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Gianfranco Donelli *Editor*

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Phytochemicals Against Drug-Resistant Bacterial Biofilms and Use of Green Extraction Solvents to Increase Their Bioactivity

A. C. Afonso, M. Sousa, L. C. Simões, and M. Simões

Abstract

Antimicrobial resistance has become one of the major global public health issues of the twenty-first century. One of the main factors in the limited action of antimicrobials is related to the ability of microorganisms, particularly bacteria, to form biofilms. These complex and well-organized communities allow the colonizing cells to acquire survival advantages over the same cells in suspension, including antibiotic resistance. A huge percentage of bacterial infections in humans are associated with biofilms, and many of them are chronic. Therefore, there is an urgent need to develop new products effective in controlling or eradicating biofilms. Plant secondary metabolites (phytochemicals) have demonstrated their potential as antibacterials against planktonic cells and sessile communities when used alone or in synergy with other molecules. This chapter covers recent advances in the activity of phytochemicals against biofilms, particularly those formed by drug-resistant bacteria. In addition, taking into account that the extraction step is crucial for the successful development of new bioactive compounds, the use of novel solvents that increase the phytochemical effect, such as natural deep eutectic solvents (NADES), as well as the recent applications of these solvents as antimicrobials are discussed.

Keywords

Antibiofilm · Antimicrobial resistance · NADES · Phytochemicals

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1 Introduction

The increasing incidence of drug-resistant pathogens in the last few years has led to a growing demand for new molecules with antimicrobial potential. Plants represent a rich source of a myriad of molecules with pharmacological interest. However, before they can be used in the most diverse applications, phytochemicals must be

extracted from plants; being the use of "Generally Recognized as Safe" (GRAS) solvents essential (Huang et al. 2015; WHO 2018). A new generation of solvents, NADES, constitute mixtures of inexpensive and easily available components that follow a green policy and have numerous advantages over conventional solvents (Dai et al. 2013b). Nevertheless, there are still many species to be identified, mainly in tropical forests, and consequently many molecules to be isolated and studied. It is estimated that only ~6% of the approximately 300,000 species of higher plants in tropical forests have been investigated pharmacologically and only ~15% phytochemically (Cragg and Newman 2013). For many years, plants have been one of the main sources of new molecules with bioactive properties in the pharmaceutical industry (Clardy and Walsh 2004); and, before that, ancient literature reveals that plants, most often crude extracts, have been used in many cultures over the years for various therapeutic purposes, including as antimicrobial agents for the treatment of infectious diseases (Prakash et al. 2020). In this sense, plants appear as a source of chemotherapeutics in the form of their secondary metabolites with the ability to fight bacterial infections including those related to biofilms (Anand et al. 2019, 2020). Plant secondary metabolites or phytochemicals exhibit a non-specific (multi-target) mode of action differing from the properties of conventional antibiotics. Their structural diversity, combined with a multi-target function, can help to overcome the resistance problem and modulate biofilm formation (Borges et al. 2016).

Hereupon, in this chapter, we will focus on recent discoveries about the application of phytochemicals against drug-resistant bacterial biofilms. The use of NADES for antimicrobial therapy will be further described.

2 Phytochemicals – Biological Functions and Chemical Diversity

Phytochemicals are products of the secondary metabolism of plants composed of a variety of chemical molecules responsible for imparting colour, flavour, aroma and texture to plants (Barbieri et al. 2017). This high variety of molecules is related to defence/stress mechanisms from free radicals, viruses, bacteria and fungi, which arose due to evolutionary selection (Barbieri et al. 2017).

The defence chemicals produced by plants can be classified as phytoanticipins or phytoalexins (Simões et al. 2012). The difference between both is based on how they are produced and not on their chemical structure. In fact, in a certain plant species, a chemical can function as a phytoalexin, and as phytoanticipin in a different species (Junghanns et al. 1998). Phytoanticipins are low molecular weight products that are present in plants in an inactive form or are produced from pre-existing constituents after a microbial attack (VanEtten et al. 1994). These phytochemicals, such as glucosinolates, cyanogenic glucosides, and saponin glycosides, are normally stored as less toxic glycosides in the vacuoles or cell walls of plant cells. If the integrity of the cell is broken when penetrated by a microorganism or due to other damage, the glycoside meets hydrolysing enzymes present in other compartments of the cell, releasing a toxic aglycone (Osbourn 1996). Phytoalexins are also low molecular weight products, but they are produced in response to elicitors, such as a microbial stimulus, producing a complex mixture of secondary metabolites to control the invader (Poulev et al. 2003). Molecules synthesized *de novo* involve the activation of certain genes and enzymes required for their synthesis (Kuc 1995). Phytoalexins are chemically diverse and may include chemical classes such as simple phenylpropanoid derivatives, alkaloids, glycosteroids, flavonoids, isoflavonoids, various sulphur products, terpenes and polyketides (Hammerschmidt 1999).

The use of phytochemicals to treat microbial infections started to grow in the last three decades with the increasing ineffectiveness of conventional antibiotics (Simões et al. 2012). Meanwhile, thousands of phytochemicals with different mechanisms of action have been described as antibacterial molecules (Barbieri et al. 2017). However, and as mentioned above,

only a small fraction of the plant species known so far has been evaluated for the presence of antimicrobial molecules and, therefore, it is necessary to increase efforts in plant collection and screening for the development of new antimicrobials (Simões et al. 2012). According to the existing literature, phytochemicals with antibacterial activity belong mainly to the following chemical subclasses: simple phenols, phenolic acids, quinones, flavones, flavonols, tannins, coumarins, terpenoids, essential oils, alkaloids, lectins, polyketides, polyamines, isothiocyanates, sulphides, thiosulphinates, glycosides, phenanthrenes, and stilbenes, among others (Simões et al. 2009). Phytochemicals are determined as antimicrobials based on susceptibility tests that produce the minimum inhibitory concentration (MIC), usually in the range of 100–5000 μg/mL (Simões et al. 2009). The majority of phytochemicals have a weak or narrow activity (Tegos et al. 2002), needed to be used in concentrations that are too high to be clinically relevant and for use in monotherapy (Simões et al. 2009). Comparatively, conventional antibiotics produce MICs of 0.031–512 μg/mL (Tegos et al. 2002). Nevertheless, the combination of antibiotics and phytochemicals has been studied. These studies show modifications of resistance mechanisms in bacteria, suggesting the combined use of phytochemicals and antibiotics to increase the activity and decrease antibiotic doses (Santiago et al. 2015; Touani et al. 2014). Similar to what happens with the antibacterial activity against planktonic cells, plants can also support populations of bacteria adhered to their surface, being able to produce phytochemicals that attenuate the development of the biofilm through specific mechanisms (Morris and Monier 2003). In this respect, phytochemicals represent a natural antimicrobial strategy with a significant impact on the formation and development of bacterial biofilm, as demonstrated by many authors and which will be described in the following section of this chapter.

3 Extraction of Phytochemicals

The extraction of molecules from natural products is one of the main steps in the isolation of phytochemicals with bioactive properties (WHO 2018). First of all, the selection of a good extraction solvent is essential for the development of selective and effective extraction and isolation methods capable of targeting specific molecules in plant matrices (Hikmawanti et al. 2021). Unfortunately, most secondary plant metabolites are not water soluble due to their lipophilic nature (Kongkham et al. 2020). That said, when performing the extraction, the solvent must meet the following criteria: high selectivity (polarity according to the target compound), safety (low toxicity, non-explosive and non-flammable), neutral, easy to separate from the target compounds, low viscosity (allows easy mass transfer), low boiling temperature (prevents the degradation of compounds) and economic (as cheap as possible) (Hikmawanti et al. 2021). Some of the conventional organic solvents used in plant molecules extraction include petroleum ether, n-hexane, toluene, diethyl ether, chloroform, dichloromethane, ethyl acetate, acetone, n-butanol, ethanol and methanol (Abubakar and Haque 2020). However, the use of organic solvents has some limitations, such as their toxicity, danger and volatility, leading to their presence in the atmosphere as pollutants (Cvetanović 2019). Furthermore, they remain in the extracts, impairing their purity. The need for additional purification as well as the use in large amounts in some types of extraction strategies (e.g., maceration and percolation) increases the costs. Furthermore, in some extraction processes, there is low selectivity, which leads to the presence of unwanted components in the obtained extracts, thus reducing their biological potential (Cvetanović 2019). In addition to the diversity, the proportion of accompanying compounds present in small amounts can determine the bioactivity direction of compounds or fractions isolated from natural sources such as plants.

The use of green solvents is proving to be a good alternative to overcome some of the limitations related to the extraction step. Green solvents are an attractive alternative since they present some advantages over these conventional and they meet the "green" criteria, i.e., they are environmentally friendly and relatively safe (Chemat et al. 2019a). Green solvents are easy to prepare, once they are synthesized with environmentally friendly materials and procedures; less hazardous; low energy consuming; recyclable; biodegradable; and cheap (Hikmawanti et al. 2021). Among them, NADES have attracted special attention from the scientific community (Chemat et al. 2019a).

3.1 NADES

Natural deep eutectic solvents, also known as NADES, are solutions of Lewis or Brønsted acids and bases that form eutectic mixtures, which may be solid or liquid and that at a particular composition present a high melting point depression becoming liquids at room temperature (Ghobadi et al. 2020; Paiva et al. 2014; Si and Misra 2020). Being a new generation of solvents, NADES are mixtures of inexpensive and easily available components: natural hydrogen-bond donors (Dai et al. 2013a). NADES are bio-based deep eutectic solvents (DES), mostly composed of one or more primary metabolites like organic acids (e.g., malic acid, citric acid, lactic acid, and succinic acid), sugars, alcohols, amines, amino acids, and quaternary ammonium salts; but can also be formed by secondary metabolites such as terpenes and vanillin, or biopolymers (Abbott et al. 2004; Dai et al. 2013b; Wils et al. 2021). Table 1 presents some of the main examples of NADES components.

NADES were introduced in 2011 by Choi et al. (2011). The authors hypothesized that the metabolites that occur in large amounts in plant cells form a third type of liquid, one separate from water and lipids. Considering what was already known about plant metabolomics data, the authors observed a clear parallel with synthetic ionic liquids (ILs). Thus, these cellular constituents emerged as perfect candidates to make ILs and DES. By 2018, more than one hundred and fifty combinations of NADES had been designed (Vanda et al. 2018).

NADES have similar characteristics to ILs but have some advantages over them. They are biodegradable, low flammable, nontoxic, cheap, safe, biocompatible, environmentally friendly, *with a wide polarity range*, and sustainable (Erdocia et al. 2021; Kohli 2019; Kua and Gan 2019). In addition, these compounds present high selectivity as solvents and have a virtual zero vapour pressure (Vanda et al. 2018). However, NADES have a high viscosity, which can be a problem, especially in large-scale processes, although their viscosity may be adjusted (Hikmawanti et al. 2021; Liu et al. 2018; Vanda et al. 2018). Because of the highly flexible structural and chemical characteristics of NADES, they are commonly referred to as "designer solvents" and have a wide potential for application (Ivanović et al. 2020). This includes catalytic, separation, extraction and electrochemical processes, solubilization of non-water-soluble compounds for pharmaceutical purposes, and the delivery of drugs and cosmetic formulations (Dwamena 2019; Ferraz et al. 2011; Ivanović et al. 2020). Moreover, they are also used for food and agrochemical purposes (Faggian et al. 2016; Vanda et al. 2018; Wils et al. 2021).

In short, NADES are good alternatives to organic solvents and ILS due to all the advantages they have over these extraction methods (Chemat et al. 2019b; Murador et al. 2019). Furthermore, it turns out that some bioactive metabolites from plants (e.g., piperine (Gorgani et al. 2017), curcumin (Modasiya and Patel 2012), and baicalin (Jakab et al. 2019)) show difficult adsorption in the gastrointestinal tract, low solubility, poor stability, and compromised bioavailability, when obtained by conventional extraction methods (Brglez Mojzer et al. 2016; Hikmawanti et al. 2021). However, when NADES are used in the phytochemical extraction processes, the solubility, stability, bioavailability, bioactivity, and shelf life of these non-nutritive products from plants increase (da Silva et al. 2021; Dheyab et al. 2021; Hikmawanti et al. 2021; Murador et al. 2019).

Table 1 Examples of NADES components

NADES components					
Element 1	Element 2	Element 3	Element 4	Molar ratio	References
Choline Chloride	Glucose (1) Citric Acid (2) Sucrose (3) Tartaric Acid (4) Xylose (5)	–	–	(1) molar ratio: 1:1 (2) molar ratio: 1:1; 2:1 (3) molar ratio: 1:1; 4:1 (4) molar ratio: 2:1 (5) molar ratio: 2:1; 3:1	Paiva et al. (2014)
Choline Chloride	Glycerol (6) 1,2-propanediol (7)	–	–	(6) molar ratio: 1:2 (7) molar ratio: 1:1	Mulia et al. (2015)
Choline Chloride	Urea (8) Lactic Acid (9) Malonic Acid (10) 1,4 Butanediol (11) Xylitol (12)	–	–	(8) molar ratio: 1:2 (9) molar ratio: 1:2 (10) molar ratio:.1:1 (11) molar ratio: 1:5 (12) molar ratio: 2:1	Garcia et al. (2015)
Glycerol	Xylitol (13) Maltose (14) Fructose (15) Sucrose (16) Glucose (17) Sorbitol (18) Malic Acid (19)	–	–	(13) molar ratio: 2:1 (14) molar ratio: 3:1 (15) molar ratio: 3:1 (16) molar ratio: 3:1 (17) molar ratio: 3:1 (18) molar ratio: 2:1 (19) molar ratio: 1:1; 2:1	Jin et al. (2019)
Lactic Acid	Glucose (20)	–	–	(20) molar ratio: 1:2	Jin et al. (2019)
Choline Chloride	Urea	Glycerol (21)	–	(21) molar ratio: 1:1:1	Garcia et al. (2015)
Betaine	Water	Sucrose (22) Citric Acid (23) Malic Acid	– – Glucose (24)	(22) molar ratio: 2:6:1 (23) molar ratio: 1:6:1 (24) molar ratio: 1:9:1:1	González et al. (2017)

Note The numbers (1)-(24) in the "Element 2" column correspond to the numbers (1)-(24) in the "Molar Ratio" column

4 Antibiofilm Activity of Phytochemicals

Biofilm formation is involved in more than 80% of bacterial infections in humans (Simões et al. 2009). Therefore, there has been a growth in research to identify better strategies for the inhibition and eradication of biofilms (Borges et al. 2016; Fu et al. 2021; Vuotto and Donelli 2019). Biofilm formation is a complex process involving different stages that can be targeted by antibiofilm agents. Some of the well-studied stages of biofilm development include: (i) attachment of bacterial cells to a suitable biotic/abiotic surface; (ii) development of the biofilm structure, including the production of extracellular polymeric substances (EPS); (iii) maturation; and (iv) dispersion (Boles and Horswill 2008). The early stages are highly critical in biofilm development and targeting them, acting on specific mechanisms, appears to be a promising strategy for inhibiting biofilm formation (Mishra et al. 2020). For instance, blocking bacterial adhesion, reducing motility and exopolysaccharide production, and interfering with quorum sensing (QS) are some of the main mechanisms by which natural antibiofilm agents act.

Most studies describing antibiofilm properties concerning plants were carried out with crude extracts and are extensively well described in several comprehensive reviews (Borges et al. 2016; Mishra et al. 2020; Ta and Arnason 2015). In a recent study, Krzyżek et al. (2021) found that *Chelidonium majus* and *Corydalis cheilanthifolia* extracts, highly composed of isoquinoline alkaloids, add antibiofilm and

antimicrobial-enhancing activity of against multidrug-resistant *Helicobacter pylori*. However, specific compounds responsible for these activities are typically unknown. That said, in this chapter, we will describe the most recent studies mainly on pure the molecules identified with antibiofilm activity. A compilation of some studies from the last 5 years on the antibiofilm activity of phytochemicals is provided in Table 2.

4.1 Inhibition of Bacterial Adhesion

Biofilm development at the initial stages of bacterial adhesion can be outlawed by interfering with the forces (e.g., Van der Waals force of attraction, electrostatic attraction, sedimentation and Brownian movements) which are responsible for the support of bacterial attachment to various surfaces (Roy et al. 2018); suppressing genes related to biofilm formation (Adnan et al. 2020); and essential metabolic functions (Sandasi et al. 2010). Members of Enterobacteriaceae express curli, an amyloid fibre on the cell surface that helps in the attachment to substrates and cell aggregation and enhances biofilm formation as well as cellular invasion (Tursi et al. 2020). For instance, phloretin, ginkgolic acid, and phytochemicals from Malaysian plants help in the regulation of curli and pili genes (Johari et al. 2020; Lee et al. 2014a, b). Vikram et al. (2013) reported that a citrus sterol b-sitosterol glucoside inhibited *E. coli* O157:H7 biofilm formation and motility by suppressing flagellar operon *flhDC* (Vikram et al. 2013). There are reports on the anti-adhesive properties of *Capsicum baccatum* extracts on *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* (Von Borowski et al. 2019)*;* and extracts from various *Eugenia* spp. on *Candida albicans* (de Sardi et al. 2017). Other studies identified phytochemicals, especially polyphenols such as 7-epiclusianone, tannic acid, and casbane which prevent cell surface attachment (Adnan et al. 2020; Carneiro et al. 2010; Nagaoka et al. 2008; Payne et al. 2013). For instance, *Adiantum philippense* crude extract was screened for its effect on bacterial adhesion and biofilm formation against common food pathogens (*Escherichia coli*, *Staphylococcus aureus*, *P. aeruginosa*, and *Shigella flexneri*). The extract restrains biofilm at the initial stages by targeting adhesin proteins, deforming the pre-formed biofilms, and obstructing EPS production. Several bioactive phytochemicals were categorized from *A. philippense* crude extract using HR-LCMS (High Resolution Liquid Chromatograph Mass Spectrometer); and the molecular docking of these identified phytochemicals was interrelated with the active site residues of the adhesin proteins, IcsA, Sortase A, OprD, EspA, and FimH from *S. flexneri, S. aureus, P. aeruginosa,* and *E. coli*, respectively. As a result, the interactions of these key residues with antibiofilm agents could help to inhibit the crucial step in the biofilm formation process, i.e., adhesion (Adnan et al. 2020). Recently, Klančnik et al. (2020) evaluated the anti-adhesion activities against *Campylobacter jejuni* NCTC 11168 (on polystyrene microtiter plates) for carvacrol (0.25 × MIC) and thymol (0.25 × MIC). The adhesion of the untreated control *C. jejuni* was 6.77 ± 0.40 log CFU/well. A reduction of 38% of *C. jejuni* adhesion was observed for thymol (4.19 ± 0.76 log CFU/well), while for carvacrol a reduction of 31% (4.67 ± 0.70 log CFU/well) was observed (Klančnik et al. 2020).

4.2 Motility Inhibition

Microorganisms can be divided as motile and non-motile ones. These have differences in structural and behavioural patterns, which influence their adhesion to the substrate surface. Unlike non-motile bacteria, motile bacteria have a chemotaxis transducer, that is, a protein that detects physical and chemical changes in the surrounding environment, and a flagellum, which is a surface appendage that gives bacteria active locomotion. As the diffusion coefficient of motile bacteria is more than 3 times higher than non-motile cells, it contributes more to the rate of adhesion, increasing the number of adherent cells per unit area (Zheng et al. 2021). Researches have documented the presence and activity of bacterial flagellum linked to the formation of suspended cell

Table 2 Recent studies on phytochemicals with antibiofilm activity. Adapted from Afonso et al. (2021)

Phytochemical	Chemical class	Biofilm-forming pathogen	Mechanism(s)	Dose/effect	References
Carvacrol	Phenol	*Salmonella enterica* serovar Enteritidis	Inhibition of the expression of genes involved in QS (*luxR, luxS*, *qseB*, *sdiA*) and biofilm formation (*csgA*, *csgB, csgD*, *flhD*, *fliZ*, and *motB*)	n.e.	Guillín et al. (2021)
		Salmonella enterica serovar Typhimurium		n.e.	Guillín et al. (2021)
		Campylobacter jejuni NCTC 11168	Anti-adhesion activity by targeting the efflux pumps CmeDEF, CmeGH, and Cj1687	31% biofilm inhibition	Klančnik et al. (2020)
(+)-nootkatone	Terpenoid	*Staphylococcus aureus* SJTUF 20758	Suppression of the biofilm-related genes, *sarA, icaA, agrA*, *RNAIII,* and *spa*	25 μg/mL (47% biofilm inhibition)	Farha et al. (2020)
α-mangostin	Xanthone	*Pseudomonas aeruginosa*	n.d.	2 μg/mL (biofilm inhibition)	Sanpinit et al. (2020)
		Staphylococcus epidermidis	n.d.	2 μg/mL (biofilm inhibition)	Sanpinit et al. (2020)
Vanillic acid	Methoxybenzoic acid	*Yersinia enterocolitica*	Interference with bacterial motility; changes in the physico-chemical properties of cell surfaces which alter the adhesion potential; and interference with QS	400 μg/mL	Sivasankar et al. (2020b)
Tannic acid	Tannin	*Salmonella enterica* serovar Paratyohi A		400 μg/mL	Sivasankar et al. (2020a)
		Salmonella enterica serovar Typhimurium		400 μg/mL (16% of cell-surface hydrophobicity inhibition; 52% EPS production inhibition)	Sivasankar et al. (2020a)
Lutein	Carotenoid	*Pseudomonas aeruginosa* PAO1	n.d.	20 μg/ml (61% biofilm inhibition)	Sampathkumar et al. (2019)
Quercetin	Flavonoid	*Pseudomonas aeruginosa* PAO1	Transcriptional changes associated with QS and expression levels of *lasI*, *lasR, rhlI,* and *rhlR* significantly reduced	250 μg/ml (43–78% biofilm inhibition)	Vipin et al. (2019)
		Pseudomonas aeruginosa PAO1		16 μg/ml (51% biofilm inhibition)	Ouyang et al. (2016)
Gallic acid	Phenolic acid	*Ralstonia solanacearum*	Interference with bacterial motility	3 mg/mL (85% biofilm eradication)	Sowndarya et al. (2020)

(continued)

Table 2 (continued)

Phytochemical	Chemical class	Biofilm-forming pathogen	Mechanism(s)	Dose/effect	References
		Pseudomonas aeruginosa PAO1	Interference with bacterial motility; changes in the physico-chemical properties of cell surfaces which alter the adhesion potential; interference with QS; and hinder the biofilm formation process	750 µg/ml (10% biofilm inhibition)	Bali et al. (2019)
Thymol	Phenol	*Salmonella enterica* serovar Enteritidis	Inhibition of the expression of genes involved in QS (*luxR, luxS*, *qseB*, *sdiA*) and biofilm formation (*csgA*, *csgB, csgD*, *flhD*, *fliZ*, and *motB*)	n.e.	Guillín et al. (2021)
		Salmonella enterica serovar Typhimurium		n.e.	Guillín et al. (2021)
		Campylobacter jejuni NCTC 11168	Anti-adhesion activity by targeting the efflux pumps CmeDEF, CmeGH and Cj1687	38% biofilm inhibition	Klančnik et al. (2020)
		Klebsiella pneumoniae KPMA19	n.d.	50 µg/ml (80.2% biofilm reduction)	Maheshwari et al. (2019)
		Escherichia coli ECM4	n.d.	50 µg/ml (78.6% biofilm reduction)	Maheshwari et al. (2019)
		Enterobacter cloacae ENM36	n.d.	50 µg/mL (83.9% biofilm reduction)	Maheshwari et al. (2019)
Curcumin	Curcuminoid	*Pseudomonas aeruginosa* PAO1	Interference with bacterial motility; changes in the physico-chemical properties of cell surfaces which alter the adhesion potential; interference with QS; and hinder the biofilm formation process	93.7 µg/mL (26.7% biofilm inhibition)	Bali et al. (2019)
Pyrogallol	Phenol	MRSA		750 µg/mL (38.5% biofilm inhibition)	Bali et al. (2019)
Luteolin	Flavone			375 µg/mL (83.9% biofilm inhibition)	Bali et al. (2019)
p-coumaric acid				28% biofilm reduction	Kot et al. (2018)
Trans-cinnamaldehyde	Cinnamaldehyde	*Enterococcus faecalis*	Downregulation of the QS *fsr* locus and downstream *gelE*	0.47 mM	Ali et al. (2021)

(continued)

Table 2 (continued)

Phytochemical	Chemical class	Biofilm-forming pathogen	Mechanism(s)	Dose/effect	References
		MRSA	Interference with bacterial motility; changes in the physico-chemical properties of cell surfaces which alter the adhesion potential; interference with QS; and hinder the biofilm formation process	42% biofilm reduction; 79% inactivation	Kot et al. (2018)
Berberine		*P. aeruginosa* PAO1	Interaction with the QS signal receptors *LasR* and *rhIR*	0.625 mg/mL (71.7% biofilm inhibition)	Aswathanarayan and Vittal (2018)
		Salmonella enterica serovar Typhimurium		0.019 mg/mL (31.2% biofilm inhibition)	
Ferulic acid	Hydroxycinnamic acid	MRSA		27% biofilm reduction	Kot et al. (2018)
Allyl isothiocyanate	Isothiocyanate	*Escherichia coli* CETC 434	Interference with bacterial motility, changes in the physico-chemical properties of cell surfaces which alter the adhesion potential to PS, and interference with QS; and hinder the biofilm formation process	1000 μg/ml (87% biofilm prevention)	Borges et al. (2016)
		Pseudomonas aeruginosa ATCC 10145		1000 μg/ml (99% biofilm prevention)	Borges et al. (2016)
		Staphylococcus aureus CETC 976		1000 μg/ml (96% biofilm prevention)	Borges et al. (2016)
2-phenylethyl isothiocyanate	Isothiocyanate	*Escherichia coli* CETC 434		1000 μg/ml (100% biofilm prevention)	Borges et al. (2016)
		Pseudomonas aeruginosa ATCC 10145		1000 μg/ml (93% biofilm prevention)	Borges et al. (2016)
		Staphylococcus aureus CETC 976		1000 μg/ml (90% biofilm prevention)	Borges et al. (2016)

n.d. not determined; **n.e.** not specified

aggregates or surface-associated biofilms (Laganenka et al. 2016). It was also found that flagellar motility stimulates transient cell-surface contacts and overwhelms electrostatic repulsive forces at interfaces, and is, therefore, necessary for bacterial self-aggregation and irreversible surface adhesion that initiates biofilm formation (Gutman et al. 2013; Houry et al. 2010; Lemon et al. 2007). Nevertheless, the presence of flagella is not restricted to the beginning of biofilm formation. For example, in *Listeria monocytogenes* biofilms, it has been suggested that flagellum-mediated motility was necessary for biofilm growth through the recruitment of cells from the planktonic phase (Lemon et al. 2007). In another study, mature *E. coli* biofilms were observed to have more dramatic vertical structures when the strains were more motile, and flatter structures for strains with impaired motility (Wood et al. 2006).

Motile bacteria are mainly Gram-negative bacteria, showing higher cell adhesion and growth rates than Gram-positive bacteria (Zheng et al. 2021). Gallic acid isolated from an agricultural by-product (cashew nutshell) was investigated against the soil-borne plant pathogen *Ralstonia solanacearum* and was found to eradicate 85% of mature biofilms. In addition, sub-MIC of gallic acid was found to inhibit both swimming and twitching motility of about 93% and 63% respectively (Sowndarya et al. 2020). Vanillic acid exhibited significant motility inhibition of *Yersinia enterocolitica* as well as anti-QS activity, inhibition of EPS production. Moreover, vanillic acid increased the sensitivity of *Y. enterocolitica* towards antibiotics (Sivasankar et al. 2020b).

4.3 Inhibition of EPS Production

After adhesion to the surfaces and the establishment of a dense population, the microorganisms start to form a self-produced matrix of extracellular polymeric substances (EPS) (Simões et al. 2009). This matrix is composed of polysaccharides, proteins, glycoproteins, glycolipids, and in some cases, extracellular DNA (Flemming et al. 2007). EPS confers numerous advantages to biofilms, most of them related to protection. The matrix produced by EPS around microbial cells has the capability of protecting them against antimicrobial compounds and heavy metals; retain water, protecting microbes and the environment against drought (Costa et al. 2018). In addition, EPS keeps cells close together, which results in higher concentrations of signalling molecules produced by the cells, in sufficient quantities capable of promoting changes in cell behaviour and the activation of several genes (Klančnik et al. 2020).

Plant extracts have shown a promising role in decreasing the content of biofilm EPS. *Adiantum philippense* L. crude extract restrained biofilm at the initial stages by targeting adhesin proteins, deforming the pre-formed biofilms, and obstructing EPS production (Adnan et al. 2020). A chloroform extract of turmeric exhibited antibiofilm effects against diverse bacteria (*E. coli, Enterobacter cloacae, Klebsiella pneumoniae, S. aureus,* and *Bacillus subtilis*). The antibiofilm effects were attributed to changes in adhesion ability, motility, EPS production, and cell surface hydrophobicity. Regarding the effect on EPS, analysis with Fourier transformed infrared spectroscopy (FTIR) indicated a loss of vital functional groups of polysaccharides and proteins from the EPS after exposure to a chloroform extract (Hayat et al. 2018). Furthermore, the hydrophobic behaviour was altered, with the strains of *K. pneumoniae* and *E. cloacae* exhibiting a hydrophilic character after the addition of the extract, when compared to the control cells. These results should be analysed bearing in mind the putative effects of the solvent used. In a different study, cells treated with the phytochemical tannic acid (TA) showed 38–43% and 35–50% of inhibition in cell surface hydrophobicity and EPS production respectively. FTIR analysis also showed considerable changes in proteins and peptides (Sivasankar et al. 2020a).

4.4 Quorum Sensing Inhibition

QS represents bacterial cell-cell communication that coordinates the expression of phenotypes and regulates physiological activities, which promotes pathogenesis and bacterial resistance to antimicrobial compounds. These include the production of virulence factors, secondary metabolites, and the formation of well-organized microbial communities, such as biofilms (Borges et al. 2014; Jakobsen et al. 2012). Indeed, QS systems are integrated into some processes important to biofilm formation and differentiation (Borges et al. 2015). So, acting on biofilms by interfering with QS can be an alternative to the ineffective conventional biofilm control strategies (McLean et al. 2004). By interfering with cell-cell communication, the intrinsic resistance of the biofilm and the ability to form robust structures may be compromised, causing cell dispersion (Hogan and Kolter 2002). In QS, small signalling molecules, called autoinducers (AI), mediate the ability to sense the size of a bacterial population

(Eberhard et al. 1981). Oligopeptides and N-acylhomoserine lactones (AHL) are major AI molecules involved in intra-species communication in Gram-positive and Gram-negative bacteria respectively, whereas boronated diester molecules (AI-2) are involved in inter-species communication among both Gram-positive and Gram-negative bacteria (Eberhard et al. 1981; Lyon and Novick 2004). Several screenings have shown that phytochemicals are a large and interesting repository of QS inhibitors (QSI) (Castillo-Juárez et al. 2015), offering a vast chemical diversity with structural complexity and biological activity (Borges et al. 2015; Vattem et al. 2007). Therefore, phytochemicals with QS inhibition activity can be promising tools to help the treatment of bacterial infections, including those that are biofilm-related (Borges et al. 2016).

Different studies have reported that phytochemicals (i.e., *trans*-cinnamaldehyde, carvacrol, thymol, eugenol, etc.) were able to inhibit biofilm formation due to the ability to downregulate biofilm-associated gene transcription, at sub-inhibitory concentrations (Kongkham et al. 2020). For instance, *trans*-cinnamaldehyde inhibits biofilm formation of *Enterococcus faecalis* by significant downregulation of the QS *fsr* locus and downstream *gelE* (Ali et al. 2021). In agreement, *trans*-cinnamaldehyde also inhibits the biofilm formation of *Cronobacter sakazakii* by reducing the expression of the transcription regulation gene *luxR*, without inhibiting bacterial growth (Amalaradjou and Venkitanarayanan 2011). Shukla and Bhathena (2016) also found that tannin-rich crude extracts from six Indian medicinal plants (*Phyllanthus emblica*, *Terminalia bellirica*, *Terminalia chebula*, *Punica granatum*, *Syzygium cumini*, and *Mangifera indica*) showed quorum-quenching (QQ) properties against *Chromobacterium violaceum* and *S. aureus* at sub-inhibitory concentrations (Shukla and Bhathena 2016). Likewise, sub-inhibitory concentrations of berberine inhibited biofilm formation and QS regulated phenotypes in the bacterial pathogens *P. aeruginosa* PA01 and *Salmonella enterica* serovar Typhimurium. The study showed that berberine interacted with the QS signal receptors, *lasR* and *rhlR* (Aswathanarayan and Vittal 2018). Moreover, compounds from essential oils such as carvacrol and thymol could inhibit the expression of genes involved in the QS mechanism (*luxR, luxS*, *qseB*, *sdiA*) and biofilm formation (*csgA*, *csgB, csgD*, *flhD*, *fliZ*, and *motB*) of *Salmonella enterica* serovar Enteritidis and *Salmonella enterica* serovar Typhimurium (Guillín et al. 2021).

In vitro studies have confirmed synergistic interactions of phytochemicals and antibiotics with valuable effects on QS inhibition. For example, Vipin et al. (2020) evaluated the efficacy of antibiotics (levofloxacin, ceftriaxone, gentamycin, tobramycin, and amikacin) in combination with quercetin, a flavonoid capable of targeting QS, against biofilm-forming *P. aeruginosa* strains isolated from catheter-associated urinary tract infections. They observed a synergistic effect that resulted in the inhibition of biofilm formation and cell viability (Vipin et al. 2020). In a different study, combinatorial action of 2,3-pyrazine dicarboxylic acid derivatives (QS inhibitor targeted against the quorum regulator LuxO) along with antibiotics (doxycycline, tetracycline, chloramphenicol, and erythromycin) was evaluated. The results indicated biofilm inhibition at very low concentrations (IC_{50} varying between 1 μM and 70 μM). In addition, the compounds synergistically enhanced the effect of the antibiotics against a multidrug-resistant clinical isolate *of V. cholerae* (Hema et al. 2016).

5 NADES against Biofilms and Planktonic Cells

It is well known that plant metabolites have been largely extracted and investigated because of their effects on many illnesses (Dias et al. 2012). However, it is necessary to properly choose the solvent to develop methods of extraction and purification of natural compounds from plants with high selectivity and efficiency (Abubakar and Haque 2020; Grozdanova et al. 2020; Hikmawanti et al. 2021; Sixt et al. 2016). When these compounds are extracted with NADES there is a better performance in terms of extraction, but also a

consequent increase in their stability, bioactivity, solubility, and bioavailability (Chaves et al. 2020; Grozdanova et al. 2020; Hikmawanti et al. 2021). NADES have been widely used in the extraction of biomolecules from various biomass sources (Eppink et al. 2021; Wils et al. 2021). There are already some published works that point to the success of macromolecule extraction with NADES; but there is little information regarding the antibiofilm activity of macromolecules extracted with NADES (Choi and Verpoorte 2019; Jablonský et al. 2019; Nava-Ocampo et al. 2021). However, it is known that the solubilization of biofilm macromolecules destabilizes its structure and assists in its removal (Nava-Ocampo et al. 2021). A very recent study developed by Nava-Ocampo et al. investigated the application of NADES as potential agents for eradicating biofilms through their dissolution, destabilization or structural breakdown (Nava-Ocampo et al. 2021). Seven different NADES were evaluated before and after biofilm treatment through attenuated total reflection FTIR and fluorescence excitation-emission matrix spectroscopy. The results obtained allowed the authors to verify that two of the seven NADES tested – Lactic Acid:Glucose:Water (LA:Glc:W), in a molar ratio of 5:1:3, and Betaine:Urea:Water (B:U:W), in a molar ratio of 1:1:10 – were able to solubilize bovine serum albumin (BSA) and EPS from the biofilm as well as revealed to have a very significant impact on the shape, size, and structure of the biofilm. In addition, the treatment of the biofilm with B:U:W resulted in a 69% decrease in weight and the treatment with LA:Glc:W led to a 48% reduction, whereby B:U:W has shown to be more efficient as a structural biofilm disintegrator (Nava-Ocampo et al. 2021).

Moreover, NADES seem to be effective as antimicrobial agents against planktonic cells and as enhancer elements of the antimicrobial activity of plant extracts (Grozdanova et al. 2020; Radošević et al. 2018; Wikene et al. 2017). Curiously, a study carried out by Grozdanova et al. (2020) demonstrated that NADES may be able to improve the antimicrobial action of bioactive agents from plants. In that work, four NADES – Choline Chloride:Glycerol (XXGly) (molar ratio 1:2), Citric Acid:1,2-Propanediol (CAPD)(molar ratio 1:4), Choline Chloride:Glucose plus 30% of Water (XXGlH)(molar ratio 5:2) and Choline Chloride:1,2-Propanediol (XXPD)(molar ratio 1:3) – were used as solvents for the extraction process of two medicinal plants – *Sideritis scardica* and *Plantago major*. The extraction efficiency was determined by measuring the total amount of phenolic compounds and flavonoids extracted, and the toxicity and antimicrobial activity of the extracts were evaluated. The results revealed that XXGlH had the highest extraction efficiency of phenolic compounds among all the NADES evaluated. However, XXGlH extracts proved to be inactive against all the microorganisms tested – *Bacillus cereus*, *E. coli*, *S. aureus*, *P. aeruginosa*, *L. monocytogenes*, *S.* Typhimurium, *Streptococcus pyogenes*, *Y. enterocolitica,* and the fungus *C. albicans*. Plant extracts containing the NADES CAPD and XXGly revealed to have a very high and interesting antimicrobial effect against the *S. pyogenes*, *E. coli*, *S. aureus,* and *C. albicans*, being the extract with CAPD the most effective. Since the CAPD extract revealed a higher antimicrobial activity when in the presence of *S. scardica* components, the authors speculated that the presence of citric acid in CAPD and the occurrence of some synergistic effects with the constituents of *S. scardica* may play a preponderant role. Despite that, reduced cytotoxicity and genotoxicity were observed for all NADES and extracts with antimicrobial action. This study reaffirms the premise that NADES are excellent extraction solvents, namely for the extraction of bioactive compounds from medicinal plants, constituting a very promising alternative to traditional solvents such as water-alcohol(s) mixtures.

6 Conclusions and Future Perspectives

The occurrence of biofilm-based infections and the associated antibiotic resistance requires innovative therapeutic approaches. This is a major concern for medicine and public health, given the World Health Organization predictions of

the number of deaths related to antibiotic resistance by the year 2050. Phytochemicals have attracted great attention, as they can be used as a source of new scaffolds for the development of new therapeutic molecules or as adjuvants to existing antibiotics. Furthermore, due to their great structural diversity, phytochemicals are associated with a multi-target mode of action, without imposing selective pressure on the bacteria. Phytochemicals, as antibiofilm agents, have been studied under *in vitro* and *in vivo* conditions. However, to date, no drug developed has been approved by the FDA, most of which have failed phase II and phase III of clinical trials (Lu et al. 2019). The possible reason for that may be related to the availability in the human body after administration, which diminishes the effectiveness of the molecules. It is believed that the choice of solvent may influence some parameters such as the bioactivity and bioavailability of the molecule. So, it is necessary to choose a suitable extraction solvent that enhances the bioactive properties. NADES are excellent candidates for this purpose, having been shown to improve phytochemical activity and some studies have even shown that these solvents can be effective as antimicrobials against biofilms and planktonic cells. Another flaw in research on antibiofilm phytochemicals is the precise nature of the mechanisms of action. To overcome this gap, the adoption of multidisciplinary approaches, including computer-based methodologies (i.e., molecular docking), may seed the development of new antibacterial and antibiofilm products.

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In Vitro Potential Virucidal Effect Evaluation of Xibornol on Human Adenovirus Type 5, Human Rhinovirus Type 13, Human Coronavirus 229E, Human Parainfluenza Virus Type 1, and Human Respiratory Syncytial Virus

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Abstract

The availability of virucidal compounds to reduce the impact of respiratory viruses is a relevant topic for public health, especially during the recent coronavirus disease (COVID-19) pandemic. Antimicrobial properties of Xibornol are known since the 1970s, but its activity on viruses is currently little explored. In this study, Xibornol activity at a fixed concentration of 0.03 mg/100 ml has been evaluated on five respiratory viruses (*Human Adenovirus 5*, *Human Rhinovirus type 13*, *Human Coronavirus 229E*, *Human Parainfluenza Virus type 1,* and *Human Respiratory Syncytial Virus*) through *in vitro* experiments based on adapted European standard UNI EN 14476-20019. The experiments were carried out under two different environmental conditions, one with the addition of fetal bovine serum to simulate an *in vivo* condition (dirty condition) and the other without the addition of any organic substances (clean condition). The viral abatement of Xibornol (expressed as Log_{10} reduction – LR) was statistically significant under both clean and dirty environmental conditions. Namely, in clean condition, LR ranged from 2.67 to 3.84, while in the dirty one the abatement was slightly lower (from 1.75 to 3.03). *Parainfluenza Virus* and *Human Adenovirus* were most resistant compared to the other viruses. The obtained data confirmed Xibornol activity and its use as topic substance for viral inactivation to prevent upper respiratory tract disease.

Keywords

Cell culture tests · Respiratory viruses · Upper respiratory tract diseases · Virucidal activity · Xibornol

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1 Introduction

Respiratory viruses have a public health relevance, since the majority of respiratory diseases are caused by viruses compared to other pathogens, increasing morbidity and mortality in the worldwide population (Roth et al., 2018). From the end of 1990s, several re-emerging and new respiratory viruses (i.e., various subtypes of *Avian influenza* and *coronavirus*) continue to challenge medical and public health systems (Liu et al., 2016). Such biological agents are a heterogeneous group that infect all aged people, carrying from acute-mild illnesses limited to the upper airways, to severe disease with interstitial pneumonia and serious systemic problems (Hodinka, 2016; Elrobaa and New, 2021). The recent pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) highlighted the importance of face masks, social distancing, hand washing, environmental disinfection, and vaccination in reducing the virus spreading among the susceptible hosts (WHO, 2020; Leung, 2021). Moreover, various therapeutic approaches have been also widely investigated, such as the evaluation of virucidal compounds for the inactivation of viral particles in the upper respiratory tract, before cells infection (Rabie, 2021; Rabie, 2022). The Xibornol (6-isobornyl-3-4-xylenol) is a chemical compound, whose antimicrobic properties are well known since the 1970s (Capponi, 1969; Morandini et al., 1985; Fabbri et al., 1988). Xibornol appears as white crystalline powder, slightly soluble in water and freely soluble in methanol and other organic solvents as acetone and Toluene. It is stable after light exposure as well as after thermal and humidity treatment while it seems slightly sensitive to acid and basic stress. It is produced by the reaction of 3,4-dimethylphenol and camphene which are condensed in presence of tin tetrachloride: Xibornol is obtained through the rearrangement of camphene into isoborneol, followed by attack on 3,4-dimethylphenol. Xibornol is marketed as spray formulation at a concentration of 3% for the topical treatment of upper respiratory tract inflammations, infections and, in general, for mouth hygiene (Italian Medicines Agency, 2016).

However, there is a paucity of information on virucidal properties of Xibornol owing to the lack of international standard procedures to test lipophilic drugs insoluble in water (Damery and Cremieux 1989), and comparisons of Xibornol effectiveness among structurally and taxonomically different respiratory viral are missing.

To overcome such technical constrain, standards on other antiseptics were adopted for the *in vitro* study of Xibornol. In particular, the European standard UNI EN 14476 2019 (*Virucidal activity of chemical disinfectants or antiseptic products for instruments, surfaces or hands, which form a physically stable homogeneous preparation when diluted with hard water or with water*) has been adapted by diluting Xibornol at different concentrations in a non-ionic oil-in-water surfactant called Labrasol (Caprylocaproyl Polyoxyl-8 glycerides) (Cirri et al., 2007; Verani et al. 2017). The obtained data on a limited number of viruses (*Human Adenovirus, Human Coronavirus, and Human Rhinovirus*) was also already published in the patent of 2019 (Bigini, 2019).

The aim of this work was to improve the knowledge on viral abatement of Xibornol by testing and elaborating all the available data related to the *in vitro* experiments and considering a fixed xibornol concentration (3%). The tested viruses were chosen on the basis of high circulation in humans (Shi et al., 2022) and their clinical relevance for respiratory diseases (Table 1), namely:

- *Human Adenovirus type 5* (HAdV-5) that has high stability to physical and chemical agents, thus being able to survive long time outside the host.
- *Human Rhinovirus type 13* (RhV13) is transmitted through close contact and respiratory secretions;
- *Human Coronavirus 229E* (HCoV-229E), commonly considered as surrogates of pandemic coronaviruses.

Table 1 Classification and general features of the selected respiratory viruses

Family	Genus	Species	Envelope	Diameter (nanometers)	Genome	Diseases	References
Adenoviridae	*Mastadenovirus C*	Human adenovirus type 5	No	60–90	DNA	Respiratory tract diseases eyes and gastrointestinal infections	Knipe et al. (2013)
Coronaviridae	*Coronavirus*	Human Coronavirus 229E	Yes	100–150	RNA	Common cold	Knipe et al. (2013)
Picornaviridae	*Rhinovirus*	Human Rhinovirus type 13	No	20–30	RNA	Upper respiratory infections	Knipe et al. (2013)
Paramyxoviridae	*Respirovirus*	Human Parainfluenza Virus type 1	Yes	150–250	RNA	Respiratory infections	Hodinka (2016)
Paramyxoviridae	*Pneumovirus*	Human Respiratory Syncytial virus strain 18,537	Yes	150–250	RNA	Bronchiolitis and pneumonia in children under the age of 5 and the elderly	Hodinka (2016)

– *Human Parainfluenza Virus type 1* (PIV), which circulation is common throughout the year, but mainly in autumn.
– *Human Respiratory Syncytial Virus strain 18,537* (RSV) that infects children under the age of 5 and the elderly.

2 Materials and Methods

Experimental phases were performed according to European Standard UNI EN 14476–2019 adapted as cited above (Bigini, 2019).

2.1 Xibornol Solution

Xibornol, kindly provided by Abiogen Pharma spa (Pisa, Italy), was supplied dissolved in Labrasol solution at concentrations 3% (g/100 ml). The obtained Xibornol is a racemic mixture of two enantiomers (1R, 2S, 4S) and (1S, 2R, 4R). Indeed, the molecule of Xibornol contains three asymmetric centers (positions 1, 2, and 4 of the bicycloheptane ring) but only the formation of the two isomers occurs owing to the mechanisms of involved reactions in the synthetic process. The final active substance has a crystalline form which is stable from a thermodynamic point of view. The formation of the right polymorph is controlled routinely during the process, together with the chiral purity, the assay ($\geq$ 99.0%), and the impurity profile which are monitored for the release and stability of the active pharmaceutical ingredient (API).

2.2 Cell Cultures Viral Replication

Tested viruses were obtained by American Type Culture Collection (ATCC) and propagated in susceptible cell lines. In particular, HAdV-5 (ATCC VR-5) and RhV13 (ATCC- VR-286) were propagated and assayed on HeLa cell line (ATCC CCL-2); HCoV-229E (ATCC- VR-740) on MRC-5 (ATCC CCL-171) cell line; PIV (ATCC VR-94) on LLC MK-2 cell line (supplied by Pisa Hospital Virological Unit); and RSV (ATCC VR-1580) was seeded on Hep-2 cell line (ATCC CCL-23). For each virus a small volume with 0.1 multiplicity of infection (MOI) was absorbed on 25-cm^2 flasks for 1 h at 37 °C in 5% CO_2 atmosphere. After adsorption, Minimum Essential Medium (MEM) with 2% Fetal Bovine Serum (FBS), 10% L-glutamine, and 0.125% gentamycin was added and the flasks were incubated for 2–3 days. The typical viral-specific cytopathic effect (CPE) was revealed by observation on microscope (Carducci et al., 2009).

2.3 Infectivity Quantification

Each viral suspension was tenfold diluted (10^{-1}–10^{-4}) in MEM supplemented with 1% L-glutamine and 0.125% gentamycin. Then, each dilution was seed into five wells, each of them received 75 µl of sample, 75 µl of MEM supplemented with 0.125% gentamycin and 0.1% HEPES buffer to stabilize pH, and 50 µl of each susceptible virus cell suspension (see Sect. 2.1) (approximately 10^6 cells/ml). Plates were covered and incubated at 37 °C under 5% CO_2 for 5 days. Examination for cytopathic effects was performed with inverted light microscopy. The highest dilution producing a cytopathic effect in 50% of the inoculated cells was determined using the Spearman-Karber formula (Hamilton et al., 1977; Verani et al., 2020, Ramakrishnan, 2016), and the results were expressed in 50% tissue culture infective dose per milliliter ($TCID_{50}$/ml). The minimum detectable limit for this procedure was $10^{1.12}$ $TCID_{50}$/ml.

2.4 Xibornol Effect on Cell Lines

To choose the right concentration for the subsequent tests, cytotoxicity assays were preliminarily performed to evaluate the effect of Xibornol on the different cell lines, using the methodological approach of European Standard UNI EN 14476–2019. Serial dilution of each supplied Xibornol 3% solution was prepared

using Labrasol and seeded into 25-cm^2 flasks with a confluent cell monolayer. To verify a possible toxic effect of the diluent, Labrasol was seeded without Xibornol. Moreover, a negative control for each cell line was also prepared. After 24-h incubation period, the cell morphology was observed under inverted microscope to verify the integrity of the monolayer. All tests were analyzed in triplicate. The obtained data allowed to define the cytotoxic concentrations (CC) of Xibornol by considering cells death or morphological modifications and the highest dilution that revealed no effect.

2.5 Xibornol Effect on Chosen Viruses

The experiments were performed by considering the concentration coming from Sect. 2.4 and two different mixture virus-Xibornol, to simulate different environmental conditions (Fig. 1): the absence of interference substances (hereafter "clean conditions") allowed to evaluate the direct effect of Xibornol on virus infectivity, while the addition of FBS at 3% was applied to simulate the *in vivo* condition of the presence of various compounds with possible virucidal action, as suggested by the European Standard UNI EN 14476-2019 (hereafter "dirty conditions").

Briefly, mixtures of 10 ml were prepared with 1 ml of virus, 8 ml of Xibornol solution from Sect. 2.4 (non-toxic concentration for cell lines) and 1 ml of Labrasol for clean condition, while the dirty one was simulated by replacing Labrasol with 1 ml of FBS. Moreover, for each test, a positive control was prepared with 10 ml of virus and Labrasol instead of Xibornol. After a contact time of 15 min at a temperature of 25 °C, viral concentration was estimated by endpoint dilution assay as described above. A total of 10 combinations (type of virus and environmental condition), each one carried out in triplicate, were analyzed. The starting titers of each virus were the

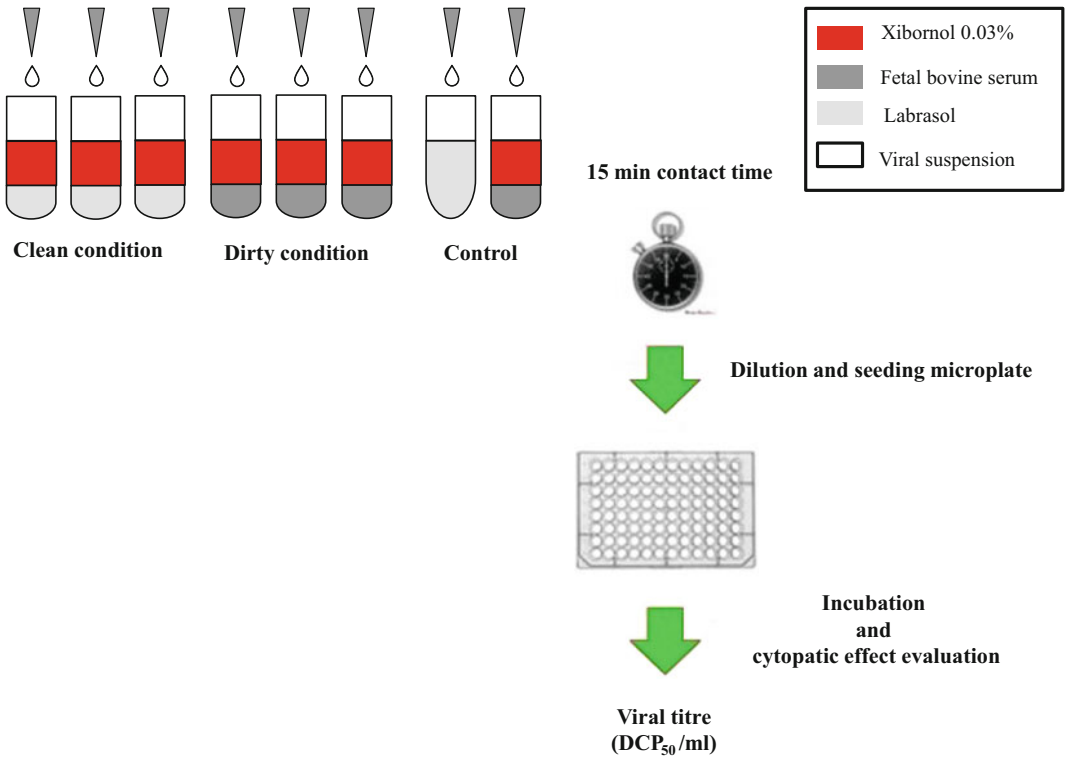

Fig. 1 Schematic flowchart of the experimental setup

following: 1.2×10^5 $TCID_{50}$/ml for HAdV-5, 7.5×10^5 $TCID_{50}$/ml for HCoV-229E, 1.5×10^4 $TCID_{50}$/ml for RhV13, 2.37×10^3 $TCID_{50}$/ml for PIV, and 3.16×10^5 $TCID_{50}$/ml for RSV.

2.6 Data Analysis

The infectivity reduction was estimated comparing viral titers obtained from untreated and Xibornol-treated samples. The abatement was expressed as logarithmic (Log_{10}) reduction (LR) calculated as $LR = Log_{10}(Nt/Nn)$, where Nt is the viral titer estimated after Xibornol treatment and Nn is viral titer obtained from untreated samples.

Unpaired Student's t-test was used to compare the effect of Xibornol on viruses' infectivity under the clean and dirty conditions. The resistance to the Xibornol was compared between the five viruses (HAdV-5, HCoV-229E, RhV13, PIV, RSV) and the two environmental conditions ("clean" and "dirty") using two-way analysis of variance (ANOVA), then post hoc comparisons using Bonferroni test were conducted to locate the differences. Values of $p \leq 0.05$ were considered as statistically significant. All statistical analyses were performed using *GraphPad Prism 5* (GraphPad, San Diego, CA, USA).

3 Results and Discussion

The role of respiratory viruses is well known and their high rate in mutations can lead to the escape from immune system and inefficacy to protect from a novel variant.

The research of effective measures to reduce the risk of infections and diseases is one of the main objectives for public health protection. Vaccination is a milestone for the viral infection prevention, but the use of antivirals is also needed to achieve this aim also by improving the knowledges of already existing and used products.

Xibornol effect is known and its efficacy on respiratory bacteria is recently studied and confirmed (Celandroni et al., 2021). Few data are available regarding the effect on viruses for its strong lipophilic nature that doesn't allow to strictly follow the European Standard on cell culture assays. To overcome such technical problems, we used a non-ionic oil-in-water surfactant, as reported by existing publication and patent (Cirri et al., 2007; Bigini, 2019), to explore the possible antiviral effect of Xibornol. In the present study, the cytotoxicity assays revealed a clear toxic effect using Xibornol 3% on the three cell lines (HeLa, MRC-5, and LLC MK-2) with concentrations ranging from 3 to 0.3 mg/100 mL responsible for a loss of viability and alteration of morphology in at least 30% on observed flasks. The cell cultures remained alive for the other tested dilutions (Table 2); therefore, the concentrations of 0.03 mg/100 mL represented the highest dilution that did not influence the replication of cell line and it was chosen for the viral survival experiments.

