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Review

Hybrid combinations containing natural products and antimicrobial drugs that interfere with bacterial and fungal biofilms

Susana A. Zacchino*, Estefanía Butassi, Estefanía Cordisco, Laura A. Svetaz

Área Farmacognosia, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531, 2000 Rosario, Argentina

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ABSTRACT

Background: Biofilms contribute to the pathogenesis of many chronic and difficult-to eradicate infections whose treatment is complicated due to the intrinsic resistance to conventional antibiotics. As a consequence, there is an urgent need for strategies that can be used for the prevention and treatment of biofilm-associated infections. The combination therapy comprising an antimicrobial drug with a low molecular weight (MW) natural product and an antimicrobial drug (antifungal or antibacterial) appeared as a good alternative to eradicate biofilms.

Purpose: The aims of this review were to perform a literature search on the different natural products that have showed the ability of potentiating the antibiofilm capacity of antimicrobial drugs, to analyze which are the antimicrobial drugs most used in combination, and to have a look on the microbial species most used to prepare biofilms.

Results: Seventeen papers, nine on combinations against antifungal biofilms and eight against antibacterial biofilms were collected. Within the text, the following topics have been developed: breaf history of the discovery of biofilms; stages in the development of a biofilm; the most used methodologies to assess antibiofilm-activity; the natural products with capacity of eradicating biofilms when acting alone; the combinations of low MW natural products with antibiotics or antifungal drugs as a strategy for eradicating microbial biofilms and a list of the low MW natural products that potentiate the inhibition capacity of antifungal and antibacterial drugs against biofilms.

Conclusions and perspectives: Regarding combinations against antifungal biofilms, eight over the nine collected works were carried out with *in vitro* studies while only one was performed with *in vivo* assays by using *Caenorhabditis elegans* nematode. All studies use biofilms of the *Candida* genus. A 67% of the potentiators were monoterpenes and sesquiterpenes and six over the nine works used FCZ as the antifungal drug. The activity of AmpB and Caspo was enhanced in one and two works respectively. Regarding combinations against bacterial biofilms, *in vitro* studies were performed in all works by using several different methods of higher variety than the used against fungal biofilms. Biofilms of both the gram (+) and gram (-) bacteria were prepared, although biofilm of *Staphylococcus* spp. were the most used in the collected works. Among the discovered potentiators of antibacterial drugs, 75% were terpenes, including mono, di- and triterpenes, and, among the atibacterial drugs, several structurally diverse types were used in the combinations: aminoglycosides, β-lactams, glucopeptides and fluoroquinolones. The potentiating capacity of natural products, mainly terpenes, on the antibiofilm effect of antimicrobial drugs opens a wide range of possibilities for the combination antimicrobial therapy. More *in vivo* studies on combinations of natural products with antimicrobial drugs acting against biofilms are highly required to cope the difficult to treat biofilm-associated infections.

Abbreviations: AA, asiatic acid; Aeth, aethiopinone; AmpB, Amphotericin B; BBR, Berberine; BEC, biofilm eradication concentration; CA, corosolic acid; Carv, carvacrol; CAS, caspofungin; Cin, cinnamaldehyde; Cip, ciprofloxacin; CLSM, confocal laser scanning microscopy; CTC, 5-cyano-2,3-ditolyl tetrazolium chloride; CV, crystal violet; DAPI, 4',6'-Diamidino-2-phenylindole; EGCg, epigallocatechingallate; EPS, exopolysaccharide; Eug, eugenol; Farn, farnesol; FCZ, fluconazole; FICI, Fractional Inhibitory Concentration Index; Gen, gentamicin; MCF, micafungin; MCZ, miconazole; MTP, microtiter plate; Naf, Nafcillin; Oxa, oxacillin; PTs, pentacyclic triterpenes; Salv, salvipisone; SBF, Specific Biofilm Formation; SEM, Scanning Electron Microscopy; SMIC, sessile minimum inhibitory concentration; Str, streptomycin; Thy, thymol; Tobra, Tobramycin; Trc, Tyrocidines; TTC, Triphenyltetrazolium chloride; UA, ursolic acid; Van, Vancomycin; XTT, 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide

* Corresponding author.
E-mail address: szaabgil@citynet.net.ar (S.A. Zacchino).

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Introduction

Several microorganisms form biofilms on living surfaces or medical devices, which constitute their mode of growth in a hostile environment (Costerton et al., 1978, 1999; Coenye and Nelis, 2010; Donlan, 2002).

