active TB, 28.1% LTBI (latent TB infection), 22.8% NPTB (non-TB pneumonia) and 24.6% healthy controls. 36.8% of all children tested had an insufficient or deficient vitamin D level. Vitamin D level was significantly lower in active TB compared to other groups ($p=0.004$)" (Buonsenso et al. 2018). In conclusion, this study also showed a clear correlation between hypovitaminosis D and active pulmonary TB.

Consequently, these researches have confirmed that, by metabolising vitamin D, the sun increases the body's immune power and it contributes to eradicate tuberculosis infection.

In the light of these results, it is clearly evident the importance of Barellai and Sclavo's intuition.

If the results achieved through the seaside hospices didn't change considerably the TB morbidity and mortality rates within the child population, it was only because it was impossible to extend these treatments to a significant number of children. The lack of drugs was also highly decisive along with the very characteristics of the disease which is still today one of the 10 main causes of death in the world.

"Globally, an estimated 10.0 million (range, 9.0–11.1 million) people fell ill with TB in 2018, a number that has been relatively stable in recent years. The burden of disease varies enormously among countries, from fewer than five to more than 500 new cases per 100.000 population per year, with the global average being around 130. [. . .] TB affects people of both sexes in all age groups but the highest burden is in men (aged $\geq$15 years), who accounted for 57% of all TB cases in 2018. By comparison, women accounted for 32% and children (aged<15 years) for 11%" (WHO 2019).

It is extremely important the data concerning the child population because it keeps on increasing: it was at 6% in 2013, it increased at 11% in 2018. Anna Galli's studies have shown that "over the last decades, TB has also emerged in the pediatric population" and "epidemiologic data on childhood TB are still limited". And "Childhood TB is considered as a sentinel of disease spreading throughout the community" (Galli et al. 2016).

The percentage of forms of tuberculosis is really significant in children, mainly in its non-pulmonaryform: especially lymphonodal TB.

In conclusion, taking up the words of the 2018 Global Tuberculosis Report of the World Health Organization, "Tuberculosis is an old disease that was once a death sentence. Effective drug treatments first became available in the 1940s, and in combination with social and economic development they allowed countries in western Europe, North America and some other parts of the world to reduce their burden of TB disease to very low levels. For most countries, however, the end of TB as an epidemic and major public health problem remains an aspiration rather than a reality" (WHO 2018).

"Leaders of all UN Member States have committed to 'ending the global TB epidemic' by 2030, backed up by concrete milestones and targets. Progress is being made. Global indicators

for reductions in TB cases and deaths, improved access to TB prevention and care and increased financing are moving in the right direction. [. . .] Nonetheless, the pace of progress worldwide and in most regions and countries is not yet fast enough" (WHO 2019).

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