Xibornol showed a marked virucidal effect under the experimental setup (Fig. 2) and, when data from all viruses were combined, the differences in virus survival between untreated and Xibornol-treated samples were statistically significant (unpaired t-test, $p < 0.0001$), under both clean and dirty conditions (Fig. 3). Overall,

Table 2 Cytotoxic evaluation on cell culture flasks

	Cytotoxic effect[a] on cell lines		
Xibornol concentration	HeLa	MRC 5	LLC MK2
3%	100%	100%	100%
0.3%	> 50%	> 30%	> 45%
0.03%	0	0	0
0.003%	0	0	0

[a]Mean percentage of cells dead or with morphological modifications on 25 cm^2 flasks

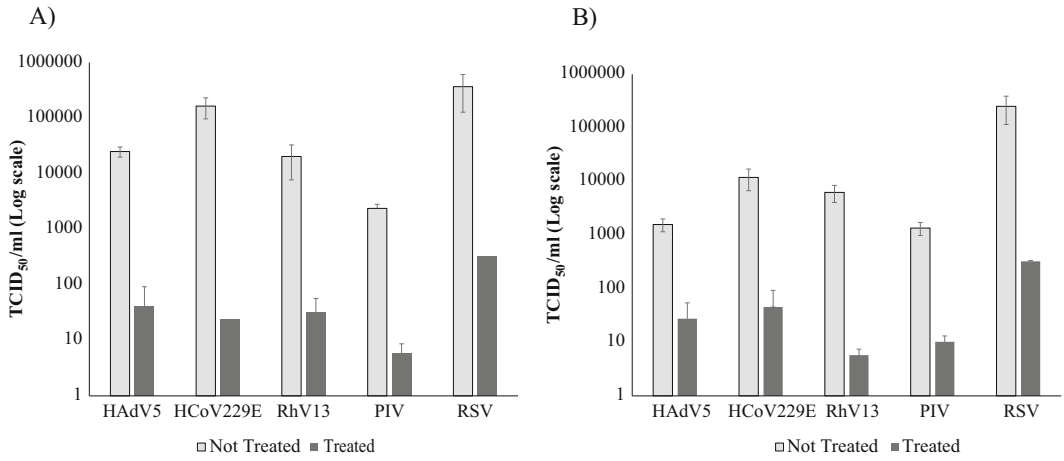

Fig. 2 Variation of viral titers for samples treated with Xibornol and untreated ones, in clean (**a**) and dirty (**b**) experimental conditions

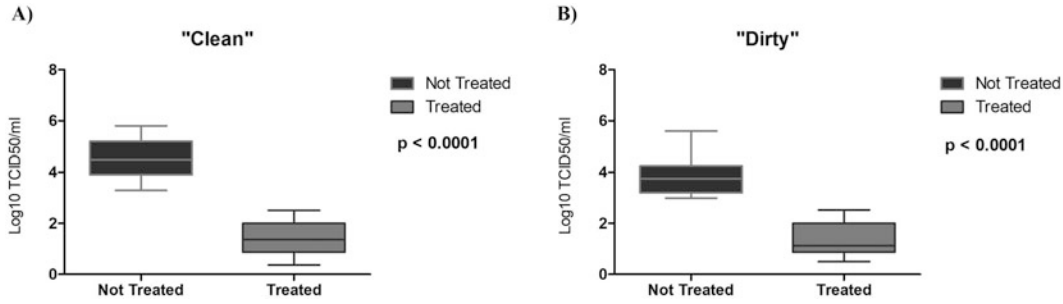

Fig. 3 Effect of Xibornol treatment under clean (**a**) and dirty (**b**) conditions (data from all viruses were combined, separately for untreated and Xibornol-treated samples). Statistical significance was determined by Student's t-test

Xibornol treatment determined at least 2 $\log_{10}$ abatement of virus infectivity, but with some differences according to the type of virus and the experimental condition (Table 3).

Regarding the effect of experimental condition on LR, the mean viral Log_{10} reduction was around 3 in clean condition and approx. 0.6 Log_{10} lower in the dirty ones and the differences of viral abatement between clean and dirty conditions were statistically significant for HAdV-5 and HCoV-229E (two-way ANOVA, $p < 0.001$). For such viruses, the abatement in dirty condition was approx. 1 Log10 higher compared to the clean condition, namely 1.05 Log_{10} for HAdV and 1.43 Log_{10} for HCoV-229E (Table 3, Fig. 4). Such results suggested the possible protective role of organic material against chemical compounds: aggregation phenomena can protect viruses when they are released in the environmental matrices, as reported in the water treatment plants where the absorption to the organic particles, not eliminated during physical treatments, protects viruses from chemical disinfection (Templeton et al., 2008).

Moreover, the comparison of LR among viruses showed statistically significant differences (two-way ANOVA, $p < 0.05$), with HAdV-5 and PIV the most resistant viruses, in both environmental conditions (Fig. 4). Such results can be supported by the biochemical and structural properties of such viruses, since non-enveloped viruses (i.e., HAdV-5) are more resistant to chemical inactivation compared to some enveloped viruses (Firquet et al., 2015),

Table 3 Viral titers and Log_{10} reductions related to different experimental conditions

		Clean condition			Dirty condition		
	Starting titer ($TCID_{50}$/ml)	Not treated (GM ± SD)	Treated (GM ± SD)	Log_{10} reduction	Not treated (GM ± SD)	Treated (GM ± SD)	Log_{10} reduction
HAdV-5 ($TCID_{50}$/ml)	1.2×10^5	$2.74 \times 10^4 \pm 1.22$	$1.78 \times 10^1 \pm 5.62$	2.80	$1.54 \times 10^3 \pm 1.32$	$1.61 \times 10^1 \pm 4.37$	1.75
HCoV-229E ($TCID_{50}$/ml)	7.5×10^5	$1.55 \times 10^5 \pm 1.53$	$2.34 \times 10^1 \pm 1$	3.84	$1.12 \times 10^4 \pm 1.51$	$3.14 \times 10^1 \pm 2.84$	2.41
RhV13 ($TCID_{50}$/ml)	1.5×10^4	$1.74 \times 10^4 \pm 2.06$	$2.15 \times 10^1 \pm 3.31$	2.81	$6.03 \times 10^3 \pm 1.40$	5.60 ± 1.34	3.03
PIV ($TCID_{50}$/ml)	2.37×10^3	$2.34 \times 10^3 \pm 1.20$	5.05 ± 1.94	2.67	$1.32 \times 10^3 \pm 1.32$	9.92 ± 1.33	2.12
RSV($TCID_{50}$/ml)	3.16×10^5	$3.16 \times 10^5 \pm 2.00$	$3.19 \times 10^2 \pm 1.01$	3.06	$2.27 \times 10^5 \pm 1.78$	$3.24 \times 10^2 \pm 1.04$	2.89
			TOTAL (mean)	**3.03**			**2.44**

GM Geometric mean (10^x, where X is the mean of log10 transformed values)
SD Standard deviation of log10 transformed concentrations

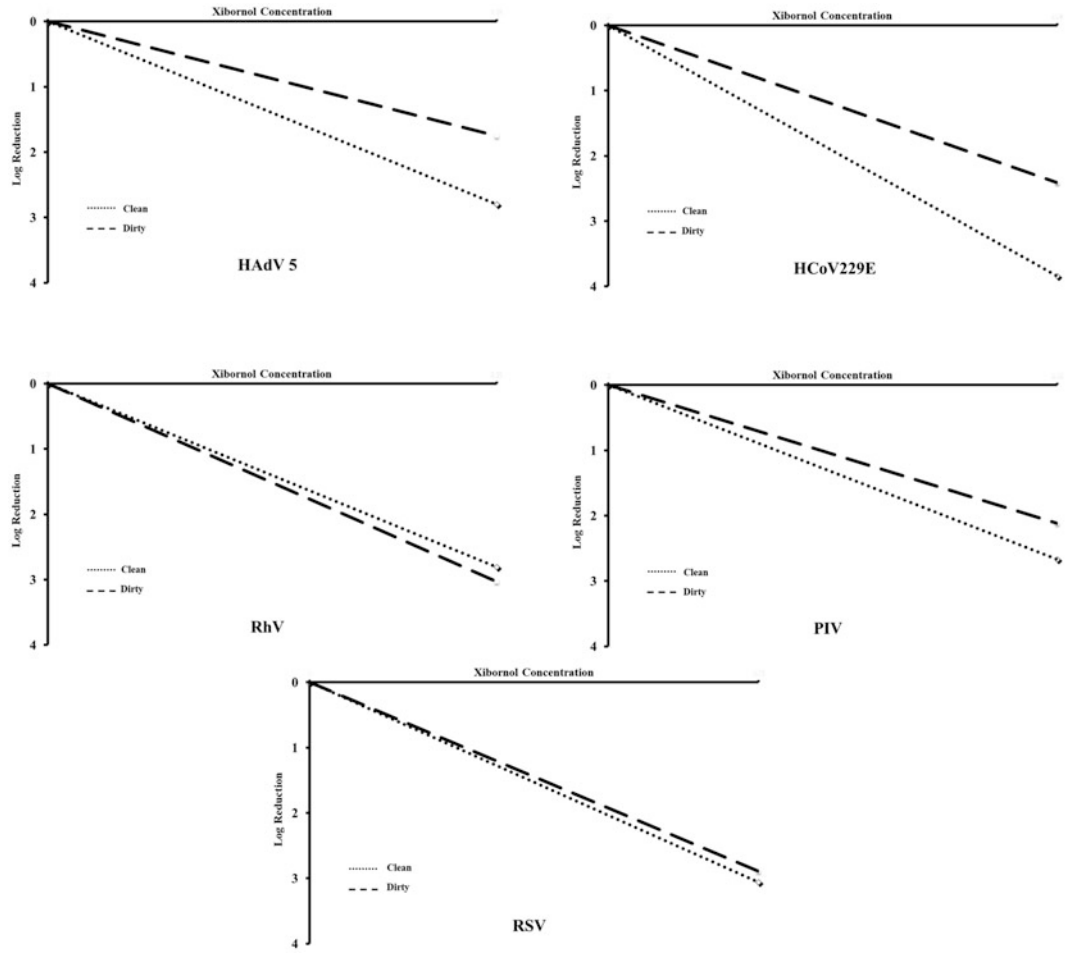

Fig. 4 Viral abatement after Xibornol treatment in clean (dotted line) and dirty (longdash line) conditions, for each virus separately

while PIV has a virion structure that provides a higher environmental stability compared to other enveloped viruses (Henrickson, 2003).

Limitation of the Study In this study, the Xibornol effect has been evaluated *in vitro* and not directly on respiratory tract mucosa. Although, *in vitro* experiments are commonly used to simulate real conditions, *in vivo* tests allow a better understanding of Xibornol action on viruses (Combe et al., 1988; Fabbri et al., 1988; Scaglione et al., 1988). Moreover, we compared the effect of Xibornol on various viruses, but through the evaluation of only one concentration of Xibornol (0.03 mg/100 ml).

4 Conclusion

The data presented in this study give a wide view on the antiviral activity of Xibornol, focusing on several representative respiratory viruses. The Xibornol effect on HCoV229E (a surrogate of SARS-CoV-2) gives some insights into the susceptibility of coronaviruses to such chemical treatment, which could be useful in the perspective of using Xibornol as a topical agent.

Although the experiments have been carried out using protocols for testing disinfectant on the environment, the obtained results support the validity of Xibornol as topical antiviral agent for human usage, also considering that its

effectiveness against viruses was maintained in the "dirty" condition tested in this paper. Clinical trials for assessing antiviral properties on oral mucous membranes are worth.

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"Man Who Won the War": Myth and Reality of Aldo Castellani's Role in Preserving the Health of Troops During the Italo-Ethiopian War 1935–1936

Luca Borghi

Abstract

The invasion of the ancient Ethiopian empire perpetrated by the Italian fascist regime in 1935–1936 deserves all the blame due to a war of aggression, a belated colonial enterprise and a bullying act of a totalitarian regime. Yet there is one aspect of that war that aroused universal admiration among contemporaries and which still deserves to be analysed today: the healthcare of troops. The Italian army, which came close to half a million men, was the largest European army that had ever fought in tropical or sub-tropical territories. Many Cassandras expected a health catastrophe, even more than a military one. But Mussolini decided to entrust Sir Aldo Castellani, the famous tropicalist doctor who had been living between Italy and England for years, with the role of Inspector General of Military and Civilian Health Services for East Africa. At the end of the seven-month victorious military campaign, the very low number of casualties recorded due to illness or injury evoked amazement and admiration. This was not just propaganda, as proved by the uncountable invitations from military and health authorities all over the world (including some of the nations that had imposed economic sanctions against Italy a few months earlier) for Castellani to reveal his secret through lectures, articles and conferences. Even US President Franklyn D. Roosevelt, who as a polio sufferer was particularly sensitive to public health issues, asked for and obtained a long private interview with Castellani, the "man who won the war".

Keywords

Aldo Castellani · Fascist propaganda · History of military medicine · History of preventive healthcare · Second Italo-Ethiopian war

The invasion of the ancient Ethiopian empire perpetrated by the Italian fascist regime in 1935–1936 deserves all the blame due to a war of aggression, a belated colonial enterprise, and a bullying act of a totalitarian regime. It was also a war that gained an ephemeral and precarious empire for Italy, at the cost of heavy economic sanctions and international political blame and isolation. All of these factors ended up throwing a reluctant Mussolini into Hitler's arms, thus laying one of the fatal premises for the Second World War.

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Nevertheless, there is an aspect of that war that, albeit with some *chiaroscuro* that we will try and analyze, aroused and still arouses admiration, which had as its main protagonist Sir Aldo Castellani.

Castellani (1874–1971) was an Italian tropicalist doctor who had acquired international fame for his youthful contribution to the elucidation of the etiology of sleeping sickness (1903) and for a number of subsequent innovations and discoveries in the field of laboratory methodologies and the world of microbiology, vaccines, and medical mycology (Borghi and Riva 2021). From 1903 to 1914, Castellani, although not a British citizen, was appointed Director of the Government Bacteriological Institute in Ceylon (now Sri Lanka) (Cook 2007, p. 197). In this capacity, he became one of the world leading experts in tropical medicine, to the point of publishing in 1910, with Albert J. Chalmers, the first edition of an influential and long-selling *Manual of Tropical Medicine* (Castellani and Chalmers 1910). During his Ceylon years, Castellani discovered the causative agent of yaws (Cook 2007, p. 197). His main and often forgotten medical contribution during the First World War was the invention of combined and polyvalent vaccines, which were successfully used in different armies against typhoid and paratyphoid fevers, cholera, and dysentery (Borghi and Riva 2021). After the war, he settled down in London where he developed a successful private practice in Harley Street, becoming the personal physician of royalty, diplomats, celebrities, and affluent people. He also played a decisive role in the founding (1923) and development of the Ross Institute and Hospital for Tropical Diseases (Cook 2007, pp. 199 ff.). Since the mid-1920s, Castellani was also appointed professor of Tropical Medicine in New Orleans, quickly becoming a point of reference in this field also in the United States (Castellani 1960, pp. 100 ff.).[1]

When, in the late 1920s, Italian fascist regime made of a colonial expansion a central point of its own agenda, the development of tropical and colonial medicine became essential tools, too: the international prestige of Aldo Castellani had no equals in Italy, so he became an essential asset of that new political strategy. The fascist regime began to do everything to bring the scientist closer to his motherland (Amalfitano 1978), as we will better see later.

Back to the 1936 Italo-Ethiopian War, a few weeks after the end of the military campaign, US *Time* magazine emphatically defined Castellani the "Man Who Won the War" to highlight the amazing results he had achieved with the healthcare of the Italian army (Anonymous 1936). The story, however, had deep roots and deserves to be reconstructed step by step.

1 The Latecomers of Colonialism

It had been 40 years now that Italy hoped to consolidate its colonial presence in the Horn of Africa and to wash away the shame of the defeats suffered at the end of the nineteenth century during the first Italo-Ethiopian War (Del Boca 2001, v. 2, p. 579 ff.). The *Belle Époque* had left the fascist regime with only two "shreds" of colonies in sub-Saharan Africa: small Eritrea and the southern part of Somaliland (Del Boca 2001, v. 2, p. 880). Not enough for the expansionist aims of Mussolini, who was looking for "a place in the sun" for Italy, alongside other colonial powers.

In the spring of 1935, while still trying to maintain close ties with France and England in the face of the growing aggressiveness of Hitler's Germany, Mussolini was creating pretexts for a clash with the Ethiopian Empire and was preparing, more or less covertly, for an invasion war (Lessona 1939).

Despite the contempt with which European nations habitually looked at the peoples of sub-Saharan Africa, Ethiopia was a relatively rich country, with a very ancient civilization and

[1] This contribution is an anticipation of a more extensive biographical research on Castellani by the author. In this paper, as well as in the full biography, I will have to rely many times on the famous autobiography Castellani published, in many editions and different countries, in the early 1960s. As every autobiographical source, it needs to be handled very carefully, trying to compare its contents and statements whenever possible with independent sources. I've tried to do so in this partial contribution, too.

deep culture. Prophet Isaiah already spoke of it with respect, back in the eighth century BC:

> Ah, land of buzzing insects, beyond the rivers of Ethiopia. Sending ambassadors by sea, in papyrus boats on the waters! Go, swift messengers, to a nation tall and bronzed, to a people dreaded near and far, a nation strong and conquering, whose land is washed by rivers (Isaiah 18, 1–2).

Furthermore, Ethiopia was a huge country, three or four times the size of Italy, and would have offered, theoretically, a boundless panorama of work and comfort for many lower-class Italians forced at home to a life of hardship (Lessona 1958, p. 143). But Ethiopia was also, since 1923, a member of the League of Nations, and the fascist regime had repeatedly promised that it would respect its territorial integrity and independence. Mussolini himself had solemnly declared back in 1922:

> The cornerstone of our political action in East Africa remains the rigorous maintenance of the integrity of Ethiopia, with which we intend to promote, both through Eritrea and Somaliland, the most intense and fruitful commercial relations (quoted in Del Boca 2001, v. 2, p. 4).

Some attempts in this direction had certainly been tried, but, after more than a decade, Eritrea and Italian Somaliland were instead rapidly becoming the base camps from which to launch a pincer attack against the empire of Haile Selassié (1892–1975).

But what did Aldo Castellani have to do with all of this?

2 An Old *Istituto Luce* Documentary

As we said, Castellani spent the first decade of the twentieth century as Director of the British Bacteriological Institute in Ceylon. Later, during the 1920s, he made London – the undisputed world capital of tropical medicine – his professional and family headquarters. Yet he never wanted to adopt British citizenship. As a consequence, at the outbreak of the First World War, he had placed himself at the disposal of the Italian authorities:

> [In] November 1914, I received orders from the Italian authorities to be ready for mobilization in the Medical Service of the Armed Forces (Naval Branch). Since I had not adopted British nationality, I had no appeal against this decree. So it has been every time Italy has gone to war – I have been recalled by the Italian Government for medical service in the naval branch. One has no say in the matter: it is conscription for life (Castellani 1960, p. 77).

Since the late 1920s the Italian fascist regime, increasingly aware of Castellani's prestige and of the strategic and propagandistic importance of colonial and tropical medicine, intensified its attempts to bring the scientist closer to the motherland, firstly (in 1929) by nominating him Senator of the Kingdom of Italy and then (in 1931) making him head of a brand new Institute for Tropical and Subtropical Diseases at the prestigious *Sapienza* University in Rome (Amalfitano 1978).

I owe the discovery of an extraordinary documentary by *Istituto Luce* to Costanza Bonelli's excellent work on the history of tropical medicine in Italy (Bonelli 2019a). This Institute, based in Rome, had been founded in 1924 for the production and distribution of documentaries intended for cinema screenings. Famous as a powerful propaganda tool of the fascist regime, the Italian *Istituto Luce* is considered the oldest public institution in the world dedicated to the production and distribution of cinematographic media for educational and informative purposes (Mignemi 1984, pp. 112–115).

This open-access movie (Istituto Luce 1936) is titled "Sanità in A.O." (Healthcare in East Africa) and most probably dates back to the second half of 1936 or early 1937 (Mignemi 1984, p. 115). In black and white, with synchronized sound, it lasts just over 18 min. I do not want to anticipate much of its contents which, in actual fact, give extraordinary visual feedback to almost everything I am going to talk about in this contribution. Here I just want to emphasize that Aldo Castellani is the absolute protagonist of the movie (Fig. 1), in the context of his Roman Institute for Tropical and Subtropical Diseases, the theater of the Ethiopian war, and the hospital ships that linked the motherland to Africa. From a biographical point of

Fig. 1 Aldo Castellani in a frame of the documentary "Sanità in A.O." (Istituto Luce 1936)

view, it is an extraordinary document. It is probably the only one that shows us Castellani – then just over 60 – "in action," and it allows us to hear his voice, a rather shrill voice, apparently, in an important sound fragment that concludes the film, of which I will give a detailed account in due course.

3 A First Exploratory Mission

On January 9, 1935, Aldo Castellani embarked from Plymouth on the transatlantic *Ile-de-France* for his annual overseas voyage (Ellis Island 1935). Ever since 1925 he had held the chair of tropical medicine in one of New Orleans' universities, firstly at Tulane and later at the Louisiana State University. Arriving in New York on the 15th, he immediately traveled to New Orleans, where he stayed as usual at the luxurious Roosevelt Hotel. That year, his American stay, with its educational and research commitments, lasted just over 2 months, if we trust him saying, with unusual accuracy, that in March he was back again in Rome to take care of the poor health of Alessandro Lessona (1891–1991) (Castellani 1960, p. 140). Since 1929, Lessona was the Italian Undersecretary at the Ministry of Colonies, and in a few months he would become Minister, after an *interim* by Mussolini himself, further proof of how much the Duce was then focused on the colonial question. After returning to Rome, Castellani went to see Lessona regularly in his office at the Ministry, obtaining a positive impression ("he had all the qualities of a good statesman"). He was a hard worker and in those weeks he was, logically, in very close contact with Mussolini. As Castellani remembered:

> One day he had a message for me. The Duce had told him that war with Abyssinia was certain. "We were beaten by the Abyssinians at Adowa [Adwa] in 1896," the Duce had said, "and we must wipe out that disgrace. We fought that battle with white troops; therefore our vindication must be carried out with white troops. However", Mussolini continued, "I am told that there is great danger in sending large masses of white soldiers to fight in a tropical zone: they die like flies from the climate and disease. Ask Castellani if he would be willing to be put in charge of the medical organization of the whole expeditionary force – army, navy, and air: I am convinced they must be under one single direction. Castellani, I feel sure, will comply with the wishes of his King and Government, and when we recall him on active service as Surgeon-General he will not refuse" (Castellani 1960, p. 140).

The following day Castellani met Mussolini and put himself at his disposal, well aware of the criticisms and risks he was probably going to face in the United Kingdom for such a decision (Castellani 1960, p. 140).

This probably happened by the end of April, because in mid-May Castellani accompanied Lessona on a first exploratory trip to Eritrea:

> Within a few days Lessona and I departed for Eritrea for a preliminary orientation work. We embarked at Naples on a small, fast steamer for Alexandria; then overland to Suez, where an Italian destroyer was waiting to take us to Massawa (Castellani 1960, p. 141).

They also had time for a short stop in Cairo, where Castellani's only daughter, Jacqueline, was living with her newly wed husband, Sir Miles Lampson, the British High Commissioner for Egypt. Here we can rely on an independent and authoritative confirmation of Castellani's account, which is also useful in trying to penetrate into his real feelings about the unexpected and uncomfortable situation he was involved in. It is a long entry, from May 21, 1935, in the *Diaries* of Miles Lampson:

> I forgot to record that Sir Aldo Castellani is passing through Cairo with the Italian Under Secretary for the Colonies on his way to Italian Somaliland[2] to advise regarding the sanitary arrangements and health of Italian troops. This is all being kept very hush and has in some respect been a little awkward, for I don't want any impression to be given that the Residency is getting mixed up with this Abyssinian business. (. . .) during tea-time came a telephone call from Aldo himself to the effect that he is arriving in Cairo at 10.15. Later. Aldo arrived duly and Jac.[3] went to bring him from the station. He seemed tired but cheerful and he and his travelling companions left three quarters of an hour later to catch their destroyer at Suez. We had a little talk in a perfectly general way about Abyssinian affairs and he told me he had no doubt that Italy meant to go for them. Regarding himself he had found it impossible not to respond to the urgent request that he should go out and have a look at the medical services and general comfort of the troops. He hoped not to be away for more than 10 days in all and would endeavour to stop off here for twelve hours on his way back (Evans 1972, p. 50).

Lampson told Castellani that, according to their own information, "the health conditions of the troops in Somaliland were lamentable," with lack of drinking water, no landing facilities for supplies, etc. Possibly, the situation in Eritrea was better, but he

> made no secret that the whole adventure seemed to [him] a most risky and ill advised affair – and just at a time too when a big political crisis raged in Europe. It was difficult to understand how Mussolini had let himself in for this African commitment at a time when he surely required all his forces and resources at home in Europe. I think – concluded Lampson – there is not much doubt that Aldo is of the same view though hardly able to say so as a true patriot. At 11 o'clock he left – I am afraid not much encouraged by my account of the appalling state of the Suez Road (Evans 1972, p. 51).

One way or another, they made it to Suez and from there by ship to the port of Massawa. They then proceeded to Eritrea's capital city Asmara to meet the Governor and Commander-in-Chief, Emilio De Bono, one of Mussolini's oldest collaborators. Over the following days Lessona and Castellani travelled far into the interior, up to the border with Ethiopia, where they saw "with emotion" – in Castellani's words:

> a simple monument to the Italian soldiers who had fallen in the Battle of Adowa [Adwa] in 1896. It was the statue of a soldier pointing towards Abyssinia, and the inscription read: "Italians, remember we died for our country, and one day cancel this defeat by victory" (Castellani 1960, p. 141).

That first inspection did not last more than a week. Lessona and Castellani were already back in Cairo on May 31. Lampson diligently noted:

> 5 o'clock. Aldo Castellani arrived back from Eritrea. So his trip has been a very hurried one. He has seen a vast number of hospitals and tells me that conditions are not so bad as he expected. He makes no bones about the Italians being out for Abyssinia's blood, though I fancy from his general attitude that he regards it as a great pity. He stops with us tonight (Evans 1972, p. 51).

They all dined at the Italian Legation, where Lampson and Lessona had a long conversation

[2] Here Lampson probably mistook Italian Somaliland with Eritrea that was the real target of the voyage.

[3] Castellani's daughter Jacqueline.

about their respective countries' divergent positions on the Ethiopian affair. Lampson could not but record the irritation of Lessona and his Italian companions "at the critical attitude adopted by Great Britain and especially by the British press towards Italian going on vis-à-vis Abyssinia," but also underlined the difficulty in reconciling the Italian attitude with her League of Nations obligations (Evans 1972, pp. 51–52). Lessona, for his part, recorded the friendly tone of that conversation, facilitated by Castellani's kinship with Lampson, and wrote that Lampson, while not commenting on his proposal that England should imitate France in a more favorable attitude towards that imminent colonial campaign, would surely report it to his government (Lessona 1958, pp. 142–143).

On a more private note, Lampson added in his Diary:

> It had been very hot all through dinner so on our way back we decided to take a turn out to the Pyramids in the cool of the night, which we did. Aldo Castellani makes no bones that he is very much worried. He is terribly afraid that Mussolini is going to try and rope him in. As he explained to me, they have no experts in tropical medicine in Italy and as that is his specialty, it is inevitable that they turn to him. This upsets all his arrangements and engagements in America, England and elsewhere, and the poor man is in a great stew about it. But as he said rather pathetically, if you do happen to be an Italian, it doesn't pay to run counter to what Mussolini asks of you (Evans 1972, p. 52).

4 Inspector General of Military and Civilian Health Services for East Africa

It took another couple of months before Castellani was definitively "roped in" to the affair, but he evidently did not put up much resistance. Already in the second half of August the international press anticipated the contents of a Royal Decree, dated August 30, 1935, signed by King Victor Emmanuel III and countersigned by Mussolini:

> Professor Aldo Castellani, Senator of the Kingdom, was appointed with effect from August 1, 1935, High Health Consultant for the Colonies of East Africa with the function of Superior General Inspector. . . (Regio Decreto 1935, p. 1592).

Consequently, starting from September 1, Castellani resumed his active duty in the Italian Royal Navy with the rank of Surgeon Major General (ACFM, p. 271a)[4]. In this new capacity, at the beginning of October, Castellani was ordered to leave again for Africa for a new inspection trip together with Lessona and Marshal Pietro Badoglio (1871–1956), Chief of Defense Staff, who in Mussolini's plans was soon to replace De Bono in the direction of the military campaign (Castellani 1960, p. 142).

Incidentally, we cannot but notice that while in Castellani's memoirs it looks as if Lessona, Badoglio, and he himself were during that trip more or less of equal importance ("the three of us had a fervent send-off from Rome – conquering heroes in anticipation"), in the contemporary memoirs of Lessona and Badoglio no mention is made of Castellani's presence. He certainly was with them, but not at all at the same rank (Lessona 1939, pp. 180 ff.; Badoglio 1937, p. 20).

Nonetheless, Castellani acknowledged Badoglio's interest in the health aspects of the military operation:

> Unlike many senior army officers, Badoglio firmly believed that medical preparation was all-important, and for this reason I was with him the whole time during his trip of inspection in Africa (Castellani 1960, p. 142).

The three embarked on October 10 in Naples on the transatlantic *Conte Biancamano* and reached the port of Massawa, Eritrea, on the following 17th (ACFM, p. 271a-b). About that second trip, Castellani only adds that:

> it was an interesting experience accompanying him [Badoglio] – he riding a fine black charger, and the entourage (including Lessona and myself) on mules – in the Adowa Mountains, where the famous battle had been fought. Badoglio had taken part in that battle as a young artillery officer, and gave us a fascinating lecture, pointing from his horse to the various hills, peaks, and mountain

[4] I will take this and some subsequent information from my own copy of Aldo Castellani's *Foglio Matricolare* (military registration sheet) and I will refer to it as ACFM.

> passes, describing vividly the various phases of the battle (Castellani 1960, p. 142).

Meanwhile, the military campaign, for the time being still led by De Bono, had achieved some moderate successes, including the highly symbolic conquests of Adwa, on October 6, and of the holy city of Axum in the middle of the month (Del Boca 2001, vol. 2, pp. 403–407). While Castellani was collecting data on the health situation of the Italian troops, Lessona and Badoglio had very tense meetings with De Bono, which they considered substantially inadequate for the continuation of the war, and even gathered evidence against him (Del Boca 2001, vol. 2, pp. 412–413). Lessona went so far as to write in his memoirs:

> I must confess that I would never have expected to find such a universal opposition to De Bono and such an unflattering judgment, especially in the Fascists, of the man and the general (Lessona 1958, p. 192).

The journey back to Italy of the three "inspectors" was on another ocean liner destined in those months for military purposes, the *Conte Verde*, between October 29 and November 2 (ACFM, p. 271a-b). As soon as they landed, they each wrote a report for Mussolini from the point of view of their own competence. In that of Lessona, regarding the health situation, we read only:

> It is good; the organization is being completed and perfected. But for this I refer to the report by Senator Castellani (Lessona 1958, p. 196).

After his spring trip, Castellani's attention had been focused on anti-malarial prophylaxis, and, mindful of his experiences during the First World War, he worked hard to ensure the availability of an adequate amount of quinine. He knew well that limiting action to mechanical defense measures against mosquitoes would not be enough with a constantly moving army (Castellani 1960, pp. 141–142).

But now he was responsible for the hygiene and health of nearly half a million men – a number never before reached in a colonial war fought mainly by European troops – and many were the bad omens. In previous months many Italian and European newspapers often wrote as follows:

> If there is an Italo-Ethiopian war, the Italian army will not be beaten by Abyssinian weapons, but by diseases and epidemics (Castellani 1937, p. 3).

It is therefore not surprising that, having returned to Rome from his second exploratory trip at the beginning of November 1935, Castellani had to rush "between the Ministers of War, the Colonies, the Navy, and the Air Force," having also "talks with the Red Cross authorities" (Castellani 1960, p. 143). By now he was fully aware that he was responsible for the coordination, technical guidance, and surveillance of nearly 300 hospitals and infirmaries, including 8 Navy hospital ships and 2 flying ambulances of the Air Force (Fig. 2). He then had to ensure efficient management of the radiological and dental ambulances, analysis and disinfection units, sanitary material warehouses, etc. Not to mention healthcare personnel: 2,500 doctors, nearly 200 pharmacists, 17,000 nurses, and nearly 300 military chaplains, whose important – let us say – "psycho-somatic" role did not escape Castellani. Last but not least, Castellani and his collaborators in the Roman Clinic had also to supervise the rushed basic training in tropical medicine of a large part of this staff (Castellani 1937, pp. 4–7; see also Giardina 1938, pp. 139 ff.).

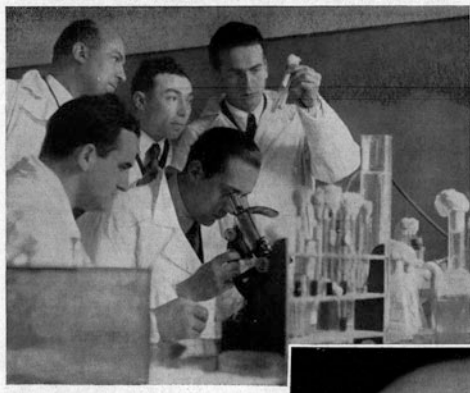
Various clips from the propaganda film we mentioned above seek to highlight this multifaceted action: actions of water purification, disinfection and disinfestation of indigenous homes, the role of radiological ambulances – of the kind already made famous during the First World War by Marie Curie herself – and the essential function of field hospitals, one of which is presented in the movie during an inspection made by Castellani (Istituto Luce 1936; see also Labanca 2015, pp. 132–133).

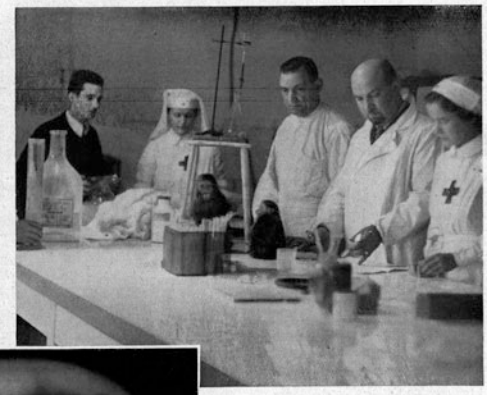
Moreover, back in Italy, Aldo Castellani became more conscious of the fact that his role, whether he liked it or not, was no longer only professional and technical but also political, therefore subject to criticism, attacks, and envy that came from the most disparate directions.

L'ILLUSTRAZIONE ITALIANA 297

SANITÀ IN AFRICA ORIENTALE

LA STRATEGIA MEDICA DI ALDO CASTELLANI





Vogliamo ricordare Leopoldo II, di felice memoria, e il suo gran discorso? È del 1876, quando fu applaudita e benedetta l'apertura della « Association Internationale pour l'exploration de l'Afrique ». Concludeva il discorso: « Aprire la civilizzazione al Continente nero che solo resta chiuso nel mistero, dissipare le tenebre nelle quali giacciono popoli interi: ecco il nobile compito che incombe al nostro secolo di progresso! ».

Da allora sono trascorsi sessant'anni. L'Italia ha ripetuto i medesimi concetti di quel Re dei Belgi, ma l'uditorio mondiale oggi è provvisto di altre orecchie e sembra che l'Italia parli a uomini di altro pianeta. Perché applaudirono tanto Leopoldo II i padri degli attuali figli che denigrano l'Italia che vuole eseguire in tutto, quanto quei nobili padri plaudirono? Si parlava allora di abbrutimento. Eppure c'è ancor oggi l'abbrutimento che allora era anche maggiore: infieriva come nessun'altra malattia, la malattia di Castellani, sconosciuta con questo nome, ma che senza quel nome significava morte ed epidemia; con quel nome significa ora profilassi e ritorno alla vita.

In quel tempo si determinò un caso — e il caso ha un sapore astrologico, per il complesso dei rapporti che esistono tra il macrocosmo e il microcosmo — che proprio in quell'anno in cui in Europa si costituiva l'anzidetta « Association » e il Re Leopoldo faceva il discorso, sotto la medesima esaltazione di pianeti, a Firenze nasceva Aldo Castellani, cioè proprio nell'epoca in cui gli Stati Europei riguardavano l'Africa con interesse, con cupidigia, e forse anche con carità cristiana.

Guardate: l'Italia contribuisce 25 anni dopo all'incirca a soddisfare gli zeli umanitari degli africanisti con un grande ingegno, il quale se trovò la via cosparsa di bei discorsi sulla fratellanza e la civiltà, una volta in Africa trovò quella via invece ostacolata dall'infierire della peste bubbonica, dagli stregoni, dalle zone miasmatiche, dalla scarsezza di provvidenze scientifiche e soprattutto dalla malattia del sonno. Ma Castellani vinse ed è divenuto il primo tropicalista del mondo.

Il generale medico, senatore Aldo Castellani che ha tracciato il piano di difesa sanitaria per le truppe metropolitane in A. O. - In alto, a sinistra: *Clinica tropicale. Identificazione difficile di un bacillo.* - A destra: *Uomini e scimmie collaborano alle prove di un siero.* - Qui sotto: *Il fattore psicologico della buona salute è l'allegria che fa buon sangue. Nel buon sangue non allignano i microbi.*

Aldo Castellani è l'espressione delle intenzioni civilizzatrici dell'Italia nella sua odierna impresa coloniale. Già la fondazione in Roma della Clinica per le malattie tropicali e subtropicali, voluta da Mussolini, non aveva altro scopo che quello di essere un centro direttivo dell'assistenza medica nelle nostre colonie e perché Castellani, dopo un lungo peregrinare all'estero, diffondesse la sua scienza e la sua esperienza tra noi. Senonché per il punto geografico della Clinica e per la fama di Castellani, essa diveniva l'unica nel Continente quindi da oltre il Mediterraneo provenivano pazienti con casi rari di malattie esotiche e il luogo diveniva mèta degli infermi d'Oriente. Una moltitudine di casi, dei più significativi nella tropocopatia venivano a costituire nel luogo delle « vere meraviglie orrende viventi » che altrove si vedono fissate solo in maschere di cera.

Decisa la nostra impresa in Abissinia, Aldo Castellani ha dinamicamente tracciato il piano di difesa medica per le truppe nazionali. Egli ha anzitutto tenuto conto che la razza latina riesce a vivere nei tropici con maggiore adattamento dell'anglo-sassone; perciò ha posto la difesa per l'acclimazione su di un piano differente da quel che poteva ritenersi opportuno per il colonizzatore anglo-sassone. Vi saranno tenui problemi differenziali che pur tuttavia apportano delle modificazioni su criteri tropicali accettati generalmente dalle scuole che hanno subito l'influenza di nazioni con vasta colonizzazione nei tropici. Aldo Castellani ha aggiornato e individualizzato i problemi dell'acclimazione generale. La preparazione è stata febbrile: si è visto Castellani affaccendarsi non solo come di consuetudine al microscopio, ma sui caschi. Gli assistenti in camice bianco con il casco in testa e termometro nel casco hanno sostato a lungo sotto il sole degli scorsi mesi estivi per registrare con esattezza e precisione la reazione al calore di vari tipi di caschi. Uno speciale studio, è stato voluto da Castellani sui colori protettivi dei raggi solari particolarmente per la protezione del capo e della colonna vertebrale. Il rancio stesso è stato riguardato con somma cura ponendo la qualità, la quantità e la specie in relazione all'esigenza del metabolismo. Non può esservi non solo in clima diverso dell'abituale ma nello speciale stato psicologico che si produce in zona di guerra. Castellani ha perciò voluto che si attuasse tra le truppe una divulgazione di medicina tropicale.

Inizia a tale scopo i « Corsi Celeri », nella Clinica, ben adatta, bene allestita. Vi presenzia il corpo sanitario civile e militare, e la gioventù. Gli universitari si entusiasmano e da loro prenderà poi forma la divulgazione. Aldo Castellani pone in rilievo che nella medicina tropicale è necessario che si diffonda la convinzione che l'empirismo, comune ancora tra noi nei villaggi, può riuscire dannoso. Nei « Corsi Celeri » non è necessaria la brillante conferenza; il corso celere è tempo del nudo diagnosticare. E si diagnostica a occhio e a tasto, analizzando e sintetizzando presto. Tutto sul vivo. Si vede e si palpa e poi si disinfettano le mani. Ma è necessario — insegna Castellani —

Fig. 2 A page from a 1936-Italian illustrated magazine about Castellani's medical strategy in East Africa (Zampetti 1936)

5 The League of Nations Sanctions Italy

For those who, like me, love to hunt for old books in Italian antique markets, it is still quite easy to find copies of *Guida dell'Africa Orientale Italiana*, published in 1938 by the *Consociazione Turistica Italiana* (the former Anglophile denomination of *Touring Club Italiano* had been forbidden for reasons we will soon understand!). This Guide, which in the first edition alone had a circulation of almost half a million copies, was intended as a tribute:

> to the Italians of every class and condition who, with their discipline ready to obey any order from the Chief, have shown how to respond to the sanctions applied by 52 states coalesced in the illusion of being able to break the will of a people determined to find its place in the Sunlight (Consociazione Turistica Italiana 1938, p. 5).

On October 7, 1935, the League of Nations condemned the Italian aggression against Ethiopia, and 4 days later 52 states decided to inflict a series of economic sanctions on Italy (Del Boca 2001, vol. 2, p. 423), which were defined and formalized on November 18: a ban on selling weapons and other goods useful to the war industry to Italy and a ban on importing Italian goods or granting loans to the Rome government (De Rosa 1982, p. 347, note).

Yet the military campaign continued victoriously, and the fascist regime was very capable in transforming the damage of international sanctions into an almost unanimous movement of national cohesion and pride, which had one of its most distinctive tones in the growing anti-British feelings. Following the example of Mussolini, for many Italians England once again became simply "perfidious Albion." It is not difficult to imagine Castellani's ambivalent state of mind. An indication of this is the fact that in his personal file, preserved in the historical archive of the Senate of the Kingdom, it is said that he signed the agenda against the sanctions but that, at the same time, it is not clear whether he was present or not in the session of December 9, 1935, when [this agenda] was approved by acclamation (ASSR 1929–1946, p. 76).

Castellani was pro-Italy, of course, but without too much enthusiasm. Meanwhile, French satirical newspapers began to ironize him who defined the state of health of the troops advancing in Ethiopia as "magnificent... perhaps better than in Italy" (Pick 1935).

However, at least on the domestic front, Castellani's work was widely appreciated and concretely supported. He himself attested at the end of the war:

> I can certify that the requests for medical personnel and material were sometimes doubled by the Head of Government and that on certain occasions the loading onto ships of medical material took precedence over the loading of war material (Castellani 1937, p. 4).

And it was not only Mussolini who intervened *sua sponte*. On December 18, 1935, the Italian Chamber of Deputies discussed and approved a bill that increased the economic annual contributions from the Ministries of Colonies and War in favor of the Clinic of Tropical and Subtropical Diseases of Rome University (Atti Parlamentari 1935, p. 2061). "Honorable comrade" Sabato Visco (1888–1971), a famous physiologist who in 1938 was the first signatory of the infamous Italian "Manifesto of racist scientists," was allowed to speak in favor of the law (Dell'Era 2020). Visco, who was not alien to hyperbole since he did not hesitate to define Castellani's Clinic as "the most important in the world," added that the new law was intended to ensure that the Clinic could "always better fulfill the new and fundamental health needs imposed by military operations in East Africa and by the work of civilization connected with them" (Atti Parlamentari 1935, p. 2061). His final praise of Castellani also allowed him a dig against the British:

> His scientific career is a whole series of discoveries of the highest importance. The English government, which has continually made use of the work of this great scientist of ours, drawing conspicuous results, knows something about it!

He provoked a rousing round of applause in the parliamentary hall by concluding as follows:

> With the current bill, the Fascist government intends to make available to Aldo Castellani the

> necessary means so that he can give his activity a faster pace and open up a wider field for it; approving it, as we will do, we will carry out highly humanitarian work, for the advantage not only of ourselves but the whole world that from our genius immensely benefited yesterday, benefits today, and will benefit tomorrow (Atti Parlamentari 1935, p. 2062).

Article 1 of the new law provided a substantial increase in contributions to the Clinic by the two Ministries involved from 50,000 to 160,000 Italian *lire* per year (Atti Parlamentari 1935, p. 2062). The law was immediately approved, with 268 votes in favor and only 3 against (Atti Parlamentari 1935, p. 2069).

It is now easier to understand why, in the *Istituto Luce* documentary on "Healthcare in East Africa," Castellani's Clinic is the main set of the first part of shooting:

> The Clinic for Tropical Diseases in Rome is a very active research center, where doctors and nurses specialize in the study of tropical diseases, under the guidance of Professor Aldo Castellani. The daily visit to the sick is used to illustrate the most interesting pathological cases to the students... (Istituto Luce 1936).

The video shows Castellani entering a ward of the clinic followed by more than 20 people. He wears a long binaural stethoscope around his neck and auscultates "a *negro* suffering from amebic colitis" (Istituto Luce 1936). Paternalistic as it is, this small detail can be hardly imagined in a contemporary Nazi propaganda film: still in 1935, Italian racism was much more similar to the Anglo-Saxon than the German one! The next scene shows the amphitheater-classroom of the Clinic where a white man and a white woman with leprosy are lying on two beds. Castellani shows how "painful sensations have almost disappeared in this kind of patient." Then, brief images of assistants studying in the library and of Castellani supervising microscope work in the histology and microbiology laboratories are shown. This is followed by microscope clips with blood preparations for some cases of sleeping sickness, whose trypanosomes are simply called "Castellanella" (Istituto Luce 1936).

Finally, this first part of the film shows some images of the "completion of the tropical diseases course," with "the last lesson for medical officers and nurses given by Professor Castellani, who is now leaving for Africa" (Istituto Luce 1936). Visco's report to Parliament stated that 16 such courses had already been completed by December 18, 1935 (Atti Parlamentari 1935, p. 2062).

The scene then shifts to a hospital ship, and another chapter of our story begins.

6 Marie-José, the Princess-Nurse

We know from his military file that Aldo Castellani embarked on December 23, 1935, on the hospital ship *Aquileia*, leaving for East Africa (ACFM, p. 271a-b). His memoirs also confirm this:

> Then I proceeded to Naples and embarked on a hospital ship, the inspection of these being one of my duties (Castellani 1960, p. 143).

It is possibly to this very journey that the images of the documentary refer. While "the hospital ship prepares to set sail," nurses and doctors set up an operating room, prepare the ward beds, and check X-ray apparatus and microscopes. Finally, a military chaplain celebrates Mass and gives the Holy Communion (Istituto Luce 1936). Castellani goes on remembering:

> In contrast to her sisterships, she was a fast boat, and we arrived in no time at the Suez Canal and the Red Sea. I visited and inspected all our ports and medical posts on the southern part of the Red Sea coast, and on the coast of the Indian Ocean down to Chisimaio at the mouth of the big, crocodile-infested Juba River (Castellani 1960, p. 143).

Castellani spent in Africa the entire first half of 1936, decisive for the ongoing war. It was during those months that he implemented those prevention and hygiene strategies which, combined with the strictly medical and surgical measures, greatly favored the maintenance of an excellent general level of health within the Italian army. All leading to the "white legend" status, which we will discuss in detail later.

In the meantime, however, we must return to Rome where, on February 27, 1936, a new training course in tropical medicine for doctors and nurses was starting, with the attendance of a very special lady student:

> The classroom of the Clinic for Tropical and Subtropical Diseases. A Clinic wanted by the Duce, with the generous contribution of the August Princes, almost as if by divination of the services that it should have rendered to us, under the guidance of one of the most competent scholars of the science of exotic diseases, H.E. Aldo Castellani. The illustrious scientist is now absent; he carries out his mission between Italy and the Eastern Africa, fighting his great battle at the behest of the Duce. An unusual energy is in the air today in the classroom as a new course for the study and assistance of tropical diseases is starting, brilliantly directed by prof. Jacono.[5] A course for students and for nurses of the Italian Red Cross. Nothing new: this is already the 9th, and yet everybody present is in nervous expectation. Here she comes in her blue Nurse's cloak, her bright smile, sure-footed and with a notebook in her hands. A young Sister – it is Maria of Piedmont - who is coming to follow the course, like the other Sisters. No special seats, no formalities, no protocol. Like the others, she sits on the benches. "It seems like being back at school," she says quite simply (Di Targiani Giunti 1937, pp. 11–12).

In 1930, Marie-José of Belgium (1906–2001) married Prince Umberto of Savoy (1904–1983), heir to the throne of Italy. At that time, the couple had the title of Princes of Piedmont, hence Marie-José as a Red Cross voluntary nurse being called the "Sister of Piedmont" (Giaconia Landi 1938). Her experience as a voluntary nurse was neither a novelty nor an exception. In fact, since the end of the nineteenth century a volunteer experience as a Red Cross nurse was almost a rite of passage for most women of the European aristocracy (Mautone 2019).

Marie-José, following the example of her mother Elisabeth of Bavaria (nicknamed the "queen nurse" by the Belgians), had dressed in the uniform of a Red Cross nurse when she was not yet 10 years old, during the First World War, when she and her mother visited the field hospital of Le Panne, Belgium, "generous with smiles and consoling words to the wounded soldiers" (Contini 1955, p. 36).

In the 2 years she spent in Turin after her marriage, Marie-José enrolled in the formal courses of the Red Cross (Contini 1955, p. 107), but after her transfer to Naples the Duchess of Aosta – who was General Inspector of the Voluntary Nurses of the Italian Red Cross – did not recognize the worth of those previous studies. In her opinion, they had been carried out a little too much "as a Princess," with too many facilities and regards (Contini 1955, pp. 153–154).

The Princess of Piedmont did not lose heart and started all over again: 2 years of regular studies, practice at the *Ospedale degli Incurabili*, and even assistance as an instrument nurse in the operating room. Gradually, her tasks had become more important, and she was given greater responsibility:

> She did not spare herself and did not spare her ladies. At eight in the morning, she was already at her work place, in the afternoon she did not allow herself to stop and, often, she only had a few hours available for a night's rest. Her ladies often felt exhausted, and begged her not to exceed, but she, without replying directly, gave the orders for the next day, at eight o'clock (Contini 1955, p. 154).

At the beginning of 1936 she decided, with the consent of her husband, to participate herself in the Ethiopian campaign and enrolled in the course on tropical diseases. The course only lasted about 10 days, but Marie-José followed it with exemplary punctuality and without shirking common obligations, such as that of signing the attendance sheet. It is said that her final exam, on March 11, was excellent (Di Targiani Giunti 1937, pp. 13–15).

The African voyage of the "Sister of Piedmont" began in Naples, Thursday, March 26, 1936, aboard the hospital ship *Cesarea* (Di Targiani Giunti 1937, pp. 16–19). In this case too, the *Istituto Luce* video helps us, capturing her just as she gets on board the ship, accompanied by her mother-in-law, Queen Elena, and her husband Umberto, while Red Cross officials and soldiers greeted her (Istituto Luce 1936).

In the meantime, Castellani had continued to carry out his role of supervisor and coordinator of healthcare for fighting troops and the civilian population. In the already mentioned documentary, we see him, in military uniform and without a gown, inspecting a small field hospital and, for the benefit of the movie camera, even stages a

[5] Igino Jacono (1889–1972) was, at that time, the main collaborator of Castellani in the Roman Clinic.

short visit to a patient with auscultation, palpation, and a final paternal slap on the cheek. Later we see him observing some colleagues setting up orthopedic traction equipment (Istituto Luce 1936).

Castellani had to travel often between the southern and northern front of the war, the Italian army trying to crush the Ethiopian counterpart as in a pincer:

> From the southern front (Somalia) I had often to go to the northern front (Tigrai and Eritrea), where Badoglio had replaced De Bono. Sometimes I went by sea via Massawa; more frequently I went by air, in military 'planes. These aeroplane trips were rather exciting because we had to fly over the whole length of unoccupied Ethiopia. Coming from Mogadishu once I remember the pilot saying breezily: "Ah well, let us hope we have enough fuel to get us to Makalle, because if we have to make a forced landing, even if we don't crash in these stony mountains, we shan't survive long: the Abyssinians don't usually bother to take prisoners. And the few they take they mutilate (Castellani 1960, p. 145).

Castellani tells in his memoirs that during a visit to Asmara, Eritrea, in March 1936, he witnessed the effects of a massacre carried out at night by the Ethiopian troops who infiltrated beyond the Italian lines. The "irregular Abyssinian troops" attacked the camp where about 80 Italian workers (Rochat 2019, p. 128), who were building a road, were sleeping. Visiting the site of the massacre a few hours later Castellani had to ascertain that the corpses of those men had all been stripped and emasculated (Castellani 1960, p. 146). The macabre account seems to be added on purpose so as not to make the reader notice too much the episode dating to the following month of April, which Castellani dismisses in a few words:

> There were accusations in the foreign press that in the battle of Makalle Badoglio used poison gas: he did not (Castellani 1960, p. 148).

We will come back to the question of gases and chemical weapons later. For the time being, we return to the southern front, where in *Chisimaio* (Kismayo, in current English), on April 14, the doctor witnessed the landing of the "Sister of Piedmont" from the *Caesarea* hospital ship. This is how that landing was told by Irene di Targiani Giunti, then National Delegate of the Voluntary Nurses of the Italian Red Cross, who was traveling with Marie-José:

> The awakening finds us standing opposite the so-called coast "of the Islands" (el Gezair), in front of the beach of Chisimaio. It is the farthest stage we must reach, beyond the Equator; we are almost on the border with the English colony of Kenya. We are anchored offshore, in the great ocean, alone; there is only another ship some distance away, the hospital ship "California". The sea in the morning is almost always calm, except when the real monsoon season arrives; now it is a period of calm. The coast of Chisimaio almost forms a natural gulf, the beach extends like a semicircle and prolongs to the surrounding islands; the island of the Serpents, where the Navy radio system is located, is the largest. Ours is the third hospital ship that has stopped here; first came the ship "Gradisca", the most powerful of all, then the "California", and today here we are with the "Caesarea"! The Authorities come on board to greet the august sister; there is also Senator Castellani, who has been in Somalia for some months; and right here in Chisimaio, he managed to tame, to quickly circumscribe a small epidemic of amoebic and bacillary dysentery, which was taking strength (Di Targiani Giunti 1937, pp. 72–73).

Aldo Castellani was practically the constant companion of Marie-José during the following 2 weeks, and it was probably here that the friendship between the young princess and the now elderly luminary of medicine, which will significantly characterize the following decades of both their lives, began. Castellani already knew the Princess's interest in exotic diseases and their treatment methods, and did not miss opportunities to cultivate that interest and give her the necessary explanations.

Marie-José was impatient to visit the sick of Kismayo. However, the shallow and sandy seabed prevented her from reaching the shore with a boat; therefore, both she and her companions, including Castellani, were carried on shoulders on a kind of gestation chair (Di Targiani Giunti 1937, pp. 74–75). The visit to the town hospital, the "Princess Maria," and to two field hospitals made the effectiveness of the measures adopted by Castellani in countering the dysenteric epidemic evident in the eyes of Marie-José. A photo of the time portrays the Princess leaving

Fig. 3 Princess Marie-José leaving one of the small field hospitals of Kismayo accompanied by Castellani. There is a writing on the wall behind them: "disinfect your hands" (Di Targiani Giunti 1938, p. 77)

one of these small field hospitals accompanied by Castellani. There is writing on the wall behind them: "disinfect your hands" (Fig. 3). Most of the 300 men hospitalized were now quite well and were cheered by the attentions of the "blonde Princess" who brought them "her sweet smile" (Di Targiani Giunti 1937, pp. 75–77).

After that, Castellani embarked on the "Caesarea" which, after a couple of days of navigation northwards, reached the Somali capital, Mogadishu, where it remained at anchor for about a week.