First structural studies of microbial biofilms

Lawrence et al. (1991) performed structural studies on microbial biofilms and found that they were highly organized hydrated structures that possess distinctive arrangements depending on the microbial species involved. They also noted that many times the biofilm was formed by more than one microbial species that aggregate one each other forming dense mats that stick to surfaces enclosed in a exopolysaccharide matrix (EPS), thus explaining the mechanisms by which microorganisms form biofilms.

Stages in the development of biofilms

The development of a biofilm involves 5 stages that were clearly explained and graphed by Stoodley et al. (2002) (Fig. 1). In stage 1 an initial attachment of microbial cells to the surface is observed; in stage 2 the EPS matrix is produced resulting in a firmly adhered "irreversible" attachment; in stage 3, an early biofilm architecture is developed and in stage 4 the biofilm reaches maturation; in stage 5, single planktonic cells are dispersed from the mature biofilm leading to the formation of a new biofilm.

Resistance of biofilms to antibiotics and antifungals

A characteristic of microbial biofilms is the markedly enhanced resistance to antimicrobial agents (Ahmad Khan and Ahmad, 2012; Costerton et al, 1999; Nickel et al., 1985; Stewart, 2002; Stewart and Costerton, 2001) possessing about 100–1000 times less susceptibility to antifungals and antibacterials than equivalent populations of planktonic cells (Gilbert et al., 2002; Seneviratne et al., 2008; Simões et al., 2009)

The mechanisms of biofilm resistance have been reviewed by Lewis (2007), who clearly explained that although most of the cells in a biofilm can show susceptibility to antimicrobial agents, a small subpopulation of cells (called persisters) stay alive, irrespective of the concentration of the antibiotic. The immune system can kill the remaining planktonic, but not the biofilm persister cells that are protected by the EPS. So, persisters cells that are contained in the biofilm can survive to both the antibiotic treatment and the immune system. When the concentration of antibiotic reduces, persister cells can grow again and repopulate the biofilm (Fig. 2).

Chronic diseases such as cystic fibrosis, native valve endocarditis, otitis media, periodontitis, and prostatitis appear to be caused by biofilm-associated microorganisms (Monroe 2007; Donlan, 2002) and thus they are considered very difficult to eradicate diseases.

The development of new antimicrobial agents with capacity of eradicating biofilms is urgently needed as alternative therapeutic options for microbial biofilm-related diseases.

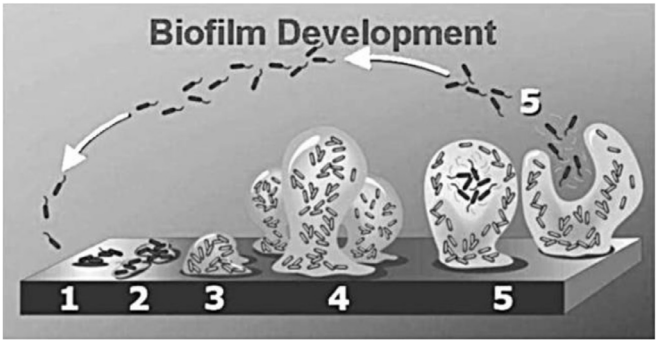


Fig. 1. The five-stage process involved in the development of a biofilm (reproduced from Stoodley et al. (2002). Ann. Rev. Microbiol. 56,187–209 (image credit: D. Davies), with permission of Prof. David Davies.