In addition to the usual visits to the sick and the hospitals, the "Sister of Piedmont" also involved Castellani in a sort of pilgrimage to the *Duca degli Abruzzi* Village, an agricultural colony founded in 1920 by Luigi Amedeo di Savoia-Aosta, the Duke of the Abruzzi, which in the 1930s had grown considerably in economic and commercial terms. The duke, who had also been one of the main benefactors of the Roman Clinic of Tropical Diseases, had died in 1933 and had wanted to be buried in his colony (Di Targiani Giunti 1937, pp. 84–85). The Princess of Piedmont, accompanied by Castellani, some other sisters, and a small retinue, covered the approximately 50 km separating Mogadishu from the *Duca degli Abruzzi* Village on an elegant white train. After the visit to the duke's tomb, the group set about a new inspection of the hospitals the duke had promoted in previous years:

> We begin the tour of the infirmaries; everything that has arisen here is the work [of the duke]. We see a small hospital for white people and a clinic for indigenous people, where His Excellency Castellani shows us some interesting cases of exotic diseases: yaws, Madura's foot, tropical ulcers (Di Targiani Giunti 1937, pp. 91–92).

Then, after a lunch offered by the new farm manager and a visit to the facilities, the group immersed itself in the noisy and colorful local market. People crowded, as is logical, around the illustrious visitor, but not even Castellani went unnoticed:

> All around were women with swollen bellies, old men bent over with the weight of years and illnesses, little girls with their skins flowered with various rashes, of which the tropics abound; and suddenly a small counseling center was formed around Senator Castellani. The interpreters, who are not lacking, were ready to give the needed explanations (Di Targiani Giunti 1937, pp. 93–94).

In the following days, Castellani again accompanied the Princess ashore for some inspections to health facilities in the city of Mogadishu (which then had about 50,000

inhabitants) or on visits of a different nature. Yet much of the time was spent on board, where other injured or sick people had been embarked, and Castellani was struck by the commitment that Marie-José put into fulfilling her care duties (Di Targiani Giunti 1937, pp. 95–104).

The next step of the journey was the ancient coastal town of Hobyo (Obbia, in Italian), where the *Caesarea* arrived on the morning of Friday, April 24. If we give credit to Castellani's memoirs, an episode happened there that seemed to confirm a notable "clinical eye" of the *Sister of Piedmont*:

> The *Cesarea* put into a place called Obbia, and I went ashore. As I knew the Princess was keen on seeing tropical cases, I asked the doctor in charge of the local hospital to collect a few typical ones for her to inspect the following day. Next day the Princess went ashore with Marchesa Targiani, two other nurses, another doctor, and myself. At the hospital the doctor in charge, forgetting in his excitement her command to be called Sister Maria, said: "Your Royal Highness, I have a most wonderful case of madura foot which I am sure will interest you" - and he brought forth the patient. Sister Maria looked at the huge, knobbly, hypertrophic foot: "This is not madura foot," said she; "it is elephantiasis verrucosa." The doctor's face was a study of bewilderment and consternation; he looked entreatingly at me, but I could not help him, because it *was* a case of elephantiasis verrucosa (Castellani 1960, p. 143).

In the following days *Caesarea*'s journey proceeded north and then, having entered the Gulf of Aden, west. Finally, after having skirted the English Somaliland, the hospital ship entered the Red Sea and headed for Massawa, Eritrea, without further stops (Di Targiani Giunti 1937, pp. 111–114). Castellani went ashore wherever there was some health post to be inspected, and for which measures had to be taken. Meanwhile, Maria of Piedmont and the other Voluntary Nurses remained mostly on board, where they took care of the sick and the injured in the wards and in the operating theaters:

> Our teacher H.E. Castellani helps us a lot. He is always good-natured with everyone, keeps us good company, and he too works on his favorite studies (Di Targiani Giunti 1937, p. 114).

In Massawa, after another 4 days of inspections and visits to the civilian and military hospitals of the city, while increasingly enthusiastic news of a probable and imminent conquest of Addis Ababa arrived from the front, the paths of Castellani and the Princess of Piedmont separated, at least for the time being. There was still time for a last joint visit:

> There is still a poor infirmary, almost lost, which is located in Otumlo, near Moncullo, and is dedicated to [Tommaso] De Cristoforis, the hero of Dogali. It is in a malarial and deserted area, near the work sites for road construction. The infirmary is entrusted to two doctors, true apostles, also affected by malaria, who work in extremely difficult conditions, maintaining great order and admirably caring for the sick. There are forty-nine hospitalized; the visit of the Sister of Piedmont, who leaves many small gifts to everyone, is a celebration. We are again accompanied by Prof. Castellani, who has a special regard for this little hospital and for these doctors (Di Targiani Giunti 1937, pp. 120–121).

That same afternoon of Sunday, May 3, 1936, the *Caesarea* hospital ship set sail from Massawa to return to Italy. The landing in the port of Naples of Marie-José and all the crew, exactly 1 week later, was once again immortalized in the often-cited documentary "Sanità in A.O.".

A few days earlier, Badoglio had sent Mussolini this telegram:

> Today, May 5, at 4 pm, at the head of the victorious troops, I entered Addis Ababa (Del Boca 2001, vol. 2, p. 689).

The war for the conquest of Ethiopia had ended, officially at least, with Italian victory. Castellani was asked to stay in Africa for a few more weeks to supervise the civilian medical organization (Castellani 1960, p. 149).

7 Children of a Lesser Empire

As hoped for by Mussolini, the Ethiopian capital had been conquered "before May 11, the date of a new meeting of *the Sanhedrin of Geneva*" (Del Boca 2001, vol. 2, pp. 681–682), as he mocked the League of Nations, which would thus find itself faced with a fait accompli.

Virtually no obstacles stood in the way of the last advance towards the Ethiopian capital by the approximately 20,000 men, half Italians and half

Africans, under the command of Marshal Badoglio. Such ease made the Italian general underestimate the risks that would follow the conquest of Addis Ababa, when his forces would instead be completely insufficient to "garrison the capital, the surrounding region and the communication routes, which will be immediately interrupted by the [Ethiopian] partisans," leaving the city isolated for a long time (Del Boca 2001, vol. 2, p. 681).

Addis Ababa, abandoned on May 2 by Emperor Haile Selassie, had fallen into chaos, with looting, devastation, and massacres, especially against the Europeans present in the city. Things got to the point that France asked Mussolini to occupy the capital as soon as possible, to restore order and defend the European personnel barricaded in diplomatic representations (Del Boca 2001, vol. 2, pp. 683–687). The *Duce*, obviously satisfied that this request came precisely from one of the nations that a few months earlier had voted on economic sanctions against Italy, decided to use an iron fist and sent the following order to Badoglio:

> Occupied Addis Ababa, Your Excellency will give orders so that: 1) all those in the city or surroundings, who are caught with weapons in hand, be summarily shot; 2) all the so-called young Ethiopians, cruel and pretentious barbarians, moral authors of the looting, be summarily shot; 3) those who have participated in violence, looting, fires be shot; 4) those who, after 24 hours, have not delivered firearms and ammunition be summarily shot. I await a word confirming that these orders will – as always – be carried out (Quoted in Del Boca 2001, vol. 2, p. 686).

Mussolini's orders were harshly carried out – contemporary French sources estimated that at least 1,500 people were summarily executed in the hours following the capture of the capital – and order in the vast forest-city was quickly restored (Del Boca 2001, vol. 2, p. 701).

It is not clear whether Aldo Castellani arrived in Addis Ababa before May 9, the day on which Mussolini, in front of a huge cheering crowd in *Piazza Venezia*, Rome, announced the proclamation of the new Italian Empire. The Duce declared that from that moment on "the territories and peoples that belonged to the empire of Ethiopia are placed under the full and entire sovereignty of the Kingdom of Italy" and that "the title of Emperor is assumed by the King of Italy for himself and for his successors" (quoted in Del Boca 2001, vol. 2, p. 709). It was an act that definitively and fatally sealed the destinies of the fascist regime and the ruling house of the Savoy, while further aggravating relations between Italy and the United Kingdom (Del Boca 2001, vol. 2, pp. 709–711).

Castellani's first impressions upon arriving in the Ethiopian capital were of a less political and more personal nature:

> Addis Ababa, when I was there, was a sprawling city with several hills thickly covered with very tall trees of the eucalyptus family. The seeds, I was told, had been sent from Australia many years before as a gift to the Emperor Menelek. At Addis Ababa, I had a pleasant surprise: I discovered that one of the two American hospitals run by an American religious body for the benefit of the indigenous population was in the charge of an old student of mine from Tulane University, in New Orleans. He had been in Abyssinia, with his wife and child, for two years. I lunched with him, and he told me that, in the interval between the retreat of the few Abyssinian regular troops and the entry of the Italians, his house had been raided for two days by the rabble, which went for all 'whites' regardless of nationality. Only the British Legation was not molested: it was guarded by a company of Indian troops, transported there from Somaliland before the war started (Castellani 1960, p. 148).

In the early days, Castellani was housed in the recently built Imperial Palace, which unfortunately had been ravaged by the mob. He was assigned none other than the room of the deposed Emperor and was able to sleep on the "immense four-poster bed under a canopy bearing his coat of arms, the lions of Judah" (Castellani 1960, p. 150).

On May 20, 1936, Badoglio and Graziani took turns in command of the Italian occupation troops. Officially, Badoglio was only taking temporary leave in Italy, but in reality he had no intention of returning to Africa. Castellani, in the previous months, had occasionally to take care of the health of the marshal, who was 3 years older than him, having been born in 1871. Badoglio appeared very tired and had even confided to him his intention to resign. This was Castellani's "medical" reaction:

> He was talking of resigning. "Nonsense," I said. "I'll prescribe a tonic medicine that will put you right in no time. Take a tot of whisky every day at sundown". Whisky was unobtainable in the Tigrai and extremely scarce in Eritrea; so a military 'plane flew to Port Sudan on the Red Sea, where, as in every self-respecting British colony, that 'tonic medicine' was plentiful. The plane came back with two cases of 'Black and White', and the treatment was a great success. Badoglio was not a drinker, although like most Italians, he liked a glass of wine at meals, and he was a very moderate eater (Castellani 1960, p. 146).

Once he decided to return to Italy, Badoglio asked Castellani to travel with him, but the doctor had to decline because, as we said, he had been asked to stop for a few more weeks to supervise the healthcare organization of the new colony. However, he too went to the airfield where Badoglio welcomed Rodolfo Graziani, who had flown in from Harar. The two hugged and appeared to be on friendly terms. Badoglio left for Italy the next day and it was he who proposed that Mussolini appoint Graziani Viceroy of Ethiopia (Castellani 1960, pp. 148–149).

Castellani was also on good terms with Graziani, who had him stay in his own villa, often invited him to lunch and made an exception for him when he prohibited other Italian officers and officials from socializing with the staff of the diplomatic representations of countries that had voted for economic sanctions against Italy (Castellani 1960, pp. 149–150).

In those days Castellani must have felt the growing sense of unease in which the new Viceroy found himself. Ethiopia was anything but pacified, and he, at the head of too few troops to control such a vast territory, had been left in a much more difficult and precarious situation than Badoglio had wanted him and all of Italy to believe (Del Boca 2001, vol. 2, pp. 733–735).

We can only imagine, in the absence of explicit sources, that Castellani's activity, during the further six weeks he spent in Africa, was as hectic as ever. There is a fleeting but positive testimony to him in the memoirs of the well-known American missionary doctor Thomas A. Lambie (1885–1954), who at that time headed the Ethiopian Red Cross in Addis Ababa:

> It was impossible to get any definite word about anything from the Italians. For a while we had Sir Aldo Castellani, the great tropical-medicine expert, and while he was there things went somewhat better for us; but he soon left (Lambie 1939, p. 250).

Perhaps by now he too felt a certain nostalgia for Italy and England. We know for sure that, on Sunday, July 5, 1936, he embarked, probably in Massaua, on the destroyer *Cesare Battisti* (ACFM, p. 271a-b). He arrived in Naples exactly 1 week later. To welcome him and the approximately 800 veterans traveling on the same ship were, among others, the heir to the throne, Umberto of Savoy, and Castellani's old friend, Hélène of Orléans, Duchess of Aosta (Hanson 2018, p. 313).

For Castellani, his office "in Eritrea-Somalia-Ethiopia as Inspector General of the Military and Civil Health Services for the Eastern Africa" (ACFM, p. 272b) officially ended. The Italo-Ethiopian War, however, apparently won in just 7 months, was actually just beginning. The time for the long-awaited colonization of the fertile Ethiopian territories would practically never come (Del Boca 2001, vol. 2, pp. 725 ff).

8 The White Legend and the Black Legend

The opinion that Castellani had made a decisive contribution to the success of the military campaign, managing to avoid the heavy "collateral damage" that many had prophesied in terms of the health of the Italian troops, began to circulate in the press all over the world a few days after the taking of Addis Ababa. In its June 8 issue, the American magazine *Time* contained the article from which I took inspiration for the title of this essay, the "Man Who Won the War":

> The man who won Italy's war with Ethiopia was last week spotted by doctors as being an Anglicized Italian, Knight Commander of the Order of St. Michael and St. George, Sir Aldo Castellani, whose wife is English and whose only daughter two years ago married British High Commissioner for Egypt, Sir Miles Lampson. Italy's No. 1 enemy in Ethiopia was disease and Sir Aldo is a world-famed specialist in tropical diseases (Anonymous 1936).

This opening, followed by a brief biographical-professional profile of Castellani with references to his activities in Uganda and Ceylon, and also in the service of the American universities of New Orleans, seemed enough to give credit to the astounding health-related results that had accompanied the second Italo-Ethiopian conflict (and which appeared to be based on an early official report by Castellani himself):

> He had set up six air-conditioned hospital ships for sunstroke cases. He proceeded to inoculate every Italian to land at Massawa or Mogadiscio with the vaccine he himself had discovered in British employ for prevention of typhoid, paratyphoid and cholera. Sir Aldo shipped to East Africa tons of quinine for malaria, tons of serum tubes for tetanus, gas gangrene and snake bite, and 18,000 hospital cots. He covered suspected water holes with petroleum, fumigated camps, provided good drinking water, dotted Eritrea with hospitals and laboratories. The Italian Army fought under unprecedentedly thorough medical care (Anonymous 1936).

Apparently, the scientific and personal prestige of "big, jovial Sir Aldo" made his report credible, to the point that the anonymous author of the article dismissed with a subtle irony the catastrophic data that the adversaries' propaganda had instead disseminated a few months earlier:

> Last winter world headlines told of Italian hospital ships unloading thousands of Italian sick in secret sick dumps on the island of Rhodes, of countless Italian crosses on the plains of Eritrea. Sir Aldo smiled. Last week, arriving in Addis Ababa, he made his health-report on the Italo-Ethiopian War. Malaria: "a few deaths". Dysentery: one epidemic in southern Somaliland, no deaths. Typhus, typhoid fever, relapsing fevers: no deaths. Beriberi and scurvy: no white cases. Cholera and plague: not one case. Chief mortality was, next to Ethiopian bullets, from sunstroke which was eliminated last November by prompt treatment of the first symptoms (Anonymous 1936).

The report of a United Press International (Harnett and Ferguson 2003) correspondent from Addis Ababa, James L. Rohrbaugh, published during Castellani's return trip to Italy, seemed to confirm the news announced by *Time*, but giving it even greater prominence thanks to the comparison with what had occurred simultaneously in the Ethiopian army:

> In the Abyssinian Army, diseases were very numerous; more than half the cases were dysentery. Scurvy destroyed the army on the Southern Front; small-pox decimated the army of Mulughieta on the Northern Front. At Dessie, pneumonia was raging. The terrible disease typhus, was passing from one camp to another, killing the victims in a few days. Malaria and relapsing fever were common. (. . .) The Red Cross doctors tried in vain to help the soldiers: they were only able to carry out their work in small zones. The army was destroyed to a great extent by disease and hunger. (. . .) It is obviously no exaggeration to say that one of the prime reasons for Italian success was the continuous health of its armies, due to the efficiency of their medical service (quoted in Castellani 1937, p. 16).[6]

This narrative, which obviously put his own work in an excellent light, was much appreciated by Castellani, who constantly quoted it in the countless lectures and conferences that he gave on this topic in the following years.

The first of these lectures was probably the one not precisely dated but of which we have a filmed summary in the above-mentioned *Istituto Luce* documentary. It took place, probably in the second half of July 1936, in the auditorium of the Roman Clinic of Tropical Diseases, crowded with Castellani's collaborators and students, as well as a large group of Red Cross Voluntary Nurses. The video summarized in just under 5 min an account that was probably much longer, as well as rather technical, considering the audience present:

> Ladies and gentlemen, here I am back among you after a year in Africa. What were the results of the campaign in the healthcare field? I feel I can say: wonderful! In this as in any other field, thanks to the directives given by the Head of Government. . . (Istituto Luce 1936).

Castellani, after quoting Rohrbaugh on the state of health of the Ethiopian army, compared the mortality from disease that had been recorded among the French troops in Madagascar in 1895 (25%), among the British troops in the Boer War

[6] *United Press Red Letter, New York, July 11, 1936*, quoted also, for example, in Castellani 1939, p. 150. I was unable to find the original text except in numerous publications by Aldo Castellani himself: what makes it credible is above all the fact that no one seems to have ever denied it.

of 1900 (3%), and the allied ones, still mainly British, in the expedition against the Germans in the Horn of Africa in 1917 (4%). The Italian expeditionary force in Ethiopia, by far the largest army of European troops that had ever fought in Africa, was made up, as we know, of about half a million men. In the light of previous experiences, one could have expected – Castellani noted – between 15,000 and 25,000 deaths from illness. Instead, there were only 599: 0.10% of the total. Castellani, who had begun with the Roman salute, emphatically concluded the lecture by shouting: "Long live the King! Salute to the Duce!", followed by a standing ovation from the audience (Istituto Luce 1936).

An even more solemn occasion, as well as textually better documented (Castellani 1936a), was the conference that Castellani held on July 31, 1936, at the Italian Institute of Public Health – today the *Istituto Superiore di Sanità* – a few hundred meters from his clinic. On this second occasion, the Sister of Piedmont was also present, and Castellani publicly praised her for the "shining example" she had given during the campaign (Castellani 1936a, p. 4).

From that moment on, and for at least 3 years, Castellani received a large number of invitations around the world to illustrate the rationale and practical measures that had made his "health miracle" possible (Castellani 1939).

In 1944, former Italian Ambassador to France, Vittorio Cerruti (Pastorelli 1980), placed "among the auspicious memoirs of [his] diplomatic mission in Paris," which lasted from 1935 to 1937, the conference held by Castellani at the invitation of the French Ministry of Public Health, in the Aula Magna "Richelieu" of the Sorbonne University, on the evening of Saturday, February 20, 1937 (Anonyme 1937; Gauducheau 1937, p. 113):

> When, after the plain exposition of your wise prescriptions, you pulled out a piece of paper from your pocket and read the figures relating to the very rare cases that occurred, the elected public who filled the vast hall to the rafters applauded with an interminable ovation the distinguished scientist who had achieved such a success (ASSR 1929–1946, p. 66).

The elderly *Marshal of France*, Louis Franchet d'Espèrey, was also present at the conference. Unable to get out of his wheelchair, he asked Castellani to join him at the end of the conference so that he could shake his hand. According to Cerruti's memory, his words were more or less as follows:

> You have rendered an invaluable service not only to your country, but to humanity, a service for which all Powers having territories in Africa will be grateful to you. During our campaigns in Morocco, we lost thousands and thousands of soldiers to infectious diseases, while you carried the war in Ethiopia to an end without suffering any loss of this kind. It's a great feat! (ASSR 1929–1946, p. 66).

We can imagine that a diplomat, with well-known anti-Nazi sentiments such as Cerruti, would have tried to use that renewed climate of esteem and sympathy for Italy on the French side to encourage rapprochement between the two governments. Unfortunately, things moved quickly in a completely different direction, and Cerruti himself was recalled to Italy in October 1937 and had to retire shortly after that (Pastorelli 1980).

Meanwhile, for Castellani, the expressions of esteem multiplied, such as that of the *Academie Royale de Medecine de Belgique,* which made him an honorary member as early as December 1936 (Vanbreuseghem 1973, p. 69).[7] The greatest satisfaction, however, as he himself admitted, was when the President of the United States, Franklin Delano Roosevelt, wanted to receive and meet him personally at the White House in the spring of the following year:

> I had an even greater honor in March 1937, while in New Orleans, when I received a telegram from the White House, inviting me to call on the President of the United States, Franklin D. Roosevelt, in Washington. I accepted with eagerness. I went to the White House and saw the President in his office, where he kept me talking for about half an hour; no one else was present. He certainly gave me the impression of being a man with a prodigious brain. He asked me many questions about the medical organization adopted during the Ethiopian War, and in the end said that he would like me to

[7] Castellani had been a correspondent member since 1925.

> give a lecture on the subject in Washington, to the Army and Navy medical officers and to the Public Health service officers (Castellani 1960, p. 273).

If Castellani is one of the only two sources I found about his private audience with President Roosevelt (see also Anonimo 1937), the lecture he delivered in Washington on April 9, 1937, at the auditorium of the Department of Commerce, under the auspices of the Surgeon-Generals of the United States Army and the United States Public Health Service, is well documented (Castellani 1937).

For Castellani, as well as for many scientists of his time, it was quite normal to repeat more or less the same things in a lot of different publications. Therefore, we are not surprised to note that between 1936 and 1940 at least 17 of his articles were published in which the same measures and the same results that we have mentioned were re-proposed, in a more or less extensive and detailed form, not rarely with the very same words.

Firstly, Castellani highlighted, as an expert on climate and acclimatization (Castellani 1931), that for the first time in history "such an imposing mass of white troops" was taken to fight "in a tropical zone, most of which in a torrid climate" (Castellani 1936b, p. 3). I have already talked about the massive logistical-health system set up to support the military campaign and the thousands of professionals who made it work. Now I only need to better specify the measures taken against some of Castellani's "old acquaintances": malaria, dysentery, typhus, and paratyphus. For each of these pathologies, the experience of previous colonial campaigns predicted tens of thousands of cases with thousands of deaths.

How was it possible, for example, to limit the cases of primary malaria to 1,241, and deaths from pernicious malaria to 23? Here is the explanation given by Castellani:

> With the troops continuously on the move and the area of operations being enormously extended, mechanical prophylaxis, such as mosquito nets and antilarval measures, was often impossible. From the beginning we insisted on quinine prophylaxis; every soldier received three tablets a day of quinine sulphate or bihydrochloride – each tablet containing 0.2 gm. (3 gr.) – and took them; a good example was given to the ranks by those in authority: at every meal, the Commander-in-Chief in Somaliland, General Graziani, and all his staff officers took quinine regularly (Castellani 1939, p. 142).

Are the figures provided by Castellani reliable? The first official report on the war, published in 1938, put at 2675 the total figure for malaria cases and to 81 the deaths caused by this disease (Giardina 1938, p. 156). Yet, we need to keep in mind that Castellani did not count the relapses (malaria was still a very common disease in Italy, too), the time span he considered was quite shorter than that taken into account by the official report, as we shall see later, and that most of the military campaign took place outside of the most dangerous season for malaria infections in sub-Saharan Africa (April–September) (Cairns et al. 2015). In my opinion, something similar can be argued about other diseases: Castellani's self-satisfactory data can be integrated or slightly corrected, but not completely falsified or overturned (Cosmacini 2019, pp. 92–93).

Dysentery was another risk always lurking, and Castellani knew well that the dysentery in the regions where the war was fought being mostly amebic no effective vaccine was available (Castellani 1939, p. 144).

The focus was therefore on other prophylactic measures, such as educating the troops to drink only suitably boiled or chlorinated water (if bottled mineral water was not available, albeit unprecedented quantities of it had been transported to Africa from Italy) and to carefully wash their hands "with a 2 per cent solution of lysol or lysoform after visiting the latrine, and before having their meals" (Castellani 1939, pp. 143–144). Even in this area, according to Castellani, the results were exceptional, with only 453 hospitalizations for dysentery and no fatal cases (Castellani 1939, pp. 143). Giardina (1938, p. 156) on the other side calculated 854 cases and 16 deaths: the sanitary results were however highly positive.

Against typhoid and paratyphoid, the "secret" weapons held by Castellani were obviously the

mixed vaccines developed by him during the First World War (Borghi and Riva 2021). According to the data he provided, hospitalizations for such diseases amounted to 458 cases, with 161 deaths. He added proudly:

> According to certain previous colonial wars, there might have been in Abyssinia over 50,000 cases, with several thousands deaths (Castellani 1939, p. 145).

The figures given by Giardina for typhoid and paratyphoid amount to 648 cases and 382 deaths (Giardina 1938, p. 156). We have, once again, quite higher figures than those provided by Castellani. In my opinion, even taking into account the differences in method and time frame we already mentioned, the main fault attributable to Castellani lays in the fact that, in 1939, he did not still take into account the official report by Giardina, which had been published 1 year before, to clarify or at least to explain better the different figures provided by himself and repeated so many times.

So, what was the cause of this undeniable and great success? Was it great skill? Was it expertise forged in the most diverse climatic and environmental conditions? Was it charisma or, as we prefer to say today, leadership?

The most able propagandist of the fascist regime, the Florentine Alessandro Pavolini, perhaps managed to give with a few brushstrokes – rhetorical it may be, but not for this lacking in insight and truth – a plausible explanation of how much Castellani's style had made the difference:

> Above all I will remember Castellani, the doctor. With many titles he can be named (. . .). But we'll call him a doctor, like the soldiers did. It does not matter that sleeping sickness and amoeba have ensured his immortality. The thing is that when meeting him in Massawa, dressed in yellowish raw silk, he continues to look like nothing more than a doctor. A doctor, who divides his time between Ceylon and Rome, between Kismayo and London; who has three continents as his own backyard, and an airplane as a buggy. In spite of everything, he inspires the confidence of a good old village doctor, elegant and easy-going. He has this prerogative of the Tuscan people, to familiarize and tame any place around them. Truly the whole world, for a Tuscan, is just a village. For Aldo Castellani, the tropics are the boundary of the family farm, and in his account the tsetse fly looks like an old fly that one always had at home. He carries on his shoulders the health of the largest white expeditionary force a colonial war has ever seen. (. . .) Now, among the Italian troops in Eastern Africa, mortality from disease was lower than that of the army at home. And such it will remain, much to the amazement of colonial experts around the world. How could you, doctor? Castellani shrugs. He could explain: quinine, mixed vaccines, vitamin B, vitamin C. Or, even simpler: cleanliness, trucks and not marches, a carefully studied type of helmet, a lemon for each soldier every two days, alcohol only after sunset. . . He prefers to talk about luck. Out of modesty. But actually bringing luck is the greatest merit a doctor can claim (Pavolini 1937, pp. 25–27).

Not all historians, however, accepted this reconstruction of the facts and their implications without objections. We also need to dig deeper into them.

The first official report on the "War of Africa," as previously mentioned, was published by Giuseppe Giardina, professor of hygiene at the Royal University of Rome and Inspector-General for the Public Health during the First World War (Giardina 1938, pp. 132 and 160). Giardina did not hesitate to say that the data on mortality from disease were so low that they could "seem even far-fetched" (Giardina 1938, p. 158). Nevertheless, as a conclusion of his detailed analysis, Giardina gave a total amount of 1784 deaths from disease (Giardina 1938, p. 159) and commented that such a greater number in comparison with that published by Castellani (555, says Giardina, but it is actually 599: see Castellani 1936a, p. 7) was mainly due to having taken into consideration a longer period of time: July 1935 to December 1936 (Giardina) and October 1935 to May 1936 (Castellani) (Giardina 1938, p. 158).

Giardina's conclusion was unequivocal:

> Therefore, from any point of view you look at the health issue of the war in Africa – from repatriations, as from reports of illnesses and deaths – the success achieved appears truly exceptional, indeed unique. Encouragement and guidance for the further development of colonization can well be drawn from it (Giardina 1938, p. 165).

Another, post-war and post-fascist official report on the health organization in Italian East Africa,

published in 1965 by Giuseppe Bucco and Angelo Natoli on behalf of the Italian Ministry for Foreign Affairs, unfortunately does not give any information about the mortality rate during the Italo-Ethiopian War, even if it gives detailed figures about hospitalizations for diseases that afflicted the Italian army (Bucco and Natoli 1965, p. 2).[8]

Therefore, although in disagreement with Giardina's optimistic conclusion about "the further development of colonization" which, at least in the Italian case, would have had a very ephemeral future, subsequent historians could not help but base their statistical reconstructions on his own (Del Boca 2001, vol. 2, p. 717; Rochat 2019, p. 128).

For example, one of the leading historians, and one of the most severe critics, of Italian colonialism and the war in Ethiopia, Angelo Del Boca, reports the mortality data repeated several times by Castellani, judging them to be neither complete nor definitive. After analyzing and discussing the various sources available on this topic, often fragmentary and contradicting each other, Del Boca concludes:

> in the absence of a well-grounded and definitive balance, it can only be argued, on the basis of all the figures presented so far, that the war in Africa, from 1 January 1935 to 31 December 1936, cost Italy at least 4,350 dead and the number of injured almost double that (Del Boca 2001, vol. 2, p. 717).

However, we must take into account that Del Boca's estimate brings together deaths or injuries in combat with those from disease, and that he again considers a much longer period than that to which Castellani always referred (October 1935–May 1936). Thus, even if we want to consider the victims of illness as being half of the total (and not a third as Castellani usually does), we would have a figure of approximately 2,000 deaths from disease, that is, about the 0.4% of the troops deployed. The distance from all the other previous and comparable colonial episodes remains very wide.

[8] It is not clear if these figures refers only to Italian East Africa or if they include also Libya.

The most recent historiography, on the other hand, seems to have no hesitation in recognizing Castellani's objective healthcare success during the second Italo-Ethiopian War (Bonelli 2019b), while highlighting his propaganda and, not infrequently, racist distortions:

> The data relating to the corps of indigenous troops, taken into consideration by Castellani only to testify that the spread of some infectious diseases spared the Italian soldiers, are instead absent in the speech of the tropicalist doctor. (...) An absence – that of colonial subjects – in the writings and communications of the tropicalist doctor confirms how much Castellani worked in those years thinking of the home front, and his victory over disease constituted a showcase for a display of the regime's power, over men and nature (Bonelli 2019b, p. 34).

But there is a much more serious accusation that was addressed against Castellani: that of having covered and minimized with his prestige and undisputed expertise one of the most negative and darkest episodes of the war, the use of gases and chemicals against the enemy troops and even to terrorize the civilian population.

As is well known, asphyxiating or stinging gases had been used on a large scale since the First World War, and, due to their devastating effects, they were considered, at least emotionally, much worse than traditional weapons. The strong opposition of world public opinion had pushed the League of Nations, since 1925, to propose an international treaty, still in force today, which banned any use of chemical or bacteriological weapons. Italy ratified the treaty unconditionally in 1928, while other countries, such as France and Great Britain, had signed it with the reservation that it was valid only towards the other signatory nations and on condition that such weapons were not used first by the enemies. For this reason too, all the nations had in fact continued to study and produce chemical weapons of varying nature (Del Boca 2021, pp. 70–71).

It has now been widely demonstrated that the use of gases was part of the Italian military culture of the 1930s – even though there was not always a unity of views on their effectiveness – and that they were used, and not only sporadically, during

the military campaign in Ethiopia, with full knowledge and agreement of the entire chain of command, from Mussolini to Badoglio (Del Boca 2021).

The use of mustard gas by the Italian Air Force became systematic as early as the end of 1935 to stop the Abyssinian counter-offensive on Macallé (Del Boca 2001, vol. 2, p. 490), and, although Badoglio tried to keep gas operations secret (Del Boca 2001, vol. 2, p. 493), it is hardly credible that Castellani was completely in the dark about something that was beginning to leak even in the international press. Yet, as we said, he stubbornly stated in his autobiography:

> There were accusations in the foreign press that in the battle of Makalle Badoglio used poison gas: he did not (Castellani 1960, p. 148).

Another testimony of the time brings up the name of Castellani on the question of gas and seems to attribute to him one of the propaganda tricks used by the fascist regime to reject the accusations of using chemical weapons. Emilio Faldella, head of the Ethiopia Section of the Military Information Service (SIM), the first Italian military intelligence agency operational between 1925 and 1945, remembered this episode dating back to the first months of the war:

> Taking advantage of the short stopover that the Cairo-London postal plane made in the airport of Centocelle, our agents examined the bags of correspondence. One day they discovered in the package that an English journalist was sending to a London press agency some suspicious photos depicting some Abyssinians covered with plagues. A few minutes later the photographs were on my table, and shortly after on that of Professor Castellani, at that time the best specialist in tropical diseases. Castellani observed the photos and replied that there could be no doubts: the photographed Ethiopians had been hit by blistering liquids. We looked at each other with mutual embarrassment. Then the professor added that lepers also have the same appearance, and he showed me some photographs as proof. Faced with this resemblance, I made a sudden decision. The photos will be delivered to London, but not the authentic ones, the others reproducing lepers. And when, a few days later, the English newspapers published the "tragic images", our ambassador to London, Dino Grandi, had a good time proving that it was just an "ignoble trick" hatched by anti-fascist propaganda (Del Boca 2001, vol. 2, p. 494).

If this episode is true, it certainly does not weigh well on Castellani's balance of merits. Far from it. Even without having direct responsibility, the Inspector General of the Military and Civil Health Services for East Africa lent himself to the propaganda game of the fascist regime and, even more seriously, did nothing to oppose or condemn an inhumane practice that stood at the antipodes of the humanitarian role required of a "doctor."

Despite this, for Castellani the late 1930s coincided with the apex of the prestige and admiration bestowed on him almost universally (Fig. 4). He was given credit for having been the main architect (if not the only one) of a historic reversal. For the first time in the modern era, in a large-scale military operation, mortality from disease had not exceeded that from war wounds. Indeed, it had remained significantly lower (Bonelli 2019a, pp. 292–295).

Fig. 4 Aldo Castellani's portrait by Spiro Tudor (1936). Entrance hall of Aldo Castellani's clinic in Rome. (Photo courtesy of Vincenzo Martines)

9 Conclusions

To trace back the results of something as huge as a military campaign to the actions of a single exceptional man is often naive and always misleading.

At the same time, the outstanding healthcare and medical results of the Italian army during the Ethiopian War – in a good measure thanks to Castellani's expertise – today appear undeniable, even if they should be framed and measured against the background of a war effort that was, overall, exceptional and to a certain degree unprecedented (Labanca 2015, pp. 131 ff.). Thanks to his global vision and his international connections, Castellani was able to put to the advantage of the Italian army the enormous progress that medicine in general, and tropical medicine in particular, had made during the first decades of the twentieth century and, in particular, during and after the First World War (Cook 2007).

His personal and scientific success was therefore real, even if indirectly it only fed the self-referentiality and arrogant self-sufficiency of the fascist regime which, by now, could find a compliant interlocutor only in the other totalitarian regime, whose shadow was beginning to spread all over Europe: Nazi Germany.

On a personal level, that success earned Aldo Castellani the hereditary noble title, prestigious at the time but purely formal, of Count of Kismayo, in honor of the Somali port town we have repeatedly referred to (Anonimo 1936). It also created many misunderstandings and resentments in that Anglo-Saxon world to which he had been linked for decades, both professionally and personally. A famous British lieutenant-general, Sir Adrian Carton de Wiart, who met Castellani while he was imprisoned in Italy during the Second World War, referred ungenerously to the Italian doctor in his war memoirs:

> Castellani owed much to England where, besides earning fame and gathering a knighthood, he had also gleaned a great deal of money, but from the time of the Abyssinian campaign his allegiance had swerved and he had become completely anti-British (Carton de Wiart 2007, p. 211).

In short, it seemed a good time to reconstruct this story just when the most recent Italian historiography on the Ethiopian War and on Italian colonialism in general appears to have forgotten, or largely underestimated, this medical and healthcare component (Labanca 2015; Calchi Novati 2021).

Among the few Italian doctors of that time to speak and write fluently in English, Aldo Castellani would have struggled greatly after the Second World War not to be overwhelmed, like so many others, by the collapse of Mussolini's regime. In addition to his professional prestige, he was saved by his closeness to the royal family, and above all to the last Queen of Italy, Marie-José, who had been so close to him during the Ethiopian campaign but whose reputation was rehabilitated in time thanks to an ever more open and courageous opposition to fascism (Regolo 2013). It will be alongside her and her husband, the exiled King Umberto II, that Castellani will spend the last decades of his long life as an exile himself in Portugal.

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Biofilm-Associated Infections in Chronic Wounds and Their Management

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Abstract

Chronic wounds including vascular ulcers, diabetic ulcers, pressure ulcers, and burn wounds show delayed progress through the healing process. Some of their common features are prolonged inflammation, persistent infection, and presence of biofilms resistant to antimicrobials and host immune response. Biofilm formation by opportunistic pathogens is a major problem in chronic wound management. Some of the commonly and traditionally used chronic wound management techniques are physical debridement and cleansing. In recent years, novel techniques based on anti-biofilm agents are explored to prevent biofilm-associated infections and facilitate wound healing. In this chapter, the role of biofilms formed by the ESKAPE pathogens (*Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*) and *Candida* species in delayed wound healing have been discussed. The current and emerging techniques in the detection of biofilms for the management of wounds have been focused. The limitations of the existing therapeutics and novel wound management strategies have been deliberated.

Keywords

Anti-biofilm agents · Biofilm-associated infections · Chronic wounds · ESKAPE pathogens

1 Introduction

Chronic wounds include vascular ulcers (e.g., venous and arterial ulcers), diabetic ulcers, pressure ulcers, and burn wounds which take more than 4 weeks to heal (Frykberg and Banks 2015). Infection of wounds with pathogen or their biofilms leads to wound chronicity. Due to persistent infection, wound healing gets delayed as there is a marked inflammatory response and tissue repairing is slowed preventing timely resolution. Delayed wound healing also depends on wound etiology, size of the wound, tissue location and environment, host immune response, and presence of biofilm-forming microbial species (Bjarnsholt et al. 2008). Biofilm-infected wounds show delayed closure. Under the in vitro conditions,

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microbial cells attach to the abiotic substratum, colonize it, and form a three-dimensional complex biofilm structure within EPS matrix (Bjarnsholt et al. 2013). However, in clinical forms such as the wounds, biofilms are seen as aggregates enclosed in EPS adhering to the host factors and wound tissues (Donelli and Vuotto 2014). In vivo biofilms are polymicrobial, interact with host tissue, and are tolerant to antimicrobials and host immune response (Roy et al. 2014).

A normal wound life cycle includes a trauma, an inflammatory phase that includes innate and acquired immune responses from the host, a regenerative phase and healing of the wound. The process of healing involves recurring phases along with numerous signaling mechanisms and immune components leading to a healed wound. In case of chronic wounds, the inflammatory stage is prolonged, and the repeated trauma on the wounded sites enables the infection to spread deeper into the tissues and delays healing. In the inflammatory phase of chronic wounds, the biofilm is constantly interacting with the host's immune system (Thurlow et al. 2011; Zhao et al. 2012; Xu et al. 2013). The most important phase of any wound is the inflammatory stage which involves various immune components and inflammatory cells like macrophages (M1 proinflammatory type and M2 the reparative type), inflammatory cytokines, and neutrophils which remove the damaged skin components and bacterial contamination from the wound bed and prepare them for forming granulation tissue which is required for the wound to heal properly. These inflammatory cells also recruit various other signaling molecules and cell components that produce new tissue components like connective tissues and blood vessels. The involvement of bacterial cells in the wound prevents all the processes involved in the healing process, and as the bacterial population increases, the tissues of the wound bed and its surroundings weaken and get dominated by the biofilms which actively prevents the immune cells from contacting the wound surface, thus delaying the healing process (Fig. 1). For instance, the quorum sensing signaling molecules in *Pseudomonas aeruginosa* induces chemotaxis of the neutrophils. In cases of *Staphylococcus aureus* biofilms, the matrix tends to attract the dormant microbicidal activity in the host. Such interactions between the host and bacteria in biofilms constantly hinder the healing process and contribute to the complex and non-healing of chronic wounds.

2 Clinical Evidence of Bacterial Infection and Biofilm Formation in Chronic Wounds

WHO has listed *S. aureus*, *Klebsiella pneumoniae, Acinetobacter baumannii*, and *Pseudomonas aeruginosa* as a part of the 'ESKAPE' pathogen. They are opportunistic pathogens involved in community-acquired and most recently in severe nosocomial infections. *P. aeruginosa* and *S. aureus* have been extensively studied in nosocomial infections, implant-associated infections, and chronic wounds. The ability of pathogens to form biofilms and be resistant to all known classes of drugs makes them highly prevalent in biofilm-associated infections (Maslova et al. 2021). *K. pneumoniae* are usually associated with pneumoniae, liver abscess, bacteremia, endophthalmitis, meningitis, urinary tract (UTIs), and wound/soft tissue infections, whereas the *A. baumannii* cause infections in the blood, UTIs, and wound (Perez et al. 2007; Higgins et al. 2009). The MDR *A. baumannii* and *K. pneumoniae* colonize in immunocompromised people, usually resulting from post-surgery complications or due to prevailing disease conditions. The ability of these pathogens to cause infections in various parts of the body is facilitated by their ability to adhere to diverse surfaces including tissues or catheters. Up to 13% of infections caused by *K. pneumoniae* are wound/surgical site-related infections (Magill et al. 2014). *A. baumannii*, on the other hand, are usually associated with burn wounds. Burn wounds are frequently infected faster as the integrity of the skin cells and tissues are vastly affected. The microbial flora endogenous to the patient's body colonizes and infects the wounded areas. The biofilm formation is the one main cause for non-healing burn wound conditions

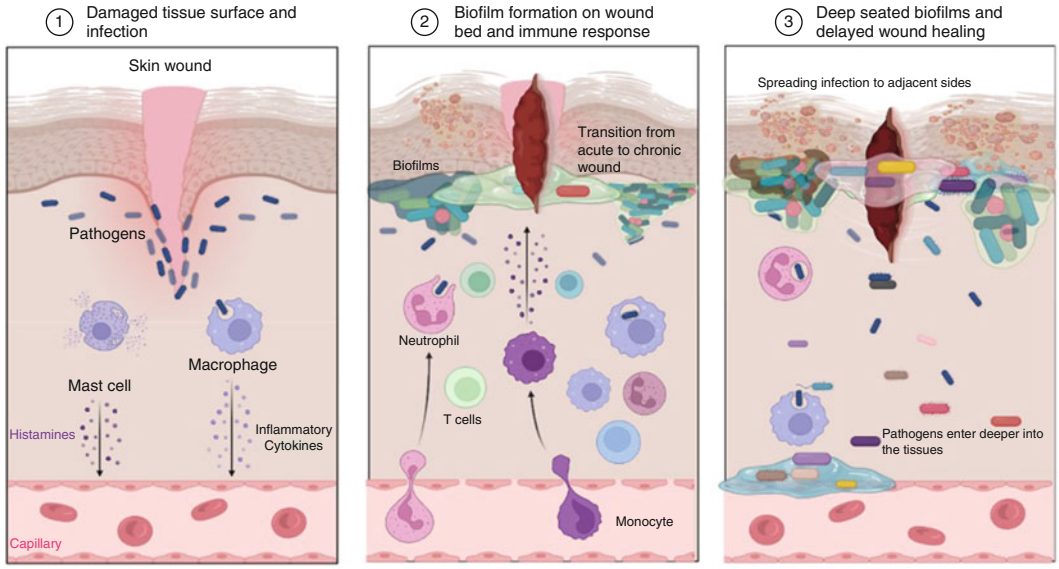

Fig. 1 Biofilm formation in chronic wounds. The growth of biofilms on wound and abrasions occurs in stages and the immune cells modulate wound healing by promoting cellular cross-talk via secreting signaling molecules, including cytokines, chemokines, and growth factors, and for a successful wound healing, a delicate balance in this process needs to be maintained which is delayed when the pathogens begin to develop biofilms. Stage 1: The initial damage in the skin tissues leading to a wound and infection by pathogens which triggers the primary immune response, that is, the macrophages, inflammatory cytokines, and mast cells. Stage 2: Development of biofilms in and around the wound bed with elevated levels of T-cells and B-cells along with monocytes, neutrophils, and PMNs. Here the virulence factors of the pathogens play an important role by their stress response mechanisms and development of thick slimy matrix called biofilms which act as a protective layer against the immune cells of the host. Stage 3: Pathogens dominate over the host's primary immune response and which initially was a basic tissue damage turns into a non-healing chronic wound and now becomes a challenge to control. There is a significant reduction in the immune cells, and the pathogens are seen to be penetrating deeper into the tissues and at the same time the infection spreads to adjacent surfaces increasing the challenge to tackle such chronic infected wounds

which also induces chronic inflammations (Wolcott 2015). *K. pneumoniae* is also isolated from wounds of burn patients (Keen et al. 2010). An epidemiological study has shown that 15.1% of *K. pneumoniae* hospital isolates are from cutaneous lesions (Lee et al. 2017). The hypervirulent phenotypes and aggressive capsular serotypes of *K. pneumoniae* strains are frequent in severe skin and soft tissue infections (SSTI) (Keen et al. 2010). Similarly, *A. baumannii* are associated with soft tissue infections due to mechanical trauma and are isolated from severe burns or wounds (Davis et al. 2008; Johnson et al. 2007). *A. baumannii* have been reported in osteomyelitis and in the skin and soft tissue infections (Vanegas et al. 2015). Biofilms produced by these pathogens act as important virulence factors and provide a protective atmosphere for them to survive. It has been proven that biofilms are involved in chronic infections such as cellulitis, necrotizing fasciitis, and diabetic foot ulcers (Hassan et al. 2011).

Open wounds due to the absence of protective skin covering have microbes from both the endogenous or exogenous sources. During the initial stages of chronic wound formation, the host's immune cells either kill or restrict the proliferation of these microbes (Percival et al. 2015a). In certain situations, the microbes adhere to the wound surface and start proliferating resulting in biofilm formation. Once the biofilm develops, it will resist the antimicrobial action and host immune response. As the biofilms mature, they are difficult to be treated or eradicated and the wound is now

considered to be in biofilm infected state. At this stage, a specialized treatment regime is required to manage the wounds. Biofilm formation delays wound healing and inflammation increases in clinically infected wounds (Percival et al. 2015b). Therefore, prevention of biofilm formation is more effective than treatment of chronic biofilm-infected wounds. Here, we present instances and clinical evidence of biofilm formation in wounds and their role in delaying wound healing.

The early evidence of biofilm formation in wounds were obtained by in vivo studies. In one such study, the presence of biofilm in wounds were confirmed in cyclophosphamide-treated mice inoculated with *S. aureus* on the cut wounds in the skin. Biopsy specimens stained with ruthenium red staining and examined by microscopy showed *S. aureus* with fibril-like structures colonizing cut wound and entering into the subcutaneous tissue within 1 h. Further, the cells enclosed in membrane-like structures were observed at 3 h and these formed clusters of colonies by 6 h. Within 12 h, the membrane thickness increased and the inflammatory cells, polymorphonuclear leukocytes, and macrophages surrounded the clusters at the end of the 36 h. Finally at 60 h, there was degeneration and necrosis of the host tissues. Thus, *S. aureus* formed biofilms in dermal or subcutaneous tissues in a neutropenic condition (Akiyama et al. 1996). The role of bacterial biofilms in wound colonization and infection was studied using a porcine model. A wound isolate *S. aureus* was inoculated into partial thickness wounds and further treated with the topical agent mupirocin antimicrobial cream and triple antibiotic (neomycin, bacitracin, and polymyxin) ointment. After 48 h of inoculation, biofilms in wounds were observed using light, scanning electron, and epifluorescence microscopy. Though the topical agents effectively reduced planktonic *S. aureus,* they had limited efficacy in treating biofilms. The study demonstrates *S. aureus* ability to form firmly adherent microcolonies and extracellular matrix on wounds and exhibit resistance to antimicrobials in comparison to their planktonic phenotype (Davis et al. 2008). The biofilms' role in delayed healing was demonstrated in a murine cutaneous wound model. Delayed re-epithelialization by *S. aureus or S. epidermidis* biofilms was observed in a splinted cutaneous punch wounds in normal C57Bl6/J mice. Re-epithelialization was considerably delayed due to biofilms, which was further confirmed by using controls including mutants deficient in biofilm formation and peptides inhibiting biofilm formation (Schierle et al. 2009). The presence of biofilms in the pathogenesis of chronic wound was confirmed in a quantitative animal model. Green fluorescent protein (GFP)-labeled *S. aureus* were inoculated into dermal punch wounds prepared on New Zealand rabbit ears. Within 24 h post inoculation, mature biofilms and persistence of viable *S. aureus* at different time intervals post wounding were observed. Further, the presence of inflammatory markers, persistent low-grade inflammatory response, and decreased epithelial migration and granulation of tissue confirmed the role of biofilms in chronic wound pathogenesis (Gurjala et al. 2011). Methicillin-resistant *Staphylococcus aureus* (MRSA) have shown to form dense biofilm communities on wound surface after 24 h of inoculation in a murine superficial infection wound biofilm model. Treatment with topical antimicrobial agents had more efficacy at 4 h after inoculation in comparison to 24 h post inoculation (Roche et al. 2012a). Delayed wound healing by MRSA has been confirmed in non-healing ulcers using a porcine model. MRSA strain was isolated from healing impaired wounds and used for inoculating in subsequent studies. On examining wound biopsy samples microscopically, multiple microcolonies of bacteria were observed. The passaged MRSA colonized faster and formed larger sized biofilms than the parent MRSA (Roche et al. 2012b).

The *P. aeruginosa* bacterial biofilms in wounds were observed in an in vivo pig model. *P. aeruginosa* were inoculated into partial-thickness wounds made on three pigs with a dermatome and covered using polyurethane or plastic cover slip. At 72 h, wounds were flushed thrice with sterile saline to remove non-adhering bacteria and treated with surfactant solution by

scrub technique. Both the flushed and scrubbed samples were inoculated on *Pseudomonas* isolation agar. The cover slips and wound curettage were removed and stained with Congo red to detect EPS. It was observed that the EPS was present as a thick, dark red to yellow-orange amorphous matrix confirming the presence of biofilm. Wounds treated with saline or surfactant showed two distinct populations of bacteria in the wound with the non-adherent population having quantitative variation in wounds, whereas the adherent bacteria required a critical mass (Serralta et al. 2001). Both wildtype and quorum sensing (QS)-deficient mutant *P. aeruginosa* strains inoculated in a thermally injured mice formed biofilms within 8 h of inoculation. Electron and confocal scanning laser microscopy showed colonization of cells in burned tissue, and blood vessels and adipose cells in the vicinity. The study showed that *P. aeruginosa* independent of QS mechanism forms biofilms in specific host tissues (Schaber et al. 2007). Previously, it was shown that PAO-R1, a lasR isogenic mutant which is defective in the virulence factors LasB and LasA elastase synthesis and produces exotoxin A and alkaline proteases at low concentrations, was less lethal compared to the parent strain, PAO1, in a burned mouse model. However, after 8 h of inoculation, both the strains were found in equal numbers at the inoculated site, but less numbers of PAO-R1 were found to disseminate to the liver and spleen in comparison to PAO1 microorganisms. It is implied that the QS-regulated lasR gene is involved in its dissemination from the infected to the other regions (Rumbaugh et al. 1999). GFP-tagged *P. aeruginosa* biofilms in full-thickness wounds were observed in the backs of Sprague-Dawley (SD) rats as early as 8 h post infection as confirmed by fluorescence microscopy studies (Kanno et al. 2010). *P. aeruginosa* PA01 biofilms in wounds of diabetic mice were studied. It was inoculated into biopsy wounds (6 mm punch) of diabetic (db/db) mice 2 days post wound forming and dressed using semiocclusive for 2 weeks. After 28 days, control wounds were epithelialized, whereas the ones with biofilms failed to close. On further examination, extensive inflammatory response, necrosis, and hyperplasia were found in biofilm-infected wound samples. Bacteria were predominant in the scabs above the wound bed and were fewer in number within the wound tissue (Zhao et al. 2010). The colonization and biofilm formation of *P. aeruginosa* in SD rats and Walker-Mason rat scald burn model have been confirmed. The burn wounds inoculated with *P. aeruginosa* were sampled on 1st, 3rd, 7th, and 11th day. Robust biofilms were observed. The expression of genes involved in alginate and pellicle production which are the constituents of biofilm matrix were upregulated within the burn eschar, while those of proteinases and siderophores were upregulated in the wound tissues. There was no recorded response of neutrophils to the pathogen in the eschar and circulating blood in comparison to control burn (Brandenburg et al. 2011). Similarly, *P. aeruginosa* PAO1 biofilms have been observed in chronic wound due to third-degree burn in C3H/HeN and BALB/c mice. Post 4 and 7 days of PA01 infection, polymorphonuclear cells were predominant with high degree of inflammation. PNA-FISH and DAPI staining confirmed the presence of clusters and inflammation in the vicinity of the inoculated site. The wounds in BALB/c mice had higher PAO1 burden initially and interleukin-1beta concentration than in C3H/HeN mice at day 7 (Trostrup et al. 2013). In another study, PA01 biofilm formation resulted in repressed neutrophil response in wounds, while C3H/HeN mice exhibited systemic inflammatory control and showed faster healing than the BALB/c mice (Trostrup et al. 2017). VEGF levels were suppressed in BALB/c wounds when compared to C3H/HeN wounds, after 4 and 7 days of infection. Further, PMN-mediated inflammation and necrosis was detected in BALB/c wounds. *P. aeruginosa* biofilm suppresses central but induces peripheral VEGF levels and thus modulates wounds and reduces host response which could lead to biofilm-infected necrosis (Trostrup et al. 2018).

Multiple bacterial species in chronic wound biofilms have been demonstrated but with limited understanding of such microbe interactions and infections. *S. aureus* UAMS-1 strain and *P. aeruginosa* PAO1, or both, were inoculated

into dermal punch wounds (6 mm) in New Zealand rabbit ears. *S. aureus* UAMS-929, a biofilm-deficient mutant, was used in mixed-species infection. In the polybacterial wounds, both the wildtype formed biofilms but PAO1 were predominant. In comparison to the monospecies infection, polyspecies biofilm infection delayed healing and increased IL-1β and TNF-α expression. UAMS-929 showed less wound impairment and decreased cytokine expression. Thus, in comparison to monospecies infections, mixed-species biofilms have a significant influence on wound healing dynamics (Seth et al. 2012a). In an in vivo experiment, biofilm formation between species in chronic wound pathogenesis have been studied. In New Zealand white rabbit ears, dermal punch wounds were made and inoculated with *K. pneumoniae, S. aureus, P. aeruginosa*, and an EPS-deficient *P. aeruginosa*. Though all species formed biofilms in the wounds, the presence of biofilms of *P. aeruginosa* in wounds impaired healing and increased inflammatory response. EPS-deficient *P. aeruginosa* had less influence on the similar endpoints in comparison to the biofilm forming strains (Seth et al. 2012b). *P. aeruginosa* PA01 inoculated in 2-day-old wounds in diabetic db/db mice formed biofilms and delayed wound healing by 4 weeks, while the control wounds without biofilm healed by 4 weeks. It was observed that 64% of the biofilm-inoculated wounds healed in 6 weeks. In the healing process, with the decrease in *P. aeruginosa* numbers, the *S. aureus* population increased. *P. aeruginosa* was predominant in scabs than in wound beds. In case of delayed wound healing, tissues, proliferative epidermis, lacking vascularization and high concentration of inflammatory cytokines and hypoxia-inducible factors were observed (Zhao et al. 2012).

Diabetic and non-diabetic mice were infected with *P. aeruginosa*. Wounds exhibit delayed clearance and closure in diabetic mice. Though administration of insulin enhanced the health of diabetic mice, it could not clear infections or improve wound healing. The insulin-treated diabetic mice had a higher prevalence of *P. aeruginosa* biofilms with increased tolerance to gentamicin treatment. The study emphasizes the diabetic wound environment in promoting biofilm formation and ability of insulin to influence antimicrobial treatment (Watters et al. 2013). Similar studies in TallyHo mouse, a polygenic model of type 2 diabetes, showed that biofilm infection in its wounds resulted in reduced expression of TLR 2, TLR 4, IL-1β, and TNF-α. Though bacterial numbers and neutrophil intrusion in biofilms-infected wounds post 3 days wounding were same, neutrophil oxidative burst activity was lesser in diabetic wounds. After 10 days, the bacterial burden increased in biofilm-infected wounds with delay in epithelization. It has been implied that the failure to recognize infection by the TLR pathway resulted in low cytokine production. Further, the lack of host immune response increased the susceptibility of diabetics to chronic wound infection and ulceration (Nguyen et al. 2013). Wound healing is delayed in diabetic mice treated with insulin. In db/db diabetic mice with *P. aeruginosa*-infected chronic wounds, the mechanism by which insulin treatment interferes with c-di-GMP signaling has been studied. In the nondiabetic mice, the high levels of intracellular c-di-GMP increased biofilm formation in wounds. However, in the wounds of insulin-treated diabetic mice, biofilm formation was more significant compared to untreated diabetic mice or nondiabetic mice. In wounds inoculated with the low c-di-GMP expression strain PAO1/P_{lac}-*yhjH*, there was an increase in IL-4 RNA expression in diabetic mice treated with insulin than in untreated diabetic or nondiabetic mice. Similarly, an early and high expression of IFN-γ was observed only in insulin-treated diabetic mice. It was inferred that insulin influenced biofilm development by increasing c-di-GMP levels and the inflammation period causing delayed wound healing (Wei et al. 2019).