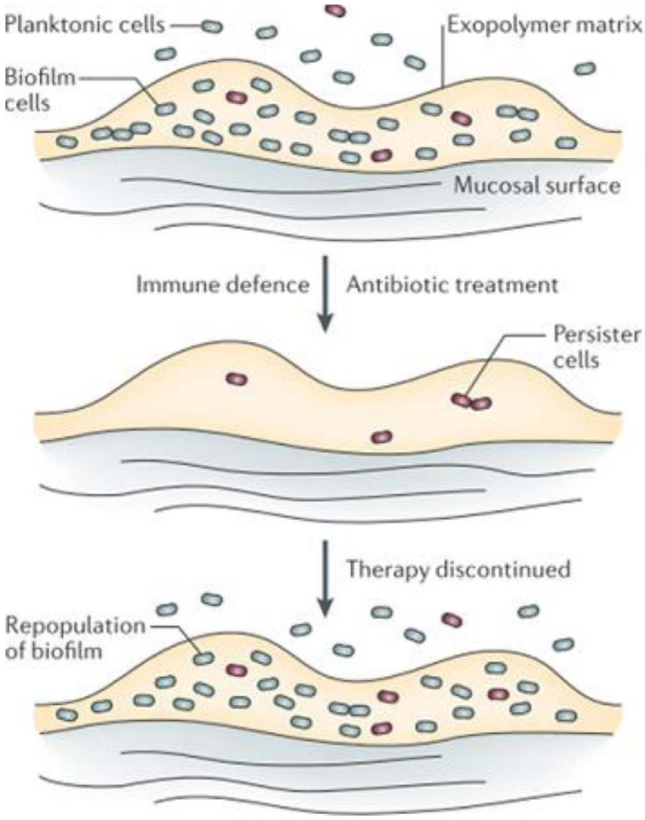


Fig. 2. Model of biofilm resistance to killing based on persister survival. Initial treatment with antibiotic kills normal cells (coloured green, please see the on line version) in both planktonic and biofilm populations. The immune system kills planktonic persisters (coloured pink), but the biofilm persister cells (coloured pink) are protected from the host defences by the exopolymer matrix. After the antibiotic concentration is reduced, persisters resuscitate and repopulate the biofilm and the infection relapses. Reproduced from Lewis (2007), Nature Publishing Group license # 4063660297428 which was a modification of a Fig. appeared in Lewis, 2001. American Society for Microbiology license # 4063670074620. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Natural products with capacity of eradicating biofilms when acting alone

In the last years, many efforts have been made in the exploration of new and effective natural compounds with antibiofilm effects on their own (Bink et al., 2011). So, the sesquiterpene *tt*-farnesol (Farn) showed a modest effect against *Streptococcus mutans* and *Streptococcus sobrinus* biofilms (Koo et al., 2002); the polyphenols epigallocatechingallate (EGCg) and ellagic acid reduced in 30 and 50% respectively the *Burkholderia cepacia* biofilm formation (Huber et al., 2003); the phenylpropanoid cinnamaldehyde (Cin) decreased the *Escherichia coli* biofilm formation in the Specific Biofilm Formation (SBF) assay (Niu and Gilbert, 2004) and the monoterpenephenol carvacrol (Carv) inhibited the biofilm development of *S. aureus* and *S.* Typhimurium (Knowles et al., 2005). Also EGCg at sub-MIC concentrations decreased the EPS production and thus inhibited the biofilm formation of 20 ocular isolated *Staphylococcus* spp including *S. aureus* and *S. epidermidis* (Blanco et al., 2005); the sesquiterpenephenol xanthorrhizol reduced 60% of adherence of *S. mutans* cells (Rukayadi and Hwang, 2006) and the diterpenoide salvipisone (Salv) prevents *S. aureus* and *S. epidermidis*

cells adhesion and biofilm formation at very low concentrations (Kuzma et al., 2007). In turn, the flavonoglycoside baicalin (baicalein glucuronide) demonstrated to be able to suppress the development of *C. albicans* biofilms with a mode of action that involves the induction of cell death *via* apoptosis (Wang et al., 2015).

From the above works, it is clear that several type of compounds, mainly terpenoids and phenols are the natural structures that demonstrated capacity for inhibiting biofilms at any of their formation steps when acting alone. However, as highlighted by Simões et al. (2009), the activity of phytochemicals on biofilms can be favourable but also unfavourable as was shown by EGCg that does not inhibit *Pseudomonas syringae* biofilms but, instead, induce biofilm formation (Tomihama et al., 2007). In addition, no natural product has been discovered to date that can completely eradicate biofilms on its own.

Combination of low molecular weight natural products with antibiotics or antifungals as a strategy for eradicating microbial biofilms

As a consequence of this lack of promissory antibiofilm natural compounds, a new strategy has emerged. It consists on combining natural small-size molecules with antifungal or antibacterial drugs in order to find mixtures that can be developed as hybrid therapies for eradicating established microbial biofilms or preventing its formation (Bink et al., 2011). This approach focuses on the enhancement of the activity of known antimicrobial compounds, by natural enhancers, that are compounds that can increase the antibiofilm activity of an antimicrobial agent at concentrations in which they had not antibiofilm activity.

The 'enhancement' (Garo et al., 2007) of antibiofilm effects of antimicrobial drugs, also known as 'potentiation' (De Cremer et al., 2015), or 'repurposing' of antimicrobial drugs (Delattin et al., 2014) takes advantage of the safe toxicity profile and the known dosing regimens of existing drugs, thus making the possible cost of reformulating them, and performing new clinical trials, considerably lower than that for the development of a new drug from the very beginning (Delattin et al., 2014).