A cyclophosphamide-induced neutropenia murine wound model was used to study the biofilm behavior of MDR *A. baumannii* in wounds. Full-thickness wound in the skin covering thoracic spine was prepared and dressing was applied for 7 days. The wounds infected with *A. baumannii* and treated with placebo did not heal beyond 21 days, whereas uninfected wounds

healed within 13 days (Thompson et al. 2014). Similarly, in cyclophosphamide-induced neutropenia skin and soft tissue infection (SSTI) porcine model, inoculation with an extensively drug-resistant strain *A. baumannii* AB5075 showed that the pathogen persisted post 7 days of inoculation on the wound bed and in the dressing with proliferation up to 10^6 CFU/g ($\log_{10}6$). SEM and PNA-FISH confirmed biofilm formation in wound tissues (Zurawski et al. 2019).

Co-infection of *S. aureus* USA300 and *P. aeruginosa* in porcine partial thickness wound model led to the mixed-species biofilms delaying re-epithelialization by suppressing keratinocyte growth factor 1 expression and co-existence of both species in cutaneous wounds. In presence of *P. aeruginosa*, the virulence factors Panton-Valentine leukocidin and α-hemolysin of USA300 were induced (Pastar et al. 2013). In a similar study with porcine full-thickness chronic burn wound infected with *P. aeruginosa* PAO1 and MRSA *S. aureus* strain USA300, monospecies-affected wounds had hyper-proliferative edge, while coinfected wounds were large in size and showed high neutrophilic inflammation and retention of necrotic tissues (Chaney et al. 2017). Chronic wound specimens from 77 subjects having diabetic foot, pressure, and venous leg ulcers and acute wounds from 16 subjects were obtained. Molecular studies from chronic wounds confirmed the prevalence of diverse polymicrobial communities forming biofilms and strictly anaerobic bacteria that were not revealed by culturing technique to be present in high numbers (James et al. 2008). Similarly, in comparison to standard culturing methods, application of peptide nucleic acid-based fluorescence in situ hybridization (PNA FISH) for identification of bacteria in nonhealing or chronic wound showed the presence of *P. aeruginosa* aggregated as microcolonies in an alginate matrix (Kirketerp-Møller et al. 2008). Sections from chronic wounds of venous leg ulcers, pressure ulcers, and diabetic foot ulcers by FISH showed distinct microcolonies of *P. aeruginosa* (Bjarnsholt et al. 2008). Wound biopsy specimens from venous leg ulcers observed by PNA-FISH and confocal laser scanning microscopy (CLSM) showed bacterial aggregates of *P. aeruginosa* and *S. aureus* in these wounds. *P. aeruginosa* were located in the deeper regions of chronic wounds (Fazli et al. 2009). The bacteria in the wounds were present as large aggregates. *P. aeruginosa*-infected wounds had recruited more neutrophils in comparison to *S. aureus*-infected wounds (Fazli et al. 2011). Biofilms have also been visualized in ulcers of the burn wound by using carbohydrate-specific stains. Polymicrobial invasion of burn wound implies the need for timely excision and closure (Kennedy et al. 2010). CLSM examination of soft tissue samples from patients undergoing deep debridement revealed densely aggregated *S. aureus* and other bacteria within EPS matrix and surrounded by host-cell debris (Neut et al. 2011). Tissue samples obtained from chronic wounds of 15 patients by culturing technique revealed the presence of three frequently isolated bacterial species. While pyrosequencing confirmed bacterial diversity with an average of 17 genera. Data from microbial community profiling of chronic wounds have shown an increased presence of Gram-negative rods, Gram-positive cocci, and anaerobes with lower populations of *Propionibacterium* (Han et al. 2011). Wounds are predominant with facultative and obligate anaerobes, while only 36% are aerobes. Some of the strict anaerobes frequently isolated are *Fusobacterium magna*, *Anaerococcus vaginalis*, and *Peptoniphilus indolicus*. The presence of these microbes is dictated by hypoxic or anoxic and the reduced redox potential in the wounds (Vuotto and Donelli 2015). Samples from chronic wounds in diabetic mice have shown that high oxidative stress (OS) levels play a critical role in initiation and development of biofilms and chronic wound (Kim et al. 2019). Diabetics chronic wounds have high OS levels. The healing and chronic wound microbiome in a *db/db* $^{-/-}$ mouse model was sequenced for ITS gene. Healing wounds had diversified and dynamic microbiome which did not develop into bacterial biofilms. *Cutibacterium acnes, Achromobacter* sp., *Delftia* sp., and *Escherichia coli* were predominant in healing wounds. Whereas chronic

wounds with high OS levels had less bacterial diversity and were colonized by biofilm forming *P. aeruginosa, Enterobacter cloacae, Corynebacterium frankenforstense*, and *Acinetobacter* sp. *P. aeruginosa* and *Staphylococcus xylosus* were prevalent during early injury. Thus, high OS in the wound alters and reduces diversity in bacterial wound microbiome and promotes establishment of bacteria derived from the skin microbiota (Kim et al. 2020). Biopsy samples from non-infected chronic venous leg ulcers revealed the identity and localization of bacterial species in multi-species biofilms and the predominant species was *P. aeruginosa*. Use of CLSM showed individual species clustering in a mixed-species biofilms, whereas FISH helped in determining bacterial load (Malic et al. 2009). The polymicrobial bacterial infection in contributing to wound chronicity was determined by dynamics and temporal analysis of chronic wounds (n = 167). Microbial communities shifted from *Pseudomonas* or *Staphylococcus* predominant to a highly variable one. Further, 80% of wounds had common or dominant species infecting wounds at subsequent time points which were low during early sampling time (Tipton et al. 2017). The role of microbiomes in wound chronicity has been studied in a two-cohort microbiome genome investigation. TLN2 and ZNF521 genotypes are involved in inter-patient variation of *P. aeruginosa* and *S. epidermidis*. In *P. aeruginosa*-infected wounds, microbial diversity is lowest. In future therapy and precision medicine, the identification of genetic determinants for wound microbiomes in patients will be useful as genetic variation determines cellular adhesion phenotypes (Tipton et al. 2020). Similarly, microbiomes in healer and non-healer MRL/MpJ (MRL) mice were studied. Further, the transfer of healing phenotype was evaluated by gut microbiome transplantation (GMT). The C57BL/6 J (B6) mice with an earpunch of 2.0 mm was orally administered with MRL/MpJ cecal contents during weaning and as adults. B6 mice with MRL-transplanted microbiota had enhanced ear hole closure and MRL-vehicle mice healed well when compared with B6-vehicle mice which healed poorly. GMT prior to ear punch led to significant ear hole closure. Further, the offsprings of transplanted mice healed faster control mice. Several microbiome clades found included Firmicutes, Lactobacillales, and Verrucomicrobia. Transplantation of MRL mice with B6 cecal had no effect on MRL healing. Thus, microbiome has a role in tissue regeneration in MRL mice and healer trait can be incorporated in non-healer mice by GMT (Velasco et al. 2021).

3 Diagnosis and Management of Biofilms in Chronic Wounds

Considering both the visual and clinical indicators, an algorithm has been designed to detect the wound biofilms which is hoped to facilitate the recognition of the biofilms and lead to optimal management of chronic wound infections. The simple algorithm can guide clinicians for designing strategies for treating nonhealing wounds. The algorithm takes into account many factors such as if the wound is failing to heal, if appropriate clinical diagnostics and procedures are undertaken, presence of slough or necrotic tissues, signs of local infection or inflammation, wound responds to topical and systemic antimicrobial intervention. Based on the assessment, if there are signs of role of biofilms in the wound infection, biofilm-based management is initiated which includes periodic wound debridement and appropriate antimicrobial therapy. Otherwise, the treatment is continued with standard protocol of care. The algorithm needs to be applied after addressing other wound-related factors. It should be used along with clinical finding and standard protocols of care (Percival et al. 2015c). Guidelines for treatment of chronic wounds having biofilm infections are necessary to aid in identifying biofilms for their effective management and improving patient care. Recognition of biofilms in chronic wounds will help clinicians in managing patients optimally. A stepped-down treatment approach will help the clinicians in wound management (Schultz et al. 2017). In Table 1, we have listed certain standard procedures and techniques involved in

Table 1 Detection and diagnosis of biofilm-associated wound infections

Methods	Techniques	Advantages	Limitations	References
Microbiological assays	Culture techniques	Standard, well-established techniques. Easy and routinely used	Possibility of manual errors. Not suitable for detecting biofilms present in deeper layers of tissues	Li et al. (2014)
	Sonication	Mechanical transfer of cultivable bacteria; highly sensitive compared to tissue sampling; does not identify non-biofilm forming bacteria	Lower specificity compared to tissue sampling techniques and is time consuming.	Trampuz et al. (2007)
	Dithiothreitol treatment	Chemical transfer of the (cultivable) bacteria from the surfaces of prosthetics and catheters etc.; specific and sensitive; easily available; efficient in identification of *S. epidermidis*; more efficient than sonification; can differentiate between viable and non-viable bacteria	Fails to identify the peri-prosthetic infections	Drago et al. (2013)
Molecular assays	PNA-FISH	Is able to detect VBNCs (viable but nonculturable) colonies; Rapid detection (<24 h); highly accurate (16s RNA based identification)	Does not distinguish between host and pathogen antigens; Cannot distinguish between planktonic and biofilm-forming bacteria	Cerqueira et al. (2008)
	16 s rRNA PCR	Rapid detection; several VBNCs can be identified	The antibiotic sensitivity cannot be determined; cannot differentiate between viable and non-viable bacteria and also between planktonic and biofilm-forming bacteria	Davies et al. (2004)
	FRACS, PRADS, PRAPS	Can identify various VBNCs; rapid detection(<24 hours)	Cannot differentiate between the viable and non-viable bacteria; sensitivity of the antibiotic cannot be determined	Brandenburg et al. (2011)

(continued)

Table 1 (continued)

Methods	Techniques	Advantages	Limitations	References
	RNA sequencing	Highly sensitive and precise; can differentiate between species in mixed biofilms; can differentiate between biofilm forming and planktonic bacteria; can detect antibiotic sensitivity	Possible manual errors; needs expert handling	Pozzi et al. (2012), Bauer et al. (2013), Tan et al. (2015), Castro et al. (2017), Garalde et al. (2018)
	Tag-encoded FLX amplicon pyrosequencing	Targets the 16S rRNA; can identify multiple VBNC colonies; rapid detection (<24 h)	Cannot distinguish between viable and non-viable and biofilm-forming and planktonic bacteria; cannot detect antibiotic sensitivity	Dowd et al. (2008)
Imaging assays	CLSM and SEM	Is a non-invasive method; can detect biofilms in biopsy tissues; suitable for surface biofilm identification	Expensive, high maintenance; and needs experts for handling	Brandenburg et al. (2011)
Sensor based assays	Bacterial species specific and EPS sensors	Can rapidly identify bacterial species in a biofilm	Specific to only one species; cannot identify all pathogens in cases of mixed culture biofilms	Ngernpimai et al. (2019), Anju et al. (2021)
	Environmental sensors	Can rapidly identify bacterial species in <1 h	Not useful in cases of mixed culture biofilms	Ngernpimai et al. (2019), Poma et al. (2020), Anju et al. (2021)
	Enzyme sensors	High sensitivity; rapid detection (<1 h)	Does not detect dynamic processes; unable to distinguish between active and inactive stages of biofilms	Ciani et al. (2012), Poma et al. (2020), Mota et al. (2021)
Morphological assays	Tissue sampling	Reliable method for pathogen identification; and can access deeply established biofilms through biopsy	Does not differentiate between viable and non-viable pathogens	Rhoads et al. (2012), Bjarnsholt et al. (2013)
	Wound blotting (nitrocellulose membrane)	Non-invasive; precise evaluation of wound beds; absorbs proteins and mucopolysaccharides onto the membrane for analysis; highly sensitive	Time consuming; cannot differentiate between viable and non-viable bacteria	Schultz et al. (2017), Minematsu et al. (2013), Kitamura et al. (2014), Percival et al. (2015), Nakagami et al. (2017)

diagnosing chronic wound biofilms. Further, in this section, we will discuss clinical evidence based conventional and novel non-conventional strategies for managing biofilm infections in chronic wounds.

Silver when comes in contact with the moisture in wound is ionized into its active form which is responsible for antimicrobial properties. Silver denatures bacterial proteins by attacking the groups like the thiol, imidazole, and sulfhydryl groups by forming reactive oxygen species (ROS) which in turn oxidizes DNA precursors. Silver at 100–150 g/L inhibits *P. aeruginosa* and *S. proteamaculans* biofilms. Therefore, silver is used in the wound dressings along with iodine (Percival et al. 2016). Silver has high growth-inhibitory activity in planktons under both in vitro and in vivo conditions. Similarly, its antimicrobial efficacy on in vitro biofilms has also been noted. However, its application in wound dressing for clearing biofilms in in vivo environment and in the chronic wound environment requires clinical evidence (Percival and McCarty 2015). Similar to silver, iodine binds to the thiol and sulfhydryl groups, blocks the electron transport chain, and decreases oxygen supply for the aerobes. The povidone iodine (PVP-I) formulation can completely eradicate biofilms of a 7-day mixed *Pseudomonas* and *Staphylococcus* cultures (Percival et al. 2016).

PHMB has previously exhibited microbicidal activity in chronic wounds and burns. The efficacy of a PHMB-containing biocellulose dressing, Suprasorb X + PHMB, was evaluated for biofilm treatment in non-healing wounds. In this cohort study, 28 patients with non-healing wounds that were locally infected or highly colonized showing clinical signs of biofilm were treated with Suprasorb X + PHMB dressing. At 24 weeks, around 12 wounds showed complete healing, epithelialization, and no drainage. Ten patients showed reduction of the biofilm and all reported reduced pain after using dressing. Thus, PHMB in biocellulose dressing reduced biofilm formation in the chronic wounds and promoted healing (Lenseilink & Andriessen 2011). Irrigation solution of propyl-betaine and polyhexanide (PHMB), Ringer's solution, octenidine dihydrochloride, hypochlorous acid, and octenilin were tested on biofilms of MRSA in a porcine wound model. Wounds that were inoculated with MRSA were dressed with polyurethane for 24 h and observed for biofilm development. The wounds were irrigated twice daily for 3 days. In comparison to all the solutions, PHMB irrigation of wounds reduced biofilm formation in MRSA by 97.85% and 99.64% at 3 days and 6 days, respectively (Davis et al. 2017). PHMB solution has been used as wound cleanser to reduce bacteria and biofilms in venous leg ulcers. The randomized study was performed in 44 patients with venous leg ulcers for 6 weeks. In the intervention group, wounds were cleaned with PHMB, whereas 0.9% saline solution was used in control group. Tissue fragments showed that though both PHMB and saline solution reduced bacterial infection in venous leg ulcers, however, biofilms were not reduced on cleaning with either of the solutions (Borges et al. 2018).

Recent studies in wound care and biofilm management confirm that PVP-I has broad antimicrobial activity, good antibiofilm activity with no resistance, and lower cytotoxicity and promotes wound healing in comparison to PHMB and silver. Thus, PVP-I is a good therapeutic for treating biofilm infection in wounds (Alves et al. 2021).

Surfactant-based wound gel was evaluated on a tolerant 3-day biofilm in an ex vivo porcine skin explant model. Dressings were done with the gel directly or with moistened gauze daily. Daily wiping with moistened gauze initially decreased bacterial load, but biofilm was observed on the third day. Whereas, the application of surfactant-based wound gel eliminated functional biofilms in the tissue sample. Further, clinical evidence is required for their usage in clinically infected chronic wounds (Yang et al. 2017). A 1% silver sulphadiazine was incorporated in surfactant-based dressing and tested on chronic wound patients ($n = 226$). In the first group of 88 patients with standard treatment, 73% showed improvement, while 60% showed healing only after 17 weeks of treatment. However, 86% of the second group treated with the surfactant-based biomaterial dressing were healed. An improved

compliance with reduced pain and a favorable side-effect was observed (Zolb and Cech, 2016).

Biofilm-disrupting wound gel was studied for therapeutical efficacy in a randomized, open-label clinical trial with 43 patients having chronic and recalcitrant wounds. They were treated with biofilm-disrupting wound gel or a broad-spectrum antimicrobial ointment for 12 weeks. Wound size reduced by 71% on using biofilm-disrupting gel, whereas it reduced only by 24% on application of antimicrobial ointment. Wound closure in half of the patients was achieved by 12 weeks on using gel in comparison to 17% for the ointment usage. Adverse events in two patients occurred only in case of the patients who received antimicrobial ointment treatment (Kim et al. 2018).

A study determined if degradation of biofilm matrix enhanced wound healing. Next Science Wound Gel (wound gel) were evaluated for disrupting biofilm and topical antibiotics in a randomized controlled trial. The wound gel degraded EPS in biofilm when directly applied to the wound bed on alternate days alone or with topical antibiotics. Wounds healed by 53% on applying topical antibiotics and by 80% on using wound gel, whereas when both were used together, it showed healing by 93%. Thus, targeting and disrupting the biofilm matrix enhances wound healing (Wolcott 2015). Similarly, in patents with diabetic foot ulcers having chronic biofilm infections, the application of concentrated surfactant gels for 6 weeks reduced microbial load and improved healing in comparison to the standard of care (SOC) treatment. Wound swabs, DNA sequencing, and qPCR determined the total microbial load and diversity. SEM, PNA-FISH, and CLSM were used to study biofilms in tissue specimens. The application of gel resulted in reduction in total microbial load in seven samples. *Corynebacterium* sp. and *Streptococcus* sp. decreased in numbers, whereas *Staphylococcus* sp., *Finegoldia* sp., and *Fusobacterium* sp. increased in numbers from 0 to week 6. SOC and the gel affected the microbial loads in chronic biofilm infections (Malone et al. 2021).

Cadexomer iodine (CI) was studied for its effect on glycocalyx production in isolates of *S. aureus* from furuncle lesions in mice. *S. aureus* and glycocalyx were absent in the dermis around the cadexomer iodine beads, while they were soaked up by the cadexomer beads and detected within them. I directly destroys and collapses glycocalyx by dehydration. Iodine kills *S. aureus* in biofilms. Thus, I can be used to eradicate biofilm and prevent exacerbation in wounds (Akiyama et al. 2004). It had high anti-biofilm efficacy in *P. aeruginosa* and MRSA compared to simple or conventional wound dressings as seen in an ex vivo mouse model. It had better activity against MRSA biofilm than silver dressings or the antibiotic mupirocin. CI-based topical products can be used to treat bacterial biofilm in chronic wounds (Fitzgerald et al. 2017).

CI reduced the microbial load of chronic non-healing diabetic foot ulcers with biofilm infection. SEM and FISH were used to detect the presence of biofilm on tissue punch biopsies obtained pre- and post treatment. Of 16 participants, 11 patients exhibited significant reductions in microbes post treatment (Malone et al. 2017). In a similar study, treatment with CI of 18 diabetic foot ulcers resulted in 14 patients showing reduced microbial load after 6 weeks of treatment. The total microbial load in diabetic foot ulcers with chronic biofilm infections reduced, and its microbial composition and diversity was affected (Malone et al. 2019). CI in comparison to silver carboxymethylcellulose dressings were effective against MRSA biofilm in mouse wounds, ex vivo pig skin, and in a pig wound model. Treatment with CI reduced biofilms as observed with microscopy and histopathology of wound tissue (Roche et al. 2019).

Negative pressure with instillation (NPWTi) is used as an adjunct to treat infected wounds. NPWTi and saline or various antimicrobial solutions was tested on mature *P. aeruginosa* biofilm in an ex vivo porcine skin explant. Six cycles with 10 min exposure to instillation solution and 4 h of negative pressure at −125 mmHg for 24 h showed that NPWTi with saline significantly reduced bacterial load by 1–7-log CFUs compared to other instillations. SEM showed disrupted EPS matrix and damaged bacterial

cells. NPWTi with active antimicrobial agents kills bacteria and enhances biofilm removal (Phillips et al. 2013). A porcine skin explant biofilm model was tested with antimicrobials such as silver gel, iodine, polyhexamethylene biguanide, honey, ethanol, moisture dressings (cotton gauze), cadexomer beads, sodium carboxymethylcellulose and calcium alginate fiber. Of all the tested antimicrobials, silver gel and CI dressings effectively reduced mature biofilm. Similar results were seen in in vivo pig burn wound model on 24 h exposure to silver dressings. Therefore, the microbicidal wound dressing's antibiofilm efficacy is affected by time of exposure, number of applications, moisture level, and agent formulation (sustained release) (Phillips et al. 2015).

In a porcine skin explant model infected with *S. aureus* and *P. aeruginosa*, the solutions of 4% w/v melaleuca oil, polyhexamethylene biguanide, chlorhexidine, povidone iodine, and hypochlorous acid were exposed for 15 min against 3-day mature biofilms. Repeated instillation of hypochlorous acid reduced microbial load. Further, melaleuca oil was applied for 15 min for 7 days in 10 patients with biofilm-associated diabetic foot ulcers. However, it had no effect on microbial load. Short exposure to topical antimicrobials is ineffective against in vivo microbial biofilms (Johani et al. 2018).

1% PVP-I irrigation reduced *P. aeruginosa* bacterial load on the surface and within the wound tissue in comparison to saline irrigation in SD rats. It also promoted wound re-epithelialization (Kanno et al. 2016).

Commercial wound care products containing EDTA include RescuDerm® and Biostep®. RescuDerm is a water-soluble gel and is composed of 0.1% EDTA, acetic and citric acid, and carbopol. It can inhibit *P. aeruginosa* and *S. epidermis* biofilms. RescuDerm was able to prevent *P. aeruginosa* forming biofilms in wounds in rats. Similarly, Biostep contains matrix metalloproteinases disrupting collagen dressing. EDTA chelates calcium and magnesium ions from biofilms disrupting their structural integrity, and removes iron required for virulence and pathogenicity (Martineau and Dosch 2007a, b). EDTA and silver in combination have significant antibiofilm activity and such a formulation has been patented (Percival et al. 2005).

Some of the non-conventional therapy that could find potential application based on in vivo studies are discussed here. Phage therapy using *Klebsiella*-specific phage, Kpn5, was tested in burn wound in mice infected with *Klebsiella pneumoniae* B5055. Phage Kpn5 hydrogel (m.o.i. 200) applied on the burn wound site significantly reduced mortality by rescuing mice from *K. pneumoniae* B5055 infection when compared with wound treated with silver nitrate and gentamicin (Kumari et al. 2011). Topically administered bacteriophage decreased *S. aureus* and *P. aeruginosa* load and improved healing in rodent and porcine model but were not effective in inhibiting *A. baumannii* (Mendes et al. 2013). However, a bacteriophage isolated from hospital sewage exhibited inhibitory activity against clinical strains of *A. baumannii* and formed plaques in beta-lactamases strains of *A. baumannii* lawns. Its efficacy was evaluated against *A. baumannii* infection in wounds of diabetic rats. There was decrease in infection and epithelization period in the mice sprayed with phage in comparison to antibiotic-treated uncontrolled diabetic rats (Shivaswamy et al. 2015). Phage vABWU2101 and tigecycline in combination exhibited synergistic inhibitory effect on planktonic and biofilms of MDR *A. baumannii* (Wintachai et al. 2022). Mixture of lytic anti-*Pseudomonas aeruginosa* bacteriophages was tested along with SOC in burn wound patients. When topically administered for 7 days, the combination of 12 lytic anti-P aeruginosa bacteriophages (PP1131) at 1×10^6 PFU decreased bacteria in burn wounds but at a slower rate compared to 1% sulfadiazine silver emulsion cream SOC (Jault et al. 2018).

Silver nanoparticles (AgNPs)-loaded hydrogels have exhibited superior wound healing and antibacterial activity under in vivo conditions when compared to silver sulfadiazine cream (Dermazin®). AgNPs were localized in the skin epidermal layers as confirmed by transmission electron microscopy (Mekkawy et al. 2017). Copper nanoparticle suspension and a drug combination of copper and zinc nanoparticles when

applied locally could rapidly eliminate bacteria from purulent wound in rats (Babushkina et al. 2015). Antimicrobial Zinc Oxide (ZnO) nanoparticles can enhance wound healing. ZnO nanoparticles reduced *S. aureus* wound infection in mice. Superficial and depth bacterial load were reduced on topical treatment with ZnO nanoparticles resulting in accelerated healing (Daghdari et al. 2017).

The role of low-level laser therapy (LLLT) in promoting wound healing in animals and humans is well known. However, the role of LLLT in biofilm removal for wound healing is being increasingly studied. Blue light has intrinsic antimicrobial activity and does not require addition of photosensitizers. Blue light at 415 nm was used to treat *P. aeruginosa* burn infections in mice. On exposure to blue light at 55.8 J/cm^2 for 30 min, microbial load was reduced in infected mouse burns without damage to mouse skin. It also increased the survival rate from 18.2% to 100% in *P. aeruginosa* burn infections (Dai et al. 2013). In a similar study, blue light inactivated *A. baumannii* or *P. aeruginosa* biofilms in infected burn wounds in mice. An exposure of blue light of 360 J/cm^2 and 540 J/cm^2 24 and 48 h, respectively, inactivated biofilms (Wang et al. 2016). In the pilot studies, exposure of patients to light at 870 nm/930 nm and energy higher than 100 J/cm^2 doses was an efficient therapeutic approach. Blue light has exhibited photoinactivation of planktonic *P. aeruginosa, E. coli,* and methicillin-sensitive *S. aureus* strains. It can be used for photoinactivation of biofilms at 170 J/cm^2 or more (Percival et al. 2015d). The photodynamic therapy (PTD) for biofilm eradication, uses a photosensitizer (usually a dye) that is taken up by the bacteria, which is then subjected to irradiation causing the excitement of the photosensitizer leading to production of ROS, including hydroxyl radicals and singlet oxygen. Currently, porphyrin derivatives, phthalocyanines, chlorins, and porphycenes are used as photosensitizers against bacteria (Percival et al. 2014). The photosensitizers have shown effective results in in vitro studies, proven to improve wound healing in chronic wounds, and are non-toxic when administered in proper doses.

Negative-pressure wound therapy (NPWT) was used for wound treatment in rabbits infected by *P. aeruginosa*. A sterile gauze dressing was used as control. In vivo biofilms were reduced as observed with microscopy using concanavalin A conjugated to Alexa Fluor® 647 stain. In vivo studies showed that NPWT reduced bacterial load, virulence factor expression, and eDNA content in biofilms. It could help clinicians in the treatment of infected wounds (Wang et al. 2018). Hyperbaric oxygen therapy (HBOT) was tested in vitro and in vivo for removing biofilms. In vitro study showed a significant reduction in cell viability at 30 and 90 min of HBOT. In vivo data show reduced bacterial load after 1 week of HBOT in patients. However, in patients' with chronic wounds, certain anaerobic bacteria and fungi evolved between treatments. Further studies on mechanisms of HBOT for biofilm treatment is required for clinical application in patients with chronic wounds (Sanford et al. 2018).

Electroceutical principles have been used to manage wound biofilm infection. The US FDA has approved the use of wireless electroceutical dressing (WED). It was evaluated against polymicrobial biofilms of *P. aeruginosa* PAO1 and *A. baumannii* 19606 in porcine chronic wounds. Weak electric field showed biofilm inhibitory activity. On applying WED, biofilm formation was prevented within 2 h of infection. WED disrupted biofilm aggregates and increased closure by reinstating skin barrier function in wounds. It affected the expression of mvfR (pqsR), rhlR, and lasR genes in *P. aeruginosa*, and miR-9 and silencing of E-cadherin required for skin barrier function. It reduced biofilm-induced persistent inflammation by preventing activation of nuclear factor kappa B and cytokines. This pre-clinical mechanistic study of electroceuticals seems promising in treating wound biofilm infection (Barki et al. 2019).

Probiotic *Lactobacillus plantarum* has shown in vivo antibacterial and wound healing activity in a burned-mouse model infected with *P. aeruginosa*. It inhibited *P. aeruginosa* colonization, improved tissue repair, and enhanced phagocytosis at 10 days (Valdez et al. 2005). Lactobacilli with a plasmid encoding antimicrobial

peptide CXCL12 applied topically on wounds in mice enhanced healing by increasing dermal cells and macrophages with increased TGF-β expression. Lactic acid reduced the local pH which inhibited the activity of peptidase CD26 and enhanced CXCL12 availability. Treatment with CXCL12 increased wound healing in hyperglycemic or peripheral ischemic mice and in human skin wound models (Vagesjo et al. 2018). A local prophylactic application of probiotic *Lactobacillus plantarum* prevented death in a mouse with burn wound sepsis caused by *P. aeruginosa*. Probiotic therapy reduced TNF-α and interleukins IL 6 and IL 10 in the mouse liver. Thus, it could be used as therapy in complicated burn injury (Argenta et al. 2016). Immobilized *L. plantarum* in calcium alginate films reduced growth of VIM-2-metallo-β-lactamase-producing *P. aeruginosa* in burn wound in rats. Freeze-dried calcium alginate films of *L. plantarum* were viable up to 6 months at 4 °C (Brachkova et al. 2011). Lactobacilli showed anti-adhesion activity in *P. aeruginosa*. *L. plantarum* supernatants increased the fibroblasts numbers, re-epithelialization, and thickness of epidermis and dermis layers in wound region. Thus, *L. plantarum* can treat *P. aeruginosa* infection in a second-degree burn and reduce inflammation (Abootaleb et al. 2021).

The natural compounds have been evaluated for treating biofilm-associated infections. Usnic acid is one such natural compound – a secondary metabolite obtained from lichens. Usnic acid is known to show a significant activity in inhibiting biofilms produced by Gram positive *Staphylococcus epidermidis* and *S. aureus* infections (Francolini et al. 2019). Usnic acid and sodium salt topically applied exhibited wound healing properties. They showed decrease in number of inflammatory cells, increase in fibroblasts and granular tissues along with vascular regeneration. Two of the usnic acid enamine derivatives displayed low cytotoxicity and high rates of wound healing under in vitro and in vivo conditions (Francolini et al. 2019; Bruno et al. 2013). The combination of standard antibiotics with other bioactive compounds are shown to reduce the biofilm formation. A study published in 2010 shows the use of N-acetylcysteine (NAC) in combination with ciprofloxacin at various concentration proved to disrupt the biofilm produced by *P. aeruginosa* at significantly high levels (Zhao and Liu 2010). The use of clarithromycin for the period of 5–6 days with other antibiotics eradicated extracellular polysaccharide and helped in penetration of antibiotics to act on *P. aeruginosa* (Yasuda et al. 1993). The use of metal chelators like EDTA are also active in disrupting the biofilm production (Banin et al. 2006).

Voriconazole can be bound with zinc by coordination-binding. These metal–natural frameworks with built-in voriconazole can inhibit biofilms of *Candida albicans* by preventing aggregation of cells resulting in higher dispersal. In the acidic environment of biofilm, voriconazole dissociates from the metal–natural framework and kills fungi (Su et al. 2020). Development of electrochemical scaffold for the generation of hypochlorous acid and slow release of it in the affected area is proven to inhibit the production of biofilms and inhibit the fungal growth (Zmuda et al. 2020).

Enzymes such as purified beta-N-acetylglucosaminidase and dispersin B can breakdown mature biofilms of *S. epidermidis* and other bacterial species. Polymeric matrices containing dispersin B and cefamandole nafate antibiotics have shown synergistic action by hydrolytic activity against EPS and antimicrobial activity against bacteria and thus prevented bacterial colonization (Donelli et al. 2007). Such polymer matrix containing dispersin B and antibiotics could be used to develop dressings for treating biofilm-associated wounds.

4 Conclusion

The complexity of bacterial biofilms in chronic non-healing wounds and their increased tolerance to conventional therapeutics makes their treatment challenging. However, early and precise detection of biofilms in the wound environment can result in efficient intervention and treatment. Antimicrobial treatment in conjunction with novel approaches are promising for debridement,

biofilm eradication, and improved wound healing. In the future, success can be achieved in effective biofilm-infected chronic wound management only when the proof-of-concept and in vivo studies are translated and applied into clinical settings.

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Relevance and Importance of Biofilms in the Resistance and Spreading of *Campylobacter* spp. Within the Food Chain

Efstathios Giaouris

Abstract

Campylobacter spp. are Gram-negative microaerophilic and non-spore-forming bacteria that primarily live as commensal organisms in the gastrointestinal tract of many domestic and wild birds and mammals. These frequently provoke foodborne illness, being responsible for the most common cause of acute bacterial gastroenteritis worldwide. Although these are typically fastidious and slow-growing microorganisms which are sensitive to desiccation and other stresses (e.g., extreme pH, freezing, UV, disinfectants), these can still survive outside their host for long periods, with their attachment to surfaces and the formation of and/or inclusion into biofilms to be considered quite important for their environmental survival and widespread dissemination. In this chapter, the most representative studies on the existence, relevance, and importance of biofilms in the resistance and spreading of *Campylobacter* spp. within the food chain are presented. Hopefully, such accumulated and focused knowledge is expected to highlight the problem and trigger more effective ways for its mitigation, improving the safety of our food supply and protecting public health.

Keywords

Antimicrobial resistance · Biofilm · *Campylobacter* spp. · Food safety · Stress response

1 Introduction

Campylobacter spp. are Gram-negative, spirally curved, or rod-shaped microaerophilic and non-spore-forming bacteria that primarily live as commensal organisms in the gastrointestinal tract of many domestic and wild birds and mammals (Costa and Iraola 2019). The genus is currently comprising more than 40 officially described species (https://www.bacterio.net/genus/campylobacter), with most of them being able to move via (unipolar or bipolar) flagella, while certain prefer anaerobic conditions for growth or are even aerotolerant (Epping et al. 2021; Hansson et al. 2018). Survival of these bacteria at room temperature is poor; these always grow above 30 °C and are best cultured in vitro at 42 °C (i.e., the temperature in the avian gastrointestinal tract) on nutritional basal media supplemented with 5–10% blood in an atmosphere with reduced oxygen (5–10% O_2) and elevated carbon dioxide (1–10% CO_2) concentrations (Hakeem and Lu

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2021). Despite their demanding nature, *Campylobacter* species are frequently provoking foodborne illness, being responsible for the most common cause of acute bacterial gastroenteritis worldwide, with *C. jejuni* and *C. coli* causing around 90 and 10% of human campylobacteriosis infections, respectively (Fitzgerald 2015). In Europe, the last available data reveal that in 2020, campylobacteriosis was the most reported human zoonosis (as it has been since 2005), with 40.3 cases per 100,000 population, 21.0% of patients requiring hospitalization, and a case fatality ratio of 0.05% (EFSA and ECDC 2021). Similarly, this is also the most prevalent foodborne bacterial infection in the United States (with 0.8 million cases of campylobacteriosis annually occurring) and in many other countries as well (Kaakoush et al. 2015).

Foods implicated in campylobacteriosis usually include raw or under-cooked poultry, raw dairy products (e.g., unpasteurized milk), and contaminated produce, while outbreaks have also been occurred following the consumption of contaminated water (Heimesaat et al. 2021). Although *Campylobacter* spp. are largely considered as commensal bacteria in birds, these may still achieve in their intestine a colonizing population of up to 10^9 CFU/g of cecal contents, altering their gut microbiota and possibly interfering with bird performance and welfare (Awad et al. 2018). Transmission to humans occurs through the fecal-oral route and is most often associated with the consumption and/or handling of contaminated poultry, with the outcome of the disease to depend on the immune status of the host and the virulence potential of the implicated *Campylobacter* strain (Tegtmeyer et al. 2021). The symptoms of campylobacteriosis are normally manifested as mild and self-limiting gastroenteritis and generally include diarrhea, vomiting, fever, and abdominal pain. However, in some severe cases, treatment with antibiotics is required, while alarmingly in a minority of patients (ca. 0.07%), this infection is additionally a precursor of more serious illness, including immunoreactive complications, such as Guillain-Barré and Miller Fisher syndromes, provoking chronic and potentially fatal paralysis (Hansson et al. 2018).

Biofilms are microbial communities developed on surfaces or interfaces (e.g., air-liquid) and embedded in a 3D matrix of self-produced extracellular polymeric substances (EPS), mainly including exopolysaccharides, proteins, and nucleic acids (Carrascosa et al. 2021). Their development involves the initial attachment of planktonic (free-swimming) microorganisms to a surface, followed by the replication and production of EPS, formation of microcolonies, maturation (i.e., development of the characteristic biofilm architecture), and detachment (Fig. 1). Biofilm formation offers significant ecological advantages to the enclosed microorganisms and particularly protection from adverse environmental conditions (e.g., antibiotic/disinfectant actions, immune response). Thus, a considerable amount of evidence shows that the cells living in a biofilm can become up to 1,000-fold more resistant than their planktonic counterparts, making their elimination a hard challenge (Giaouris and Simões 2018). In the last decades, biofilm formation by pathogenic microorganisms has attracted much attention, mainly in the medical and food processing fields, due to its potential risks, including antimicrobial resistance, persistence, and virulence induction (Koo et al. 2017; Yuan et al. 2021). Indeed, pathogenic biofilms formed on food contact surfaces are of considerable interest in the context of food hygiene, since these can result in the cross-contamination of foods leading to the transmission of diseases (Alvarez-Ordóñez et al. 2019).

Although *Campylobacter* spp. are typically fastidious and slow-growing microorganisms which are sensitive to desiccation and other stresses (e.g., extreme pH, freezing, UV, disinfectants), these can still survive outside the avian intestinal tract for long periods, especially under refrigerated and moist dark conditions, until they are able to reach a new host (Bronowski et al. 2014). Indeed, it has long been considered that the attachment to abiotic surfaces and the formation of and/or inclusion into biofilms should play a key role in the environmental survival and

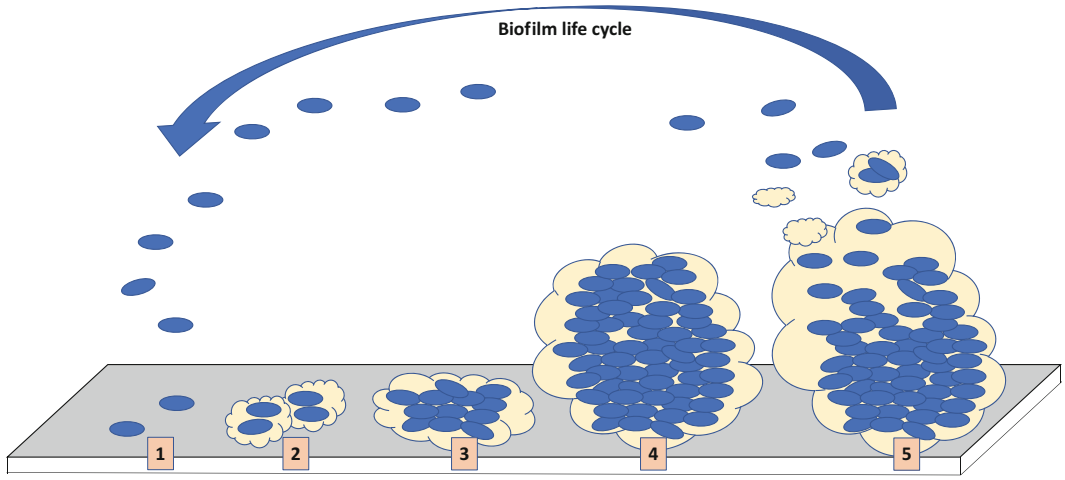

Fig. 1 Stages of biofilm life cycle: (1) initial attachment; (2) replication and production of EPS; (3) formation of microcolonies; (4) maturation (development of biofilm architecture); (5) detachment

transmission of these pathogens (Buswell et al. 1998; Lamas et al. 2018; Tram et al. 2020a) (Fig. 2). It is worth noting that such a surface-associated lifestyle is known to induce a considerable readjustment in the transcriptome and proteome of the *C. jejuni*, with hundreds of genes being differentially expressed in comparison to planktonic cells (Püning et al. 2021; Sampathkumar et al. 2006; Tram et al. 2020b). At the same time, comparative genomic analyses have shown that the gene contents of *C. jejuni* strains isolated throughout the broiler meat production chain that efficiently form biofilms are different from those that do not, suggesting that biofilm formation might be a lineage-specific property for this pathogen (García-Sánchez et al. 2019; Kudirkienė et al. 2012). In addition, the adhesion of *Campylobacter* to host gastrointestinal epithelial cells is a prerequisite for colonization, and this is mediated by several adhesins exposed on the bacterial surface, such as the fibronectin-binding outer membrane proteins (Bolton 2015). With regard to the food industry, like with other bacterial pathogens, biofilm growth by *Campylobacter* on typical food contact surfaces, such as stainless steel and plastics, frequently found in food processing environments (and in poultry houses as well), contributes to the survival and persistence of these bacteria outside the host, under environments that would otherwise be harmful to them (Nguyen et al. 2012). These biofilms are thus believed to act as a source of constant food contamination and contribute to the very high number of human *Campylobacter* infections (Teh et al. 2014).

In the next sections of this chapter, the most representative studies on the existence, relevance, and importance of biofilms in the resistance and spreading of *Campylobacter* spp. within the food chain will be summarized. Such accumulated and focused knowledge is expected to highlight the problem and hopefully trigger more effective ways for its mitigation.

2 *Campylobacter* Biofilms as a Stress Adaptation and Survival Mechanism

It is generally acknowledged that biofilms play a key role in the survival of microorganisms under unfavorable environmental conditions, while their formation by bacterial pathogens is also considered as a significant virulence mechanism in relation to their dissemination and pathogenesis (Bai et al. 2021). This seems also the case for the intestinal pathogen *C. jejuni*, whose biofilms are considered as an important contributing factor in its persistence and spreading through the food chain (Bronowski et al. 2014; Murphy et al.

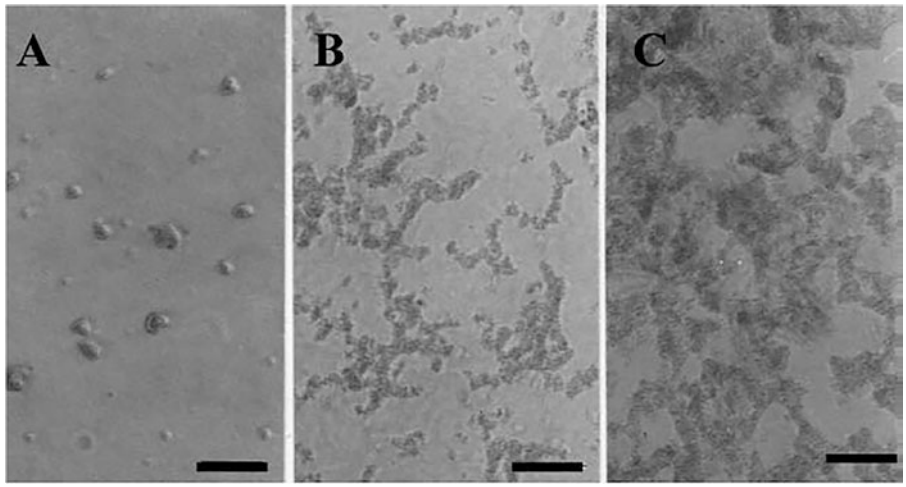

Fig. 2 Time course of biofilm formation by *C. jejuni* strain 81–176 on a glass coverslip incubated in Brucella broth under microaerobic conditions at 37 °C for (**a**) 2 h, (**b**) 4 h, and (**c**) 6 h under static conditions. Bar =5 mm. (Reproduced with the permission of the Japanese Society of Veterinary Science from Moe et al. 2010)

2006). Thus, the ability of this pathogen to form biofilms or more likely be included in those formed by other species, such as *Pseudomonas* and other food-related bacteria, is probably the answer which explains the paradox associated with this fastidious bacterium, which is on the one hand quite vulnerable to environmental stresses, while on the other hand the incidence of human *Campylobacter* infections continues to be enough high and in some cases has even progressively increased (Facciolà et al. 2017). Interestingly, early investigations that compared transcriptional and translational expression profiles between sessile and planktonic *C. jejuni* have revealed that the immobilized bacteria alter their cellular priorities away from metabolic, motility, and protein synthesis capabilities toward an emphasis on iron uptake, membrane transport, and oxidative stress defense, confirming this way some common adaptive responses that some other biofilm-forming bacteria are also presenting (Sampathkumar et al. 2006).

2.1 Oxidative Stress Defense

Oxidative stress defense (aerotolerance) is one of the most studied adaptive responses that *C. jejuni* is exhibiting upon its inclusion in biofilms, with oxygen-enriched conditions to have been shown to stimulate in this bacterium the overexpression of membrane proteins involved in both the biofilm initiation and virulence (Sulaeman et al. 2012). To this direction, several studies have revealed that biofilm formation by this microaerophilic bacterium increases under aerobic conditions, suggesting that this could be a response behavior to oxidative stress by providing a local microaerobic environment (Oh et al. 2016; Reuter et al. 2010; Sulaeman et al. 2012; Turonova et al. 2015). This microenvironment can protect *C. jejuni* from oxygen inactivation, extending its survival even in low-nutrient media, at ambient temperatures or lower, and under normal aerobic atmospheric conditions (Buswell et al. 1998; Joshua et al. 2006). In such a relevant study evaluating the heterogeneity among 90 *C. jejuni* and 21 *C. coli* isolates with respect to biofilm formation capacities under either microaerobic or aerobic atmosphere and resistance to various stresses that can be encountered within the food chain (aerobic stress, refrigeration, freeze-thaw, heat, peracetic acid, and osmotic stress), a high prevalence (63%) of hyper-aerotolerant isolates was observed, which also presented a high endurance to the other physical stresses and in parallel a high biofilm formation capacity which was further

enhanced under aerobic conditions, advocating thus for an association between stress adaptation and biofilm formation (Mouftah et al. 2021).

Reactive oxygen species (ROS) are molecules containing at least one oxygen atom and one or more unpaired electrons (due to incomplete oxygen reduction), such as the superoxide anion (O_2^-) and the hydroxyl radical ($^{\bullet}OH$), and whose excessive formation is known to contribute to oxidative stress, causing damage at both the molecular and cellular level (Jakubczyk et al. 2020). Like other bacteria that should survive exposure to different levels of ROS, *C. jejuni* contains several regulatory proteins involved in the oxidative stress response (Kim et al. 2015). Two such proteins are the MarR-type transcriptional regulators RrpA and RrpB (*r*egulator of *r*esponse to *p*eroxide) which have been shown to control both oxidative and aerobic stress responses in this bacterium (Gundogdu et al. 2015). Analysis of 3,746 *C. jejuni* and 486 *C. coli* genome sequences showed that *rrpA* is present in over 99% of *C. jejuni* strains, while the presence of *rrpB* is restricted to specific multilocus sequence typing (MLST) clonal complexes. On the other hand, *C. coli* strains lack both these genes. Interestingly, a *rrpA* mutation resulted in enhanced biofilm formation in *C. jejuni* independent of *rrpB* status (Gundogdu et al. 2016).

Oh et al. (2016) compared ROS accumulation inside *C. jejuni* biofilms developed on stainless steel surfaces between aerobic and microaerobic conditions after having observed that this bacterium developed more biofilms under the former conditions. As expected, they found that ROS accumulated at a substantially higher level under aerobic conditions. Interestingly, however, the addition of an antioxidant (i.e., 1 nM N-acetyl cysteine) not only significantly reduced the levels of total ROS but at the same time reduced biofilm formation (Fig. 3), suggesting that aerobiosis enhances biofilm formation in *C. jejuni* via oxidative stress. This is indeed stress that this bacterium unavoidably encounters upon being found, for instance, in water and air following its excretion from host animals. In another similar study, the role of this stress in biofilm formation by *C. jejuni* was investigated in mutants defective in catalase (KatA), superoxide dismutase (SodB), and alkyl hydroperoxide reductase (AhpC), proteins that are all involved in oxidative stress defense (Oh and Jeon 2014). Biofilm formation was found to be considerably increased in the *ahpC* mutant compared to the wild type and *katA* and *sodB* mutants, whereas at the same time another strain overexpressing *ahpC* presented reduced biofilm formation. As expected, ROS accumulation was higher in the *ahpC* mutant than the wild type, while the antioxidant treatment of this mutant reduced biofilm formation to the wild-type level. In another study by the same team, the effect of ferrous (Fe^{2+}) or ferric (Fe^{3+}) iron on biofilm formation was investigated in 70 *C. jejuni* isolates from raw chicken. Regardless of the innate levels of biofilm formation in each strain, iron induced biofilm formation in all of them, also increasing the levels of total ROS inside the sessile communities (Oh et al. 2018). It was also found to increase the levels of extracellular DNA (eDNA) and polysaccharides in these biofilms. As expected, the treatment with either an iron chelator (deferoxamine mesylate) or an antioxidant (N-acetyl cysteine) inhibited the iron-mediated increase of ROS generation and prevented biofilm enhancement.

2.2 Antimicrobial Resistance

Although most *Campylobacter* infections are mild and self-limiting, antimicrobial therapy using antibiotics is required to treat the most severe and/or prolonged infections, with fluoroquinolones and macrolides being included in the drugs of choice in those cases (Shen et al. 2018). However, as with many other bacteria of clinical significance, *Campylobacter* spp. have developed multiple mechanisms for resistance to deal with the pressure provoked by the antimicrobial use in both veterinary and human medicine, as well as within food processing (Shen et al. 2018; Tang et al. 2017). These generally include alteration or replacement of antimicrobial targets, modification or inactivation of antimicrobials, and reduced drug accumulation by efflux pumps and/or membrane modifications, with some of

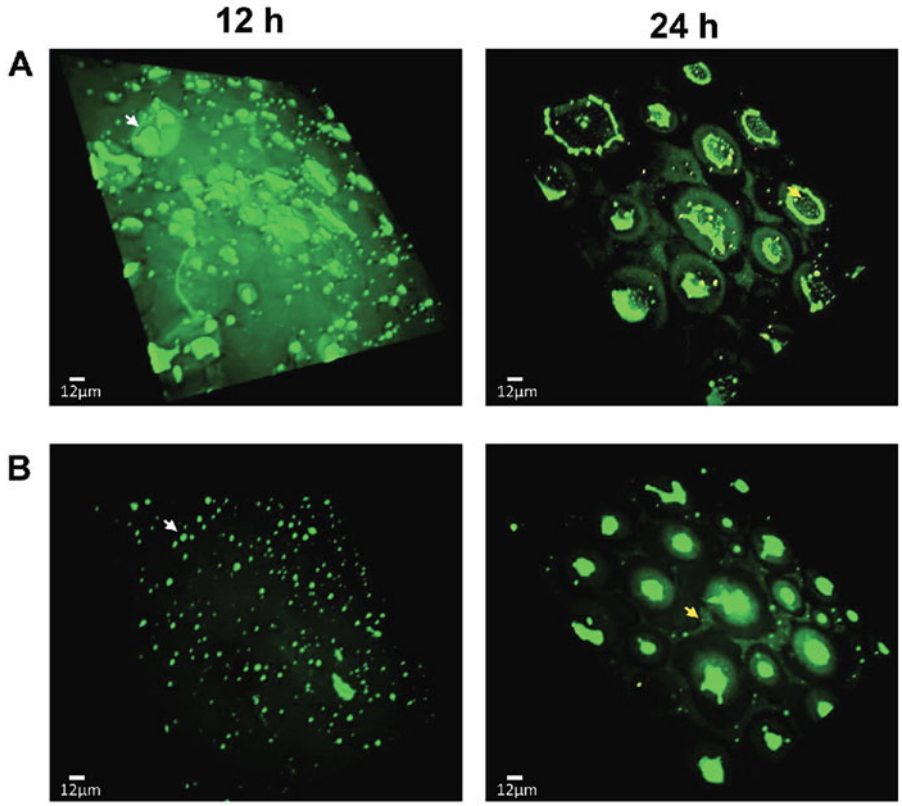

Fig. 3 Confocal microscopy of *C. jejuni* biofilms at 12 h and 24 h of development under aerobic conditions in the absence (**a**) and presence (**b**) of an antioxidant (1 nM N-acetyl cysteine). (Oh et al. 2016; © 2016 Taylor & Francis)

these mechanisms to alarmingly give rise to multidrug resistance (Christaki et al. 2020). Besides all these mechanisms, the inclusion inside a biofilm structure is undoubtedly a universal stress response and survival strategy for most microorganisms, including *Campylobacter* spp. (Yan and Bassler 2019). In such a study aiming to unravel the survival strategies exploited by *C. jejuni* through the poultry processing chain, 45 slaughterhouse isolates, belonging to 6 different pulsed field gel electrophoresis (PFGE) profiles, were phenotypically characterized for biofilm formation, virulence, and antimicrobial resistance, in combination with whole genome sequencing (WGS) (García-Sánchez et al. 2019). Results revealed that those strains which could form biofilms belonged to three multi-locus sequence types (MLST), out of the six that were totally revealed (following WGS), while all the isolates were resistant to the fluoroquinolones nalidixic acid and ciprofloxacin, as well as to tetracycline and two common detergents used in the slaughterhouse (at the lower recommended concentration by suppliers). Alarmingly, all the isolates were also positive for almost all the 68 virulence-associated genes studied, with still a combination of several changes in the genomes of some isolates probably explaining their ability to form biofilms and persist better in the environment (García-Sánchez et al. 2019).

Rossi et al. (2021) compared the susceptibilities of both planktonic and biofilm cells of 35 *C. jejuni* strains, previously isolated from chicken carcasses (of which 85.7% presented high phylogenetic distinction), to five classes of antibiotics, exhibiting different mechanisms of action (i.e., ciprofloxacin, erythromycin, tetracycline, meropenem, and colistin). Results showed

that all strains (100%) in biofilms were resistant to erythromycin, meropenem, and colistin, whereas the resistance ratios for planktonic cells were 34.3%, 8.6%, and 80%, respectively, following the specifications and cutoff points described by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (available at https://eucast.org/clinical_breakpoints/). At the same time, 71.4% (25/35) of the isolates were multidrug-resistant, with more than 85% of strains being resistant to ciprofloxacin and tetracycline even in their planktonic form. Alarmingly, strains that were susceptible as planktonic cells were found to increase biofilm production (on 96-well polystyrene microtiter plates for 48 h at 37 °C under microaerophilic conditions) upon the presence of the antimicrobial, except for tetracycline which was the only drug that showed an antibiofilm effect. However, the latter was quite probably due to its use in a concentration (i.e., 32 μg/mL) that was higher than its minimum inhibitory concentration (MIC). In agreement, another study that investigated the biofilm matrix in *C. jejuni* following culturing with antibiotics in subinhibitory doses showed that quinolones (particularly nalidixic acid) and tetracyclines enhanced the formation of biofilms and at the same time increased the stress tolerance of the pathogenic cells (Efimochkina et al. 2018). In another similar study investigating the effect of six antibiotics with different modes of action (ampicillin, ciprofloxacin, erythromycin, nalidixic acid, rifampicin, and tetracycline) on biofilm formation by seven *C. jejuni* isolates from poultry, exhibiting different antibiotic resistance profiles, it was found that the presence of most of the tested antibiotics resulted on the one hand to the reduction of biofilm formation by those strains that were resistant to them but on the other hand to its increase in sensitive strains (Teh et al. 2019a).

Zhang et al. (2017) tested a total of 206 *Campylobacter* isolates from chicken farms and live poultry markets in Central China (of which 166 are *C. jejuni* and 40 *C. coli*), for genotypic diversity (by MLST analysis), antibiotic susceptibility to a total of 11 antibiotics (by the disk diffusion method), and biofilm-forming ability (on 24-well polystyrene tissue culture plate for 48 h at 37 °C under microaerobic conditions). All the isolates were resistant to norfloxacin, ciprofloxacin, and cefazolin, while a widespread resistance to fluoroquinolones, β-lactams, and tetracyclines was also noticed. 64.6% of the isolates (133/206) were able to form biofilms, with this ability however to vary among different genetic branches, suggesting its mazy genetic background. Interestingly, those biofilm-producing strains were also more resistant to six of the tested antibiotics (i.e., ampicillin, neomycin, sulfamethoxazole, amikacin, clindamycin, and erythromycin) compared to the biofilm-deficient strains.

Fluoroquinolones are broad-spectrum antibiotics which are routinely used to treat campylobacteriosis and some other undiagnosed diarrheal infections as well as in animal husbandry. They work by inhibiting the function of the DNA gyrase which is essential for chromosome replication and function (Majalekar and Shirote 2020). However, bacteria can display resistance to this class of antibiotics by acquiring mutations in some of those genes involved in DNA supercoiling. At the same time, the World Health Organization (WHO) has included *Campylobacter* spp. in the list of 12 bacteria for which new antibiotics are urgently needed due to the rapid increase in fluoroquinolone-resistant strains (WHO 2017). Alarmingly, acquisition of fluoroquinolone resistance in this bacterium has also been shown to result in increased biofilm formation under aerobic conditions and higher pathogenicity (Whelan et al. 2019). This agrees with previous knowledge on the positive association of the relaxation of DNA supercoiling on the attachment to and invasion of human epithelial cells by *C. jejuni* (Scanlan et al. 2017). At the same time, the frequencies of fluoroquinolone resistance (FQ^R) have been shown to be higher in *C. jejuni* cells dispersed from biofilms compared to planktonic cells, suggesting that *Campylobacter*-harboring biofilms may release *Campylobacter* cells with increased FQ^R (Bae and Jeon 2013). In addition, the increased occurrence of fluoroquinolone-resistant *C. jejuni* chicken isolates even in countries that do not allow for large-scale

use of fluoroquinolones in livestock suggests that resistance to these antibiotics may confer a transmission benefit to this bacterium beyond just defense from antibiotics.

It is largely known that antimicrobial resistance (AMR) genes can be rapidly disseminated between various microorganisms by horizontal gene transfer (HGT). Interestingly, HGT has been shown to be significantly higher inside *C. jejuni* biofilms compared to planktonic populations even in the lack of any antibiotic selective pressure (Bae et al. 2014; Ma et al. 2021). Its frequencies were related to biofilm cultivation time and amount of eDNA, but rather strangely not to biofilm biomass, cell density, or bacterial metabolic activity, and were also found to increase between *C. jejuni* with different genomic backgrounds (Ma et al. 2021). Alarmingly, HGT mutants were shown to be spontaneously released from the biofilm structures into the supernatant cultures, pointing out the spreading of AMR to broader niches. Although HGT was also detected in *Escherichia coli-C. jejuni* biofilms, this was not observed inside *Salmonella enterica-C. jejuni* biofilms. However, the latter cannot be also excluded since the traditional plating assay that those authors used could not have detected transformants that had been entered the viable-but-non-culturable (VBNC) state (Ma et al. 2021). This is indeed a state that has been recognized as a stress adaptation strategy adopted by many different bacterial species to cope with harsh conditions, such as those encountered within food chain (Dong et al. 2020).

Numerous previous studies with several bacteria have shown that eDNA is progressively released during biofilm formation, thereby being a crucial structural component of those sessile communities promoting their establishment (Campoccia et al. 2021). The significance of eDNA that is released by bacterial lysis in response to aerobic and/or starvation stress in biofilm formation by *C. jejuni* was shown in the study of Feng et al. (2018). In that study, the deletion of the stress response genes *spoT* (encoding for the stringent response regulator) and *recA* (encoding for the DNA repair system) enhanced biofilm formation under either aerobic or starvation conditions by stimulating cell lysis and eDNA release. At the same time, the exogenous addition of genomic DNA from either *Campylobacter* or *Salmonella* also increased biofilm formation in a concentration-dependent manner, whereas the enzymatic degradation of DNA by DNase I disrupted *C. jejuni* biofilm. The importance of eDNA on biofilm formation and stress tolerance of *C. jejuni* was also shown by the study of Svensson et al. (2014). It is also worth noting that the latter authors have also shown that a *C. jejuni* mutant, which carried a deletion in the sensor kinase of the CprRS two-component system (*Campylobacter* planktonic growth regulation), formed enhanced biofilms, while at the same time proteomic analyses revealed the upregulation of several stress tolerance proteins in this mutant (Svensson et al. 2009). Alarmingly, eDNA in *C. jejuni* biofilms has also been shown to contribute to the spread of antimicrobial resistance through the transfer of antibiotic resistance genes and their acquisition by susceptible cells by natural transformation (Brown et al. 2015).

Biocides are routinely used in the food industry to ensure food safety and improve the microbiological quality of the products. However, their improper use can result not only in the appearance of contamination problems but also in the emergence of biocide-resistant microorganisms, due to selective pressure, as well as sometimes to the emergence of cross-resistance to unrelated antimicrobials such as clinically important antibiotics (Maillard 2018). In such a relevant study, Techaruvichit et al. (2016) showed that *C. jejuni* was not only able to exhibit adaptation to five biocides used in the food industry (trisodium phosphate, acetic acid, sodium hypochlorite, and two commercial biocide formulations) following its growth in the presence of sub-lethal concentrations of these agents, but at the same time, this adaptation enhanced biofilm formation and induced resistance to kanamycin and streptomycin, two of the nine total antibiotics that were tested. With respect to the seven remaining antibiotics, although the adapted cells remained sensitive, most of the inhibition zones of their cultures were still smaller than those of the non-adapted cells.

3 Enhanced Survival of *Campylobacter* Within Mixed-Species Biofilms

Although most of the in vitro studies on *Campylobacter* biofilms have been executed using strains that have been left to attach to surfaces and form biofilms under well-defined mono-species conditions, in real food production environments, as well within the host, this bacterium is more likely to encounter on surface cells of several other species as well, which either are simply attached or have already been enclosed in complex sessile structures. Indeed, there are several studies that have shown that *C. jejuni* can survive better in mixed-species biofilms than in mono-species ones, in particular under adverse for planktonic growth environmental conditions (Indikova et al. 2015; Teh et al. 2014). In one of the earliest studies in this field, Trachoo et al. (2002) investigated the viability of a chicken carcass isolate of *C. jejuni* in biofilms of three Gram-positive chicken house isolates (P1, Y1, and W1) and a *Pseudomonas* sp. isolated from a meat processing plant, using a cultural method in combination with a direct viable count (DVC) approach (via an indirect fluorescence antibody technique). Two-day biofilms of P1, Y1, and *Pseudomonas* spp. on polyvinyl chloride (PVC) coupons were found to significantly increase the attachment of *C. jejuni* compared to W1 and controls without preexisting biofilm. At the same time, the viability of sessile *C. jejuni* cells decreased with time (during incubation at either 12 or 23 °C over a 7-day period), but this happened with remarkably higher rate on surfaces without a preexisting biofilm. In addition, the comparison of agar plating and DVC results revealed that *C. jejuni* have entered a VBNC state within the biofilm (Trachoo et al. 2002). The positive influence of co-existence of some other bacterial populations on the attachment to surfaces and/or subsequent survival of *Campylobacter* has also been demonstrated in some other studies (Klančnik et al. 2020; Lehtola et al. 2006; Sanders et al. 2007). However, this co-existence may be sometimes neutral or even detrimental to the attachment and/or survival of *C. jejuni* depending on the species and strains involved, as well as the prevailing environmental conditions (Hanning et al. 2008; Teh et al. 2019b).

Ica et al. (2012) investigated the survival strategies of *C. jejuni* in either mono-species or dual-species conditions with *Pseudomonas aeruginosa*, a strict aerobic opportunistic pathogenic bacterium and strong biofilm former, that is also recognized for its ubiquity, multidrug resistance, and association with hospital-acquired infections and some serious illnesses (Thi et al. 2020). By using a simplified flat-plate aerated flow reactor in combination with live/dead staining and confocal laser scanning microscopy (CLSM), these authors observed that the two types of biofilms presented significantly different structures and activities. Thus, cells from monoculture biofilms were alive but not culturable after 5 days of biofilm growth (on a microscope coverslip), something that was probably related to the exposure to dissolved oxygen (DO). In addition, these cells did not consume any measurable quantity of oxygen as this was determined using a custom-made DO microelectrode. On the contrary, *C. jejuni* cells remained in a culturable state when these were grown together with *P. aeruginosa*, probably due to their limited exposure to DO, given that this was consumed by their biofilm partners (through aerobic respiration) and at the same time unaffected by higher flow rates (2.5 mL/min). Altogether, the results of this study clearly indicate that mixed-culture biofilms may provide an environmental shelter that may prove crucial for the survival of *C. jejuni* (Ica et al. 2012). In some later similar co-culture biofilm studies employing the same two bacterial species, it was again verified that the localized microaerophilic environment generated by *P. aeruginosa* enhances the sessile growth and persistence of *C. jejuni* (Culotti and Packman 2015; Scheik et al. 2021). Interestingly, such metabolic commensalism between the microaerophilic cells of *C. jejuni* and the aerobic cells of *Pseudomonas* spp., resulting in the extended survival of the former at ambient atmospheric oxygen tension, had also been shown in an older study, that time with planktonic cultures

(Hilbert et al. 2010). Enhanced survival of *C. jejuni* cells upon exposure to aerobic stress by forming mixed-culture biofilms with some other bacterial species with importance to food, such as *Staphylococcus aureus* and *Salmonella enterica*, has also been shown (Feng et al. 2016; Karki et al. 2021). It is worth noting that the higher content of EPS that was found in some of these dual-species biofilms, compared to the mono-species ones, may also explain this survival enhancement (Feng et al. 2016).

4 Conclusions and Future Perspectives

Undoubtedly, the results of the studies presented in this chapter clearly indicate that *Campylobacter* spp. can form biofilms on various surfaces, such as stainless steel, glass, and plastics, that can be encountered within the food production chain and elsewhere (e.g., in water distribution systems, medical establishments, etc.), and more importantly, these sessile structures can protect their microaerophilic and otherwise fastidious cells from various stresses, such as aerobic and antimicrobial exposure. Although in most of these studies biofilms were left to be formed under microaerophilic conditions (e.g., in the presence of 5% O_2 and 10% CO_2), in growth media containing (5–7% v/v) defibrinated (horse or sheep) blood, and at an elevated temperature (i.e., 37–42 °C), it is evident that *Campylobacter* cells may still survive for extended periods in minimal media and/or at lower temperatures and under normal atmospheric conditions either by being included in pre-established biofilms of some other bacterial species or by forming together with them mixed-culture sessile communities. This capability surely consists of an important microbiological risk for food safety and should be always considered by all those involved in food production and distribution, which should try to develop and apply more effective ways to get rid of these widely disseminated bacteria. In the last years, high-throughput omics approaches have significantly increased our knowledge on the molecular basis of biofilm development, together with those subcellular mechanisms which may be responsible for stress adaptation and resistance; however, the quite complex biological backgrounds of these interrelated phenomena require further dedicated research, preferably under conditions that should try to imitate better those probably encountered within the food chain. With respect to the latter, multi-species biofilms formed on industrial surfaces under food-relevant environmental conditions (e.g., in the presence of food residues and normal atmospheric oxygen tension and/or at lower incubation temperatures) are expected to offer knowledge that might be of higher relevance to the real problem. Hopefully, this will later trigger more effective ways for its mitigation and, thus, substantially contribute to the protection of public health.

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Magnitude and Molecular Characterization of Extended-Spectrum β-Lactamase Genes among *Klebsiella pneumoniae* Isolates in a Large Tertiary Hospital in Ethiopia

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Abstract

Background Extended-spectrum β-lactamases (ESBLs)-producing *Klebsiella pneumoniae* is reported worldwide increasingly. However, studies on ESBLs are still scarce in Ethiopia. Therefore, the current study aimed to determine the magnitude and resistance patterns of ESBL-producing *K. pneumoniae* as well as the frequency of ESBL-encoding genes.

Methods A cross-sectional study was conducted from September 2018 to February 2019 at Tikur Anbessa Specialized Hospital, Addis Ababa, Ethiopia among a total of 132 non-duplicate *K. pneumoniae* isolates. Phenotypic detection of ESBL production was done using Combined Disc Test. ESBL-encoding genes of $bla_{\text{CTX-M}}$, bla_{TEM}, and bla_{SHV} were detected through multiplex PCR.

Results The magnitude of ESBL production was 102/132 (77.3%). ESBL positive isolates were 100% resistant to ceftriaxone, cefotaxime, and cefuroxime. Co-resistance of ESBL-positive isolates to other non β-lactam antimicrobials was high to trimethoprim-sulfamethoxazole (96.1%) followed by tetracycline (75.5%) and gentamicin (73.5%). However, these isolates showed high susceptibility to amikacin (96.1%) and meropenem (89.2%). From the total ESBL-positive isolates, 82.6%, 73.5%, and 75% carried $bla_{\text{CTX-M}}$, bla_{TEM}, and

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bla$_{SHV}$ genes, respectively. The majority 78/102 (76.5%) of ESBL-positive isolates harbored all three types of ESBL genes simultaneously.

Conclusions The magnitude of ESBL-producing *K. pneumoniae* isolates was very alarming in the study area. The co-occurrence of *bla*$_{CTX-M}$, *bla*$_{TEM}$, and *bla*$_{SHV}$ genes is high, demanding large-scale studies to evaluate the presence of antimicrobial resistance super-clones. ESBL-producing isolates showed high resistance to most of the antimicrobials, needing phenotypic detection of ESBL regularly for better management of patients.

Keywords

Antimicrobial susceptibility · ESBL genes · Ethiopia · Extended-spectrum-beta-lactamase · *Klebsiella pneumoniae*

1 Introduction

Antibiotics play a critical role in reducing the morbidity and mortality associated with infectious diseases. However, antibiotic resistance is a problem of deep scientific concern both in hospital and community settings (Alanis. 2005). Beta-lactam antimicrobial agents exhibit the most common treatment for bacterial infections because of their broad-spectrum activities and better safety profiles (Shaikh et al. 2015). The persistent exposure of bacterial strains to a multitude of β-lactams has induced dynamic and continuous production and mutation of β-lactamases, especially in Gram-negative bacteria (Shah et al. 2004). Based on the amino acid sequence homology (ambler classification system), β-lactamases are classified into four major classes (A to D). Class A, C, and D β-lactamases require serine at their active site which is known as Serine β-lactamases (SBLs), while class B requires zinc as a cofactor for enzyme activity which is known as metallo-ß-lactamases (MBLs) (Behzadi et al. 2020).

Extended-spectrum-beta-lactamases (ESBLs) are β-lactamases belonging to class A and D; capable of hydrolyzing penicillins, first, second, and third-generation cephalosporins, and aztreonam (but not the cephamycins or carbapenems) and inhibited by β-lactamase inhibitors such as clavulanic acid (Harada et al. 2008). Moreover, many ESBL-producing bacteria are also resistant to other antimicrobial agents such as aminoglycosides, trimethoprim, and quinolones (Rawat and Nair. 2010; Zeynudin et al. 2018; Ahmadi et al. 2022). For instance, in Eastern, South-Western Europe, and Mediterranean countries, ESBL-producing *K. pneumoniae* showed exceeding 50–60% non-susceptibility for third-generation cephalosporins, fluoroquinolones, and aminoglycosides (Navon-Venezia et al. 2017). This is because ESBL genes are mostly located on plasmids harboring other resistance genes, leaving only a few effective drugs available for treatment (Rawat and Nair. 2010; Navon-Venezia et al. 2017), worsening the management of patients.

While *K. pneumoniae* have many plasmids that harbor most of the antibiotic-resistant genes including ESBLs, it serves as a worldwide source for the inter- and intra-species spread of multidrug resistance via horizontal gene transfer (Navon-Venezia et al. 2017; Wyres and Holt. 2018). The rapid increase in the frequency of drug resistance among *K. pneumoniae* isolates, especially the frequency of ESBL-producing strains (World Health Organization. 2014; Padmini et al. 2017), requires close monitoring of susceptibility patterns of these bacteria in different settings.

There are more than 200 different ESBL types (Paterson and Bonomo. 2005). In Africa, some studies characterize ESBLs and CTX-M-15, which is a variant of CTX-M, being the most common (Storberg. 2014). Likewise, the predominant ESBLs reported in East Africa were CTX-M-, TEM-, and SHV-types (Sonda et al. 2016). Studies on molecular characterization of ESBL-encoding genes are still scarce in Ethiopia. Therefore, the current study aimed to determine the magnitude of ESBL production and to describe the resistance patterns as well as the

frequency of bla_{CTX-M}, bla_{TEM}, and bla_{SHV} genes among *K. pneumoniae* isolates.

2 Materials and Methods

2.1 Study Population

A total of 132 study participants who were visiting Tikur Anbessa Specialized Hospital (TASH), Addis Ababa, Ethiopia and became culture positive for *K. pneumoniae* at any time during the visit from September 2018 to February 2019 were enrolled using a convenience sampling technique. The identification of *K. pneumoniae* was done using culture, Gram stain, and biochemical characteristics of the bacteria. *K. pneumoniae* is Gram-negative and rod-shaped, lactose fermenter, indole negative, gas and acid producer, hydrogen sulfide negative, citrate positive, mannitol fermenter, malonate positive, lysine decarboxylase positive, urea slow producing, and non-motile (Cheesbrough 2006). Socio-demographic characteristics and clinical information of the study participants were obtained using a well-designed questionnaire and from their medical records by health workers.

2.2 Antimicrobial Susceptibility Testing

The Antimicrobial Susceptibility Testing (AST) was performed on Muller-Hinton agar (Oxoid, UK) using the modified Kirby–Bauer disc diffusion technique (Cheesbrough. 2006) by using the following antimicrobial discs, Tetracycline (30 μg), Gentamicin (10 μg), Amikacin (30 μg), Ciprofloxacin (5 μg), Amoxicillin-clavulanate (20/10 μg), Piperacillin-tazobactam (100/10 μg), Trimethoprim-sulfamethoxazole (SXT 1.25/23.75 μg), Aztreonam (30 μg), Cefoxitin (30 μg), Cefuroxime (30 μg), Ceftriaxone (30 μg), Ceftazidime (30 μg), Cefotaxime (30 μg), Cefepime (30 μg), and Meropenem (10 μg) (Oxoid, UK) and (BD, USA). The results were read and interpreted after 16–18 h of incubation at 35 ± 2 °C (CLSI 2018).

2.2.1 Screening Test for Extended-Spectrum β-Lactamase (ESBL)

The ESBL screening test was performed by the standard disc diffusion method using Ceftazidime (30 μg), Cefotaxime (30 μg), Ceftriaxone (30 μg), and Aztreonam (30 μg) (BD, USA) antimicrobial discs as recommended by Clinical and Laboratory Standards Institute (CLSI) guidelines. Isolates that showed an inhibition zone size of ≤22 mm for Ceftazidime (30 μg), ≤ 25 mm for Ceftriaxone (30 μg), ≤27 mm for Cefotaxime (30 μg), and/or ≤ 27 mm for Aztreonam (30 μg) were considered as potential ESBL producers and selected for confirmation for ESBLs production using Combined Disc Test (CDT) as recommended by CLSI, 2018 guidelines (CLSI 2018).

2.2.2 Phenotypic Confirmatory Test for ESBL

Those isolates that fulfilled the screening test were confirmed for ESBL production using CDT using Cefotaxime-clavulanate (30/10 μg) and Ceftazidime-clavulanate (30/10 μg) (BD, USA) along with third-generation cephalosporins (Cefotaxime and Ceftazidime) on Mueller-Hinton agar plate. After 16–18 h of incubation at 35 ± 2 °C, a ≥ 5-mm increase in zone diameter for either antimicrobial agent tested in combination with clavulanate versus the zone diameter of the agent when tested alone is considered as positive for the ESBL production (CLSI 2018).

2.2.3 DNA Extraction

The bacterial DNA was extracted by the boiling lysis method as previously used by El-Badawy et al. (2017). Briefly, 3–5 fresh colonies of the bacteria were suspended in 300 μL of DNase-free water in a 1.5 ml Eppendorf tube. After vortexing, suspensions were boiled for 15 min at 94 °C and placed at −20 °C for 10 min. Then placed at room temperature for 1 min and centrifuged at 14000 rpm for 5 min at room temperature. The supernatant was then transferred to a new Eppendorf tube and was stored at −20 °C till amplification. The quality and quantity of the

extracted DNA were measured using Nanodrop (Thermo Scientific, US) as indicated elsewhere (Ranjbar et al. 2017).

2.2.4 Molecular Characterization of ESBL Genes

Multiplex Polymerase Chain Reaction (PCR) was done as it is much more accurate, time-consuming, and economic in comparison with Monoplex PCR technique (Khonsari et al. 2021). Specific primers were used to detect genes encoding ESBLs (bla_{CTX-M}, bla_{TEM}, and bla_{SHV}) as shown in Table 1. Briefly, the PCR was performed with approximately 300 ng template DNA, 0.2 µM of each primer, and 7.5 µl of 2 × QIAGEN Multiplex PCR Master Mix (QIAGEN, Germany) in a final volume of 15 µl. The amplification was performed in a thermocycler (Biometra, Germany) with cycling parameters including initial denaturation at 95 °C for 15 min followed by 35 cycles each of denaturation at 94 °C for 30 s, annealing at 58 °C for 90 s, extension at 72 °C for 90 s, and final extension at 72 °C for 10 min. The amplicon was visualized by electrophoresis in 1.5% agarose gel after staining in ethidium bromide. A 100 bp ladder molecular weight marker (Promega, US) was used to measure the molecular weight of amplified products. The amplicon was visualized and its size was determined under UV trans-illuminator (Bio-Rad, US). Figure 1 shows the laboratory work flowchart based on scientific recommendation (Behzadi and Gajdács. 2021).

2.3 Data Quality Assurance

The reliability of the study findings was guaranteed by implementing quality control measures throughout the whole process of

Table 1 Primers used for detection of ESBL genes in *K. pneumoniae* isolates

Gene	Primer		Nucleotide sequence 5′----------3′	Amplicon size (bp)	Reference
bla_{CTX-M}	CTX-M	F	CGCTGTTGTTAGGAAGTGTG	754	Hassan et al. (2013)
		R	GGCTGGGTGAAGTAAGTGAC		
bla_{TEM}	TEM	F	TTTCGTGTCGCCCTTATTCC	404	
		R	ATCGTTGTCAGAAGTAAGTTGG		
bla_{SHV}	SHV	F	CGCCTGTGTATTATCTCCCT	294	
		R	CGAGTAGTCCACCAGATCCT		

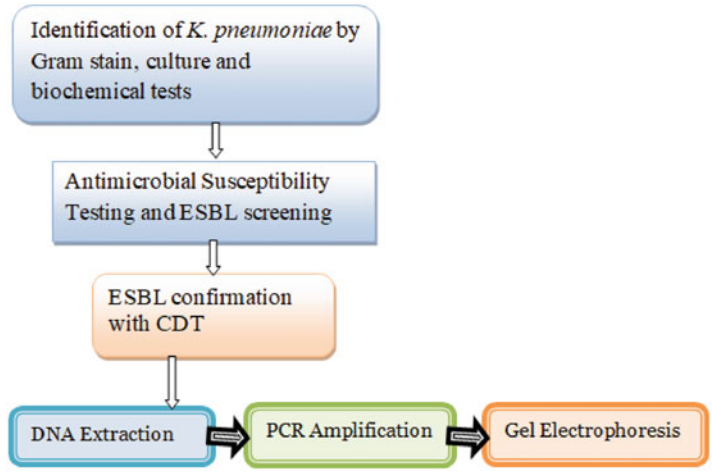

Fig. 1 Laboratory work flowchart. ESBL: Extended Spectrum B-lactamase, CDT: Combined Disc Test, PCR: Polymerase Chain Reaction

the laboratory work. *K. pneumoniae* ATCC® 700603 and *Escherichia coli* ATCC® 25922 were used as ESBL positive and negative control strains respectively during CDT. Known $bla_{\text{CTX-M}}$, bla_{TEM}, and bla_{SHV} genes were used as positive controls in molecular detection. Each primer pair was checked in Monoplex PCR before multiplexing.

2.4 Data Entry and Analysis

Data was checked, cleaned, and double entered into Epidata software version 3.1 (The EpiData Association, Denmark), and then it was exported to Statistical Package for Social Sciences (SPSS version 25.0, I BM Corp., USA) software for analysis. Binary logistic regression was used to compare categorical variables, and P-value <0.05 at 95% confidence interval was considered as statistically significant.

3 Results

3.1 Magnitude of ESB-Producing *K. pneumoniae* Isolates

Of the total 132 *K. pneumoniae* isolates, 128 (97%) of the isolates were identified as potential ESBL producers with a zone of inhibitions of cefotaxime ≤27 mm, ceftriaxone ≤25, ceftazidime ≤22 mm, and/or aztreonam ≤27 mm. Among the potential ESBL producers, the large majority 102/128 (79.7%) were ESBL positive with CDT. As shown in Fig. 2 generally, the ESBL positivity rate from the total isolates was 102/132 (77.3%). Figure 3 shows ESBL positive and negative results of CDT.

3.2 Association of Socio-demographic and Clinical Characteristics with ESBL Production

As shown in Table 2, the proportion of ESBL-producing *K. pneumoniae* isolates in females 39/49 (79.6%) was comparable with their male counterparts 63/83 (75.9%). Age and ward type has been associated with ESBL production in bivariate analysis. A higher proportion of *K. pneumoniae* isolates from patients in ICUs 37/46 (80.4%) and pediatric wards 42/53 (79.2%) were ESBL producers compared to those from medical wards 4/9 (44.4%). Similarly, ESBL producers were more frequently encountered among patients less than 5 years 64/74 (86.5%) than those 18 to <45 years of age (64.0%). Age and ward types were subjected to multivariate analysis. However, none of them were associated with ESBL production in multivariate analysis.

3.3 Specimen-Wise Distribution of ESBL-Producing *K. pneumoniae* Isolates

As displayed in Fig. 4, blood was the major specimen from which ESBL-producing *K. pneumoniae* were isolated 54/102 (52.9%)

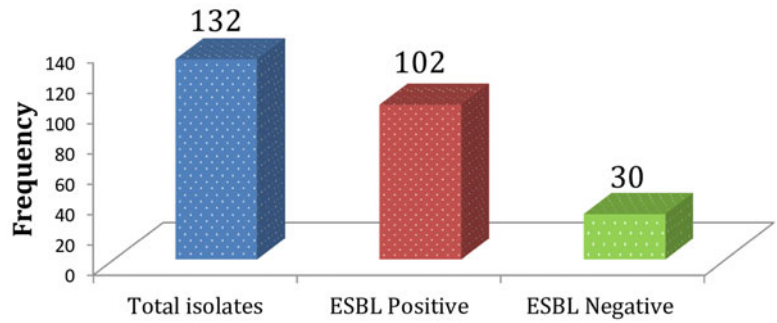

Fig. 2 Frequency of ESBL-producing and non-producing *K. pneumoniae* isolates

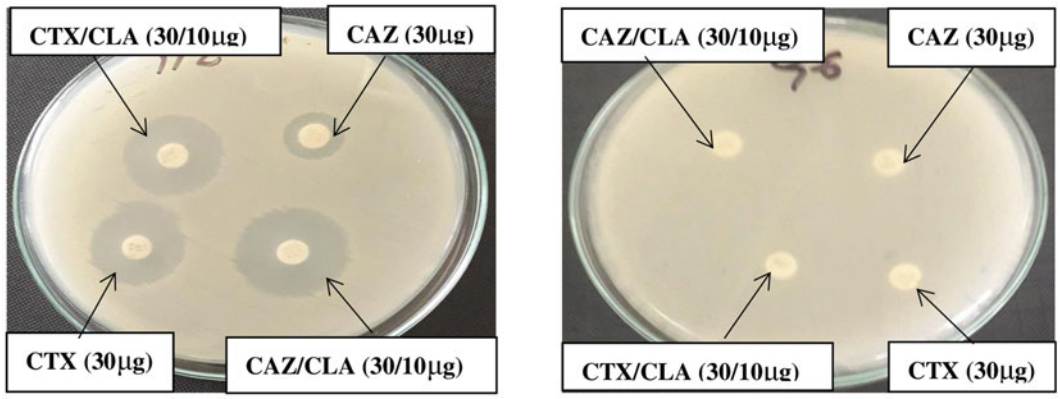

Fig. 3 ESBL positive (left) and negative (right) *K. pneumoniae* isolates with Combined Disc Test (CDT). CTX: Cefotaxime, CAZ: Ceftazidime, CTX/CLA: Cefotaxime/ clavulanate, CAZ/CLA: Ceftazidime/ clavulanate

Table 2 Association of age, sex, patient setting, and ward type with ESBL production

	Extended-spectrum β-lactamase		Bivariate analysis		Multivariate analysis	
Variables	Positive n (%)	Negative n (%)	P-value	COR (95%CI)	P-value	AOR (95%CI)
Sex						
Male (n = 83)	63(75.9)	20(24.1)	0.626	0.81 (0.34–1.9)		
Female (n = 49)	39(79.6)	10(20.4)	R			
Age group						
<5 years (n = 74)	64(86.5)	10(13.5)	**0.017***	3.6 (1.26–10.33)	0.116	3.86 (0.72–20.77)
5 to <18 years (n = 20)	12(60.0)	8(40.0)	0.783	0.84 (0.25–2.83)	0.959	0.95 (0.15–6.11)
18 to <45 years (n = 25)	16(64.0)	9(36.0)	R			
≥45 years (n = 13)	10(76.9)	3(23.1)	0.420	1.88 (0.41–8.63)	0.474	1.93 (0.32–11.6)
Patient setting						
Inpatients (n = 120)	94(78.3)	26(21.7)	0.363	1.81 (0.5–6.48)		
Outpatient (n = 12)	8(66.7)	4(33.3)	R			
Ward type						
ICUs (n = 46)	37(80.4)	9(19.6)	**0.033***	5.14 (1.14–23.1)	0.421	2.15 (0.33–13.8)
Pediatric ward (n = 53)	42(79.2)	11(20.8)	**0.038***	4.77 (1.09–20.82)	0.445	2.21 (0.29–16.77)
Medical ward (n = 9)	4(44.4)	5(56.6)	R			
Surgical ward (n = 8)	7(87.5)	1(12.5)	0.086	8.75 (0.74–103.8)	0.106	7.83 (0.65–94.9)
Emergency (n = 2)	2(100.0)	0(0.0)	0.999			
Orthopedics (n = 2)	2(100.0)	0(0.0)	0.999			

R; Reference, *, Significant P < 0.05, COR; Crude Odds Ratio, AOR; Adjusted Odds Ratio, CI; Confidence Interval, ICUs; Intensive Care Units

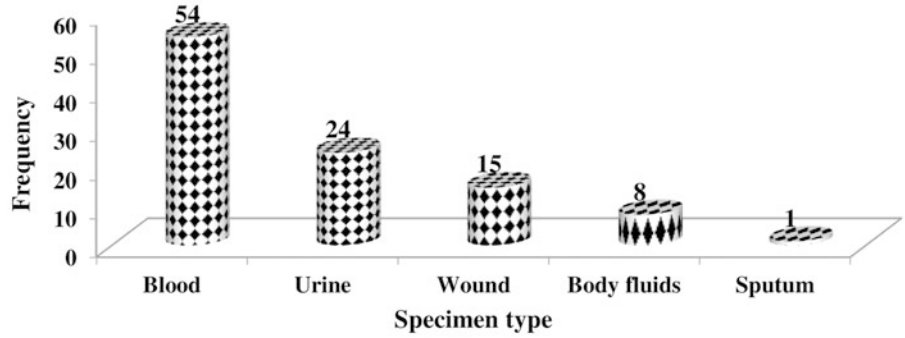

Fig. 4 Specimen-wise distribution of ESBL-producing *K. pneumoniae* isolates

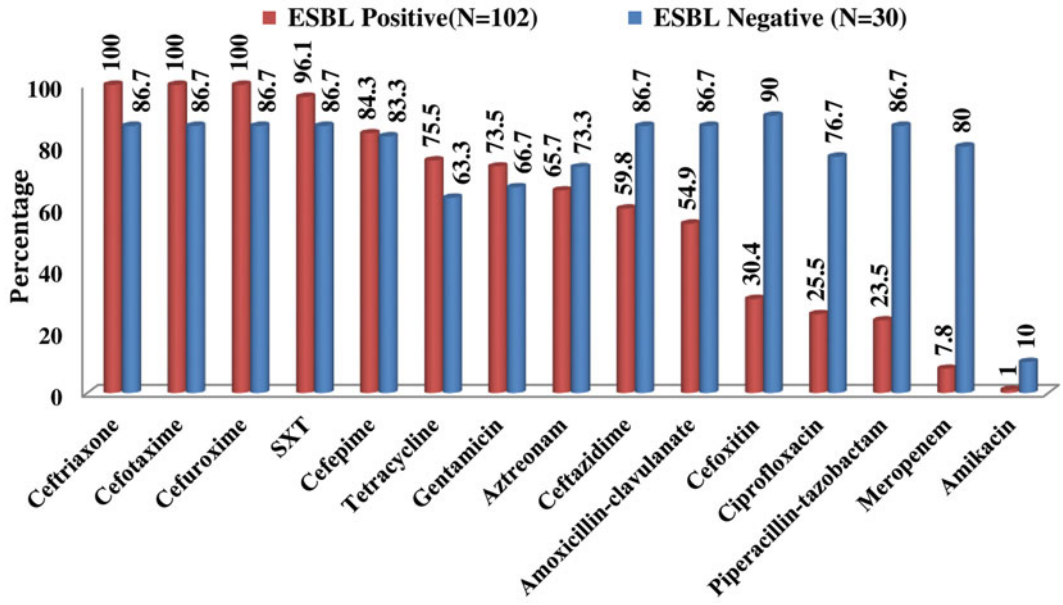

Fig. 5 Antimicrobial resistance patterns of ESBL-producing and non-producing *K. pneumoniae* isolates. SXT: Trimethoprim-sulfamethoxazole

followed by urine 24/102 (23.5%). While only one ESBL-producing *K. pneumoniae* was recovered from sputum.

3.4 Antimicrobial Resistance Patterns of ESBL-Producing and Non-producing *K. pneumoniae* Isolates

The antimicrobial resistance patterns of ESBL producers and non-producers were checked. As shown in Fig. 5, ESBL-positive isolates were 100% resistant to two third-generation cephalosporins namely ceftriaxone and cefotaxime. Co-resistance of ESBL-positive isolates to other non β-lactam antimicrobials was higher to trimethoprim-sulfamethoxazole 98 (96.1%) followed by tetracycline 77 (75.5%), gentamicin 75 (73.5%), aztreonam 67 (65.7%), and amoxicillin-clavulanate 56 (54.9%). However, these isolates showed high susceptibility to amikacin 98 (96.1%) and meropenem 91 (89.2%). ESBL-negative isolates showed high resistance to meropenem (80.0%) and cefoxitin (90.0%). Additionally, 26/30 (86.7%)

Table 3 Distribution of bla_{ESBL} genes among ESBL-positive *K. pneumoniae* isolates

Gene	Frequency (n = 102)	Percentage
Total ESBL gene-positive	101	99.0
bla_{CTX-M} total	97	95.1
bla_{TEM} total	89	87.3
bla_{SHV} total	88	86.3
bla_{CTX-M} only	3	2.9
bla_{TEM} only	1	1.0
bla_{SHV} only	2	2.0
bla_{CTX-M}, bla_{TEM}, and bla_{SHV}	78	76.5
bla_{CTX-M} and bla_{TEM}	9	8.8
bla_{CTX-M} and bla_{SHV}	7	6.9
bla_{TEM} and bla_{SHV}	1	1.0
Negative for bla_{CTX-M}, bla_{TEM}, or bla_{SHV}	1	1.0

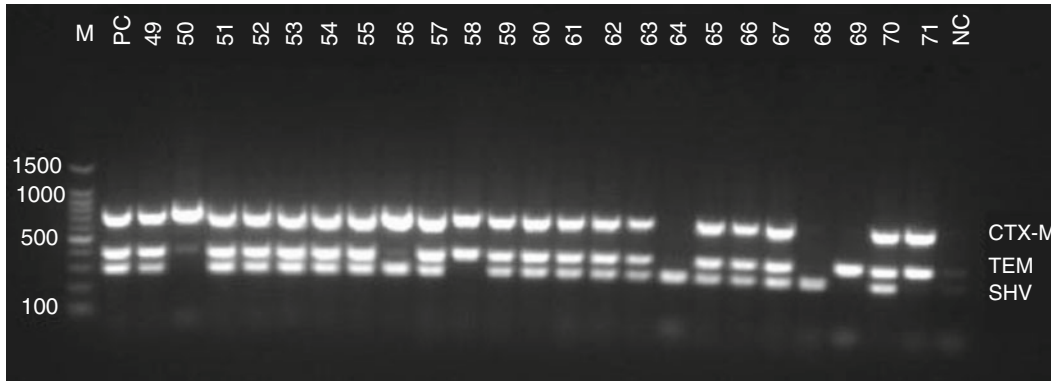

Fig. 6 Agarose gel electrophoresis of PCR products for ESBL genes. Lane M: 100 bp DNA ladder; PC: Positive control; Lanes 49–71: *K. pneumoniae* isolates; NC: Negative control

of ESBL negatives were resistant to piperacillin-tazobactam, amoxicillin-clavulanate, ceftriaxone, cefotaxime, ceftazidime, cefuroxime, and trimethoprim-sulfamethoxazole (Fig. 5).

3.5 Frequency of ESBL Genes Among *K. pneumoniae* Isolates

The ESBL genes of bla_{CTX-M}, bla_{TEM}, and bla_{SHV} were simultaneously amplified by multiplex PCR. It was revealed that from the total ESBL-positive *K. pneumoniae* isolates, 99.0% carried one or more *bla* genes. From the total ESBL-positive isolates, 95.1%, 87.3%, and 86.3% carried bla_{CTX-M}, bla_{TEM}, and bla_{SHV}, respectively (Table 3). The majority of 78 (76.5%) ESBL-positive isolates harbored all the three types of genes and 17 (16.7%) had a combination of two genes, while only 6 (5.9%) had a single type of ESBL gene. The co-existence of two genes was observed with bla_{CTX-M} and bla_{TEM} gene in nine (8.8%) isolates followed by bla_{CTX-M} and bla_{SHV} in seven (6.9%) isolates, bla_{TEM} and bla_{SHV} in one isolate. Figure 6 shows the gel image of the bla_{CTX-M} (754 bp), bla_{TEM} (404 bp), and bla_{SHV} (294 bp) genes.

4 Discussion

In this study, the magnitude of ESBL-producing *K. pneumoniae* from the total isolates was 77.3%. This finding is in close harmony with other studies in Ethiopia at which ESBL-producing

K. pneumoniae was 78.6% in Addis Ababa (Teklu et al. 2019) and 76% in Bahir Dar (Moges et al. 2019). Similarly, a comparable finding was reported from other countries such as 72.7% in Uganda (Kateregga et al. 2015) and 81.39% in Iraq (Aljanaby and Alhasnawi. 2017). Conversely, it is higher than studies from India 48.27% (Shashwati et al. 2014) and Iran 52% (Ahmadi et al. 2022). The observed difference might be due to differing geographic locations. Moreover, it could be due to the frequent use of broad-spectrum β-lactams in the current study area (Gutema et al. 2018) which creates selective pressure and results in the emergence of stable, rapidly proliferating ESBL-producing clones with continued horizontal gene transfer across genera (Lukac et al. 2015).

Furthermore, a previous study done in the same hospital where we did the current study noted that gastrointestinal colonization of ESBL-producing *K. pneumoniae* was as high as 100% in hospitalized neonates, 88% in children, and 50% in adults (Desta et al. 2016). Gut colonization with ESBL-producing bacteria can lead to extra-intestinal infections (Karanika et al. 2016). Given the majority of *K. pneumoniae* isolates in our study were recovered from inpatients and the over crowdedness of the hospital, the high magnitude of ESBL-producing *K. pneumoniae* may also be due to a lack of stringent infection control measures and effective antimicrobial resistance surveillance.

In this study, the susceptibility of ESBL-producing *K. pneumoniae* to meropenem was high (89.2%). Carbapenems are the drugs of choice to treat ESBL-producing *K. pneumoniae*, but a considerable meropenem resistance was observed in our study. Therefore, they should be used as a therapy of choice for only severe infections with ESBL-producing organisms as recommended by Tamma and Rodriguez-Baño (2017). Utilizing non-carbapenem antimicrobial for the treatment of ESBL-producing organisms intends to preserve the therapeutic value of these precious drugs.

In the present study, when the resistance rates of ESBL-producing *K. pneumoniae* isolates to β-lactam/β-lactamase inhibitor combinations were compared, resistance to piperacillin/tazobactam (23.5%) was much lower than amoxicillin/clavulanate (54.9%). It was consistent with other studies (Shashwati et al. 2014; Desta et al. 2016). ESBLs are better inhibited by the β-lactamase inhibitor tazobactam than by sulbactam and clavulanate showing clinical resistance to amoxicillin/clavulanate than piperacillin/tazobactam (Bradford. 2001). However, as reported elsewhere, β-lactam/β-lactamase inhibitor consumption had a significant temporal correlation with increased resistance to piperacillin/tazobactam in *K. pneumoniae,* suggesting that reducing the misuse of β-lactam/β-lactamase inhibitors may help to use piperacillin/tazobactam as an alternative to carbapenems (Ryu et al. 2018) for low to moderate severity infections (Tamma and Rodriguez-Baño. 2017). On the other hand, a recent review highlights piperacillin/tazobactam as a suitable alternative to carbapenems for the treatment of many invasive infections caused by ESBL producers if it is active in vitro and the dose is adjusted (Rodriguez-Bano et al. 2018). It has been indicated that the prescription of accurate, specific, and effective drugs may lead to a definite treatment (Issakhanian and Behzadi. 2019).

Of particular concern, non-ESBL-producing isolates had higher resistance rates to more than half of the antimicrobials used in our study than that of ESBL producers, which magnifies the burden of drug resistance *K. pneumoniae* in the study setting. The opposite was true in several other studies, in which ESBL producers had higher resistance rates than non-ESBL producers (Obeng-Nkrumah et al. 2013; Teklu et al. 2019). In this study, ESBL-negative isolates were highly resistant to meropenem (80.0%) and cefoxitin (90.0%). Moreover, 86.7% of ESBL negative isolates were resistant to piperacillin-tazobactam, amoxicillin-clavulanate, ceftriaxone, cefotaxime, ceftazidime, cefuroxime, and trimethoprim-sulfamethoxazole. This worrisome resistance profile of *K. pneumoniae* could be due to the circumstance that the majority (73.3%) of the ESBL-negative isolates in our study were carbapenemase producers [See additional file]. Carbapenemase-producing Gram-negatives, in particular, are resistant to all or

almost all beta-lactams, fluoroquinolones, and/or aminoglycosides concomitantly (Meletis 2016). For instance, some of the MBLs are known as important carbapenemases capable of hydrolyzing a wide range of drugs including carbapenems, cephalosporins, and penicillins (Behzadi et al. 2020).

In our study, the proportion of ESBL-positive *K. pneumoniae* was higher in those aged below 5 years than in those aged 18 to <45 years. This could be because, as it was previously reported by Desta et al. in the hospital where we did the present study, the proportion of ESBL-producing *K. pneumoniae* colonization was higher in these age groups (Desta et al. 2016), which could result in infection with these bacteria if infection control measures were not adequately implemented. A higher proportion of ESBL production was observed among those admitted to the intensive care units than those in medical wards. A study by Marra et al. also reported a higher proportion of patients with ESBL-producing nosocomial bloodstream infections in the ICUs (Marra et al. 2006). This could be due to the reason that hospitalized patients in the ICU are more likely to be exposed to a heavy load of antimicrobials, long-term hospitalization, and extensive usage of invasive devices. The current study showed that none of the variables were significantly associated with ESBL production which is in line with other studies (Kateregga et al. 2015; Camara et al. 2017).

In our study, from the total ESBL-positive *K. pneumoniae* isolates, 95.1%, 87.3%, and 86.3% carried $bla_{\text{CTX-M}}$, bla_{TEM}, and bla_{SHV} respectively, which is very alarming. This finding was comparable with a report from Tunisia at which 89%, 56.78%, and 81.35% (Alibi et al. 2015) and Côte d'Ivoire 93.3%, 86.7%, and 80.0% (Müller-Schulte et al. 2020) of ESBL-positive *K. pneumoniae* isolates carried $bla_{\text{CTX-M}}$, bla_{TEM}, and bla_{SHV}, respectively. Another comparable finding was reported from Iran indicating that the prevalence of $bla_{\text{CTX-M}}$, bla_{SHV}, and bla_{TEM} genes were 96%, 94%, and 91%, respectively (Ahmadi et al. 2022). In our study, the majority (76.5%) of ESBL-positive isolates carried three genes simultaneously which is analogous with a study from Côte d'Ivoire, 71%. This might be due to the spread of a plasmid harboring the three genes. The most globally common type of ESBL that appeared is CTX-M with their higher incidence in most locations compared to SHV and TEM (Storberg 2014; Shaikh et al. 2015). Similarly, the occurrence of $bla_{\text{CTX-M}}$ in this study is slightly higher than the other genes. The high magnitude of $bla_{\text{CTX-M}}$ in many countries could be due to the reason that cefotaxime and ceftriaxone are used worldwide (Paterson et al. 2003) which creates selective pressure. The study detects only three ESBL-encoding genes due to resource limitations.

5 Conclusions

The magnitude of ESBL-producing *K. pneumoniae* isolates was very alarming. The majority of the isolates also harbored $bla_{\text{CTX-M}}$, bla_{TEM}, and bla_{SHV} genes concurrently intensifying the problem. Meropenem and amikacin, followed by piperacillin-tazobactam, were the most active drugs against ESBL-producing isolates. ESBL-producing *K. pneumoniae* isolates showed high resistance to the rest of the drugs used in the current study. Therefore, phenotypic detection of ESBL production should be done in the hospital regularly for better management of patients and to monitor further progress in the control of antimicrobial resistance. Further large-scale studies to evaluate the presence of antimicrobial resistance superclones are warranted.

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Author contributions All authors made a significant contribution to the work reported. TA1, TA2, AM, BT, BY1 conceived and designed the experiments. TA1, AS, SS, BY2, BT took part in formal analysis and investigation; TA1, AS, SS writing – original draft preparation; BT, TA2, AS, SS, AM, BY1, BY2, AA writing – review and editing; BT, TA2, AS, SS, AM, BY1, AA, funding acquisition. All authors read and approved the manuscript. TA1 refers to Tewachew Awoke and TA2 to Tamrat Abebe. BY1 refers to Biruk Yeshitela and BY2 to Berhanu Yitayew.

Dataset The datasets supporting the conclusions of this article are included within the article and its additional files.

Ethics This study was approved by the Ethics Review Committee of the Department of Microbiology, Immunology and Parasitology, School of Medicine, College of Health Sciences, Addis Ababa University (Reference number: DERC/17/18/02-N) and AHRI/ALERT ethical review committee (Protocol number: PO12/18). A permission letter was obtained from TASH. Moreover, before commencing the study, a written informed consent/ assent was obtained from each study participant/ parent or guardian. Confidentiality was maintained for all data collected.

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Conflict of Interest Statement The authors report no conflicts of interest in this work.

Additional file Carbapenemase production among extended-spectrum β-lactamase (ESBL) positive and negative *K. pneumoniae* isolates.

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Seasonal Variation Analysis for Weekly Cases, Deaths, and Hospitalizations of COVID-19 in the United States

Tianze Xu and Yingying Cui

Abstract

Knowing the seasonality of COVID-19 helps decision-makers to take suitable interventions against the pandemic. In this study, we performed the Brown-Forsythe variance analysis on seasonal variations on different indicators based on the data on COVID-19 for the United States provided publicly by WHO. Our study finds that the seasonality of weekly cases and deaths of COVID-19 are strongly statistically supported by the data. The weekly total cases(/deaths) in winter are three to seven times(/two to three times) more than the other three single seasons. The ICU patients in winter and autumn are four to five times more than spring. The weekly hospital admissions in winter are four times more than spring. The mean of the positive rate in winter is five times more than spring. The findings of this research can be a reference in decision-making when taking interventions against the pandemic, such as taking stricter interventions in winter while considering less strict interventions in summer, etc.

Keywords

COVID-19 · Seasonality · Variance analysis

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1 Introduction

Studies have shown that continuing and timely non-pharmaceutical interventions alongside vaccinations are essential for faster recovery from COVID-19 (Arık et al. 2021a; Huang et al. 2021; Albarracin et al. 2021; Ward et al. 2022; Aranzales et al. 2021). However, the interventions involve restricting people's behavior, effecting people's life, and slowing down the economy (Sarma and Ghosh 2022; Matta 2020). How to balance between taking measures to control the spread of the virus and minimize the side effect of the interventions on the economy and people's life is a vital issue needing studying. For this purpose, the interventions with different strictness level should be considered and applied in different situations, the reason being that if the strictest interventions are applied in all situations, the economy and the people will be overly negatively influenced in the situation that is not severe, whereas, if the least strict interventions are applied in all situations, the spread of the virus will be out of control in the situation that is severe. The distinction of different situation can be done via considering multiple important factors, one of which might be seasons of a year.

By performing seasonal variation analysis of the data of the pandemic, including the cases and deaths and other indicators, we can ascertain if the pandemic situation is variable with the rotation of seasons. Many studies have been done on the

association between COVID-19 transmission rates or symptom severity and environmental parameters such as climate zone, season, meteorological variability, and air quality (Smit et al. 2020; Briz-Redón and Serrano-Aroca 2020; Pun et al. 2020; Zhu et al. 2020; IHME COVID-19 Forecasting Team 2021; Carlson et al. 2020; Yamauchi et al. 2022; Chang et al. 2022; Lowe et al. 2020; Fattorini and Regoli 2020; Ma et al. 2021; Steyn et al. 2022; Arık et al. 2021b; Baker et al. 2020; Gardner et al. 2020; Zaitchik et al. 2020; Yanamala et al. 2021; Brehm et al. 2021; Chivukula 2021; Ayanlade 2020; Redding et al. 2021; Martheswaran 2022; d'Albis et al. 2021; Edridge et al. 2020; Chen et al. 2021). Unfortunately, not a little reported evidence was often contradictory. The veracity of forecasts of COVID-19 incidence based on seasonality or other meteorological factors was often in dispute 1 or 2 years ago partly because there were misconceptions about weather and seasonality (Reyburn et al. 2011) and partly because there was no enough data (Simpson et al. 2020; De la Puerta et al. 2021).

As for the seasonality of COVID-19, the Virtual Symposium on Climatological, Meteorological and Environmental (CME) Factors in the COVID-19 Pandemic, August 4–6, 2020, admitted that the seasonality of COVID-19 was difficult to distinguish at the early phase in the pandemic and needs to be established as soon as there were enough data. The symposium's outcome also indicated the observed challengers and limitations of the then research and applications as some contradictory and non-comparable studies caused by using a wide range of methodological approaches/and inconsistent data sets. It called on that studies should attempt to meet a minimum standard of good practice in data use and methods, inclusive of spatially and temporally aligning epidemiological and CME data (Grauer et al. 2020).

It is for positively responding to this calling that we perform this research. With WHO's work in the past more than 2 years, the accumulative and daily cases and deaths on COVID-19 in most countries in the past more than 2 years are readily available (Outcome Statement 2020).

2 Method

The Virtual Symposium on CME Factors in the COVID-19 Pandemic called for timely research on the seasonality of COVID-19. In this study, we pick all the daily data on COVID-19 for the United States provided publicly by WHO (WHO COVID-19 Explorer 2022) till the day of this study to process the data and convert the daily data into weekly data and perform seasonal variation analysis on different indicators. The factor to be analyzed is season, and the treatment includes four seasons of a year. The analyzed data include weekly cases, weekly deaths, Friday's reproduction rate, ICU patients, hospitalized patients, weekly hospital admissions, and weekly positive rate.

We convert the accumulative daily total cases/deaths during the period from January 24, 2020, when the data began to be available, to March 25, 2022, when this study was done, into weekly cases/deaths of the same period. We break down the weekly data into two groups. Group 1 is for period from January 24, 2020, to January 24, 2021, and group 2 is for period from January 25, 2021, to February 1, 2022. From January 24, 2020, the first day when the daily data became publicly available, every seven consecutive days are taken as a week, and 52 weeks are taken as a year's time; thus, group 1 is for period from January 24, 2020, to January 24, 2021. Group 2 is also for 52 weeks or a year's time. For each group, the observed values of each treatment/season are the weekly data for the indicator in the season. We define spring as February to April, summer as May to July, autumn as August to October, and winter as November to January. The reason for defining the seasons in this way is to consider when the data used in the study start and end, and the seasons in the study were based on the pandemic timeline or flu seasonal.

The same method is used to convert the daily accumulative total hospitalizations during the period from July 17, 2020, the first day when such data became publicly available, to March 25, 2022, when this study was done into weekly total hospitalizations of the same period. Likewise, every seven consecutive days are taken as a week, and 52 weeks are taken as a year's time. When a week is in February to April, it is taken as spring. When it is in May to July, it is taken as summer. When it is in August to October, it is taken as autumn. When it is in November to January, it is taken as winter. Similarly, such weekly data are broken down into two groups. Group 1 is for period from July 17, 2020, to June 25, 2021, and group 2 is for period from July 2, 2021, to March 25, 2022. For the same reason of the availability of data, the same time period applies to the grouping of the following indicators: Friday's reproduction rate, ICU patients, hospitalized patients, weekly hospital admissions, and weekly positive rate. Data on such indicators are not available until July 17, 2020. So the period for them is from July 17, 2020, to March 25, 2022, when this study was done (the latest data we could have when we did this study).

For each group, the Brown-Forsythe variance analysis is performed to find if there is statistical difference in the mean values of the indicator considered among four seasons. This Brown-Forsythe variance analysis is done on the statistical software SPSS for every indicator and every group. This study does not involve conducting research on living individuals nor obtaining or using identifiable private information or identifiable biospecimens. Thus, this research does not require an IRB approval. The degree of freedom between treatments in this study is $m = 3$, and the intragroup degree of freedom n is dependent on the sample size. Brown F is calculated and denoted as BF(m, n). The significance level for Brown F is 0.01. All the confidence intervals (CI) are at significance level 0.05. The result of the analysis for each indicator is explained and is shown in a figure accordingly. In all the plots, the abscissa is season, with 1.0 representing spring, 2.0 summer, 3.0 autumn, and 4.0 winter, respectively. The ordinate is dependent on the indicator being analyzed (cases, deaths, etc.). The results are shown in the following section of this chapter.

3 Results

For the weekly total cases of group 1 from January 24, 2020, to January 24, 2021, BF (3, 25.585) = 50.300, $p = 0.000$. The 95% CI are [134321.92 ± 80480.27] for spring, [321982.54 ± 112878.61] for summer, [556221.46 ± 352380.82] for autumn, and [1316864.09 ± 321440.17] for winter, respectively. The weekly total cases in winter are four to seven times more than the other three single seasons. For the weekly total cases of group 2 from January 25, 2021, to February 1, 2022, BF (3, 11.219) = 14.643, $p = 0.000$. The 95% CI are [363449.31 ± 108539.33] for spring, [422596.92 ± 392747.00] for summer, [720562.54 ± 227318.12] for autumn, and [2679058.91 ± 1853793.83] for winter, respectively. The weekly total cases in winter are three to seven times more than the other three single seasons (Fig. 1). The seasonality of COVID-19 in terms of total cases is strongly ascertained.