In order to contribute to the development of new possible hybrid agents useful for eradicating biofilms and therefore to treat the chronic and difficult-to-eradicate infections associated to them (Katragkou et al., 2015), this review provides an overview of the low molecular weight (MW) secondary metabolites isolated from natural sources that possess the ability of *in vitro* or *in vivo* potentiating the activity of antifungal or antibacterial drugs against fungal or bacterial biofilms. The compiled studies have been published in the literature up to July 2017. Within the review, the most promissory structures were highlighted.

Materials and methods

Search strategy

The search for suitable papers was performed in internet databases (PubMed, Sciencedirect and other web pages, by using the following keywords: "biofilm", "bacterial infections", "fungal infections" "sessile cells", "secondary metabolites", "enhance", "enhancers", "synergism", "natural products", "potentiators", "repurposing", "antifungal drugs", "antibacterial drugs", "antibiofilm". Additional papers were included in our collection after surveying the references from the selected articles. The goal of this search was to explore articles that investigated the therapeutic approaches of combinations of natural low MW molecules with antimicrobial drugs against bacterial and fungal biofilms using *in vitro* and *in vivo* experimental systems. As a result of this search, seventeen papers were collected, nine on combinations of antifungal drugs with low MW natural products against fungal biofilms and eight on combinations of antibacterial drugs with low MW natural products against bacterial biofilms.

Data extraction

The information gathered from the chosen articles included: the structures of natural products; the concentrations at which they act as enhancers; the fungal or bacterial strains used to form biofilms; the *in vitro* and *in vivo* assays and the assessments of molecular mechanisms of the antibiofilm effects. The information was divided into two groups: (a) Natural products in combination with antifungal drugs against fungal biofilms; (b) Natural products in combination with antibacterial drugs against bacterial biofilms. The information is summarized in Tables 1 and 2 and each work was commented and detailed into the Results section. Previously, a detailed description of the most used methodologies to assess the antibiofilm effects of compounds alone or in combinations was added to the Results section in order to a better comprehension of the results.

Results and discussion

Methodologies most used to assess antibiofilm-activity

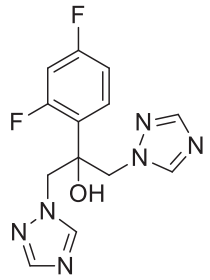
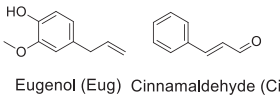
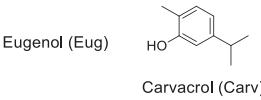
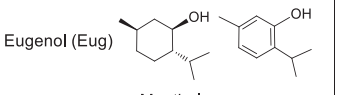
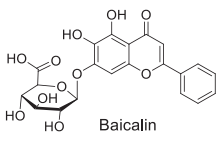
In vitro assays

The microtiter plate (MTP)-based systems are among the most frequently-used *in vitro* models for quantifying biofilms (Coenye and Nelis, 2010). In all *in vitro* assays, biofilms need to be seeded in a MTP and quantified before and after being submitted to treatments. A broad range of model systems for quantifying biofilms have been used in the last decades, such as direct microscopic enumeration, total viable plate counts, metabolically active dyes, radiochemistry and luminometry, among others (Amorena et al., 1999; Domingue et al., 1994; Gander and Gilbert, 1997; Gracia et al, 1999).

In these models, microbial cell suspensions are added to the wells of a 96-well MTP and let to stay some time, for adhesion. The medium is then aspirated from the wells and washed to remove loosely adhered cells. Culture medium is further added to each well and incubated for a proper time to obtain the complex tridimensional architecture characteristic of biofilms (Delattin et al., 2014). The growth medium is again carefully removed by aspiration without disrupting the integrity of the biofilm and thoroughly washed to remove non-adherent cells. For quantification purposes, biofilms are stained with different dyes. One is crystal violet (CV) that allows biofilm total mass quantification (Christensen et al, 1985; Stepanovic et al., 2000), but due to both the living and the dead cells are stained, it is poorly suited to evaluate killing of biofilm cells. Similar information on total biofilm biomass can be obtained with the fluorogenic dye Syto9 that is a nucleic acid stain which binds to DNA of both viable and dead cells (Boulos et al, 1999). To discriminate between living and dead cells, quantification techniques based on the metabolic activity of viable cells are available (Peeters et al., 2008). Some of the viability stains are the tetrazolium salts 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) and 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) (Gabrielson et al., 2002; Roehm et al., 1991) or 1,9-dimethyl methylene blue (DMMB) (Peeters et al., 2008). Another viability stain is Alamar Blue that is based on the reduction of resazurin to pink resorufin (which is fluorescent) by metabolically active cells (O'Brien et al., 2000). For viability assays, the colony-forming units (CFU) counting has been highly used too (Jabra-Rizk et al., 2006) but it has the inconvenience to be very time-consuming.