For the weekly total deaths of group 1 from January 24, 2020, to January 24, 2021, BF (3, 21.430) = 39.389, $p = 0.000$. The 95% CI are [8161.69 ± 5987.43] for spring, [5796.54 ± 1315.07] for summer, [6588.46 ± 2061.36] for autumn, and [19663.36 ± 2720.55] for winter, respectively. The weekly total deaths in winter are two to three times more than other three single seasons. For the weekly total deaths of group 2 from January 25, 2021, to February 1, 2022, BF (3, 37.589) = 32.183, $p = 0.000$. The 95% CI are [6265.85 ± 2331.71] for spring, [3368.38 ± 2239.26] for summer, [10835.38 ± 2600.77] for autumn, and [12982.27 ± 3363.35] for winter, respectively. The weekly total deaths in winter are two to four times more than spring and summer. That figure in autumn is nearly two to three times higher than spring and summer. That figure in winter is 20% higher than autumn (Fig. 2). The seasonality of COVID-19 in terms of total deaths is strongly ascertained.

For Friday's reproduction rate of group 1 from July 17, 2020, to June 25, 2021, BF (3, 44.228) = 8.857, $p = 0.000$. The 95% CI are

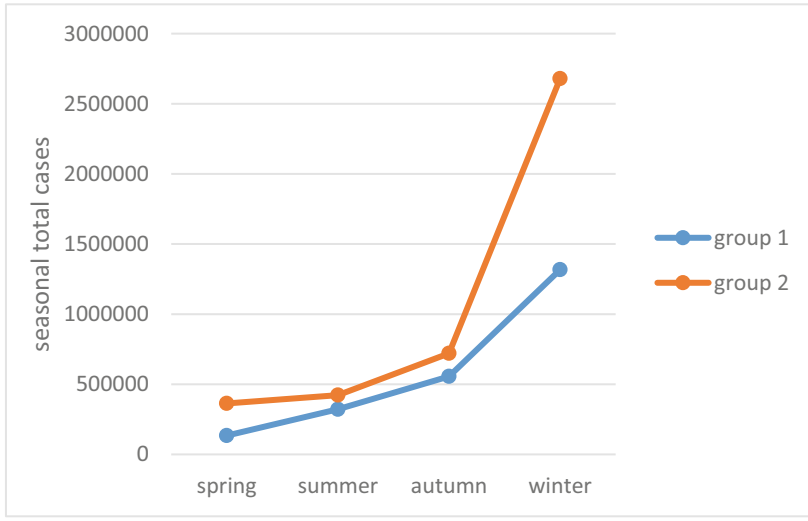

Fig. 1 The seasonal total cases of group 1 and group 2

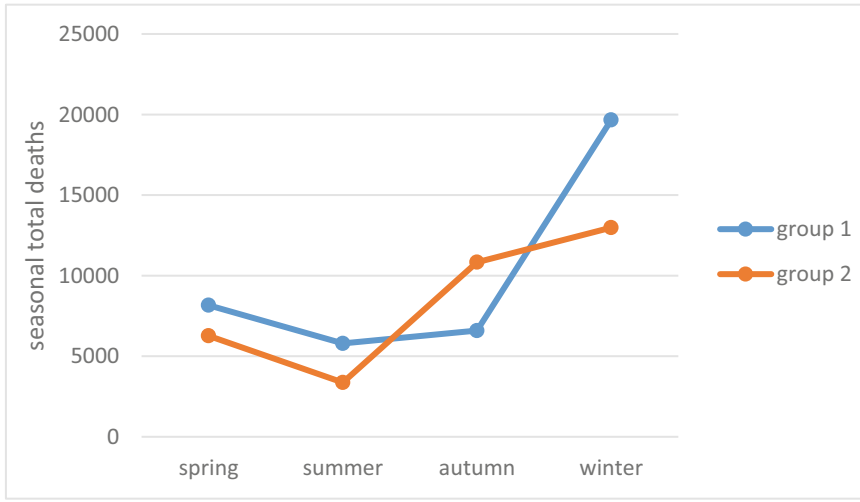

Fig. 2 The seasonal total deaths of group 1 and group 2

[0.91 ± 0.12] for spring, [0.93 ± 0.09] for summer, [1.11 ± 0.11] for autumn, and [0.93 ± 0.13] for winter, respectively. The mean of Friday's reproduction rate in autumn is about 20% higher than other three seasons. For Friday's reproduction rate of group 2 from July 2, 2021, to March 25, 2022, BF(3, 21.667) = 6.416, $p = 0.003$. The 95% CI are [0.79 ± 0.18] for spring, [1.32 ± 0.19] for summer, [0.97 ± 0.08] for autumn, and [1.02 ± 0.38] for winter, respectively. The mean of Friday's reproduction rate in summer is about 40% higher than spring. That figure in winter and autumn is about 20% higher than spring (Fig. 3). The seasonality of COVID-19 in terms of reproduction rate is not observed.

For weekly ICU patients of group 1 from July 17, 2020, to June 25, 2021, BF(3, 34.761) = 33.076, $p = 0.000$. The 95% CI are [8538.31 ± 1250.55] for spring, [9342.91 ± 4348.90] for summer, [11675.31 ± 4458.42] for autumn, and [22931.46 ± 5326.70] for winter, respectively. The ICU patients in winter are about two to

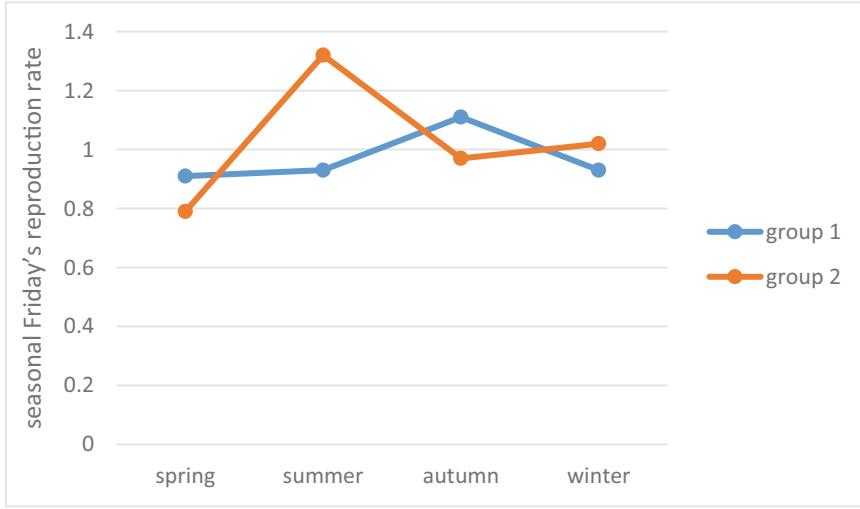

Fig. 3 Seasonal Friday's reproduction rates of group 1 and group 2

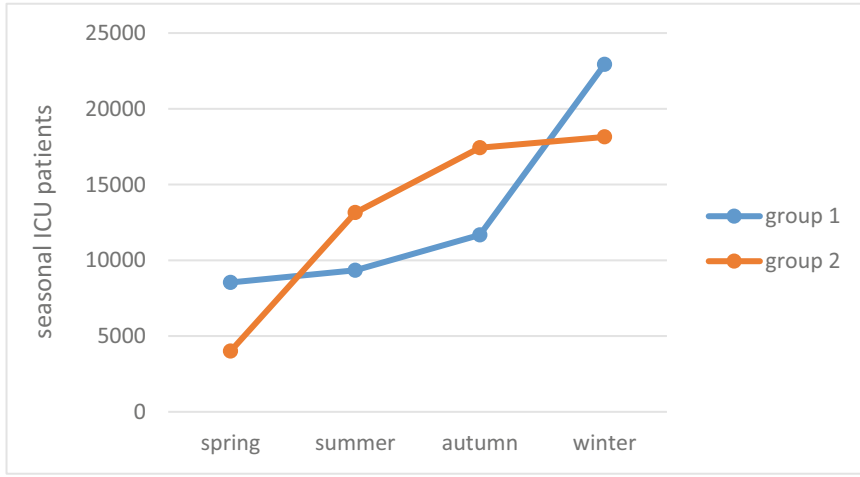

Fig. 4 The seasonal ICU patients of group 1 and group 2

three times more than other three single seasons. For ICU patients of group 2 from July 2, 2021, to March 25, 2022, BF(3, 21.748,) = 7.481, p = 0.001. The 95% CI are [4006.50 ± 1696.24] for spring, [13145.89 ± 8305.43] for summer, [17428.00 ± 5594.06] for autumn, and [18143.77 ± 5366.01] for winter, respectively. The weekly ICU patients in winter and autumn are four to five times more than spring. The weekly ICU patients in summer are more than three times more than spring (Fig. 4). The seasonality of COVID-19 in terms of weekly ICU patients is ascertained.

For weekly hospitalized patients of group 1 from July 17, 2020, to June 25, 2021, BF (3, 31.505) = 35.864, p = 0.000. The 95% CI are [33364.85 ± 6081.32] for spring, [30970.73 ± 14004.59] for summer, [44119.69 ± 21330.68] for autumn, and [97210.00 ± 26195.60] for winter, respectively. The weekly hospitalized patients in winter are about two to three times more than other three single seasons. For weekly

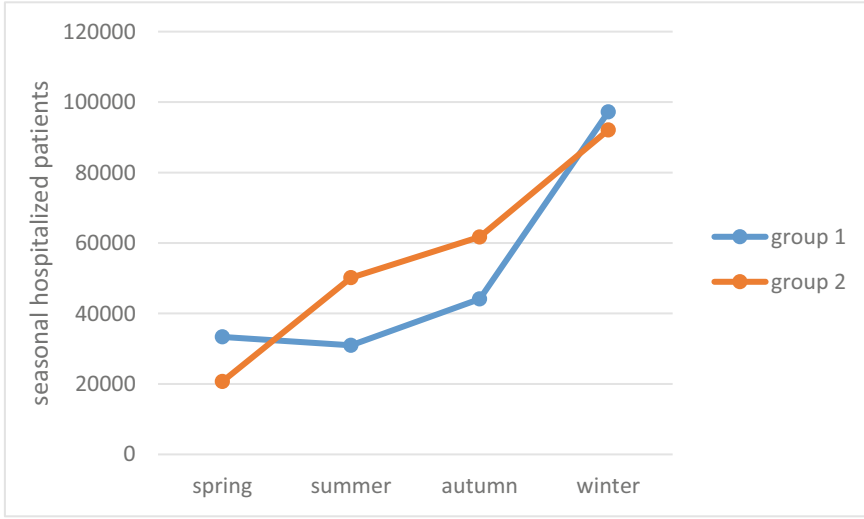

Fig. 5 The seasonal hospitalized patients of group 1 and group 2

hospitalized patients of group 2 from July 2, 2021, to March 25, 2022, BF(3, 26.592) = 9.042, $p = 0.000$. The 95% CI are [20680.00 ± 7708.49] for spring, [50160.00 ± 32647.91] for summer, [61722.08 ± 20649.57] for autumn, and [92082.77 ± 38740.52] for winter, respectively. The weekly hospitalized patients in winter and autumn are four to five times more than spring. That figure in summer is more than three times higher than spring (Fig. 5). The seasonality of COVID-19 in terms of weekly hospitalized patients per million is ascertained.

For weekly hospital admissions of group 1 from July 17, 2020, to June 25, 2021, BF (3, 29.078) = 33.240, $p = 0.000$. The 95% CI are [32898.31 ± 5818.23] for spring, [25595.80 ± 9652.33] for summer, [44086.92 ± 21266.74] for autumn, and [88290.15 ± 24695.00] for winter, respectively. The weekly hospital admissions in winter are about two to three times more than other three single seasons. For weekly hospital admissions of group 2 from July 2, 2021, to March 25, 2022, BF(3, 25.399) = 9.077, $p = 0.000$. The 95% CI are [19066.25 ± 7000.59] for spring, [49104.11 ± 29628.09] for summer, [53449.54 ± 17788.25] for autumn, and [87931.31 ± 40692.53] for winter, respectively. The weekly hospital admissions in winter are four times more than spring. That figure in summer and autumn is more than two times higher than spring (Fig. 6). The seasonality of COVID-19 in terms of weekly hospital admissions is ascertained.

For weekly positive rate of group 1 from July 17, 2020, to June 25, 2021, BF (3, 33.099) = 11.985, $p = 0.000$. The 95% CI are [0.04 ± 0.01] for spring, [0.06 ± 0.03] for summer, [0.07 ± 0.02] for autumn, and [0.10 ± 0.03] for winter, respectively. The mean of the weekly positive rate in winter is about one and a half to two times higher than other three single seasons. The weekly positive rate in autumn is about two times higher than spring. For weekly positive rate of group 2 from July 2, 2021, to March 25, 2022, BF (3, 15.739) = 12.102, $p = 0.000$. The 95% CI are [0.03 ± 0.01] for spring, [0.08 ± 0.03] for summer, [0.07 ± 0.01] for autumn, and [0.16 ± 0.09] for winter, respectively. The mean of the weekly positive rate in winter is five times more than spring. That figure in summer and autumn is more than two times higher than spring (Fig. 7). The seasonality of COVID-19 in terms of weekly positive rate is ascertained.

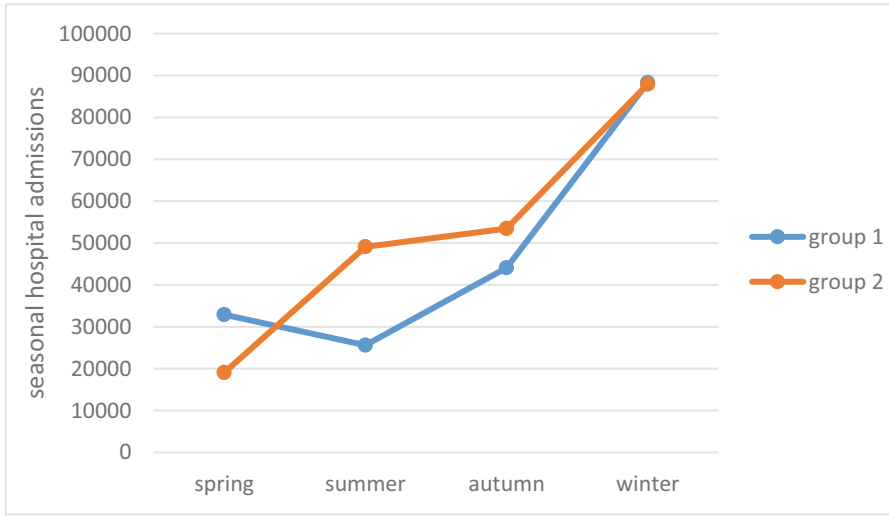

Fig. 6 The seasonal hospital admissions of group 1 and group 2

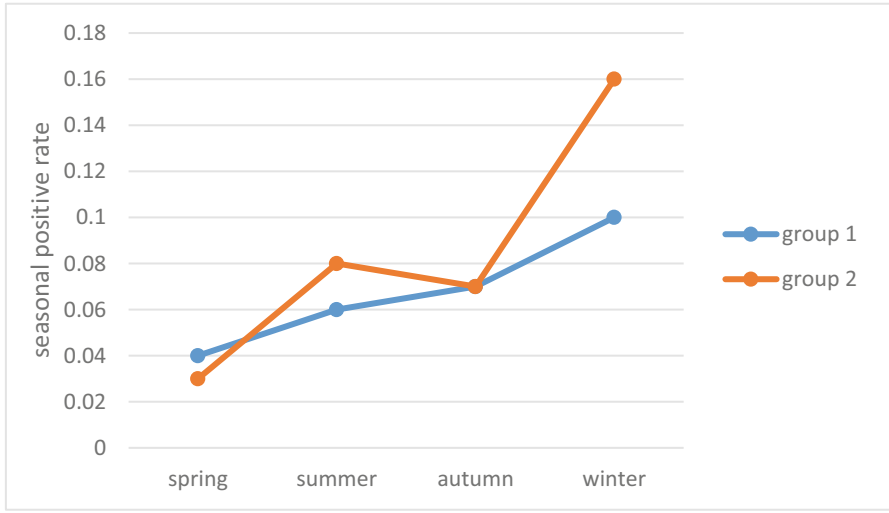

Fig. 7 The seasonal positive rate of group 1 and group 2

4 Conclusions

By performing the Brown-Forsythe variance analysis on the above indicators, the distinct seasonality of the pandemic is observed statistically on most indicators. The findings shows there are strong yearly seasonality traits in some indicators of COVID-19 in general. There are many things changing during the study period; such factors include environmental factors, host and virus factors (severity and infectivity), regional variation, the availability of vaccines and boosters, changing population attitudes and behaviors, and many others. Since the changes are relatively smaller within a year and bigger between 2 years and seasonality is observed for every single year and the 2 years' results are consistent, seasonality does exist for every single year.

There are some reasons for the seasonal patterns present in cases, deaths, hospitalizations,

etc. The first is the COVID-19 viruses are more proliferating in cold weather than in hot weather (Ganslmeier et al. 2021). The second is the viruses are very infectious and can spread among people through many channels if there are no preventive measures to cut the spreading channels. However, there are other reasons as well. The increase in cases, deaths, hospitalizations, ICU admissions, etc. observed in group 1 was partly due to the fact that vaccines were mostly unavailable during the winter season, whereas the increase in the same indicators during a winter season in group 2 was due to increase in severity and infectivity of Delta and Omicron variants that were observed in the fall/winter of the second year of the pandemic, especially among unvaccinated individuals (Stærke et al. 2022).

The preventive measures include wearing facial mask, not shaking hands, washing hands with germicide after touching something, keeping social distance, not gathering together, isolation when necessary, taking vaccine, or even lockdown of a community or a city in the most emergent cases. For all the different variants including Delta and Omicron variants, the interventions play a big role in cutting their spreading. If there are no stricter interventions in winter, the viruses will proliferate more and spread more than in hot weather (Ganslmeier et al. 2021), which can be a vicious cycle. So stricter interventions in winter are necessary to flatten the peak of the cases and deaths of the pandemic in a year. In hot days, the COVID-19 viruses are less proliferating and less spreading, so less stricter measures can be taken to prevent its spreading while alleviating to the most extent the side effect of the interventions and measures on the economy and people's life.

The limitation of this study is the factors that may influence the variations were not considered in the analysis, such as the aforementioned factors. Future study may take such factors into consideration and perform temporal and spatial analysis on the virus.

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Ethics Approval and Consent to Participate Not applicable.

Consent for Publication Not applicable.

Availability of Data and Materials The data used in this study are provided by WHO https://worldhealthorg.shinyapps.io/covid.

Competing Interests The author declares no competing interests.

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Trends of Antimicrobial Consumption in Hospital: Tackling the Hidden Part of the Iceberg with an Electronic Personalised Prescription Software for Antimicrobial Stewardship

G. Bertolino, L. Marras, V. Mureddu, M. Camboni, and A. Cadeddu

Abstract

Background The monitoring of antibiotic prescriptions is of fundamental importance in the hospital setting. Inappropriate prescriptions could cause an unjustified exposure of patients to the risk of ADR (adverse drug reactions) and increase the risk of spreading the ecological resistance of hospital microorganisms. The use of IT media is essential in antimicrobial stewardship programs.

Objective The purpose of this work was to evaluate the variation in the exposure index to antibiotics following the adoption of electronic pharmacist-controlled prescriptions in 2015.

Methods Electronic Personalised Prescription Software (EPPS) was introduced in our University Hospital in 2015. The exposure index to antibiotics was expressed per WHO methodology in DDD (defined daily dose)/100 patient days (DPD). The changes in DPDs over the 2015–2020 period were calculated as percentages and through linear regressions. The analysis was performed using SPSS® (IBM).

Results Following the introduction of EPPS, there was a progressive decline in DPDs during the 2015–2020 period from 98.9 to 65.1 ($R^2 = 0.687$, $p = 0.041$). This could mainly be linked to the decreased use of ATC class J01CR – penicillin association, including beta-lactamase inhibitors (DPD 2015 39.9; DPD 2020 11.5; variation −71.1%). Expenditure progressively decreased from € 427,000 in 2015 to € 269,000 in 2020.

Conclusion The use of EPPS was shown to be useful for pharmacists in implementing proper antibiotic dispensing practices; the avoidance of inappropriate prescriptions leads to a better monitoring of DPDs and the related expenditure which is the main goal of antibiotic stewardship programs.

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Keywords

Antimicrobial consumption · Antimicrobial stewardship · COVID-19 · Electronic personalised prescription software · EPPS · Hospital pharmacist

1 Introduction

Antimicrobial resistance (AMR) is a recognized global concern, despite the numerous efforts to combat it both in hospital settings and at the community level. Concerns about AMR as a defense mechanism against pharmacological pressure were already raised in the early 1930s at the beginning of the antibiotic era by Sir Alexander Fleming (1945).

The AMR problem was also addressed by national institutions in the early 1990s, when evidence showed that antibiotic overuse gave rise to *Enterococcus* spp. resistant to "last resort" treatment with vancomycin.

During the mid-1990s, a rapid rise in vancomycin-resistant enterococci (VRE) occurred in the United States. This initially led to recommendations to restrict intravenous vancomycin use, based on the theory that vancomycin overuse could be a risk factor in the development of VRE infections. Later, further investigations demonstrated that the risk of vancomycin-related VRE development was actually lower. In fact, studies showed that agents such as broad-spectrum cephalosporins and clindamycin appeared to increase the risk of VRE development (MacDougall and Polk 2005). Since the early 2000s, various statistical analyses have been carried out on the relation between antimicrobial consumption and AMR; in 2004 Aldeyab et al. reported that multivariate time-series analysis (ARIMA) showed significant relationships between the incidence of hospital-acquired MRSA (methicillin-resistant *Staphylococcus aureus*) and a number of potential explanatory variables, and statistically significant positive relationships were observed for the use of fluoroquinolones, third-generation cephalosporins, macrolides, and amoxicillin/clavulanic acid (Aldeyab et al. 2008). Also travel can play a role in moving resistance from one country to another (Frost et al. 2019), and old drugs such as fosfomycin and nitrofurantoin are repurposed in order to overcome the problem of AMR (dos Santos et al. 2021).

Nowadays, the new approach to AMR adopted by institutions is the so-called "One Health" concept, defined as "the collaborative effort of multiple health science professions, together with their related disciplines and institutions—working locally, nationally, and globally—to attain optimal health for people, domestic animals, wildlife, plants, and our environment" (McEwen and Collignon 2018). This plan of action refers to the need to adopt a single common strategy in limiting the use of antibiotics in hospital, community, and veterinary settings. A milestone in this field is represented by the joint reports of the European Food Safety Authority (EFSA), European Centre for Disease Prevention and Control (ECDC), and European Medicines Agency (EMA), now in their third edition (ECDC/EFSA/EMA 2021). The European Union has played a leading role in One Health antimicrobial resistance activities, particularly with regard to the regulation of antimicrobials for veterinary use and in the integrated surveillance of antimicrobial resistance and antimicrobial use (Robinson et al. 2016; McEwen and Collignon 2018; ECDC/EFSA/EMA 2021). Moreover, a One Health learning platform open access on interventions for antimicrobial resistance should be useful to a broad range of stakeholders, including health-care professionals, public health practitioners, policy makers, industries, and consumer groups (Wernli et al. 2020).

At the Italian national level, the Ministry of Health has issued a document to fight AMR divided into several points; the core concerns regional and local policies for implementing ministerial recommendations. The two objectives for the hospital sector were set at a 5% reduction in the global consumption of antibiotics and a 10% decrease in the use of fluoroquinolones in the 2016–2020 period calculated in DDD/100 days of hospitalization (National Antimicrobial Resistance Contrast Plan 'PNCAR' 2020).

The monitoring of antibiotic prescriptions is also of fundamental importance in the hospital setting, since avoiding inappropriate prescription

would lead to a decrease in the unjustified exposure of patients to the risk of ADR (adverse drug reactions) (Willemsen et al. 2009). The use of IT media is essential in antimicrobial stewardship programs, and AMC (antimicrobial consumption) control is a core strategy in such programs (NICE Guidelines 2019). The first guidelines of antimicrobial stewardship programs stress the importance of formulary restrictions and recommend benchmarking analyses (MacDougall and Polk 2005; Hegewisch-Taylor et al. 2020).

The SARS-CoV-2 pandemic led national institutions to develop strategies aimed to prevent infections. Some of these strategies could also be useful in fighting AMR (e.g., hand hygiene, etc.), and the issues must continue to receive attention, so that the progress achieved by the antimicrobial stewardship programs is not nullified.

The challenge in finding the best way to control AMR spread through the implementation of efficacious antimicrobial stewardship policies is not an easy one. The main goal is to control resistant species by restricting their use to only when really needed, as well as controlling costs. It has been demonstrated that antibiotic expenditure represents 20% of hospital pharmacy budgets (Tamma and Cosgrove 2011). Nonetheless, the best clinical practices need to be followed so that patients are guaranteed the highest care standards. Restriction of antibiotic consumption is generally accepted by physicians, but it is suitably applied only though time-saving and easily accessible systems (Buising et al. 2008). The use of Electronic Personalised Prescription Software (EPPS) designed to monitor antibiotic prescription, if well implemented, is a valuable option to optimize therapy and to ensure patient-oriented treatment. Although the use of such EPPS in antibiotic stewardship programs is an encouraging innovative field, to date there are still few reports in the literature.

The purpose of our work was to evaluate the possible variation in the antibiotic exposure index considering the 5-year period between January 2015, when the EPPS was first adopted, and December 2020.

2 Methods

2.1 Study Design and Data Collection

We retrospectively reviewed data regarding antimicrobial consumption (AMC) in an Italian Tertiary-Care University Hospital with 600 beds and about 27,000 admissions per year, during the study period January 2015–December 2020.

EPPS (Bustermed®) was gradually introduced in 2015. This software has different tools that guarantee the appropriateness and safety of prescriptions for each patient, such as a drug-drug interaction checker. The prescription of antibiotics, in particular the ATC J01 group on the WHO AWaRe "Reserve" list, is only possible on a named-patient basis in accordance with the "Watch" list (Table 1) (Sharland et al. 2018), and the physician is asked to relate each prescription to the type of infection and the antibiogram results. Hospital pharmacists are able to use the EPPS to verify and approve antibiotic therapies; the amount of medication needed is automatically calculated in the prescribed dose.

The dataset relating to consumption and expenditure for the 2015–2020 period was extrapolated from our IT management system (AMC Areas®). The exposure index to antibiotics was expressed in line with WHO methodology in DDD/100 patient-days (DPD) (Cizman and Beovic 2014; Indicators and Ddd 2021).

2.2 Statistical Analysis

Descriptive analysis of the data was carried out using median values, using the interquartile range for quantitative variables and percentage values for qualitative ones. Normality of variables was assessed with the Shapiro-Wilk test and values of asymmetry and kurtosis related to their standard error. The changes in the DPDs in the 2015–2020 period were calculated as percentages and assessed through linear regressions using the same method as ECDC reports (ECDC 2020).

Table 1 Summary table of list of antimicrobials in ATC J01 subject to limitation with personalised reasoned request "PRR"

ATC J01 4° level	Antimicrobial
J01AA08	Minocycline (i.v.[a]; o.s.[b])
J01AA12	Tigecycline[a]
J01CE08	Benzylpenicillin
J01CF04	Oxacillin
J01DD01	Cefotaxime[b]
J01DD52	Ceftazidime/avibactam[a]
J01DE01	Cefepime[b]
J01DH03	Ertapenem[b]
J01DH02	Meropenem[b]
J01DH51	Imipenem/cilastatin[b]
J01DI01	Ceftobiprole[a]
J01DI54	Ceftolozane/tazobactam
J01DI02	Ceftaroline[a]
J01EE01	Trimethoprim
J01FA10	Azithromycin[b]
J01GB06	Amikacin
J01GB03	Gentamicin
J01MA02	Ciprofloxacin[b]
J01MA14	Moxifloxacin[b]
J01MA12	Levofloxacin[b]
J01XA01	Vancomycin[b]
J01XA02	Teicoplanin[b]
J01XA04	Dalbavancin[a]
J01XB01	Colistin[a]
J01XX08	Linezolid[a]
J01XX11	Tedizolid[a]
J01XX09	Daptomycin[a]
J01XX01	Fosfomycin (i.v.)[a]

[a]Reserve list WHO
[b]Watch list WHO)

We computed linear regression to identify trends in AMC. A p value of <0.05 was considered statistically significant. All analyses were conducted using the SPSS statistical package (version 23 for Windows. SPSS, Inc. Chicago, Ill).

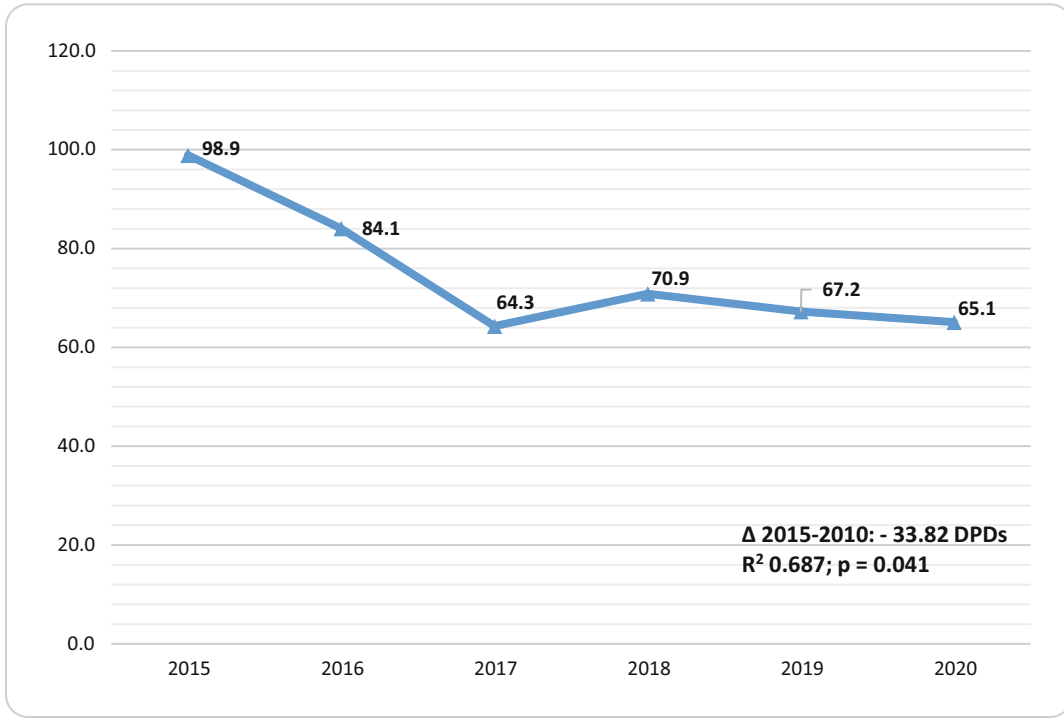
3 Results

During the 2015–2020 study period, the total consumption of antibiotics decreased significantly from 98.92 DPD in 2015 to 65.10 DPD in 2020 (-34%; $R^2 = 0.687$; $p = 0.041$) as shown in Graph 1 and Table 2.

DPD related to tetracyclines increased from a value of 0.54 DPD in 2015 to 0.75 in 2020 (+39%; $R^2 = 0.327$; $p = 0.236$). In particular, tigecycline showed an increase with a value that rose from 0.19 to 0.30 DPD during the period under review (+58%; $R^2 = 0.456$; $p = 0.141$).

The ATC class with the highest consumption was J01CR. Indeed, combinations of penicillin, including beta-lactamase inhibitors, had a DPD of 39.95, equal to 40% of the total in 2015, which then decreased significantly to 11.53 in 2020 (-71%; $R^2 = 0.58$; $p = 0.078$).

Third-generation cephalosporins followed with a 27.81 DPD in 2015 (28% of the total) which slightly increased between 2016 and 2018



Graph 1 Overall yearly trend of DPDs of antimicrobials in ATC J01

and then returned to the previous trend in 2020 with a small decrease, 25.03 DPD (−10%; $R^2 = 0.06$; $p = 0.641$). The consumption of fourth-generation cephalosporins remained constant throughout the period under review (−9%; $R^2 = 0.07$; $p = 0.613$).

Carbapenems demonstrated an overall unchanged consumption over time (−5%; $R^2 = 0.034$; $p = 0.727$). In particular, there were a decrease in the use of imipenem/cilastatin from 1.50 DPD in 2015 to 0.32 in 2020 (−79%; $R^2 = 0.859$; $p = 0.008$) and an increase in the use of meropenem, which had a value of 1.19 DPD in 2015, while in 2020 its DPD value almost doubled to 2.19 (+84%; $R^2 = 0.788$; $p = 0.018$).

Macrolides have not changed over the years (−1%; $R^2 = 0.068$; $p = 0.617$), but azithromycin had a significant increase in 2020 (+940%; $R^2 = 0.409$; $p = 0.171$).

As regards the other aminoglycosides, there was a general decrease in consumption from 1.43 in 2015 to 0.83 DPD in 2020 (−42%; $R^2 = 0.697$; $p = 0.039$). Among these, gentamicin showed the most significant decrease (−53%; $R^2 = 0.706$; $p = 0.036$).

Fluoroquinolone consumption also decreased from 13.41 DPD in 2015 to 10.70 DPD in 2020 (−20%; $R^2 = 0.265$; $p = 0.296$), and among these, ciprofloxacin showed the greatest decrease, from 4.80 in 2015 to 2.73 DPD in 2020 (−43%; $R^2 = 0.801$; $p = 0.016$).

The DPD values for vancomycin and teicoplanin also declined. The former decreased from 0.31 DPD to 0.27 in 2020 (−13%; $R^2 = 0.166$; $p = 0.422$), while the latter fell from 2.18 DPD in 2015 to 1.36 DPD in 2020 (−38%; $R^2 = 0.318$; $p = 0.244$).

Colistin and linezolid showed a constant trend over time, the former with a slight increase (+67%; $R^2 = 0.002$; $p = 0.941$), the latter with a slight decrease (−13%; $R^2 = 0.006$; $p = 0.881$).

All the antimicrobials with PRR showed a decrease from a value of 20.98 DPD in 2015 to 18.08 in 2020 (−14%; $R^2 = 0.148$; $p = 0.451$).

The consumption of latest generation antibiotics, which have entered the market in the

Table 2 Summary table of antimicrobial classes (bold) and antimicrobial drugs subject to PRR in 2015–2020 period (unit: DDD/100)

ATC – Antimicrobial	2015	2016	2017	2018	2019	2020	Δ 2015–2020	R^2	p value[a]
J01AA – Tetracyclines	0.54	0.47	0.67	0.83	0.56	0.75	+0.21	0.327	0.236
J01AA08 – Minocycline	0.02	0.00	0.00	0.01	0.00	0.00	−0.02	0.452	0.153
J01AA12 – Tigecycline	0.19	0.13	0.08	0.29	0.25	0.30	+0.11	0.456	0.141
J01BA – Amphenicols	0.00	0.00	0.00	0.00	0.00	0.00	0.00	–	
J01CA – Penicillins with extended spectrum	1.11	0.85	0.33	0.30	0.26	0.85	−0.26	0.210	0.360
J01CE – Beta-lactamase-sensitive penicillins	0.01	0.06	0.06	0.08	0.09	0.06	+0.05	0.441	0.150
J01CE08 – Benzylpenicillin	0.01	0.06	0.06	0.08	0.09	0.06	+0.05	0.441	0.150
J01CF – Beta-lactamase-resistant penicillins	0.00	0.00	0.22	0.03	0.14	0.01	+0.01	0.023	0.776
J01CF04 – Oxacillin	0.00	0.00	0.22	0.03	0.14	0.01	+0.01	0.023	0.776
J01CR – Combinations of penicillins, incl. beta-lactamase inhibitors	39.95	24.97	8.00	6.06	10.61	11.53	−28.41	0.580	0.078
J01DB – First-generation cephalosporins	1.05	0.90	0.88	1.25	1.90	1.88	+0.83	**0.740**	**0.028**
J01DC – Second-generation cephalosporins	0.00	0.00	0.00	0.00	0.00	0.00	0.00	–	–
J01DD – Third-generation cephalosporins	27.81	31.29	31.66	36.96	27.80	25.03	−2.78	0.060	0.641
J01DD01 – Cefotaxime	0.03	0.01	0.01	0.01	0.01	0.01	−0.01	0.127	0.489
J01DD52 – Ceftazidime/avibactam	–	0.00	0.00	0.01	0.01	0.00	–	–	–
J01DE – Fourth-generation cephalosporins	0.11	0.12	0.06	0.08	0.08	0.10	−0.01	0.070	0.613
J01DE01 – Cefepime	0.11	0.12	0.06	0.08	0.08	0.10	−0.01	0.070	0.613
J01DH – Carbapenems	2.69	2.46	2.31	2.35	2.94	2.56	−0.14	0.034	0.727
J01DH03 – Ertapenem	0.01	0.01	0.02	0.03	0.06	0.04	+0.03	**0.742**	**0.027**
J01DH02 – Meropenem	1.19	1.61	1.46	1.81	2.50	2.19	+1.01	**0.788**	**0.018**
J01DH51 – Imipenem/cilastatin	1.50	0.83	0.83	0.51	0.38	0.32	−1.17	**0.859**	**0.008**
J01DI – Other cephalosporins and penems	0.00	0.00	0.00	0.01	0.01	0.02	+0.02	**0.833**	**0.011**
J01DI01 – Ceftobiprole	0.00	0.00	0.00	0.00	0.00	0.00	0.00	–	–
J01DI54 – Ceftolozane/tazobactam	0.00	0.00	0.00	0.00	0.01	0.02	+0.02	**0.833**	**0.011**
J01DI02 – Ceftaroline	0.00	0.00	0.00	0.00	0.00	0.00	0.00	–	–
J01EC – Intermediate-acting sulfonamides	0.00	0.00	0.00	0.00	0.00	0.00	0.00	–	–
J01EE – Combinations of sulfonamides and trimethoprim, incl. derivatives	0.31	0.13	0.29	0.12	0.15	0.34	+0.03	0.001	0.996
J01EE01 – Trimethoprim	0.31	0.13	0.29	0.12	0.15	0.34	+0.03	0.001	0.996
J01FA – Macrolides	5.06	5.07	3.53	4.03	3.79	5.03	−0.03	0.068	0.617
J01FA10 – Azithromycin	0.10	0.14	0.09	0.05	0.13	1.04	+0.93	0.409	0.171
J01FF – Lincosamides	0.17	0.16	0.22	0.17	0.24	0.21	+0.04	0.397	0.180
J01GA – Streptomycins	0.00	0.00	0.00	0.00	0.00	0.00	0.00	–	–
J01GB – Other aminoglycosides	1.43	1.15	1.00	0.75	0.97	0.83	−0.61	**0.697**	**0.039**
J01GB06 – Amikacin	0.33	0.46	0.26	0.26	0.36	0.36	+0.03	0.014	0.824
J01GB03 – Gentamicin	0.95	0.63	0.65	0.48	0.58	0.45	−0.49	**0.706**	**0.036**

(continued)

Table 2 (continued)

ATC – Antimicrobial	2015	2016	2017	2018	2019	2020	Δ 2015–2020	R^2	p value[a]
J01MA – Fluoroquinolones	13.41	11.46	11.25	12.52	12.37	10.70	−2.71	0.265	0.296
J01MA02 – Ciprofloxacin	4.80	3.89	3.41	3.90	3.16	2.73	−2.06	**0.801**	**0.016**
J01MA14 – Moxifloxacin	0.04	0.03	0.00	0.04	0.02	0.01	−0.03	0.224	0.343
J01MA12 – Levofloxacin	8.57	7.54	7.84	8.58	9.18	7.95	−0.62	0.052	0.665
J01XA – Glycopeptide antibacterials	2.49	2.27	1.39	1.52	2.00	1.63	−0.86	0.365	0.204
J01XA01 – Vancomycin	0.31	0.49	0.34	0.32	0.36	0.27	−0.04	0.166	0.422
J01XA02 – Teicoplanin	2.18	1.78	1.05	1.19	1.63	1.36	−0.82	0.318	0.244
J01XA04 – Dalbavancin	0.00	0.00	0.00	0.00	0.00	0.00	0.00	–	
J01XB – Polymyxins	0.03	0.08	0.05	0.05	0.06	0.05	+0.02	0.002	0.941
J01XB01 – Colistin	0.03	0.08	0.05	0.05	0.06	0.05	+0.02	0.002	0.941
J01XD – Imidazole derivatives	2.43	2.36	2.19	3.18	3.17	3.09	+0.66	0.618	0.064
J01XE – Nitrofuran derivatives	0.00	0.00	0.00	0.00	0.00	0.00	0.00	–	–
J01XX – Other antibacterials	0.31	0.27	0.41	0.59	0.24	0.45	+0.14	0.111	0.518
J01XX08 – Linezolid	0.16	0.14	0.19	0.27	0.16	0.14	−0.01	0.006	0.881
J01XX11 – Tedizolid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	–	–
J01XX09 – Daptomycin	0.00	0.03	0.07	0.15	0.00	0.19	+0.19	0.419	0.165
J01XX01 – Fosfomycin (i.v.)[a]	0.15	0.10	0.15	0.17	0.08	0.11	−0.04	0.135	0.474
Total	98.92	84.06	64.31	70.85	67.24	65.10	−33.82	**0.687**	**0.041**
Antimicrobials with PRR	20.98	18.21	17.35	18.46	19.56	18.08	−2.9	0.148	0.451

[a]Linear regression

last 6 years, such as ceftazidime/avibactam, ceftobiprole, ceftolozane/tazobactam, ceftaroline, dalbavancin, and tedizolid, did not show a significant trend. They all showed a DPD = 0 over the 6 years except for two cases: the first, ceftazidime/avibactam was used in 2018 and 2019 with a DPD value of 0.01 ($R^2 = 0.085$; $p = 0.633$), while the second, ceftolozane/tazobactam showed an increasing trend from 0.01 DPD in 2019 and 0.02 in 2020 ($R^2 = 0.833$; $p = 0.011$).

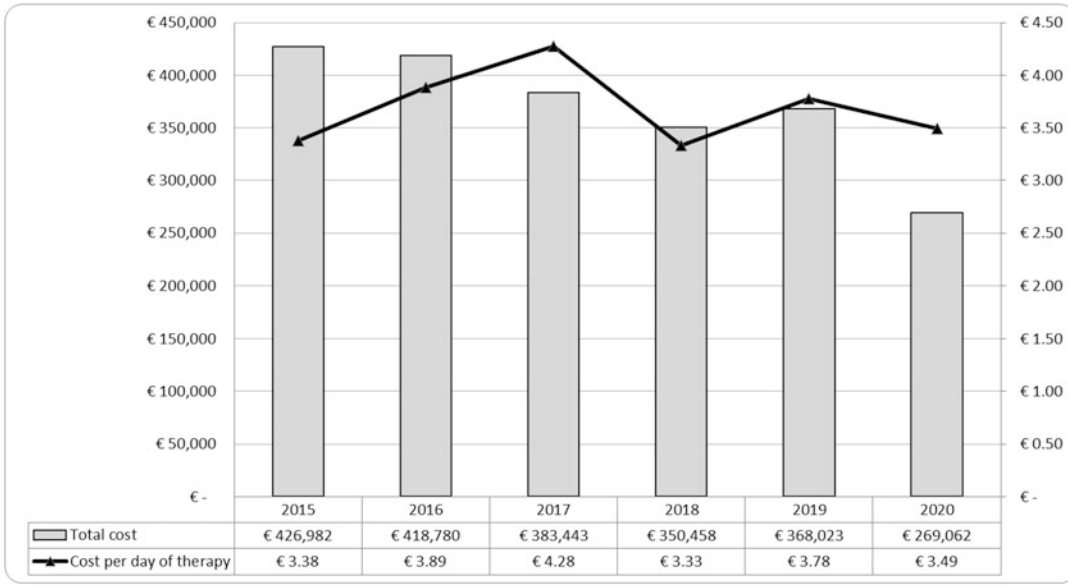
Spending decreased from €427,000 in 2015 to €269,000 in 2020, without a similar reduction in the average daily cost of therapy from €3.38 in 2015 to €3.49 in 2020 (Table 3).

4 Discussion

A significative decrease in the AMC trend during the 2015–2020 period was observed (Graph 1), and levels were found to be similar to those reported for other university hospitals in Denmark and the Netherlands in recent years (Cizman and Beovic 2014). This decline would seem to be unrelated to the COVID-19 pandemic; in fact, the annual AMC for 2020 appears to be similar to that of 2019. The only exception seems to be azithromycin which, before it was shown to be ineffective, was initially used as treatment for mild-to-moderate COVID-19 in both hospitalized patients (Hinks et al. 2021) and outpatients (Oldenburg et al. 2021). In this case the AMC increased almost ten times compared to the average consumption of previous years (Table 2).

The most significant percentage decrease occurred in ATC J01CR: combinations of penicillins, including beta-lactamase inhibitors, decreased by 71%, even though they are still among the most used molecules in hospitals, mirroring the national hospital-wide use of antibiotics (ECDC 2020), similarly to what happens at a community level where the protected penicillin prescriptions were 76.5% in 2017 (Bruyndonckx et al. 2021). Another recent point prevalence study in Emilia Romagna showed that

Table 3 Antimicrobial total cost in study period and average cost per day of therapy (calculated as total DDD/cost per year)



penicillins with beta-lactamase inhibitors and third-generation cephalosporins were the most used ("Use of antibiotics in acute care hospitals in Emilia-Romagna" 2019).

A 2008 Australian study by Buising and colleagues evaluated EEPS in clinical practice using a time-series analysis with linear segmental regression that demonstrated a decrease in AMC of third- and fourth-generation cephalosporins, but at the same time an increase in the use of piperacillin/tazobactam due to a change in the empirical therapy protocol for febrile neutropenia (Buising et al. 2008). Although several studies have reported that an increase in AMC results in a rise in antimicrobial resistance (Aldeyab et al. 2008; Willemsen et al. 2009; Malik and Bhattacharyya 2019; ECDC/EFSA/EMA 2021), an Italian study showed that an increasing trend in ATC J01CR – combinations of penicillins, including beta-lactamase inhibitors – and ATC J01DH, carbapenems, results in a decreasing rate of *S. aureus* MRSA and of total ESBL (extended-spectrum beta-lactamase)-positive *Enterobacteriaceae* (Mascarello et al. 2017).

A slight drop in AMC was recorded for antibiotics (Table 1) dispensed by personalized prescriptions (from 20.98 to 18.08 DPD from 2015 to 2020); this can be explained by the fact that, although not computerized, these molecules were subject to stringent prescription regulations even before 2015.

During the study period, there were a significant increase in the use of first-generation cephalosporins and a decrease (although not significant) in the AMC of third-generation cephalosporins. An increase in the AMC of tigecycline was observed, possibly linked to an increase in MDR (multidrug-resistant) strains, which will be confirmed in the future by antibiogram analysis carried out by the microbiology laboratory; this observation seems confirmed by evaluating the 67% increase in the use of colistin.

The use of carbapenems remained stable from 2015 to 2020 with a slightly nonsignificant decrease and with a shift over time demonstrated by the decrease in the use of imipenem/cilastatin and ertapenem and an increase in the AMC of meropenem.

There was a 20% drop in the use of fluoroquinolones, an interesting finding in light of the recent safety notice issued by Italian Medicines Agency (AIFA) in April 2019. This result is in line with what was found in a 2012 study regarding the impact of a pharmacist on a multidisciplinary team in an antimicrobial stewardship program (ASP) (Magedanz et al. 2012). This result together with the abovementioned overall decrease in AMC is important since it respects the goals of the national plan to combat AMR (National Antimicrobial Resistance Contrast Plan 'PNCAR' 2020).

An important limitation in the study is the current unavailability of microbiological data which would allow a comparison with hospital ecology and therefore with the resistance observed in hospital infections. Moreover, hospital guidelines of antimicrobial therapy have not yet been published and are being finalized after a delay in publication due to the COVID-19 pandemic. Probably also the introduction of rapid molecular diagnostics on FilmArray (BioFire – BioMerieux) in 2019 contributed to decreasing the use of broad-spectrum antibiotics as fluoroquinolones and combinations of penicillins, including beta-lactamase inhibitors in favor of an earlier targeted therapy.

The use of personalized therapy management software could help the pharmacist in the correct management of antibiotic therapy in the wards, avoiding potential improper prescriptions and thus leading to a reduction in hospital DPD and hopefully also in expenditure. The drop in spending is most likely due to the implementation of regional tenders; the decline recorded in 2020 is instead attributable to the decrease in hospitalization due to the SARS-CoV-2 pandemic. Italy is currently the fifth European country in terms of consumption of antibiotics at the hospital level (ECDC 2020); a recent visit by the ECDC confirmed that there are many improvements to be made to the national health system. In detail, the ECDC experts identified the following critical issues in the national health system regarding the problem of AMR: little sense of urgency about the current AMR situation from most stakeholders and a tendency by many of them to avoid tackling the problem; lack of institutional support at national, regional, and local levels; lack of professional leadership at each level; lack of accountability at each level; and lack of coordination of the activities between and within levels (ECDC 2017). Such negative assessments underline how important antimicrobial stewardship projects are for the correct use of this important class of drugs, especially in light of the new possibilities introduced by the rapidity of new diagnostic tests regarding identification and antibiogram which could considerably limit the use of empirical antibiotic therapy (Idelevich and Becker 2019).

Spending decreased to €269,000 in 2020, but probably due to the fewer hospitalizations in the pandemic period as confirmed by the similar average daily cost of therapy from €3.38 in 2015 to €3.49 in 2020. Costs are an important topic in antimicrobial stewardship; in fact, it cannot be seen as an expense but as an investment, also in the light of the new antibiotics recently put on the market. Various economic strategies have been hypothesized to keep these resources beyond the logic of the market (Morel et al. 2020), and a review of papers that have tried to estimate the costs of AMR per patient episode showed that additional costs varied from less than US$5 to more than US$55,000 (Roope et al. 2019).

The future development of this work will also involve the company microbiology laboratory for the verification of the incidence of MDR infections with the future possibility of preparing a defined corporate antimicrobial stewardship program and defining its goals in line with the international literature (Tamma and Cosgrove 2011). Several studies have been performed to evaluate other aspects to add into an ASPs: the impact of specialist consultation to improve patient outcome (Menichetti et al. 2018), the importance of using alternative strategies in the future such as the use of vaccines against MDR germs given their reduced possibility of generating AMR (Micoli et al. 2021), and the use of new biomarkers (especially in the ICU where AMC was generally higher) such as procalcitonin and proadrenomedullin in escalation and de-escalation therapy (Moniz et al. 2021; Piccioni et al. 2021).

5 Conclusion

A significative decrease in AMC during the 2015–2020 period was observed after the introduction of the computerized prescription system, an aspect which has received little attention in the international literature. The use of these technologies is essential for the correct management of the antibiotic resource. Furthermore, the advent of artificial intelligence in the health field will lead to an ever greater integration between man and machine, which can no longer be postponed over time. This integration between big data coming from computerized medical records, laboratory analyses, and management of antibiotic therapy will be fundamental for the future development of new antimicrobial stewardship programs.

In other countries, hospital pharmacists also have a clinical role in monitoring adherence to antimicrobial therapy which has a good impact on patient outcome with a corresponding decline in the incidence of hospital-onset *Clostridium difficile* infection (from 1.18 cases to 0.9 cases per 1000 patient days) probably due to the lower AMC (Siegfried et al. 2017). Another quasi-experimental study assessed the impact of the work of a dedicated pharmacist in an ASP project in an emerging country, highlighting a 25% reduction in antibiotic consumption and 69% reduced costs compared to the baseline (Magedanz et al. 2012). The most critical aspect of an EEPS system for our experience is the training of the staff, who must always be updated and able to manage and request therapies correctly; it is therefore necessary to schedule periodic retraining courses. Last but not least, the achievement of the objectives set by the national plan to combat AMR persuades us to persevere on this path, although more studies are needed in our care setting to assess the incidence of MDR germ infections and *Clostridium difficile* infection rates. Such studies will be necessary to verify that the decrease in AMC is also reflected in a better ecological situation in hospitals.

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Microbiota and Thyroid Disease: An Updated Systematic Review

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Abstract

Studies analyzing the relationship between microbiota composition and the thyroid have been increasing rapidly in recent years, and evidence has recently come to light about the involvement of the gut microbiota in various aspects of thyroid pathology. Recently, besides studies analyzing the microbiota composition of different biological niches (salivary microbiota or thyroid tumor microenvironment) in patients with thyroid disorders, some studies have been carried out in peculiar subcategories of patients (pregnant women or obese). Other studies added a metabolomic insight into the characterization of fecal microflora in an attempt to enlighten specific metabolic pathways that could be involved in thyroid disorder pathogenesis. Lastly, some studies described the use of probiotics or symbiotic supplementation aimed at modulating gut microbiota composition for therapeutic purposes. The aim of this systematic review is to analyze the last advancements in the relationship between gut microbiota composition and thyroid autoimmunity, extending the analysis also to nonautoimmune thyroid disorders as well as to the characterization of the microbiota belonging to different biological niches in these patients. The overall results of the present review article strengthen the existence of a bidirectional relationship between the intestine, with its microbial set, and thyroid homeostasis, thus supporting the newly recognized entity known as the gut-thyroid axis.

Keywords

Differentiated thyroid cancer · Graves' disease · Hashimoto's thyroiditis · Hypothyroidism · Microbiota

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1 Introduction

Despite the possible influence of intestinal bacteria on the thyroid gland that has been hypothesized about one century ago by a surgeon

named Harries (1923), most of the studies concerning the analysis of gut microbiota composition in humans with thyroid disorders have been published in the last 10 years. In fact, current technological know-how allows the identification of bacterial species, which could not be made until a few years ago (Köhling et al. 2017).

Studies analyzing the relationship between microbiota composition and the thyroid have been increasing rapidly in recent years; in this regard, several comprehensive reviews have been published in the last couple of years summarizing evidence on the involvement of the gut microbiota in various aspects of thyroid pathology, including the effects of microbial metabolites on thyroid function or its role on immune system activation (Fröhlich and Wahl 2019; Docimo et al. 2020; Bargiel et al. 2021; Fernández-García et al. 2021; Virili et al. 2021). In particular, peculiar fecal microbial signatures have been described in patients with thyroid autoimmune disorders (Su et al. 2020a, b; Liu et al. 2020), and dysbiotic states have been observed in patients with different thyroid functional conditions (Zhou et al. 2014; Lauritano et al. 2007). Furthermore, the presence of bacterial sulfatase and beta-glucuronidase enzymatic activities has been demonstrated in some gut bacteria, leading to the hypothesis of their involvement in thyroid hormone enterohepatic recycling (Hazenberg et al. 1988; Virili and Centanni 2017).

If previous studies were mostly based on the composition of fecal microbiota in patients suffering from different thyroid disorders, the spectrum of research has been significantly expanded today in relation to different topics. In fact, besides studies analyzing the microbiota composition of different biological niches (salivary microbiota or thyroid tumor microenvironment) in patients with thyroid disorders, some studies have been carried out in peculiar subcategories of thyroid patients (pregnant women or obese). Other studies added a metabolomic insight into the characterization of fecal microflora to enlighten specific metabolic pathways involved in the pathogenesis of thyroid disorders. Even more recently, some studies described the use of probiotic or synbiotic (prebiotic and probiotic mix) supplementation in an effort to modulate gut microbiota composition for therapeutic purposes.

The exponential increase in the number of these studies and the existence of only one systematic review, also limited to fecal microbial analysis in patients with thyroid autoimmunity (Gong et al. 2021), prompted us to systematically review this topic by updating our previous review on gut microbiota and thyroid autoimmunity (Virili et al. 2021). For this purpose, we extended the analysis to nonautoimmune thyroid diseases and to microbiota composition at the level of extraintestinal biological niches in patients with thyroid disorders.

2 Methods and Material

2.1 Review Conduction

The present systematic review was performed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Page et al. 2021).

2.2 Search Strategy and Inclusion Criteria

The literature was examined independently by three authors (I.S., S.C., C.V.).

Studies have been sought on PubMed and Web of Science using multiple combinations of the following keywords: thyroid carcinoma, goiter, goitre, Hashimoto's thyroiditis, Graves' disease (GD), Graves' orbitopathy, gut microbiota, microbiota, and microbiome. Original articles potentially eligible for the present analysis were identified; original articles on the relationship between thyroid autoimmune disorders (Hashimoto's thyroiditis, Graves' disease, and Graves' orbitopathy), thyroid function alterations (hypothyroidism and hypothyroidism), thyroid cancer, goiter, and microbiota composition from different biological niches were included in the present study. The beginning date limit was

January 1, 2020, and the search was updated until April 30, 2022. The research was not language restricted. We also examined the reference list of the selected articles to find further studies and extend the search (Fig. 1).