Inhibition of biofilm formation or biofilm eradication by a compound on its own. To test the inhibition capacity of a compound to biofilm formation, media containing a range of two-fold dilutions of the tested compound is added to the wells of the MTP at the zero hour of biofilm formation (immediately after the adhesion phase). After incubation at the temperature and time needed for the development of biofilms, the compounds are removed, the treated biofilms are washed and the whole plate is stained with CTC, XTT or other

Table 1
In vitro interactions of low molecular weight natural compounds with antifungal drugs against fungal biofilms. Methods used and main results. FICI = Fractional inhibitory concentration Index.

Antifungal	Potentiators	Biofilm from	Ref	Method used	Results
 Fluconazole (FCZ)	 Eugenol (Eug) Cinnamaldehyde (Cin)	*C. albicans*	Ahmad Kahn and Ahmad, 2012	The activities of Eug/FCZ and Cin/FCZ were determined with the checkerboard design against established *C. albicans* biofilms FICI was determined.	FICI, Eug/FCZ = 0.25 FICI, Cin/FCZ = 0.31 Dose Reduction Index (DRI). the SMIC of FCZ diminished 32-fold
	 Eugenol (Eug) Carvacrol (Carv)		Doke et al., 2014	The activity of Eug/FCZ, Carv/FCZ and Thy/FCZ were determined with the checkerboard design against biofilm formation and established *C. albicans* biofilms. FICI was determined	*Effects on the formation of biofilms*: Carv, Eug or Thy potentiate 16-, 256- and 256- fold respectively the SMIC of FCZ at the stage of development of *C. albicans* biofilm. However, the FICI values showed synergistic effects for Carv/FCZ and Eug/FCZ (0.31 and 0.25) with indifferent effects for Thy/FCZ (1.003) *Effects on established biofilms*, Carv and Eug showed greater effects on mature biofilms in terms of the decrease of SMICs: SMIC of
					FCZ diminished > 32-fold when combined with carvacrol and > 512-fold with Eug and Thy. However, the FICIs on mature biofilms showed indifferent interaction (0.516, 1 and 1.001) for Carv/FCZ, Eug/FCZ and Thy/FCZ.
	 Eugenol (Eug) Menthol Thymol (Thy)		Pemmaraju et al., 2013	The activities of Eug/FCZ; Thy/FCZ and menthol/FCZ were determined with the checkerboard design. FICI was determined. An adherence assay was performed too	*Effects on established biofilms* $SMIC_{90}$ of FCZ decreased from 2000 µg/ml (alone) to 500 µg/ml (4-fold) when combined with Eug, Thy or menthol. Thy/FCZ, Eug/FCZ and menthol/FCZ showed FICI values = 0.31, 0.37 and 0.5 respectively. *Effects on adherence of Candida cells* It was observed 30 % viability of *C. albicans* cells after 2h of treatment with 0.05 % Thy/FCZ
	 Baicalin		Wang et al., 2015	The activity of baicali/FCZ was determined with the checkerboard design. FICI was calculated.	FICI, Baicalin/FCZ = 0.28-0-50

(*continued on next page*)

Table 1 (*continued*)

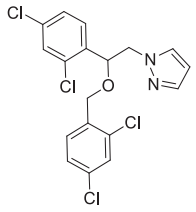
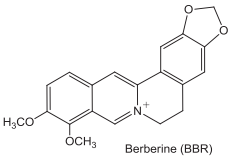
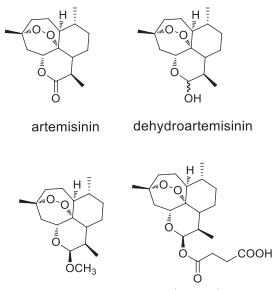
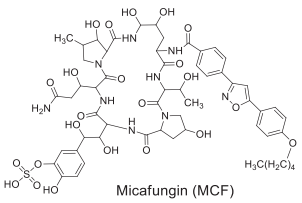
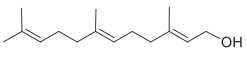
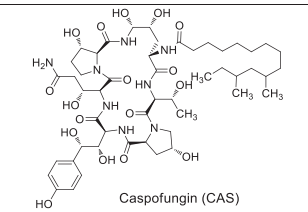
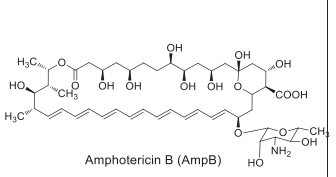
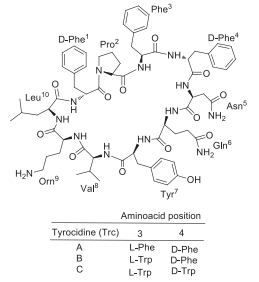
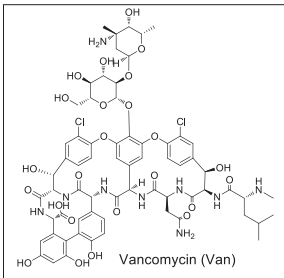
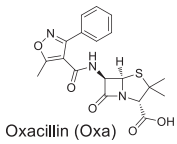
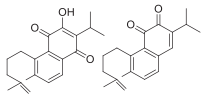
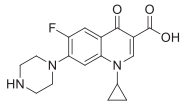
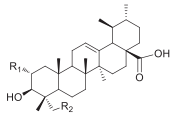
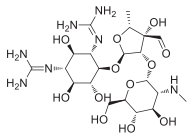
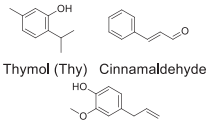
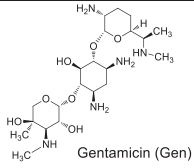
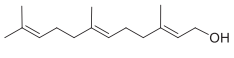
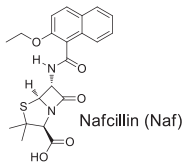
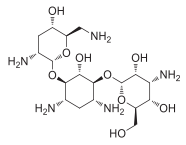
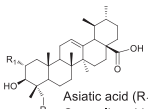
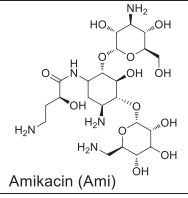
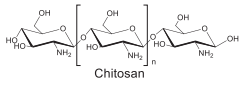
 Miconazole (MCZ)	 Berberine (BBR)	*C. albicans*	Wei et al., 2011.	The activity of BBR/MCZ, was determined with the following methods:: (i) a dual-flow chamber with two compartments where the *C. albicans* biofilms are grown. The non-stained fungal biofilms are visualized by dark field microscopy and images were analyzed with ImagePro Plus software (ii) with the checkerboard design against established *C. albicans* biofilms with XTT staining	(i) Biofim formation was almost completely inhibited (>91 %) in the presence of BBR/MCZ combination (ii) $SMIC_{90}$ decreased four-fold FICI BBR/MCZ = 0.25
	 artemisinin, dehydroartemisinin, artemether, artesunate	*C albicans*	De Cremer et al., 2015	The activity of artemisinines/MCZ, against *C. albicans* established biofilms was determined with the checkerboard design. The FICI was calculated	FICI artesunate/MCZ = 0.0069 FICI artemisinin/MCZ = 0.186 FICI dihydroartemisinin/MCZ = 0.145 FICI artemether/MCZ = 0.112
 Micafungin (MCF)	 Farnesol (Farn)	*C. albicans*	Katragkou et al., 2015	The activities of Farn/FCZ, Farn/MCF and Farn/AmpB against C. albicans established biofilms was determined with the checkerboard design. The nature of interactions was assessed using the FICI and the the Bliss Independence model. Structural changes were observed with confocal microscopy	The SMIC of FCZ anf MCF decreased 16-fold when combined with Farn FICI, Farn/FCZ = 0.5 FICI, Farn/MCF = 0.49 FICI, Farn/AmpB = 0.79 Confocal microsopy showed great distortion in the biofilm architecture with Farn-MCF and mild distortion with Farn/FCZ
- Fluconazole (FCZ)					
 Caspofungin (CAS)		*C. parapsilopsis*	Kovács et al., 2016	Interactions were assessed against established biofilms with the checkerboard design, FICI values, time-kill experiments and Bliss independence models that were built by using MacSynergy II analysis	*FICI against established 5 C. parapsilopsis biofilms* FICI, Farn/CAS = 0.15-0.50 FICI, FICI Farn/MCF = 0.09-0.5 The results were corroborated with time-kill curves and the Bliss independence models
- Micafungin (MCF)					
 Amphotericin B (AmpB)		*C albicans*	Troskie et al., 2014.	*In vitro*: checkerboard, FICI In vivo: *Caenorhabditis elegans*	*In vitro* studies FICI Trc A/AmpB = 0.23-0.41 FICI Trc B/AmpB = 0.14-0.42 FICI Trc C/AmpB = 0.28-0.35 FICI Trc A/CAS = 0.10-0.18 FICI Trc B/CAS = 0.12-0.19 FICI Trc C/CAS = 0.12-0.35 *In vivo* studies: At 3 μM, Trc A significantly enhanced the survival of *C. albicans*-infected *C. elegans* nematode treated with 0.095 μM CAS
- Caspofungin (CAS)					