2.3 Data Extraction

For each article included in this review, the above three authors independently extracted the following information: (a) author details, country, date of publication, journal name, and number of patients enrolled; (b) the number of subjects included in the control groups; (c) the type of thyroid disorder examined; (d) the biological niche in which microbiota was examined; and (e) the main findings on alpha e beta diversity and the abundance of bacteria at phylum and genus/species levels.

2.4 Study Quality Assessment

The three authors (I.S., S.C., C.V.) independently carried out a quality assessment by using the Jadad modified scale for randomized controlled trial (RCT) studies and the National Heart, Lung, and Blood Institute Quality Assessment Tool for non-RCT studies (Shen et al. 2015). The scores ranged from 0 to 12 for RCT and 0 to 8 for non-RCT (a higher score indicates a higher

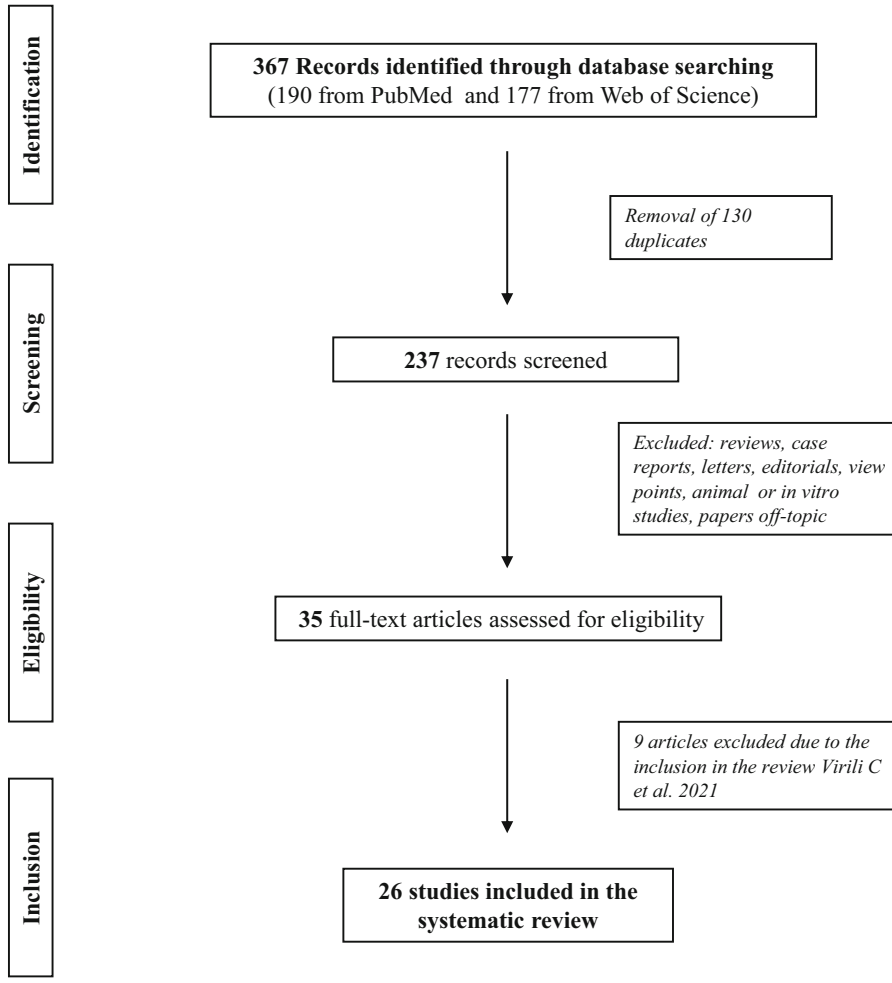

Fig. 1 Search strategy and studies' selection process

Table 1 Main characteristics of the 26 studies included in the systematic review, subdivided based on the disorders analyzed

First author	Country	Date	Journal	Quality assessment
Graves' disease				
Chang SC	Taiwan	2021.05.05	*Frontiers in Cellular and Infection Microbiology*	7/12
Jiang W	China	2021.05.03	*Thyroid*	7/12
Zhu Q	China	2021.06.02	*The ISME Journal*	6/12
Chen J	China	2021.01.09	*Journal of Endocrinological Investigation*	6/12
Yang M	China	2022.03.25	*Frontiers in Cellular and Infection Microbiology*	6/12
Yang M	China	2021.09.20	*International Archives of Allergy and Immunology*	6/12
Huo D	China	2021.09.07	*Communications Biology*	7/12
Han Z	China	2022.01.10	*Frontiers in Immunology*	7/12
Benign or malignant thyroid nodules				
Li A	China	2021.03.12	*Frontiers in Cellular and Infection Microbiology*	8/12
Ishaq HM	China	2022.03.28	*Journal of Cancer*	6/12
Yu X	China	2021.04.08	*Journal of Advanced Research*	8/12
Liu CJ	China	2021.10.06	*Journal of Microbiology*	3/12
Gnanasekar A	USA	2021.04.09	*Computational and Structural Biotechnology Journal*	6/12
Dai D	China	2021.11.30	*Journal of Translational Medicine*	6/12
Lin B	China	2022.03.08	*Frontiers in Endocrinology*	6/8[a]
Hashimoto's thyroiditis and hypothyroidism				
Talebi S	Iran	2020.05.03	*The International Journal of Clinical Practice*	6/8[a]
Su X	China	2020.06.25	*Clinical Science*	7/12
Wang B	China	2020.10.05	*America Journal of Physiology in Endocrinology and Metabolism*	6/12
Tabasi M	Iran	2020.11.05	*Metabolic Syndrome and Related Disorders*	6/12
El-Zawawy HT[b]	Egypt	2021.02.04	*The International Journal of Clinical Practice*	5/12
Dong T	China	2021.02.26	*Frontiers in Cellular and Infection Microbiology*	6/12
Cayres LC	Brazil	2021.03.05	*Frontiers in Immunology*	6/12
Wang B	China	2021.05.24	*Frontiers in Endocrinology*	6/12
DeClercq V	Canada	2021.12.09	*PLOS ONE*	6/12
Li J	China	2022.01.07	*Frontiers in Cellular and Infection Microbiology*	6/12
Cai Y	China	2022.01.19	*Frontiers in Endocrinology*	6/12

[a]Jadad modified scale for RCT
[b]This paper contains also patients with Graves' disease

quality of assessment) (Table 1). In the case of poor agreement among the three authors, a consensus discussion with the fourth author (M.C.) has been made.

3 Results

3.1 Articles Retrieved

Using the search strategy, we recruited a total of 367 records, 190 from PubMed and 177 from Web of Science. Once 130 duplicates were excluded, we analyzed 237 papers; 26 of them have been included in the present analysis. In Fig. 1 is depicted the flow chart summing up the search strategy and the steps in study selection.

The articles included in our analysis were published from 2020 to 2022; furthermore, to exclude overlaps and to make an update on the more recent evidence on the topic, we excluded the articles published in 2020 that are included in our previous narrative review, which was published in 2021 (Virili et al. 2021). Table 1 summarizes the main features of the articles included in the present systematic review.

3.2 Findings of the Articles Included

Among the 26 papers included in the present review, seven articles dealt with patients showing goiter and thyroid cancer, nine focused on those with Graves' disease and orbitopathy, while 11 analyzed hypothyroid patients with Hashimoto's thyroiditis, treated or not with oral levothyroxine. To note, one included paper encompassed both patients with Graves' disease and Hashimoto's thyroiditis.

In the following sections, we will analyze and discuss the main findings of the 26 papers included in this systematic review, subdividing them on the basis of the thyroid disorders they have.

3.2.1 Thyroid Nodules and Cancer

Although different studies have shown the correlation between microbiota composition and neoplastic diseases (Sepich-Poore et al. 2021), only a few papers have characterized microbiota composition in patients with thyroid nodules and thyroid cancer (TC).

An overview of microbiota composition in seven studies involving patients with benign or malignant thyroid nodules is shown in Table 2.

Li et al. (2021) compared gut microbial composition in 196 patients with thyroid nodules and 283 controls using whole-genome shotgun sequencing of stool samples. They described a reduced microbial richness in patients with thyroid nodules, in contrast to previous findings (Zhang et al. 2019). Microbial composition was more similar between controls and patients with low sonographic risk-featured nodules (identified by Thyroid Imaging Reporting and Data System (TI-RADS) score 2), while patients with high sonographic risk nodules (TI-RADS scores 3 and 4) presented a reduction of the typical butyrate-producing gut microbiota (*Butyrivibrio unclassified*, *Coprococcus comes*, *Coprococcus catus*, *Roseburia hominis*, *Eubacterium eligens*, and *Faecalibacterium prausnitzii*). Butyrate acts as a histone deacetylase inhibitor, inducing growth arrest in various cell types and increasing iodine uptake in thyroid follicular cells by restoring the human sodium-iodide symporter function (NIS) (Provenzano et al. 2007). Thus, authors have postulated that its deficiency could be associated with the development of thyroid cancer.

Two papers analyzed gut microbiota from stool samples of patients diagnosed with TC and healthy controls (HC) (Yu et al. 2021; Ishaq et al. 2022). Yu et al. studied 90 TC patients and 90 HC. Patients with TC exhibited decreased microbial richness and diversity as compared to HC, in contrast to previous findings (Zhang et al. 2019; Feng et al. 2019). The authors speculated that diet habits and environmental variables might justify these contradictory results since these studies were carried out in different areas of China. Noticeably, in the study of Yu et al., 62% of patients showed metastatic lymphadenopathy at presentation, a percentage significantly higher than in the previous two studies (Zhang et al. 2019; Feng et al. 2019). Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria were the four dominant phyla: no significant difference emerged in the Firmicutes/Bacteroidetes ratio between the two groups. However, TC patients showed a higher abundance of Proteobacteria, considered a microbial signature of dysbiosis (Shin et al. 2015). At the genus level, patients with metastatic lymphadenopathy were characterized by a microbial signature consisting of increased levels of g__*Fusobacterium* and *g__Alistipes*, as well as decreased levels of *g__Hungatella* and *g__Phascolarctobacterium*. *Fusobacterium* has already been reported to be associated with distant metastases in human colorectal cancer (Bullman et al. 2017), suggesting its potential role as a noninvasive biomarker of metastatic disease.

A smaller sample, 16 euthyroid TC patients and 10 HC, was studied by Ishaq et al. (2022). Richness and alfa diversity were increased in TC patients as compared with healthy subjects, depicting significant gut bacterial overgrowth in the study group. This result was in keeping with previous findings (Zhang et al. 2019; Feng et al. 2019). At the level of phyla, Firmicutes and Verrucomicrobia were significantly increased, while Bacteroidetes were reduced in TC patients. At the genus level, patients with thyroid cancer

Table 2 Benign and malignant thyroid nodules

First author	Sample	Subjects	Bacterial α diversity	Bacterial β diversity	Phyla level	Genus/species level
Li A	Stool	196 TN (thyroid nodule) pts 283 HC	–	PCoA significant difference between TN and HD	–	**Reduced:** *Butyrivibrio unclassified*, *Bacteroides plebeius*, *Coprococcus comes*, *Coprococcus catus*, *Roseburia hominis*, *Eubacterium eligens*, *Anaerotruncus unclassified*, *Faecalibacterium prausnitzii*, and *Barnesiella intestinihominis*
Ishaq HM	Stool	16 TC pts 10 HC	Shannon and Simpson augmented	UPGMA significant difference between TC and HD	**Augmented:** Firmicutes Verrucomicrobia **Reduced:** Bacteroidetes	**Augmented:** *Escherichia-Shigella*, *[Eubacterium]_coprostanoligenes*, *Subdoligranulum*, and *Ruminococcus_2* **Reduced:** *Bacteroides*, *Klebsiella*, and *Prevotella_9*
Yu X	Stool	90 TC pts 90 HC	Shannon reduced	PCoA significant difference between TC and HD	**Augmented:** Proteobacteria	**Augmented:** *Fusobacterium* and *Alistipes* **Reduced:** *Prevotella*, *Hungatella*, and *Phascolarctobacterium*
Liu CJ	Thyroid tissue and stool	25 TN (thyroid nodule) pts	Shannon and Simpson augmented in stool compared to thyroid	PCoA significant difference between samples	Proteobacteria most abundant in thyroid tissue, *Firmicutes* in stool	–
Gnanasekar A	Thyroid tissue	563 TC pts		PCoA significant difference between samples	–	**Augmented:** *M. luteus and Bradyrhizobium sp.* in tall cell papillary TC
Dai D	Thyroid tissue	30 TC pts	Shannon reduced in tumor compared to peritumor tissue	Bray-Curtis and PCoA significant difference between tumor and peritumor tissue	Proteobacteria, Bacteroidetes, Firmicutes most abundant in thyroid tissue	*Comamonas*, *Acinetobacter*, *Chryseobacterium*, *Pseudomonas*, *Microvirgula*, *Soonwooa*, and *Sphingomonas* most abundant in thyroid tissue **Augmented:** *Sphingomonas* in tumor tissue **Reduced:** *Comamonas*, *Acinetobacter*, *Microvirgula*, and *Soonwooa* in tumor tissue
Lin B	Stool Saliva	23 TC probiotic 16 TC placebo	Shannon reduced during THW in placebo group, no difference in the probiotic group			**Augmented:** *Holdemanella*, *Enterococcus*, and *Coprococcus_2* in stool in the probiotic group **Reduced:** Stool: *Fusobacterium*, *Eubacterium_ruminantium*_group, *Ruminococcus_1*, and *Parasutterella* in the probiotic group Saliva: *Prevotella_9*, *Haemophilus*, *Fusobacterium*, and *Lautropia* in the probiotic group

TC thyroid cancer, *HC* healthy controls

showed a significant increase in *Escherichia-Shigella*, *[Eubacterium]_coprostanoligenes*, *Subdoligranulum*, and *Ruminococcus_2* and a reduction in *Bacteroides*, *Klebsiella*, and *Prevotella_9* (Ishaq et al. 2022). The authors concluded that the higher taxa diversity between the control and the study patients supports the existence of dysbiosis and bacterial overgrowth, which would characterize the patients with thyroid cancer.

Stool samples of TC patients were collected prior to surgery in both studies, and abnormal thyroid function tests or autoantibodies positivity was an exclusion criterion. So far, besides patients with autoimmune thyroid disease (AITD) or altered thyroid function, even those with thyroid cancer appear to be characterized by specific gut microbiota changes.

3.2.2 Tumoral Microenvironment in Patients with Thyroid Carcinoma

Three papers focused on intratumor microbiome composition in patients with thyroid cancer (Liu et al. 2021; Gnanasekar et al. 2021; Dai et al. 2021). Indeed, recent studies found that in all types of tumor tissues, from cerebral to breast cancer, bacterial communities do exist: these bacteria are mostly intracellular and are present both in cancer and immune cells (Nejman et al. 2020). The intratumor microbiome may have a role in affecting tumor progression and in the response to antitumor treatments (An et al. 2021). Its characterization in various cancer types would be a promising therapeutic target (Nejman et al. 2020). Liu et al. (2021) analyzed the gut microbiome in 25 patients with benign ($n = 6$) and malignant ($n = 19$) thyroid nodules in tumor, paratumor, and normal tissues, showing that the bacterial microenvironment in thyroid tissues is different from the gut one. In fact, at the phylum level, Proteobacteria was the most abundant in thyroid tissues, while Firmicutes was dominant in stools. Gnanasekar et al. (2021) correlated thyroid sample microbial abundance to clinical variables and cancer-associated gene expression in 563 patients with TC. More specifically, the authors compared samples obtained from men and women, previously classified as follicular or tall cell variants of papillary thyroid carcinoma (PTC), being the last ones characterized by more aggressive behavior. The authors described that tumor tissue contained lower microbe abundance compared to adjacent normal tissue as their first finding. The main result was that *Micrococcus luteus* and *Bradyrhizobium* sp. were abundant in tall cell papillary TC (TCPTC) and were positively correlated to MACIS (distant metastasis, patient age, completeness of resection, local invasion, and tumor size) score and oncogenic pathways (i.e., p53 instability). These findings might contribute in the more aggressive TCPTC phenotype. Dai et al. (2021) characterized thyroid microbiota from 30 patients with TC, showing that microbial diversity and composition were again significantly different between peritumor and tumor tissues. In particular, tumor tissues had lower thyroid microbiota richness and diversity than matched peritumor tissues. At the genus level, the combination of *Comamonas* and *Sphingomonas* could discriminate tumor from peritumor samples with an area under the curve (AUC) of 0.981 (95% CI): the former was enriched with tumor tissues, while the latter was enriched with peritumor tissues. Moreover, the abundance of *Sphingomonas* was significantly higher in the N1 stage than in the N0 stage, potentially representing a prognostic value for TC patients at an early stage. *Comamonas* has already been associated with more metastasized lymph nodes in pancreatic cancer (Jeong et al. 2020), while *Sphingomonas* was found to be increased in patients suffering from colitis-associated cancer (Richard et al. 2018).

3.2.3 Probiotics Used in Complications of Thyroid Hormone Withdrawal

An RCT study assessed the oral-gut microbiota in postoperative thyroid cancer patients who underwent thyroid hormone withdrawal (THW) before radioiodine therapy (Lin et al. 2022). The authors enrolled 50 TC patients and randomly assigned them to receive probiotics (Bifidobacterium tetravaccine tablets containing $>10^6$ CFU/tablet *B. infantis*, $>10^6$ CFU/tablet *Lactobacillus acidophilus*, $>10^6$ CFU/tablet

Enterococcus faecalis, $>10^5$ CFU/tablet *Bacillus cereus*, and $>10^6$ CFU/tablet total bacteria) for 4 weeks or placebo during THW. The authors investigated whether probiotics could alleviate THW-related complications and whether these therapeutic effects were associated with oral-gut microbiota composition. The presence of THW complications was assessed by the thyroid symptom questionnaire (TSQ) (focusing on the items of fatigue, constipation, edema, weight gain, dry mouth) and by the plasmatic sampling of lipopolysaccharides (LPSs), lipids, glucose, and the liver and renal functions. The probiotic group showed a significantly lower occurrence of THW symptoms (lack of energy, constipation, weight gain, and dry mouth) as well as lower levels of serum LPS and lipid values (total cholesterol, triglycerides, low-density lipoprotein, and apolipoprotein A) as compared to the untreated group. Gut and oral microbial diversity was significantly decreased after THW. Probiotic treatment differently restored the gut and oral microbial diversity. Increased *Holdemanella*, *Enterococcus*, and *Coprococcus_2* while decreased *Fusobacterium*, *Ruminococcus_1*, *Eubacterium_ruminantium_group*, and *Parasutterella* were found in the gut after probiotics intervention. Lack of energy, constipation, weight gain, and dyslipidemia were seen to be related to the above microbiota. In addition, probiotics reduced oral *Prevotella_9*, *Haemophilus*, *Fusobacterium*, and *Lautropia*, which were positively correlated with the occurrence of dry mouth.

It has been **hypothesized** that microbiota could regulate plasma lipid metabolism through the short-chain fatty acid (SCFA) pathway (Nogal et al. 2021). Probiotics may reduce serum LPS levels by regulating the intestinal barrier, increasing tight junction protein expression, and reducing inflammatory markers. Therefore, the reduction in symptoms like fatigue in the probiotics group could be associated with improvement in microbiota diversity, intestinal inflammation, and barrier function and a reduction in inflammatory bacterial abundance.

3.2.4 Graves' Disease

Multiple studies have evaluated the relationship between Graves' disease, Graves' orbitopathy, and gut microbiota in both an experimental and a clinical ground in the last 2 years.

The overview of microbiota characteristics in the nine studies encompassing patients with Graves' disease is depicted in Table 3.

Chang et al. analyzed fecal microbiota in 55 GD patients and 48 healthy controls (Chang et al. 2021); patients had an average follow-up of 45.33 months and had all been treated with antithyroid drugs. No difference was observed regarding bacterial richness and alpha diversity between GD patients and controls. In keeping with previous findings, Bacteroidetes and Actinobacteria increased, while Firmicutes decreased in the GD group at the phyla level (Shi et al. 2019; Jiang et al. 2021). At the genus level, the abundance of *Bacteroides*, *Collinsella*, and *Prevotella_9* was significantly higher, while *Faecalibacterium* and the *Lachnospiraceae_NK4A136_group* were slightly lower in the GD group as compared to healthy controls. Although *Prevotella* and *Bacteroides* are known to produce SCFAs, major actors in the maintenance of gut and immune homeostasis, their increase has been associated with GD, rheumatoid arthritis, and systemic lupus erythematosus (SLE) (Ishaq et al. 2018; Su et al. 2020a, b; Yan et al. 2020; De Aquino et al. 2014; Sun et al. 2019; Mendonca et al. 2019). The genus *Collinsella* has also been characterized in patients with rheumatoid arthritis, where a strong correlation to the production of the proinflammatory cytokine IL-17A has been shown (Chen et al. 2016). Similarly, the reduction of *Faecalibacterium* has been described in various autoimmune disorders; in GD patients, its abundance has been negatively correlated with thyroid-stimulating antibodies (Cornejo-Pareja et al. 2020). Jiang et al. analyzed microbiota from the fecal samples of 45 newly diagnosed and untreated GD patients and 59 controls (Jiang et al. 2021). GD patients had reduced richness and alpha diversity and, at the phylum level, a significant reduction in Firmicutes, and an

Table 3 Main results on microbiota composition in patients with Graves' disease

First author	Sample	Subjects	Bacterial α diversity	Bacterial β diversity	Phyla level	Genus/species level
Chang SC	Stool	55 GD pts 48 HC	Shannon and Simpson no differences	–	**Augmented:** Actinobacteria Bacteroidetes **Reduced:** Firmicutes	**Augmented:** *Collinsella*, *Bacteroides*, and *Prevotella_9* **Reduced:** *Faecalibacterium* and *Lachnospiraceae_NK4A136_group*
Jiang W	Stool	45 GD pts 59 HC	Shannon and Simpson reduced	PLS-DA Significant differences between GD HC	**Augmented:** Bacteroidetes **Reduced:** Firmicutes	**Augmented:** *Bacteroides* and *Lactobacillus* **Reduced:** *Blautia*, *[Eubacterium]_hallii_group*, *Anaerostipes*, *Collinsella*, *Dorea*, *unclassified_f_Peptostreptococcaceae*, and *[Ruminococcus]_torques_group*
El-Zawawy H	Stool	13 GD pts 30 HC	Shannon no differences	Bray-Curtis no differences	**Augmented:** Bacteroidetes **Reduced:** Firmicutes	**Augmented:** *Prevotella*
Zhu Q	Stool	64 GDM pts (mild) 36 GDS pts (severe) 62 HC	Reduced in GDS	Bray-Curtis and PCoA Significant differences between GDS and GDM, HC	–	**Augmented:** *Eggerthella lenta*, *Streptococcus parasanguinis*, *Veillonella parvula*, *Fusobacterium mortiferum*, and *Streptococcus salivarius* in GDS **Reduced:** *Faecalibacterium prausnitzii*, *Butyricimonas faecalis*, *Bifidobacterium Adolescentis*, and *Akkermansia muciniphila* in GDS
Chen J	Stool	15 GD pts 14 HC	Shannon and Simpson reduced	–	**Augmented:** Proteobacteria after treatment **Reduced:** Proteobacteria and Synergistetes in GD	**Augmented:** *Streptococcus*, *Blautia*, *Lactobacillus*, and *Veillonella* **Reduced:** *Phascolarctobacterium*
Yang M	Stool	18 newly diagnosed GD pts (ND) 10 methimazole-treated GD pts (MT) 11 HC	Shannon and Simpson no differences	NMDS significant difference between the three groups	**Augmented:** Actinobacteria in ND Proteobacteria in MT **Reduced:** Firmicutes	**Augmented:** *Prevotella*, *Collinsella*, and *Bifidobacterium* **Reduced:** *Roseburia*

(continued)

Table 3 (continued)

First author	Sample	Subjects	Bacterial α diversity	Bacterial β diversity	Phyla level	Genus/species level
Yang M	Stool	191 GD pts 30 HC	Shannon and Simpson no differences	NMDS significant difference between GD and HC	**Augmented:** Actinobacteria **Reduced:** Firmicutes	**Augmented:** *Collinsella*, *Bifidobacterium*, *Pediococcus*, *Enterobacter*, etc. **Reduced:** *Roseburia*, *Dialister*, *Thermus*, etc.
Huo D	Stool	8 GD pts treated with methimazole	Shannon and Simpson reduced	Bray-Curtis no differences	–	**Reduced:** *Faecalibacterium prausnitzii*, *Ligilactobacillus salivarius*, *Lactococcus lactis*, and *Prevotella*
		9 GD pts treated with methimazole + black beans	Shannon no difference	Bray-Curtis no differences		**Augmented:** *Arabia massiliensis*, *Bacteroides cellulosilyticus*, and *Desulfovibrio* (some species) **Reduced:** *Bacillus litoralis*, *Streptococcus milleri*, and *Rothia mucilaginosa*
		9 GD pts treated with methimazole + *Bifidobacterium longum* All patients followed up for 6 months	Shannon no difference	Bray-Curtis no differences		**Augmented:** *Bifidobacterium* (some species) and *Faecalibacterium prausnitzii* **Reduced:** *Blautia hansenii*, *Clostridium estertheticum*, and *Klebsiella pneumoniae*
Han Z	Stool	8 GD pts treated with methimazole followed up for 6 months	Shannon Simpson no difference	Bray-Curtis no differences	–	**Reduced:** *Prevotella*, *Streptococcus pneumoniae*, *Selenomonas ruminantium*, and *Enterobacter hormaechei*
		10 GD patients treated with methimazole + berberine followed up for 6 months	Shannon Simpson no difference	Bray-Curtis significant difference		**Augmented:** *Lactococcus lactis* **Reduced:** *Prevotella*, *Tannerella forsythia*, and *Chryseobacterium indoltheticum*

HC healthy controls, *GD* Graves' disease, *NMDS* nonmetric multidimensional scaling

increase in Bacteroidetes has been observed, as compared with the controls, in agreement with what was reported by Chang SC et al. At the genus level, GD patients had greater numbers of *Bacteroides* and *Lactobacillus* and fewer *Blautia* and *Collinsella* than control patients. Although *Lactobacillus* is considered a beneficial probiotic, some strains of this genus may be involved in triggering AITD through molecular mimicry because they share the same amino acid sequences of thyroid peroxidase (TPO) and thyroglobulin (Tg) (Kiseleva et al. 2011). *Blautia*, instead, is a butyrate producer, an SCFA that promotes the differentiation of Treg in the intestine. Finally, the authors examined the relationship between microbiota and thyroid function tests, including triiodothyronine (T3), thyroxine (T4), thyroid-stimulating hormone (TSH), thyrotropin receptor antibody (TRAB), thyroglobulin antibody (TGAB), thyroid peroxidase antibody (TPOAB), and thyroid microsomal antibody (TMAB). *Blautia* levels positively correlated with TPOAB and TMAB levels, while *Bacteroides* negatively correlated with TPOAB and TMAB levels. El-Zawawy et al. (2021) analyzed fecal microbiota in 13 GD patients and 30 healthy controls; most of the patients were newly diagnosed cases and only three of them were on antithyroid treatment. Dietary habits between patients and controls were markedly different. The patients did not show any significant difference in terms of richness and alpha and beta diversity. At the genus level they described an increase in *Prevotella*. Moreover, TRAb showed a significant positive correlation with Bacteroidetes and Firmicutes. Furthermore, Zhu et al. (2021) analyzed fecal microbiota from 62 healthy donors and 100 GD patients, divided on the basis of biochemical and clinical thyroid parameters in patients with mild (n = 36) and severe disease (n = 64). Interestingly, the authors found that patients with mild GD presented microbiome features more similar to HD, and that patients with severe GD presented a specific gut microbiome signature in terms of taxonomic and metagenomic analysis that could represent a useful noninvasive diagnostic tool.

3.2.5 Effect of Antithyroid Drugs

Two manuscripts analyzed gut microbiota composition in GD patients and its changes during antithyroid drug treatment (Chen et al. 2021a, b; Yang et al. 2022a, b). Chen et al. (2021a, b) showed that the abundance and diversity of gut microbiota were significantly reduced in GD patients (15 pts), while they were increased after antithyroid treatment; thus, the author speculated that the alteration of microbiota composition found in GD patients might be an effect of hyperthyroidism and might not necessarily be involved in the pathogenesis. At the phyla level, the ratio of Firmicutes/Bacteroidetes was not different between patients and healthy donors. It was documented as a reduction in Proteobacteria and Synergistetes in GD patients and a significant increase in Proteobacteria after treatment. At the genus level, increased relative abundance in *Streptococcus*, *Blautia*, *Lactobacillus*, and *Veillonella* has been observed, while *Phascolarctobacterium* decreased significantly. After treatment, relative abundance in *Ruminococcus*, *Streptococcus*, and *Blautia* decreased significantly, while abundance in *Phascolarctobacterium* increased significantly. Moreover, the authors correlated different bacterial abundances with clinical thyroid-related parameters. They stressed the finding that TRAb levels were negatively correlated with Synergistetes and *Phascolarctobacterium* and were positively correlated with *Lactobacillus* before treatment and *Ruminococcus* after treatment. They concluded that Synergistetes and *Phascolarctobacterium* probably have a protective role in the pathogenesis of GD, while *Ruminococcus* and *Lactobacillus* possibly represent a trigger in the immune mechanism of GD. According to studies in SLE patients, Synergistetes may be involved in regulating the synthesis and secretion of autoantibodies by modulating the balance of Th17/Treg (Chen et al. 2017; Lopez et al. 2016). Conversely, Yang et al. (2022a, b) did not show a significant difference in terms of richness and alpha diversity in their study population, consisting of 18 newly diagnosed GD patients, ten methimazole-treated patients, and 11 healthy controls. The most

represented bacterial phyla in the stool samples of the three groups were Firmicutes (64.7%), Bacteroidetes (23.5%), Actinobacteria (7.0%), and Proteobacteria (4.5%). The newly diagnosed group presented an increase in Actinobacteria and a reduction in Firmicutes as compared to HC, while treated patients showed an increase in Proteobacteria. At the genus level, they showed an increase in *Prevotella* abundance in newly diagnosed GD patients compared to healthy people, in agreement with previous studies (Ishaq et al. 2018; Su et al. 2020a, b). An increase in *Collinsella* abundance has also been observed in untreated GD patients, which change was reverted with methimazole treatment; this bacterial genus has been related to the production of the proinflammatory IL-17 (Chen et al. 2016). A reduction in the SCFA-producing *Roseburia* and an increase in *Bifidobacterium* in untreated GD patients as compared to healthy people were similarly observed, in line with the results reported in the literature (Song et al. 2019; Sun et al. 2020); this change was reverted with methimazole therapy. The relative abundance of this bacterium was positively correlated with the levels of TRAb, TgAb, and TPOAb. As already mentioned for *Lactobacillus*, also *Bifidobacterium* may be involved in triggering AITD through molecular mimicry (Kiseleva et al. 2011).

These results are similar to those described by the same group on a larger study sample where the dosage of vitamin D and serum IL17 was also included; vitamin D levels negatively correlated with serum IL17 and TRAb values, although no significant correlation with gut microbiota emerged (Yang et al. 2022a, b).

3.2.6 Leaky Gut in Patients with Graves' Disease

Most autoimmune diseases are associated with a gut barrier dysfunction (leaky gut), which allows bacterial and/or antigen translocation into the systemic circulation. Several biomarkers have been validated for the assessment of intestinal barrier integrity and bacterial translocation: (a) intestinal fatty-acid-binding protein (I-FABP) and diamine oxidase (DAO) are cytosolic proteins in intestinal epithelial cells, which are immediately released into the circulation when the intestinal epithelium is disrupted; (b) zonulin, a physiological regulator of intestinal permeability, reversibly opens tight junctions between intestinal epithelial cells; and (c) lipopolysaccharide (LPS) and D-lactate, which are components of bacterial membranes, act as indicators of the translocation of parts of gut-resident bacteria in the systemic circulation (Zheng et al. 2021). Indeed, Zheng et al. (2021) investigated correlations between a leaky gut and GD by measuring LPS, I-FABP, zonulin, D-lactate, and DAO in 91 patients with GD and 44 healthy controls. The serum levels of LPS, I-FABP, zonulin, and D-lactate were significantly higher in the patient group. Moreover, higher circulating LPS levels were associated with more severe hyperthyroidism, higher TRAb concentrations, and a worse course of both hyperthyroidism and orbitopathy. These results make clear the association between the development of GD and a leaky gut. They speculate that bacterial translocation may trigger thyroid autoimmunity via molecular mimicry and that circulating LPS may cause a pro-inflammatory status by activating Toll-like receptor 4 (TLR4) and initiating NF-kB signaling pathways (Benvenga and Guarneri 2016; Chow et al. 1999).

3.2.7 Microbiome Modulation in Graves' Disease Patients

Two articles analyzed the role of probiotic and prebiotic compounds in improving the therapeutic effect of methimazole in patients with Graves' disease. Both these studies were carried out in the same laboratory in Haikou, China. The first trial (Chen et al. 2021a, b) compared the effect of these compounds on 25 patients with Graves' disease, subdivided into three arms of treatment: eight patients treated with methimazole alone, nine with methimazole and 100 g of black beans per day, and nine with methimazole and probiotic *Bifidobacterium longum* (2×10^7 CFU) per day. Black beans were chosen because, in Chinese medicine, they are used as a dietary **prescription** in hyperthyroid patients to alleviate their symptoms; *Bifidobacterium longum* is known for its anti-inflammatory and immune-regulating

properties (Chen et al. 2021a, b). To note, the authors underlined the effect of methimazole in improving thyroid function and in changing significantly the abundance of bacterial species. The concomitant administration of black beans limited these microbial variations, while in the third group, it was observed the increase of several species of *Bifidobacteria* and a lower amount of *Blautia hansenii* and *Klebsiella pneumoniae*. These variations were linked to increased production of propionic and butyric acids, which are beneficial microbial metabolites. In the group supplemented with *Bifidobacterium longum*, besides thyroid function improvement, it has been recorded a dramatic drop in TRAb levels to normal levels. The second study (Han et al. 2022) was carried out to evaluate the effect of berberine added to methimazole in ten patients (as compared to methimazole alone in eight patients) in restoring thyroid function. Berberine is a natural cationic alkaloid usually found in the rhizomes of many medicinal plants (Habtemariam 2020). Because of its prebiotic activity, it was able to modulate gut microbiota composition in Graves' diseases patient, increasing the abundance of the beneficial *Lactococcus lactis* and decreasing the pathogen *Enterobacter hormachei*. However, its coadministration with methimazole showed a small adjuvant effect than the one exerted by methimazole alone (Han et al. 2022).

3.2.8 Hashimoto's Thyroiditis and Hypothyroid Patients

Hypothyroidism often recognizes inflammatory etiopathogenesis, both acute and chronic, and its more common leading cause worldwide is HT (Taylor et al. 2018; Ragusa et al. 2019). Previous studies dealing with the role of human microbiota in the pathogenesis and clinical outcomes of HT were mostly carried out in China (Virili et al. 2021). The more recent studies provided novel evidence about this topic by analyzing Brazilians, Caucasians or Afro-descendants, and Egyptians, thus widening the geographical distribution of the population examined (Cayres et al. 2021; El-Zawawy et al. 2021). The microbiota characteristics of 8/11 studies encompassing patients with Hashimoto's thyroiditis/hypothyroidism are depicted in Table 3. The remaining three papers, taken into account in this review, have been not included in the table, due to the different design of the studies, since the analysis at phyla and genus/species levels were not included (Talebi et al. 2020a, b; Wang et al. 2021; Li et al. 2022) (Table 4).

Cayres et al. conducted their observations on stool samples of 40 Brazilian patients with HT as compared to 53 healthy controls (HC) (Cayres et al. 2021). In contrast with a previous study, they observed a reduction of *Bacteroides* and an increase of *Bifidobacterium* in HT subjects as compared to HC. However, the serum concentrations of TSH and FT4, as well as those of antithyroid antibodies, were not available for all subjects, thus rendering it hard to identify a possible role of thyroid dysfunction/autoimmunity on microbiota composition. Despite that, the authors found a positive correlation between TSH levels and *Clostridium coccoides* and *Clostridium coccoides-Eubacteria rectale* and an inverse correlation between *Roseburia* and FT4 levels, but this correlation has not been studied in all HT patients due to the unavailability of data in some patients of the study group. Furthermore, it was observed that *Lactobacillus* (belonging to the Firmicutes phylum) were significantly higher in levothyroxine (LT4)-replaced subjects (33/40 HT were on LT4 therapy), but the authors did not observe correlations between LT4 dose, TSH level, and microbiota composition. Therefore, these data confirm previous evidence describing the presence of a possible imbalance in the composition of the gut microbiota in HT patients, with or without hormone replacement therapy (Lauritano et al. 2007; Patil 2014; Yao et al. 2020). More importantly, this study showed an increase in gut permeability, measured as an increase in serum zonulin levels, in HT patients. The values of zonulin showed an inverse correlation with interleukin 2 and a direct correlation with interferon gamma. Furthermore, using a food-frequency questionnaire (FFQ), the authors tried to find a relationship between the composition of the gut microbiota in correlation with the nutritional habits of the study group. There

Table 4 Main results on microbiota composition in patients with Hashimoto's thyroiditis/hypothyroidism

First author	Sample	Subjects	Bacterial a diversity	Bacterial b diversity	Phyla level	Genus/species level
Tabasi et al.	Stool	23 H obese, 79 HC obese	No differences	No differences	/	/
Su et al.	Stool	50 H, 40 HC	Chao/Ace: increased richness Shannon/ Simpson: reduced diversity	Bray-Curtis: significant difference HvsHC	**Augmented:** Firmicutes/ Bacteroidetes ratio **Reduced:** Bacteroidetes	**Augmented:** *Veillonella* and *Paraprevotella* **Reduced:** *Neisseria* and *Rheinheimera*
Dong et al.	Salivary	20 SH, 20 HC	Chao: increased richness Simpson: reduced diversity	PLS-DA/PCoA Genus level: significant difference SHvsHC	/	**Augmented:** *Granulicatella*
El-Zawawy et al.	Stool	7 HT, 30 HC	No differences	No differences	**Augmented:** Bacteroidetes **Reduced:** Firmicutes and Firmicutes/ Bacteroidetes ratio	**Augmented:** *Prevotella*
Cayres et al.	Stool	40 HT, 53 HC	/	/	/	**Augmented:** *Bacteroides*
DeClercq et al.	Salivary	127 LT4, 44 HND	Shannon: increased diversity Eveness: increased	No differences	/	**Reduced:** *Bacillus*
Cai et al.	Stool	27 SH P, 28 HC P	Shannon/ Sobs: reduced richness and diversity	PCoA/Bray-Curtis: no differences	/	**Augmented:** *Prevotella* and *Haemophilus*
Wang et al.	Stool Salivary	30 H P, 31 HC P	Chao/Ace: no differences Shannon/ Simpson: no differences	ANOSIM/ PCoA: – Intestinal: ns – Oral: significant difference H PvsHC	/	**Intestinal Augmented:** *Roseburia*, *Lachnospira*, *Prevotella*, and *Parabacteroides* **Oral Augmented:** *Prevotella* and *Neisseria*

HC healthy controls, *HND* healthy no-drug users, *SH* subclinical hypothyroid, *H* hypothyroid, *HT* Hashimoto's thyroiditis, *ns* not significant, *P* pregnant, *LT4* levothyroxine replaced

are currently no studies investigating this aspect. Notably, the FFQ revealed that there was a significant difference between the two study groups in daily protein consumption (20 HT versus 9 HC), and *Bacteroides* species inversely correlated with animal-derived protein consumption.

El-Zawawy et al. (2021) compared the fecal microflora of 20 subjects affected by thyroid autoimmune disease (AITD) and 30 healthy controls. At the phylum level, they observed an increase in Bacteroides and a decrease in Firmicutes in AITD subjects. Notably, among the Bacteroides,

the main genus was *Prevotella*. However, no differences were observed in alpha and beta diversity in the two groups. Nevertheless, the following data are affected by many biases, which may not allow definitive conclusions about HT: (1) the study involves only seven HT subjects, (2) the HT patients were both affected by overt and subclinical hypothyroidism, (3) three to seven of the HT patients were smokers, and (4) AITD and HC groups significantly differ in dietary habits (healthy diet in HC and diet rich in fat and animal-derived food in AITD). These environmental biases are able to influence significantly microbiota composition (Rothschild et al. 2018).

Su et al. (2020a, b) analyzed gut microbiota composition in a group of 52 untreated nonautoimmune hypothyroid subjects compared with a group of 40 HC. The authors observed a significant difference in alpha and beta diversity between the two groups. Notably, the intestinal bacterial communities of the study group (hypothyroid) were characterized by greater richness and lesser diversity. At the phylum level, the *Bacteroides* genus and Firmicutes/Bacteroides ratio were reduced and increased, respectively, in this study group. Furthermore, at the genus level, they observed that in hypothyroid subjects, *Veillonella* and *Paraprevotella* were significantly reduced, while *Neisseria* and *Rheinheimera* were increased. These data suggest a condition of intestinal dysbiosis in the hypothyroid group, which is consistent with previous studies (Lauritano et al. 2007; Liu et al. 2020). By metagenomic analysis, it was observed that the structural differences between the two groups were also related to functional differences in the gut microbiota. Notably, these data revealed that the ability to produce SCFA was significantly reduced in hypothyroid subjects, and there was a significant increase in the serum level of LPS. On this ground, Su et al. (2020a, b) transplanted the intestinal flora (FMT) of 10 primary hypothyroid patients and 10 healthy controls in 20 male pathogen-free mice, randomly subdivided in two groups (10 vs. 10). The authors observed a significant increase in serum LPS and a decrease in SCFA synthesis as well as in serum total thyroxine levels in mice transplanted with fecal samples from hypothyroid patients.

Dong et al. (2021) carried out the first study on salivary samples collected from 20 HC and 20 subjects affected by subclinical hypothyroidism (SH). Significant alpha and beta diversity characterized the oral microbiota from these two groups. The SH group showed greater richness without the identification of a dominant species. Analysis at the phylum level revealed a similar composition between the two groups but a different distribution of 45 taxa. In the SH group, the more prevalent taxonomic species were Lactobacillales at the order level; Porphyromonadaceae, Carnobacteriaceae, and Spirochaetae at the family level; and *Granulicatella*, *Treponema*, and *Streptobacillus* at the genus level. The *Granulicatella* genus in oral cavity is normally associated with healthy subjects, but some evidence supports the correlation with clinical metabolic conditions (Si et al. 2017). Therefore, clinical and biochemical metabolic aspects were analyzed, describing significant differences in HOMA-IR, fasting serum insulin, total cholesterol, and triglycerides between the two groups; however, no differences were observed in the expression of metagenomic bacterial pathways.

3.2.9 Modulation of Gut Microbiota in Hypothyroid Patients

A recent paper by Talebi et al. (2020a, b) studied the effect of synbiotic (an association of pro- and prebiotics) supplementation in hypothyroid subjects. This double-blind placebo-controlled study includes 56 Iranians with primary hypothyroidism (both autoimmune and nonautoimmune) replaced with the same dose of LT4 for at least 1 year, taken regularly 30 min before breakfast. The study sample was randomly divided into the placebo group ($n = 27$) and the synbiotic group ($n = 29$). All subjects were asked to maintain the same nutritional habits and physical activity. The symbiotic capsules contained *Lactobacillus casei*, *L. acidophilus*, *L. rhamnosus*, *L. bulgaricus*, *Bifidobacterium breve*, *B. longum*, and *Streptococcus thermophilus* and were taken for 8 weeks after breakfast; the same was done with the placebo capsules. After 8 weeks, the only significant

difference observed was the improvement of constipation in the supplemented group. The same author (Talebi et al. 2020a, b) has also carried out in the same population a comparative study showing a lower TSH concentration and levothyroxine requirement and a higher serum FT3 in patients supplemented with symbiotic and levothyroxine as compared to patients treated with levothyroxine alone.

3.2.10 Microbiota Composition in "Special Hypothyroid Populations"

In the past, no studies have observed the different composition of the microbiota in particular hypothyroid populations (i.e., pregnant, obese), while some evidence was provided on the relationship between intestinal microbiota and pregnancy outcomes or metabolic disorders, such as obesity (Sekirov et al. 2010; Qi et al. 2021).

Recently, Tabasi et al. (2021) analyzed serum metabolic biomarkers, cytokines, and the gut microbiota in 23 (M/F:4/19) Iranian obese subjects affected by hypothyroidism as compared with 79 HC. Unfortunately, no significant difference was observed between the two groups. To date, only four studies by a group from Zhengzhou University (China) have focused on the microbiota in pregnant women with thyroid dysfunction. The first study published by Wang et al. (2021) focused on the incidence of small intestinal bacterial overgrowth (SIBO) in 224 pregnant women with SH and 196 HC pregnant, both groups in the third trimester of gestation. The diagnosis of SIBO was based on a lactulose breath test. These authors observed a significant increase in SIBO occurrence and serum C-reactive protein (CRP) levels in the study group, mainly associated with intestinal constipation. Interestingly, in each group, SIBO was directly correlated with TPO-Ab and TSH levels while indirectly with body mass index (BMI) and FT4 levels. A second study by Li et al. (2022) analyzed blood samples and methane hydrogen breath tests of 30 pregnant women with SH and 30 pregnant HC, both groups in the third trimester of gestation. As in the previous study, the incidence of SIBO was significantly higher in the study group, and the presence of SIBO positively correlated with total cholesterol and TSH levels and negatively correlated with glycocholic acid levels and maternal BMI. The pregnancy outcome was also analyzed, which showed that maternal serum lipid levels, hypertensive disorders, and newborn body weight were significantly different in the two groups, this finding being possibly associated with hypothyroidism and not with microbiota composition.

A similar population (27 women with SH and 28 HC pregnant women in the third trimester of gestation) from the previous study was analyzed by Cai et al. (2022), who obtained the same results on clinical pregnancy outcomes and biochemical data. Despite a similar alpha and beta diversity in intestinal microflora in the two groups, it has been observed that the *Prevotella* and *Haemophilus* genera were significantly higher in the study group, while *Blautia* was significantly lower than in HC. In SH women, glycerophospholipid (GP) metabolism was upregulated, while sphingolipid (SL) metabolism was downregulated. Furthermore, *Blautia* correlated positively with SL and FT4 levels and negatively with GP; *Prevotella* correlated positively with TSH and CRP levels and negatively with GP; GP negatively correlated with newborn body weight and length. This study showed that the gut microbiota composition and lipid profile of pregnant women with SH differ significantly from those of HC subjects; unfortunately, the authors did not provide information on the etiology of thyroid dysfunction. Subsequently, the same group studied the composition of the fecal and salivary microbiota in 30 hypothyroid women compared with 31 HC women in the third gestational trimester (Wang et al. 2020). Hypothyroidism was not a pregestational condition, and LT4 therapy was administered to hypothyroid patients, though it is not specified whether before or after specimen collection. The oral and intestinal microbiota showed no significant differences in alpha diversity. However, significant differences appeared in the composition of the oral microbiota: the oral cavity of women with HC had relative abundance in Firmicutes, Leptotrichiaceae, and *Actinobacteria*, while

in the hypothyroid group, there was a higher relative prevalence of Gammaproteobacteria, Pasteurellaceae, *Prevotella*, and *Neisseria*. Alpha and beta diversity was similar in the gut microflora but was relatively increased in Pasteurellales, Porphyromonadaceae, *Roseburia*, *Lachnospira*, *Prevotella*, and *Parabacteroides* in the study group, and *Clostridium* and *Blautia* were observed in the control group. The gut microbiota of hypothyroid pregnant women showed increased metabolic pathways of structural molecules and flagellar assembly. Furthermore, the authors observed that oral Gammaproteobacteria and Pasteurellaceae correlated positively with CRP and negatively with FT4 and TSH and with weight gain during pregnancy; oral Firmicutes correlated negatively with CRP, while oral and intestinal Pasteurellaceae showed a positive correlation. Levothyroxine treatment did not change the significantly higher CRP level and poor clinical outcomes of pregnancy in hypothyroid women, as has been observed in previous studies. Again, it is unclear whether it is due to changes in microbiota composition, altered thyroid function, or both.

Since the subjects of these four studies belonged to the same research structure, it is possible that they are burdened by the overlapping of the different study groups and by selection bias.

3.2.11 Microbiota and Levothyroxine Treatment

A recent cross-sectional study by De Clercq et al. (2021) examined the impact of drugs on the oral microbiota of salivary samples from 1214 Canadian subjects, divided into drug users and nonusers. Alpha and beta diversity, richness, and evenness were similar in the two groups. Among the 127 patients taking thyroid hormones, 60 subjects declared to assume only LT4 (SD), whereas 71 subjects assumed LT4 and other medications (MD). Alpha diversity differed significantly among no-drug users, SD and MD, although no differences were observed in beta diversity. Nevertheless, in subjects taking LT4 were identified several genera significantly enriched as compared with no-drug users (Veillonellaceae, *Bacteroides*, *Prevotella 6*, *Tannerella*, *Bergeyella*, *Bacillus*, *Mycoplasma*); on the contrary, *Bacillus* was significantly depleted.

4 Concluding Remarks

There are several differences in microbiota composition between patients with different thyroid disorders and healthy subjects, often confirmed in more than one study and sometimes between the same category of thyroid patients. The overall results of the present review article **strengthen** the existence of a bidirectional relationship between the intestine, with its microbial set, and thyroid homeostasis, thus supporting the newly recognized entity known as the gut-thyroid axis. However, before definite conclusions could be drawn about their clinical meaning, the accuracy of patients' selection and their geographic and ethnic roots is mandatory. In fact, since microbiota composition may be shaped by genetic and lifestyle features (i.e., variations in dietary habits, drug use), further studies on patients coming from other countries should be carried out. These studies should be multicentric, involving a large number of patients and possibly including metabolomic analysis.

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Taxus wallichiana Zucc. (Himalayan Yew): A Medicinal Plant Exhibiting Antibacterial Properties

Vibha Bhardwaj

Abstract

Taxus wallichiana Zucc. or the Himalayan yew is a gymnosperm growing along the Himalayan region of India and adjacent countries. Traditionally, this plant was extensively used by indigenous people for folk medicines for treating various diseases such as fever, headache, diarrhoea, fractures, problems of the nervous system etc. It is also practiced in the Unani system of medicine. The plant is rich in various bio-organic compounds and natural products, such as hydrocarbons, glycosides, flavonoids, phenol, tannins, terpenoids etc. In this research work, an effort has been made to highlight the valuable properties of *T. wallichiana*. The present study was undertaken to evaluate the secondary metabolites (flavonoids, glycosides, phenols, saponins, tannins, terpenoids) and antibacterial potential of methanol extracts and the subsequent fractions of the leaves and fruit of *Taxus wallichiana* Zucc. In order to rationalise traditional use, methanol extracts from the leaves and fruit of *Taxus wallichiana Zucc*. were tested against five bacteria using the agar well diffusion method. Ciprofloxacin was used as a standard. All extracts and fractions displayed significant anti-microbial effects. *Taxus wallichiana* leaves and fruit methanolic extracts showed a maximum zone of inhibition with *Bacillus subtilis*, which is 18 ± 0.0 mm, and *Staphylococcus aureus*, 19 ± 0.2 mm. The methanolic extracts of the leaves of *Taxus wallichiana* tested positive for glycosides, flavonoids, phenol, tannins and terpenoids, whereas the *T. wallichiana* fruit tested positive for flavonoids, saponins and terpenoids. According to the research findings, it was identified that the methanol extract of *Taxus wallichiana* exhibited quite high antimicrobial activity as well as secondary metabolites, and with this quality, together with lots of its other values, this plant can very well become a source of medicine for the better management of a large number of diseases, including cancer, and value-added products.

Keywords

Agar well diffusion assay · Antibacterial · Microorganisms · Multidrug resistant · *Taxus wallichiana*

Abbreviations

ATCC	American Type Culture Collection
C	ciprofloxacin

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E	extract
h	hours
MDR	multidrug resistant
SD	standard deviation
T. wallichiana	*Taxus wallichiana*

1 Introduction

Globally, infectious disease is the major cause of death, accounting for approximately one-half of all deaths in tropical countries (Iwu et al. 1999). New therapeutic agents and strategies are demanding issues to cope with infectious diseases. Low-income people, especially from small remote villages and native communities, use folk medicine for the treatment of common infections. These plants are ingested as decoctions, teas and juice preparations to treat respiratory infections or as a poultice and are applied directly on the infected wounds or burns (Gonzalez 1980; Kaul 1997). Exploration in herbal medicine has increased in developing countries as a way to rescue ancient traditions as well as a substitute solution to the health problems in cities. Therefore, with the increasing acceptance of traditional medicine as an alternative form of health care, the screening of medicinal plants for active compounds has become very important (Bhardwaj 2022). The Himalayan region has been a rich source of medicinal plants for millions of populations inhabiting the mountain ranges and those around them. The Himalayan region is rich in floral diversity, and plants are extensively used by the local people for their daily needs, such as for thatching and shelter, fuel, fodder, household items and medicines (Rana et al. 2019; Pala et al. 2019).

Taxus wallichiana Zucc. is a member of the family Taxaceae, which is commonly known as the Himalayan yew (Shrestha et al. 1997). *Taxus wallichiana* is one such gymnosperm that grows in the Himalayan region. It is a small to medium-sized evergreen tree with a height of 10–28 m (Juyal et al. 2014). The plant is widely distributed in Asia, and its occurrence spans from Afghanistan to the Philippines and is widely distributed in the Himalayan regions of India and adjacent countries (Hussain et al. 2013). *T. wallichiana* is traditionally used by the local people of the Indian subcontinent for the cure of a number of ailments. In India, tincture prepared from the aerial part of the plant is traditionally used for the treatment of several diseases of the central nervous system, such as hysteria, grittiness, biliousness, epilepsy and nervousness. The plant also forms one of the components of the popular Unani drug 'Zarnab', which is known to possess sedative and aphrodisiac properties (Sharma and Garg 2015). *T. wallichiana* is also used indigenously by the people of Nepal for curing respiratory problems, bronchitis and cancer (Gaire and Subedi 2011). The leaves of *T. wallichiana* are also used to prepare herbal tea for the cure of epilepsy and indigestion (Aboutabl 2018). *T. wallichiana* is also reported to have immuno-modulatory, antibacterial, antifungal, analgesic, anti-pyretic and anti-convulsant activities (Rahman et al. 2013).

However, through a literature survey, it was revealed that no significant work has been done on the antibacterial and phytochemical activities of *Taxus wallichiana Zucc*. Keeping this knowledge in view, the present study was undertaken to investigate the antibacterial and secondary metabolite potential of *Taxus wallichiana Zucc.*

Botany and Uses

Taxus wallichiana (Himalayan yew) is the first ever Himalayan species of *Taxus* to be discovered by Joseph Gerhard Zuccarini in 1843, hence giving him the authority of the species and associating the code, Zucc., after his name while naming Taxus wallichiana. Initially, *Taxus baccata* (European yew) was the first *Taxus* species to be identified by Carl Linnaeus in 1753. *Taxus wallichiana* is a dioecious tree species (Yang et al. 2009). The stems are fluted with spreading branches. The barks are thin, reddish brown and scale like (Bhuju and Gauchan 2018). The leaves are dark grey in colour, glossy green above, paler beneath, linear, 2–3.8 × 0.3 cm in length, coriaceous, flattened and arranged in two vertical opposite rows. The cones are

Fig. 1 (**a**) Leaves, (**b**) fruit of *Taxus wallichiana*

axillary and sessile. The male cones are solitary, axillary and sub-globose; their bracts are empty; and they have ten stamens. The female cones are solitary with a few imbricate scales surrounding an erect ovule. The ovules are surrounded at the base by a membranous cup-shaped disc. The fruit has a bright red disc (Fig. 1b) and is succulent, enlarged and 7–8 mm in length. The seeds are olive green in colour, and they are dispersed by birds and animals. The growth of the trees is extremely low, with 12–14 annual rings per 2.5 cm radius and a girth increment of 0.4–1.3 cm per year (ENVIS). Details about the species' outline can be found at http://www.worldbotanical.com/Nomenclature.htm# (Nomenclature, Keys and Descriptions for species of Taxus with discussion and citation of specimens studied).