Table 2
In vitro interactions of low molecular weight natural compounds with antibacterial drugs against bacterial biofilms. Methods used and main results. FICI = Fractional inhibitory concentration Index.

 Vancomycin (Van)		*S. epidermidis*	Pammi et al., 2011	- Checkerboard design followed by XTT staining - The median-effect method of Chou (Chou, 2006) with constant ratio combinations (1:1, 1:2, 1:4 and 2:1. Combinations Indexes (CI) were calculated by using Calcusyn software (BIOsoft, Cambridge, UK). A CI < 1, indicates synergy, CI= 1, additivism and CI >1, antagonism.	Farn/Naf and Farn/Van showed synergis at most equipotent and non-equipotent drug-dose ratios at 75 % and 90 % effects of the combinations with CIs between 0.07 and 0.35.
 Oxacillin (Oxa)	 Salvipisone (Salv) Aethiopinone (Aeth)	*S. aureus* *S. epidermidis*	Walencka et al., 2007	Oxa was tested in combination with Salv or Aeth, against *S. aureus* and *S. epidermidis* biofilms and, after 24 h, the pre-formed biofilms were stained with MTT. The color intensity was measured with a microtiter plate (MTP) reader.	*S. aureus* and *S. epidermidis* biomass were reduced by Oxa-Salv and Oxa-Aeth in about 40 % while the partners alone were not so effective.
 Ciprofloxacin (Cip)	 Asiatic acid (R_1=R_2=OH) (AA) Ursolic acid (R_1=R_2=H) (UA)	*E. coli*	Wojnicz et al., 2015	*E. coli* biofilms were grown on two different surfaces: polystyrene pieces and a silicone-coated latex Foley catheters with or without Cip + AA and Cip + UA. After 96 h, the staining with 4',6'-diamidino-2-phenylindole (DAPI) or triphenyltetrazolium chloride (TTC) allowed the analysis by	Cip + UA inhibit the biofilm formation on microtiter plates but not on catheters. Cip + AA showed weak activity of biofilmd in both surfaces. Instead, Cip +AA and Cip + UA exhibited strong inhibitory capacity of mature biofilms.
Antibiotic	**Potentiators**	**Biofilm from**	**Ref**	**Methods**	**Results**
 Streptomycin (Str)	 Thymol (Thy) Cinnamaldehyde (Cin) Eugenol (Eug)	*L. monocytogenes* *S.* Typhimurium	Liu et al., 2015	- Checkerboard design and FICI determinations on mature biofilms. Staining with CV and MTT. - Scanning Electron Microscopy (SEM) - Immunofluorescence	Against *L. monocytogenes* FICI Str/Thy = 0.375 FICI Str/Cin = 0.312 Against *S. typhimurium* FICI Str/Cin = 0.375 FICI Str/Eug = 0.375 *Results of SEM* The architecture of *S.* Typhimurium showed few or scattered cell aggregates *Immunofluorescence* A greater green fluorescence was observed in biofilms treated with combinations than with the partners alone
 Gentamicin (Gen)	 Farnesol (Farn)	*S. aureus*	Jabra-Rizk et al., 2006	Established *S. aureus* biofilms were exposed to various concentrations of both agents and the biofilm colonies were counted at 0, 4 and 24 h post-treatment	Gen 10 μg/ml (2.5x MIC)/Farn at 22 μg/ml (100 μM) at 24 h reduce *S. aureus* biofilms in more than 2 log units
 Nafcillin (Naf)					