In our previous study, we analysed the potency of cedar deodar for antibacterial and secondary metabolite potential (Bhardwaj 2022). Also, we investigated that ghaf and mangrove have potential for antioxidant and antimicrobial properties (Bhardwaj 2021a, b, c). In addition, we investigated the nutraceutical and anti-microbial properties of ghaf (Al Ghais et al. 2020a, b, c; Bhardwaj 2021d, e). Therefore, to continue our further research and also meet the increasing demand for anti-microbial agents, we explored natural sources and alternative strategies to search for new anti-microbial agents. Hence, the objective of the study was to seek the anti-microbial activity of the methanolic extract of *T. wallichiana* and also secondary metabolites. This probe was carried out as an awareness of the medicinal value of the plant.

2 Material and Methods

2.1 Plant Material Collection

Samples of the leaves and fruit of the Himalayan yew plant were collected from Chail in Himachal Pradesh, India, in the month of December 2021, at an altitude of 2250 m above sea level, and were placed in plastic bags. The leaves and fruit of *T. wallichiana* were washed with water, dried at 45 °C for 6 h and then crushed into powder with a mixer (Bhardwaj 2021b).

2.2 Preparation of the Extracts

The powdered samples, 5 g, were extracted with 25.0 ml of methanol, followed by a continuous hot extraction method, and were stirred well and kept for incubation in closed containers. The tubes were centrifuged at 4000 rpm for 30 min, then the supernatant extract was transferred for drying for 10 min and, finally, residue of the leave sample was obtained. Weighed accurately 0.1 g of residue of leaves sample in test tube and added 1.0 mL of methanol [10% (w/v) solution]. The final concentration of extracts (residue with methanol) used for further experiment. All the extracts were then stored at 4 °C in a refrigerator for further analysis as crude methanolic extracts (Al Ghais et al. 2020b; Bhardwaj 2021d).

2.3 Chemicals

The chemicals used in the present investigation were of analytical grade and high purity, obtained from Merck and HiMedia. The standard kits and reagents used for analysis were purchased from Germany and the USA.

2.4 Test Organisms

In the present study, the bacterial strains used were *Bacillus subtilis* (ATCC 6633), *E. coli* (ATCC 8739), *Salmonella enterica* (ATCC 14028), *Staphylococcus aureus* (ATCC 6538) and *Pseudomonas aeruginosa* (ATCC 27853), obtained from the American Type Culture Collection (ATCC), to determine their antibacterial activity. The bacterial strains were procured from LTA srl Italia. Pure culture of bacteria was maintained at 4 °C on nutrient agar slants.

2.5 Methodology for the Detection of Antibacterial Activity

2.5.1 Inoculums Preparation

The bacterial pure culture isolates were first grown in 5 ml of nutrient broth in sterile test tubes for 18 h before use.

2.5.2 Agar Well Diffusion Assay

The antibacterial activity of the methanolic extracts of *T. wallichiana* (leaves and fruit) was tested against isolates using the agar-well diffusion method. An aliquot of 100 µl inoculum for each bacterial isolate was evenly spread onto Muller-Hinton agar using a sterile spreader and was allowed to settle at room temperature. A cork borer of 6 mm diameter was used to punch well in agar plates to cut uniform wells. Wells were bored in agar plates. The concentration of the extracts was 10% (w/v), prepared using methanol as solvent. Subsequently, 30 µl of extracts (leaves and fruit) were poured into the wells. Ciprofloxacin 30 µg was used as a positive control. Then the plates were kept at 2–8 °C in a refrigerator to allow diffusion of the extracts into the agar and were further incubated at 37 °C for 24 h. The diameter of the zone of inhibition was measured to the nearest millimetre (Sohel 2010; Uddin et al. 2007). The formation of a clear inhibition zone of ≥7 mm diameters around the wells was regarded as a significant susceptibility of the organisms to the extract (Bhardwaj 2022). The effect was compared to those of antibiotic discs. The tests were performed in triplicates, and the mean was taken. The whole experiment was performed under strict aseptic conditions.

2.6 Phytochemical Analysis

Test for Flavonoids (Ammonia Test)
One millilitre of the extract was taken and placed in a test tube, and an ammonia solution was added (1:5), followed by the addition of concentrated sulphuric acid. The appearance of a yellow color and its disappearance on standing indicates a positive test for flavonoids.

Test for Glycosides (Keller-Kiliani Test)
Five millilitres of each extract was added, with 2 ml of glacial acetic acid, which was followed by the addition of a few drops of ferric chloride solution and 1 ml of conc. sulphuric acid. The formation of a brown ring at the interface confirms the presence of glycosides.

Test for Phenols (Ferric Chloride Test)
Next, 0.5 ml of the extract was added, with a few drops of neutral ferric chloride (0.5%) solution. The formation of a dark green color indicates the presence of phenolic compounds.

Test for Saponins (Froth Test)
One millilitre of the extract was taken and placed in a test tube, and distilled water (2 ml) was added to it. The test tube was then kept in a boiling water bath for boiling and was shaken vigorously. The existence of a froth formation during warming confirms the presence of saponins.

Test for Tannins (Ferric Chloride Test)
One millilitre of the extract was added, with 5 ml of distilled water, and kept for boiling in a hot water bath. After boiling, the sample was cooled down, and to this, 0.1% ferric chloride solution

was added. The appearance of a brownish-green or blue-black coloration confirms the presence of tannins.

Test for Terpenoids (Salkowski Test)
Five millilitres of the extract was taken in a test tube, and 2 ml of chloroform was added to it, followed by the addition of 3 ml of conc. sulphuric acid. The formation of a reddish-brown layer at the junction of two solutions confirms the presence of terpenoids.

2.7 Statistical Analysis

The tests were performed in triplicates. Data are expressed as mean. Pair-wise comparisons were performed. An experimental error was determined for the triplicate and expressed as standard deviation (SD).

3 Results and Discussion

The objective of this research was to seek the antimicrobial activity of the methanolic extract of *T. wallichiana* and also secondary metabolites. This probe was carried out as an awareness of the medicinal value of *T. wallichiana*, for its activity against selected bacterial pathogens. Three different extracts of different parts of *T. wallichiana* (leaves and fruit) were treated using methanolic extraction. Methanolic extracts were found to be more potent against human pathogens. Similar results were reported by Derwich et al. (2010), who reported that the essential leaves of *Cedrus atlantica* were active against *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Bacillus sphaericus* and *Staphylococcus intermedius*. In the present study, phytochemical analysis of the Himalayan yew extracts was done to explore their composition. The results revealed the presence of terpenoids, flavonoids, glycosides, phenols, saponins and tannins. These results are similar to the results obtained by Devmurari (2010), who reported that phytochemical studies on *Cedrus deodara* revealed the presence of alkaloids, glycosides flavonoids, triterpenoid, tannins, proteins and fixed oil. In particular, terpenoid substances are secondary metabolites that characterise *C. libani*-derived products (Kizil et al. 2002; Yilmaz et al. 2005; Loizzo et al. 2008).

3.1 Antibacterial Activity of *T. wallichiana* Extracts Against Human Pathogenic Bacteria

Table 1 summarises the results of the antibacterial activities of the extracts of *T. wallichiana*, which were evaluated on *Bacillus subtilis* (ATCC 6633), *E. coli* (ATCC 8739), *Salmonella enterica* (ATCC 14028), *Staphylococcus aureus* (ATCC 6538) and *Pseudomonas aeruginosa* (ATCC 27853) by means of the agar well diffusion method. Leaves (Fig. 2) of *T. wallichiana* showed a zone of inhibition with all five strains of microorganisms that were used. The *T. wallichiana* fruit (Fig. 3) methanolic extract showed a maximum zone of inhibition with *Bacillus subtilis*, which is 17 ± 0.5 mm, and the bark showed a maximum zone of inhibition with *Staphylococcus aureus*, 21 ± 0.6 mm (Fig. 3). All the tested extracts of the Himalayan yew fruit showed no activity against *E. coli*, *Salmonella enterica* and *Pseudomonas aeruginosa* (Table 1).

Table 1 Antibacterial activity of the methanolic extracts of *Taxus wallichiana* leaves and *Taxus wallichiana* fruit

SNo.	Microorganisms	*Taxus wallichiana* leaves	*Taxus wallichiana* fruit
1	*Bacillus subtilis* (ATCC 6633)	18 ± 0.0	17 ± 0.5
2	*E. coli* (ATCC 8739)	2 ± 0.5	No zone
3	*Salmonella enterica* (ATCC 14028)	17 ± 0.2	No zone
4	*Staphylococcus aureus* (ATCC 6538)	19 ± 0.2	21 ± 0.6
5	*Pseudomonas aeruginosa* (ATCC 27853)	1 ± 0.1	No zone

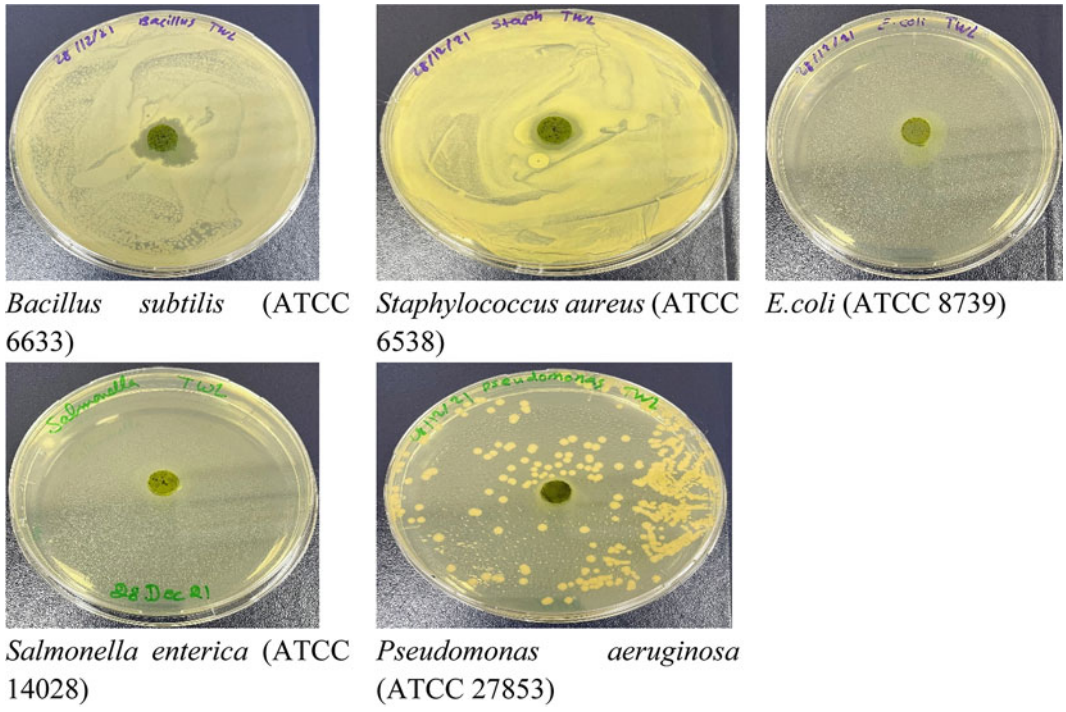

Fig. 2 Extracts of *Taxus wallichiana* leaves showed antibacterial activity, as indicated by the zone of inhibition against different microorganisms' strains

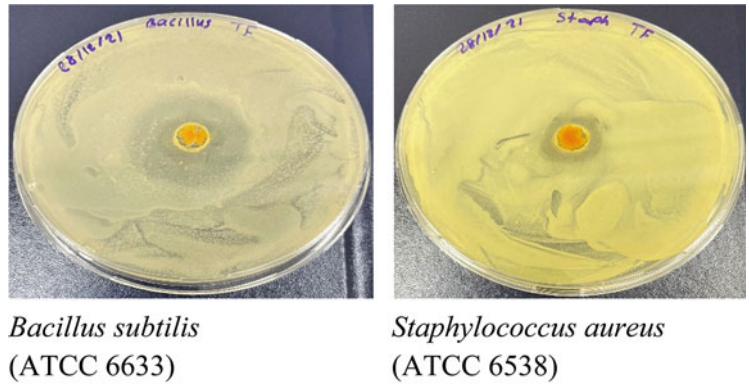

Fig. 3 Extracts of *Taxus wallichiana* fruit showed antibacterial activity, as indicated by the zone of inhibition against different microorganisms' strains

3.2 Phytochemical Screening of *Taxus wallichiana* (Leaves and Fruit)

Six phytochemicals were screened for this research work (tannins, phenolic, terpenoids, glycosides, saponins and flavonoids), as seen in Table 2; from the crude extracts obtained from *T. wallichiana* leaves and fruit (Figs. 4 and 5), the methanol crude extracts tested positive for the presence of flavonoids, terpenoids, phenol, tannins and glycosides. Similarly, the *T. wallichiana* fruit (Fig. 5) showed the presence of the phytochemicals flavonoids, saponins and terpenoids.

Table 2 Phytochemicals present in methanolic crude extracts of *Taxus wallichiana* leaves and fruit

SNo.	Phytochemicals	*Taxus wallichiana* leaves	*Taxus wallichiana* fruit
1	Flavonoids	+	+
2	Glycosides	+	–
3	Phenol	+	–
4	Saponins	–	+
5	Tannins	+	–
6	Terpenoids	+	+

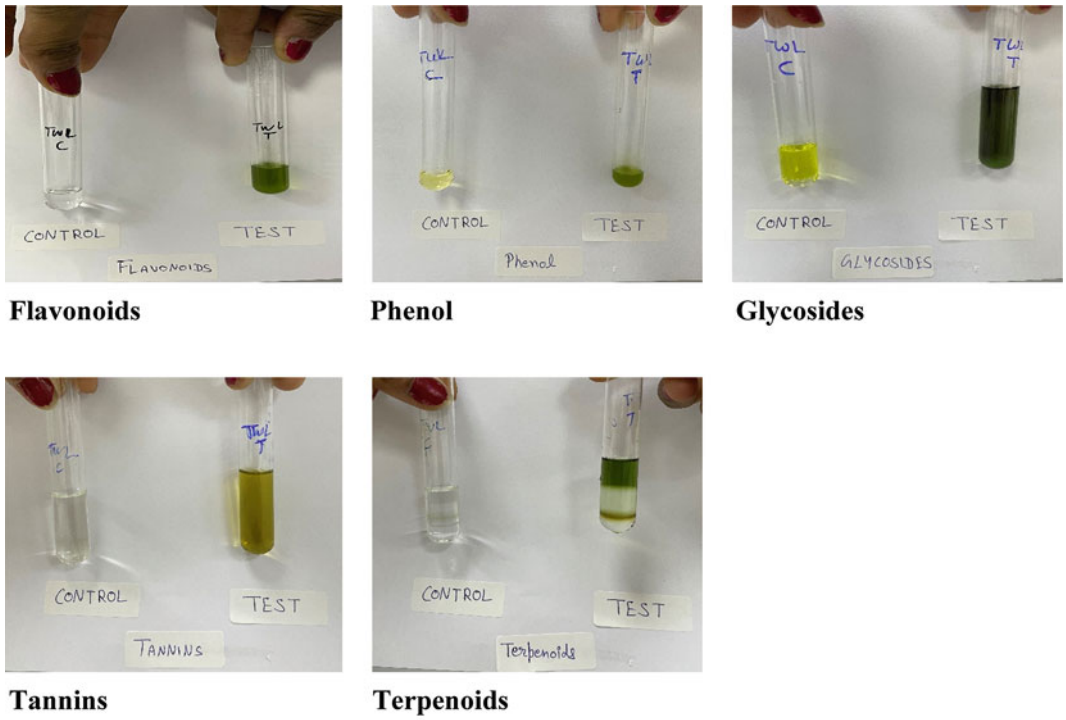

Fig. 4 Pictures show a confirmation of the phytochemicals present in the crude extracts of *Taxus wallichiana* leaves

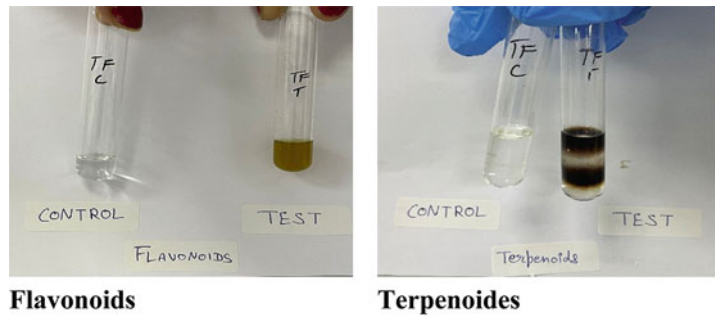

Fig. 5 Pictures show a confirmation of the phytochemicals present in the crude extracts of the *Taxus wallichiana* fruit

4 Conclusion

This probe was carried out as an awareness of the medicinal value of *T. wallichiana*, for its activity against selected bacterial pathogens. The Himalayan yew extract, particularly the methanolic extract, obtained from different parts of the tree (leaves and fruit) showed an anti-microbial effect against human pathogens, which suggests that it could be considered a safe anti-microbial agent. The broad spectrum of antibacterial activities of the Himalayan yew seems to be due to the presence of terpenes, terpenoids detected in the bioactive fractions. These promissory extracts open the possibility of finding new clinically effective antibacterial compounds as well as secondary metabolites.

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Ethics Approval and Consent to Participate Not applicable.

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Availability of Data and Materials The relevant data and materials are available in the present study.

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Different Efflux Pump Systems in *Acinetobacter baumannii* and Their Role in Multidrug Resistance

Saroj Sharma, Vaishali Kaushik, Mukta Kulshrestha, and Vishvanath Tiwari

Abstract

Several infections, such as pneumonia, urinary tract infections (UTIs), as well as bloodstream, skin, and soft tissue infections, are caused by *Acinetobacter baumannii*, a nosocomial pathogen and Gram-negative coccobacillus. Due to its resistance to a variety of medications, multidrug therapy, and occasionally pan therapies, this bacterium is a huge public health concern. Drug resistance is a big worry not only in *A. baumannii*, but it is also a major challenge in many other diseases. Antibiotic resistance, biofilm development, and genetic alterations are all linked to variables like the efflux pump. Efflux pumps are transport proteins involved in the extrusion of hazardous substrates from within cells into the external environment (including nearly all types of therapeutically relevant antibiotics). Both Gram-positive and Gram-negative bacteria, as well as eukaryotic organisms, contain these proteins. Efflux pumps may be specialized for a single substrate or can transport a variety of structurally dissimilar molecules (including antibiotics of many classes); these pumps have been linked to multiple drug resistance (MDR). There are five primary families of efflux transporters in the prokaryotic kingdom: MF (major facilitator), MATE (multidrug and toxic efflux), RND (resistance-nodulation-division), SMR (small multidrug resistance), and ABC (ATP-binding cassette). The efflux pumps and their types as well as the mechanisms of an efflux pump involved in multidrug resistance in bacteria have been discussed here. The main focus is on the variety of efflux pumps commonly found in *A. baumannii*, along with their mechanism by which they make this bacteria drug resistant. The efflux-pump-inhibitor-based strategies that are significant in targeting efflux pumps in *A. baumannii* have also been discussed. The connection of biofilm and bacteriophage with the efflux pump can prove as an efficient strategy for targeting efflux-pump-based resistance in *A. baumannii*.

Keywords

Acinetobacter baumannii · Bacteriophage · Biofilm · Efflux pump · Multidrug resistance · Nanoparticles · Synthetic and natural efflux pump inhibitors

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1 Introduction

Acinetobacter baumannii is an opportunistic, Gram-negative, cocco-bacilli (Wong et al. 2017) and is a primary cause of pneumonia, urinary tract infection (UTI), as well as meningitis (Michalopoulos and Falagas 2010). Secondary infections during the COVID pandemic have shown a prevalence of this bacteria in causing severe recurrent infections (Rangel et al. 2021). The capacity of this bacteria to persist even under the pressure of existing antibiotics has led to the emergence of multidrug-resistant strains in various hospital environments (Norton et al. 2013). *A. baumannii* is now viewed as a potential intercontinental threat. It also tops the list of critical pathogens, proposed by the World Health Organization (WHO), in recent years (https://www.who.int/news/item/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed). As far as multidrug resistance in bacteria is concerned, it is mainly contributed by four different mechanisms such as, by the reduction in membrane permeability, downregulation of porins, alteration in drug target sites, by producing antibiotic lytic enzymes, and the most popularly known mechanism is the efflux pump. Among all the popular mechanisms of resistance, the efflux pump can be recognized as the centralized target of scientific consortia. Efflux pumps are transport proteins involved in the extrusion of nearly all types of therapeutically relevant substrates/antibiotics from within cells into the external environment (Verma et al. 2021). Gram-positive and Gram-negative bacteria, as well as eukaryotic organisms, contain these proteins (Van Bambeke et al. 2000). Pumps may be specialized for a single substrate or for the transport of a variety of structurally dissimilar molecules (especially antibiotics of many classes); these pumps have been linked to multiple drug resistance (MDR). There are five primary groups of efflux transporters in the prokaryotic kingdom, which are discussed ahead (Webber and Piddock 2003). The general mechanisms of efflux pumps found in *A. baumannii*, with a specific focus on their contribution to acquiring MDR, have been covered in the text. Furthermore, this review will encompass the strategies to combat MDR in *A. baumannii* by targeting the efflux pumps. The study of the link between the biofilm-efflux pump and the bacteriophage-efflux pump as a strategy for targeting the efflux-pump-based MDR of *A. baumannii* can be a future approach to therapeutic development for the prevention of fatal, recurrent resistant infections.

2 Bacterial Efflux Pumps

Bacterial efflux pump systems are broadly categorized into five major families, namely, the ATP-binding cassette (ABC) superfamily, the resistance-nodulation-division (RND) family, the small multidrug resistance (SMR) family, the major facilitator superfamily (MFS), and the multidrug and toxic compound extrusion (MATE) family (Chung and Saier Jr 2001). Due to their high conservation, efflux pumps have been recently selected as a therapeutic target for the treatment of human infections. In the bacterial system, the major role of efflux pumps is related to the gaining of resistance against several antibiotics. Apart from that, bacterial efflux pumps also play a major role in physiological processes and the biodegradation of organic pollutants preventing bacterial populations from the toxic compounds. For instance, *Pseudomonas putida* DOT-TE1, a strain capable of resisting high concentrations of solvents, is associated with toluene tolerance due to the activity of TtgABC (a tripartite RND efflux pump). TtgABC expression is regulated by the TtgR transcriptional repressor; mutations inactivating TtgR cause the overexpression of the efflux pump, making *P. putida* DOT-TE1 more resistant to chloramphenicol, nalidixic acid, and tetracycline than the wild-type strain (Duque et al. 2001). In addition to this, efflux pumps are also capable of mediating cell-to-cell communication processes through the release of signaling molecules. For example, in *P. aeruginosa*, two interconnected quorum-sensing systems have been investigated in detail. The signal molecules in one of them are

homoserine lactones with various acyl chain modifications, acyl homoserine lactones (AHLs), while the other is the *Pseudomonas* quinolone signal (PQS). MexAB-OprM is known to export 3-oxo-C12-HSL, an AHL signal molecule with a long side acyl chain. As the quorum-sensing response is important for *P. aeruginosa* virulence, resistant mutants overexpressing MexAB are less virulent because they gather enough of this quorum-sensing signal (Evans et al. 1998). Another vital function associated with the overexpression of efflux pumps is their role in the gaining of biocide resistance. Efflux-pump-mediated biocide resistance is highly relevant to bacteria, both environmentally and clinically. They also play a major role in the interactions between plants and bacteria. For instance, the IfeAB efflux pump in *Agrobacterium tumefaciens* is engaged in the competitive colonization of alfalfa roots and can provide considerable ecological benefits to these bacteria in a flavonoid-rich environment (Palumbo et al. 1998).

2.1 Role and Importance of Efflux Pumps in Various Bacteria

Efflux pumps regulate the internal environment of bacteria by helping the microorganisms in removing toxic substances, like antimicrobial agents, metabolites, and quorum-sensing signaling molecules. These pumps serve as an integral part of the bacterial system and play a variety of functions, including their role in equipping bacteria with resistance mechanisms. This resistance, provided by efflux pumps in bacteria, continues to hamper the available chemotherapies against them. Not only this, but resistance in bacteria due to this mechanism of evasion from antibiotics by using an efflux pump system is seen as a major cause of the rising infectious diseases and biocide resistance globally (Abdi and Ghotaslou 2020). Efflux pumps play a significant role in resistance due to their ability to recognize a large number of compounds, besides their natural substrate, probably because of the physicochemical properties of the substrate, like hydrophobicity, aromaticity, and ionizable characteristics, rather than its defined chemical characteristics, as happens in the classical enzyme-substrate or ligand-receptor complex. Most antibiotics are amphiphilic molecules that possess a combination of hydrophilic and hydrophobic characteristics, which are usually easily recognizable by the bacterial efflux pump system, making their role even more significant in the resistance mechanism. The role of efflux pumps in bacteria can further be highlighted by iterating some of the latest and common resistance mechanisms that have been identified recently, for example, the efflux mechanisms in *Escherichia coli* against tetracycline, *Staphylococcus aureus* against fluoroquinolones, and *Enterococcus faecalis* against lincosamide. The efflux mechanism is an important indicator of intrinsic and acquired resistance to antimicrobial agents. Moreover, the drug-efflux mechanism can be both drug-specific and multidrug-specific. The drug-specific efflux mechanism is usually encoded by mobile genetic elements, which can be acquired for developing sufficient resistance in bacteria. Considering the multidrug efflux mechanism, it is usually chromosome-encoded and their expression is resultant of mutation in regulatory genes (Poole 2007). Efflux pumps have several functions, which are clinically and physiologically significant for different microbes for their survival in a stressful environment (i.e., in the presence of antibiotics or toxins). For instance, the *E. coli* AcrAB efflux system plays a vital physiological role in pumping out bile acids as well as fatty acids to lower their cellular toxicity (Okusu et al. 1996). Similarly, in the *Streptomyces pristinaespiralis* MFS family, the PTR pump is famous as an autoimmunity pump as it is involved in the production of pristinamycins I and II for this specific organism (Vecchione et al. 2009). In addition to the AcrAB efflux system in *E. coli*, the AcrAB-TolC system is suspected to bear a role in the transport of the calcium channel component in the *E. coli* membrane (Du et al. 2014). In addition to this, the MtrCDE system plays a defensive role in providing resistance against fecal lipids studied in *Neisseria gonorrhoeae* isolates from the rectum of patients (Rouquette et al. 1999). On the other hand, the efflux system (AcrAB) in *Erwinia amylovora* has an important role in the virulence

of this organism during colonization in the host plant and thereby provides resistance against the plant toxin (Pletzer and Weingart 2014). The role and importance of the efflux pumping system in bacteria can be seen in MexXY-OprM multidrug efflux system in *Pseudomonas aeruginosa*. The MexXY-OprM multidrug efflux system gets triggered by an antibiotic that specifically targets the ribosome via the PA5471 gene product of that organism (Morita et al. 2012).

2.2 General Mechanism of the Efflux Pump in Bacteria

With regard to the general mechanism of antibiotic resistance, it is already known that antibiotic exerts pressure that favors the emergence of resistance in a specific organism, and bacteria employ several methods to combat this antibiotic exposure. Among these strategies by bacteria to become resistant to antibiotics, an efflux pump is the most common. Efflux pumps are classified into five major families based on their similarities in amino acid composition and energy source: the resistance nodulation division (RND) family, adenosine triphosphate (ATP)-binding cassette (ABC) transporter family, multidrug and toxin extrusion (MATE) family, small multidrug resistance (SMR) family, and major facilitator superfamily (MFS) (Spengler et al. 2017; Coyne et al. 2011). Efflux pumps are known to consist of several transporters, which can be categorized according to high specificity with substrates, phylogenetic relationship, as well as energy source. For example, various efflux pumps hydrolyze ATP, which serves as the energy source, and such types of ATP-dependent pumps are referred to as primary active transporters, such as the ABC superfamily (Rees et al. 2009). Similarly, other types of pumps employ proton-motive force (PMF) or sodium-motive force (SMF) and fall in the category of secondary active transporters to discharge drugs. This whole system of transporters is said to be the antiporter system (Lekshmi et al. 2018). MFS, SMR, RND, and MATE superfamilies also fall under the category of secondary active transporters (Van Bambeke et al. 2000).

3 Efflux Pumps in Multidrug Resistance of *A. baumannii*

Efflux pumps are involved in several activities ranging from nutrient balancing to assuaging stress on a cell, pathogenesis to toxin excretion, as well as heavy metal balancing. Antibiotic efflux is not one of the major roles of efflux pumps; still, they play a key role in bacteria's innate resistance to antibiotics. According to the literature, there are five classes of multidrug efflux pumps described in *Acinetobacter* spp., which contribute to bacteria's reduced susceptibility to antibiotics (Fig. 1). Regardless of this, there are a large number of efflux pumps that are yet to be identified in *A. baumannii*.

3.1 AdeABC Efflux Pump

In *A. baumannii*, AdeABC is a well-studied and widely distributed efflux pump. Three genes, adeC, adeB, and adeC, encoding an AdeABC efflux pump, are cotranscribed, forming an operon. These genes translate proteins, namely, AdeC (outer membrane factor (OMF) component), AdeB (innermost protein or RND component), and AdeA (periplasmic protein or membrane fusion protein (MFP)). AdeA protein has a role in the membrane fusion protein, AdeB acts as a multidrug transporter protein, and AdeC plays a role as an outer membrane protein. Antibiotics in the inner membrane of the phospholipid bilayer or cytoplasm are captured by AdeB, then the substrates are transported out by AdeC, which behaves as a membrane channel protein (Xu et al. 2019; Poole 2002). A regulatory system (AdeRS) is involved in the expression of the efflux pump, which is comprised of two components—sensor kinase AdeS (Srinivasan et al. 2009) and response element AdeR.

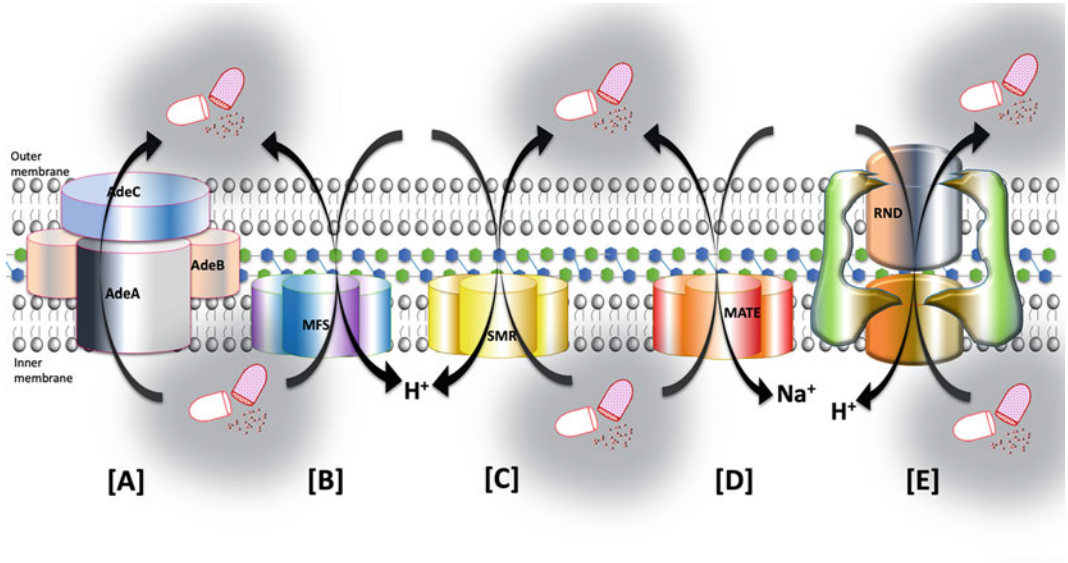

Fig. 1 Efflux pumps present in *Acinetobacter baumannii*: the *Acinetobacter baumannii* bacterial membrane consists of efflux pumps of five major families based on similarities in amino acid composition and energy source—(**a**) ATP-binding cassette (ABC) transporter family (ADeABC), (**b**) major facilitator superfamily (MFS), (**c**) small multidrug resistance (SMR) family, (**d**) multidrug and toxin extrusion (MATE) family, and (**e**) resistance nodulation division (RND) family (Sharma et al. 2019; Verma et al. 2021)

Depending on environmental conditions, AdeS is involved in the activation/inactivation of AdeR. Among RND transporters, AdeABC is polyspecific and is known to be a major contributing factor to MDR. The substrates associated with the AdeABC efflux pump are aminoglycosides, fluoroquinolones, β-lactams, chloramphenicol, trimethoprim, erythromycin, tetracyclines, and ethidium bromide (Magnet et al. 2001).

Any mutation in the efflux pump regulatory system can cause a change in the expression levels of efflux pumps. AdeABC has a role in β-lactam resistance in *A. baumannii* (Knight et al. 2016) and its role in carbapenem resistance has been identified recenlty (Verma et al. 2022). According to Zhang et al., carbapenem resistance is associated with the presence of additional blaOXA-23-like and ISAba1/blaOXA-51-like complex genes, as well as AdeABC overproduction (Zhang et al. 2021). Susceptibility tests to imipenem and meropenem in the presence and absence of well-known efflux pump inhibitors, like phenyl-arginine-naphthylamide, 1-(1-napthylmethyl)-piperazine, carbonyl cyanide 3-chlorophenylhydrazone (CCCP), (1-(1-napthylmethyl)-piperazine), and reserpine (plant derived), have been studied. Quite the opposite, a few investigations revealed a two- to eightfold increase in carbapenem susceptibility, implying the role of AdeABC in carbapenem resistance (Peleg et al. 2007; Lee et al. 2010). Even though the overproduction of carbapenemases has long been believed to be the primary cause of carbapenem resistance, still several studies have been linked to the overexpression of RND efflux pumps (Héritier et al. 2005). Previous studies have reported that AdeABC overexpression contributes to resistance to netilmicin and tigecyclines; also, AdeABC mutants of *A. baumannii* were susceptible to imipenem (Wong et al. 2009; Nemec et al. 2007; Ruzin et al. 2007). The association of tigecycline resistance with a mutation in

AdeABC regulatory components, AdeR/AdeS, has also been studied (Gerson et al. 2018; Nowak et al. 2016).

3.2 MFS (Major Facilitator Superfamily) Selective Transporter

MFS is a group of secondary active transporters that can get transferred from bacteria to humans. The MFS protein transporter family is capable of transporting a large range of substrates across the biological membrane and also plays a vital role in multiple physiological processes. MFS transporters are usually categorized into 70 families based on sequence homology. These can be classified into three main groups, which include uniporters, symporters, and antiporters (Reddy et al. 2012). Uniporters do not require external energy to function; however, they can only transport their substrates from high to low concentration, whereas symporters and antiporters can use the energy stored in their linked ion or concentration's gradient to transport substrates in the opposite direction. A study claimed that efflux is a critical mechanism of fosfomycin resistance and that AbaF is involved in *A. baumannii* fosfomycin resistance. AbaF appears to have a role in *A. baumannii* biofilm development and pathogenicity. The MFS transporter AbaF dynamically effluxes fosfomycin from the cells, making them resistant to it. Furthermore, when exposed to fosfomycin, its expression is increased. In fosfomycin-resistant clinical strains of *A. baumannii*, AbaF is expressed. In the presence of efflux inhibitors, these bacteria show a reduced susceptibility to fosfomycin (Sharma et al. 2016).

3.3 SMR (Small Multidrug Resistance) Transporter

The drug/metabolite transporter (DMT) superfamily includes proton-motive force-driven SMR transporters. They are relatively small, with only four transmembrane alpha-helical spanners (TMSs) each. As a result, different MFS transporters appear to behave as monomers referred to as SMR transporters. Furthermore, they exchange H^+ absorbed by pumping out monocationic and ethidium complexes, as well as dicationic complexes (Yerushalmi et al. 1995). The involvement of AbeS, a chromosomally encoded probable drug efflux pump of the SMR family from a multidrug-resistant strain of *A. baumannii*, in resistant strains was investigated. When the cloned AbeS gene was expressed in the hypersensitive *E. coli* host KAM32, susceptibility to different colors, antimicrobial agents, and detergents was reduced. In *A. baumannii*, deletion of the AbeS gene confirmed its involvement in conferring resistance to these complexes (Sharma et al. 2016).

3.4 MATE (Multidrug and Toxin Extrusion) Transporter

These transporters are widely found in bacteria, as well as in animals and plants. Such transporters were identified for the first time as NorM, a Na^+/cationic antiporter agent from *Vibrio parahaemolyticus* (Kuroda and Tsuchiya 2009). These pumps are responsible for excreting cationic dyes, fluoroquinolones, and aminoglycosides from the periplasmic space. As an energy source, several of these transporters were used as a gradient of Na^+ and H^+ (He et al. 2010). The AbeM protein shared over 90% sequence homology and an additional 70% identity with the sequence of ACIAD0429, which was shown to be a NorM homolog of *A. baumannii* ATCC 19606. AbeM is a multidrug efflux pump that belongs to the MATE family. The AbeM efflux pump increases the minimum inhibitory concentrations (MICs) of ciprofloxacin, norfloxacin, ofloxacin, gentamicin, triclosan, daunorubicin, doxorubicin, ethidium bromide, and rhodamine by more than four-fold. It also causes a two-fold increase in the MICs of kanamycin, chloramphenicol, erythromycin, trimethoprim, and tetraphenylphosphonium (TPPCl), which is repeatable. AbeM belongs to the MATE family of pumps, according to sequence analysis. AbeM was discovered to be a unique member of the MATE family and an H^+-coupled multidrug efflux pump (Su et al. 2005).

3.5 RND (Resistance-Nodulation-Division) Family

The MDR strains of *A. baumannii* with the highest prevalence of RND transporters are linked to both inherent and acquired multidrug resistance. Chromosome-encoded RND efflux pumps exhibit specificity to a variety of substrates, including antibiotics, detergents, biocides, dyes, etc. The RND family of transporters works as a tripartite complex made up of a membrane fusion protein, an OMF, and an RND component. The most important component of this efflux system is the RND component. It is a trimmer made of a porter domain, an OMF docking domain, and a transmembrane domain. The drug-binding pocket in this instance is part of the porter domain, which facilitates substrate recognition, binding, and transport (Blair and Piddock 2009). Depending on the functional states (such as access, binding, and extrusion), each protomer can take on various conformations, and the proton-motive force is what causes these functional rotations. The substrate is entirely extruded out of the bacterial system by the distal portion of this protein, which opens into the outer membrane channel. The outer membrane protein and the inside protein are connected by the membrane fusion protein (MFP) (Verma et al. 2021).

4 Strategies Targeting Efflux Pumps in *A. baumannii*

Drastically increasing cases of MDR in *A. baumannii* have brought attention to the importance of targeting various resistance-acquiring mechanisms. Efflux pumps, playing a significant role in the acquired resistance of *A. baumannii*, have become a potent target for resistance inhibition for coping infections. A literature survey shows various studies focusing on the involvement of efflux pumps in resistance development in *A. baumannii* (Hou et al. 2012; Lin et al. 2009; Zhu et al. 2020; Nogbou et al. 2021). Targeting efflux pumps via various approaches, like inhibitors, nanoparticles as inhibitors, biofilm-mediated targeting, and bacteriophage-mediated targeting, is a reliable approach to curbing MDR infections (Fig. 2).

4.1 Efflux Pump Inhibitors

The strategy of using efflux pump inhibitors to target MDR of *A. baumannii* is greatly beneficial as therapeutics. Various efflux pump inhibitors have been studied against *A. baumannii*, which are both synthetic and natural derivations. Inhibitory compounds known as efflux pump inhibitors (EPIs) have been identified as potential bioactive molecules for reviving the antibacterial action of antibiotics that have already lost their potency (AlMatar et al. 2021). Given the crucial role that efflux plays in mediating antibiotic resistance, it is sensible to anticipate that removing resistance-causing factors could enhance the effects of substrate drugs. Efflux could be eliminated using a variety of techniques, including redesigning antibiotics that are no longer recognized as substrates, decreasing the level of expression of efflux pump genes by interfering with genetic regulation, inhibiting the assembly of functional efflux pumps, blocking the pump to prevent substrate binding to the active site, and collapsing the energy mechanism responsible for activating these pumps (Bhardwaj and Mohanty 2012).

Furthermore, different strategies can be followed to combat efflux-pump-mediated resistance in bacteria. Efflux pump inhibitors are broadly classified into two categories: natural and synthetic. Natural efflux pump inhibitors incorporate PAβN (phenylalanine-arginine-β-naphthylamide), NMP (1-(1-napthylmethyl)-piperazine), carbonyl cyanide m-chlorophenyl- hydrazone (CCCP), verapamil, and amlodipine. On the other hand, synthetic efflux pump inhibitors include reserpine, eugenol, trans-cinnamaldehyde, epigallocatechin 3-gallate (EGCG), and polyamines. Nanoparticles and phage are also seen as futuristic alternatives to be used as potential efflux pump inhibitors (Verma et al. 2021). The most prominently used efflux pump inhibitors against MDR bacterial strains are phenylalanine-arginine β- naphthylmethyl (PAβN), 1-(1-naphthylmethyl)-piperazine (NMP), and

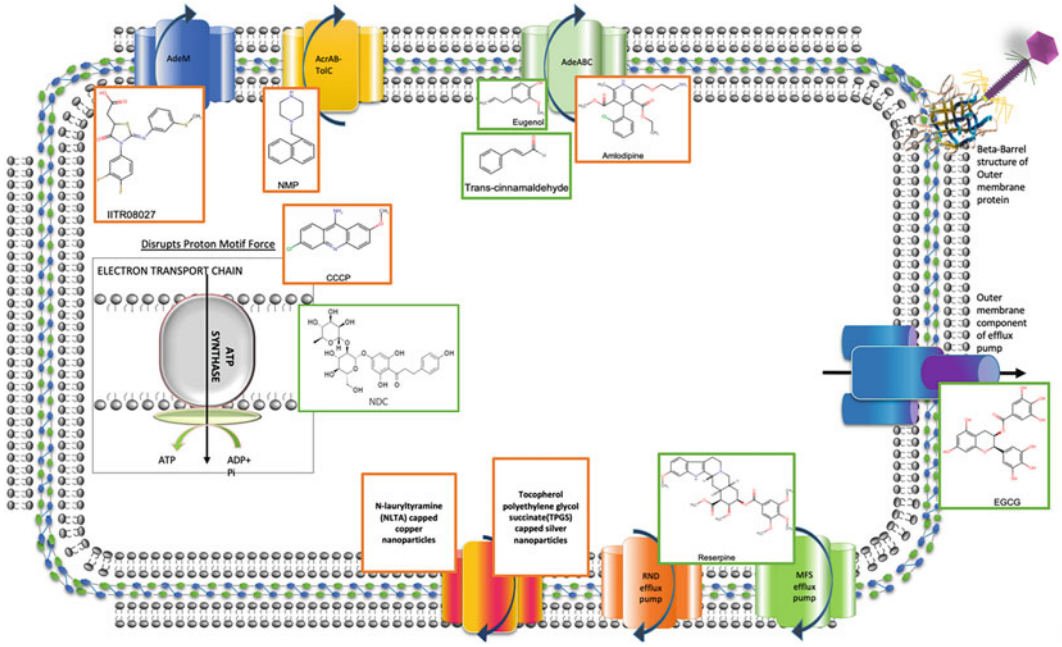

Fig. 2 Efflux pump inhibitors of *Acinetobacter baumannii*: efflux pump inhibitors against *Acinetobacter baumannii* can be broadly categorized into two main types—natural and synthetic. Natural inhibitors for efflux pumps of *A. baumannii* include PAβN (phenylalanine-arginine-β-napthylamide), NMP (1-(1-napthylmethyl)-piperazine), carbonyl cyanide m-chlorophenyl-hydrazone (CCCP), naringin dihydrochalcone (NDC), verapamil, and amlodipine, and synthetic efflux pump inhibitors include reserpine, eugenol, trans-cinnamaldehyde, epigallocatechin 3-gallate (EGCG), and polyamines. Other than this, nanoparticles and the bacteriophage have also been used as a potential therapeutic approach, which can be used as inhibitors targeting the efflux pumps of *A. baumannii* (Verma et al. 2021)

carbonyl cyanide 3-chlorophenylhydrazone (CCCP), but these inhibitors possess high toxicity on mammalian cells. Nevertheless, EPIs can still be seen as an appealing way to tackle antimicrobial resistance despite the significance as well as the difficulties they confront as treatments. But the commercial manufacturing of EPIs is nonetheless constrained by several factors, which need to be examined. The inhibitors of the efflux pump bear the potential of combatting the efflux of antimicrobial agents by blocking these pumps and, thereby, enhancing the efficacy of antibiotics against bacteria. In the future, there is a critical need to increase the number of clinical studies for these substances rather than keeping them at the laboratory level. Before clinical trials, additional thorough scientific investigations are also required to address all the positive and unfavorable characteristics of EPIs. All of this would eventually make them more accessible for the treatment of different nosocomial illnesses and aid in attracting the attention of pharmaceutical corporations to them.

4.2 The Link Between Biofilm and Efflux Pump

Efflux pumps, are one of the major causes of developing resistance in bacteria for antibiotic treatments. The extracellular polymeric substance (EPS) biofilm matrix allows bacteria populations to coexist and serves as a perfect repository for the cellular interchange of plasmids encoding antibiotic resistance, ultimately aiding in the spread of bacterial resistance (Bowler et al. 2020). Biofilms are colonial aggregates of microcolonies of bacteria, which remain together by producing extracellular polymeric substances,

comprised of polysaccharides, extracellular deoxyribonucleic acid (DNA), and secreted proteins (Kaushik et al. 2022). Biofilm formation is an intrinsic ability of *A. baumannii* isolates (Kim et al. 2021). In *A. baumannii*, the overexpression of RND efflux pumps contributes to MDR and reduces biofilm development (Yoon et al. 2015). Relative to stationary and exponential-phase cells, the expression of RND efflux genes A1S 0009, A1S 0116, and A1S 0538, as well as the MFS efflux gene A1S 1316, was upregulated in biofilms. A1S 0116 was found to be the most elevated efflux gene, whereas A1S 0009 was reported to be the least upregulated. It has been observed that the efflux genes A1S 1117, A1S 1751, and *adeT* are solely expressed in biofilm cells and not in planktonic cells. A1S 1117 (a putative gene) is a sugar transporter protein that belongs to the MFS family. *adeT* encodes an RND-type efflux pump that is implicated in aminoglycoside resistance, while AdeA is a membrane fusion protein. However, it is unclear if these efflux pumps play a role in biofilm development and are being looked at, but it is highly probable that the MFS sugar transporter is involved. Sugar efflux may be facilitated by the A1S 1117 gene, which is involved in biofilm matrix formation (Rumbo-Feal et al. 2013).

Another study was done which concluded that *adeB* overexpression and high H33342 efflux activity were found to be related to biofilm-forming activity. *adeB* is known to be correlated with tigecycline and cefotaxime resistance (Kim et al. 2021). The upregulation of AdeABC, AdeFGH, and AdeIJK EPs in mutants has been shown to substantially reduce biofilm production as compared to the wild-type (WT) strain. Furthermore, the knocking off of *adeG* and *adeJ* considerably enhanced biofilm formation, while the mutant strain lacking *adeB* was still unable to form biofilm. This leads to the inference that *A. baumannii* biofilm production necessitates the beginning and maintenance of the expression of some efflux pumps. Tet(A) and Tet(B) are the primary efflux pumps in *A. baumannii*. Tet (A) confers resistance to tetracycline, while Tet (B) confers resistance to minocycline and tetracycline. In a pathogenic strain of *A. baumannii*, a 453-bp MFS transporter-like open reading frame (ORF) was discovered, and its association with adhesion and biofilm formation was investigated. In *A. baumannii*, the identified MFS transporter-like ORF (PMT) has been associated not only with adhesion but also with biofilm development and possible extracellular DNA release (Marti et al. 2006).

When it comes to biofilm development, certain efflux pumps are more significant than others (Yoon et al. 2015). CsuA/B, CsuC, and FimA are pilus system proteins that play a role in surface colonization, bacterial adhesion, and microcolony formation in the initial stages of biofilm development. The underexpression of several genes encoding proteins CsuA/B, CsuC, and FimA was observed in mutants overexpressing the efflux genes *adeABC* and *adeIJK*. This may be the reason why mutants with high levels of AdeABC and AdeIJK pumps had poor antibiofilm abilities. The downregulation of pilus system genes might obstruct biofilm development in the initial stages. The AdeABC and AdeIJK efflux systems might indirectly control pilus gene expression via the efflux of substances that may activate regulator genes. The capacity of several *A. baumannii adeb*-deficient strains to produce biofilms on various surfaces has been studied. When compared to the wild type (WT), the adeb-deficient *A. baumannii* (AYE mutant) demonstrated antibiofilm activity on both mucosal tissue and plastic, but the *adeAB*-deficient *A. baumannii* (S1 mutant) only showed the capacity to produce biofilm on mucosal tissue. In contrast to the WT, the lack of *adeB* in *A. baumannii* ATCC 17978 improved its biofilm-forming potential. These findings revealed that different strains of *A. baumannii* respond differently to *adeB* deletion and that the involvement of the AdeB efflux pumps in biofilm formation differs across strains due to endogenous *adeABC* expression levels (Richmond et al. 2016).

In another investigation, mutants overexpressing the AdeABC, AdeFGH, and AdeIJK efflux proteins, when compared to the WT strain, showed a substantial reduction in biofilm development. Furthermore, biofilm restoration was observed upon the deletion of efflux genes *adeG* and *adeJ*, although the mutant

strain lacking *adeB* showed substantial biofilm formation defects. Quorum sensing (QS) is a phenomenon in which chemical signals generated and released by bacteria are used to communicate between similar or different species (Miller and Bassler 2001). Efflux pumps also have a correlation with QS in bacteria. In *Pseudomonas aeruginosa*, MexAB-OprM overexpression causes an increase in antibiotic and OdDHL secretion, which leads to enhanced resistance and decreased expression of QS-controlled virulence factors (López et al. 2017; Evans et al. 1998). C4HSL (homo-serine lactone) induces MexAB-OprM expression, resulting in increased antibiotic resistance, OdDHL-LasR binding specificity, and further regulation of QS-regulated gene expression (Maseda et al. 2004). The absence of the MexAB-OprM pump causes OdDHL to accumulate in the cell, limiting cell-cell communication (Evans et al. 1998).

This shows that quorum sensing and the production of biofilms in *A. baumannii* necessitate a specific efflux pump expression profile to start and maintain the production of biofilms and that some efflux pumps are more important in this regard than others (Yoon et al. 2015). The efflux pump of *A. baumannii* shows a correlation with biofilm formation. This correlation can further be investigated for a better understanding of the therapeutic significance of the links between them. Efflux pump inhibitors may also prove to be of great significance in targeting biofilm-mediated acquired resistance in *A. baumannii* isolates. This may hinder the ability of *A. baumannii* to survive in hospital environments via biofilm formations causing resistant infection, where antibiotic treatments become a failure due to the efflux of antibiotics.

5 Conclusion

Acinetobacter spp. is one of the most common opportunistic infections in hospitals around the world. This bacterium's resistance to a wide spectrum of antibiotics has resulted in higher mortality rates. The information gathered here indicates that efflux pumps play a unique role in drug resistance. Some of the inhibitors linked to these pumps have also been highlighted. Antibiotic resistance can be impacted by changes in pump behavior, according to studies. More research is needed to see if synthetic compounds of natural materials may alter these pumps. Several genes have an impact on efflux pumps. Any change in the expression of these genes could have an impact on their function. In conclusion, antibiotic resistance is the most significant problem in *Acinetobacter* spp. infection. The most significant aspect of the establishment of this method is efflux pumps. We can stop these pumps that used a variety of chemicals, which can aid in infection management. Efflux pumps are critical components of bacteria. It is necessary to pay more attention to the performance of these pumps because any changes in their performance may affect antibiotic resistance.

6 Future Prospects

EPIs are compounds that, by means of one or more methods, inhibit efflux pumps, resulting in inactive drug transport. These EPIs can be used in conjunction with antibiotics to boost their action against bacteria that express efflux pumps since this could lead to the successful buildup of an antibiotic inside the cell. Since the turn of the century, the prospect of employing EPIs to revitalize the activity of antibiotics has been under investigation (Pagès and Amaral 2009). There are numerous obstacles on the way to a commercially successful EPI. These problems range from scientific and intellectual to administrative and commercial. The financial worth of an EPI is a big roadblock in creating and promoting it. Because EPI is essentially a new chemical entity, major pharmaceutical companies tend to steer away from this arena of new chemical entities (NCEs). The challenges associated with NCEs are well known to drug professionals, but the idea of changing currently available antibiotics, which have a well-documented pharmacological profile and clinical data from a large number of patient records, takes precedence (Sharma et al. 2019). Synthesizing naturally derived EPIs is difficult due to their complicated structure and bulky

nature. While synthetic compounds are faster to make, they generally have poor solubility, toxicity, and cell permeability issues. The discovery of NCE is a time- and capital-intensive procedure. In addition, a significant amount of effort is expended in meeting severe regulatory requirements. This, along with ordinary economic returns, renders the discovery of EPIs, and NCEs in general, a financially unviable enterprise, preventing most pharmaceutical corporations from participating (Lomovskaya and Bostian 2006). The targets themselves are a big barrier for EPIs as medicinal agents. Antibiotic resistance is caused by a variety of mechanisms, including efflux pumps, but they are not usually the sole ones. Fluoroquinolone resistance is frequently mediated through efflux pumps as well as point mutations in gyrase-coding genes in bacteria, like *A. baumannii* and *P. aeruginosa*. The coexpression of several pumps and substrate redundancy exacerbate the situation. As a result, the EPI-antibiotic combinatorial therapy is case specific, raising reservations about its success in the population (Nakajima et al. 2002). The absence of preclinical and clinical evidence is one of the biggest obstacles to EPI success. To support EPI activity, there is a limited amount of information on model organisms and patient data. To take EPI research to the next level, further work is needed at the preclinical and clinical levels. Last but not least, the main disadvantage of these EPI compounds is their toxicity, which prevents them from being used in clinical settings; however, they are used to evaluate the various efflux mechanisms expressed by different pathogenic bacteria, as well as the affinity of efflux pumps for them concerning the antibiotics. Excluding GamR protein, there are several bacteriophage receptors in Gram-positive bacteria that refer to the components of the cell wall and peptidoglycan layer, as expected. Though the function of these receptors in reference to bacteriophage is not yet clear, it appears to be related to cobalt transporter proteins belonging to the ATP-binding cassette (ABC) family (Davison et al. 2005). Peptide receptors associated with porins, such as OmpC and OmpF, or channels, such as LamB and FadL, or pumps and transport systems, such as BtuB, FhuA, TonB, and TolC, are found in much higher numbers in Gram-negative bacteria (Bertozzi Silva et al. 2016). All proteins found on the outer membrane and in the LPS layer may be receptors, although receptors only account for 79% (108 out of 137) of the proteins found on the outside membrane (Kortright et al. 2020).

As a result, the interaction between receptors (porins, pumps, channels) and the capacity of bacteriophage nucleic acids to infiltrate cells become a question. It is unclear if bacteriophages use receptor structural features for penetration through the outer membrane or merely for recognition and attachment to bacteria's surfaces. Pumps located on the outer and inner membranes, such as the TolC-containing pump group and the CusCFBA copper/silver efflux system, are of special interest in this regard (Alcalde-Rico et al. 2016). At the same time, phage attachment to the porin receptor might trigger alterations in the channel's transport characteristics. Even though the kinetics of sugar binding was not altered when the lambda phage was attached to maltoporin, ion fluxes through the pores of maltoporin in the phage-receptor complex shared a new common route (Gurnev et al. 2006). These findings show that the irreversible binding of the phage to the receptor, as well as the creation of the MDR pump-phage complex, causes a shift in the manner MDR pumps expel xenobiotics.

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