(*continued on next page*)

Table 2 (*continued*)

				fluorescence microscopy	
 Tobramycin (Tobra)	 Asiatic acid (R_1=R_2=OH) (AA) Corosolic acid (R_1=OH, R_2=H) (CA)	*P. aeruginosa*	Garo et al., 2007	*P. aeruginosa* biofilms were grown on rotating disk reactors (RDRs) and the statistical interaction was calculated as follows: Log reduction (LR) for the test compound plus the antibiotic - LR for the test compound alone - LR for the antibiotic alone.	AA and CA exhibited positive interaction with Tobra against *P. aeruginosa* biofilms
 Amikacin (Ami)	 Chitosan	*L. monocytogenes*	Mu et al., 2014a	Checkerboard design, and further biofilm mass staining with (CV) and viable cells (MTT dye).	Ami/chitosan (between 3 and 180 kDa) appeared to increase *L. monocytogenes* biofilm mass Also chitosan with a high deacetylation degree (88%) produced an optimal combination effect of Chitosan/Ami. This combination inhibited not only biofilm formation but also disrupt established biofilms
Gentamicin (Gen)			Mu et al., 2014b	Checkerboard design. Fluorescence, Confocal Laser Scanning Microscopy (CLSM) and SEM	FICI Gen/chitosan < 0.15. Fluorescence microscopy, confocal microscopy and SEM showed fewer scattered cell aggregates in *L. monocytogenes* biofilms after exposure to Gen/chitosan than to Gen and chitosan each on its own.

viability stain. Aliquots of coloured cell-free supernatants are transferred to a blank MTP, and absorbance is determined spectrophotometrically at 490 nm. From the resulting colorimetric readings and after subtracting the corresponding values for negative controls, the sessile minimum concentration for a given percentage of biofilm inhibition (SMIC) is calculated for each microorganism. For example, $SMIC_{50}$ and $SMIC_{80}$ are defined as the minimum concentration resulting in a 50 and 80% reduction in absorbance that is detected when compared with control biofilms formed with the same microorganism in the absence of an antimicrobial drug (Pierce et al., 2008).

Instead, to analyze the effects on established biofilms, media with a range of two-fold dilutions of the tested compound are added to the 24–48 h mature biofilms (Doke et al., 2014) and the Biofilm Eradication Concentration (BEC) of the compound can be obtained and expressed as BEC_{50} or BEC_{80} similarly than with SMIC.

Antibiofilm effect of a combination

Checkerboard antibiofilm MTP assay. For the assessment of the combinations' antibiofilm effects, the same methodology described above is applied to combinations but with the difference that the MTP with seeded biofilms is used in a checkerboard microdilution design (Lewis et al., 2002; Rand et al., 1993). In this assay, a variable combination of the commercial antimicrobial drug and the probable potentiator is added to the wells of the MTP. The plates are prepared as follows: the bottom row (A) of a 96-well microtiter plate contains the antimicrobial drug (X) in a concentration of about four times the SMIC against the biofilm examined. Each following row (B-H) contains half the concentration of the previous one. The same procedure was carried out along the columns (1–12) with the probable potentiator Y, but considering the SMIC of Y. So, each well from A-1 to G-12 contains a unique combination of the two substances (X and Y). After incubation of the plate at proper temperature and time, the mixtures' solutions are removed from the wells, and after washed, the plate is stained with a viability stain (XTT or other). Aliquots of coloured cell-free supernatants are transferred to another blank MTP and absorbance is determined spectrophotometrically at 490 nm (Iten et al., 2009). Potentiation is determined by the Fractional Inhibitory Concentration Index (FICI) that is calculated by the following formula: $FICI = [CSMIC_X/SMIC_X] + [CSMIC_Y/SMIC_Y]$, in which $CSMIC_X$ and $CSMIC_Y$ are the SMIC values of the antimicrobial drug and the partner respectively in combination, and $SMIC_X$ and $SMIC_Y$ are the SMIC values of the components X and Y alone. The interaction was defined as high potentiation (synergism) for a FICI value $\leq$ 0.5; indifference for 0.5 FICI $<$ 4; and antagonism for a FICI value $\geq$ 4.0 (Odds, 2003). Additionally, the extent of the interaction between (X) and (Y) can be analyzed using the isobologram approach that depicts the results of the checkerboard assay and the FICI values. The x axis of the isobologram represents substance X, the y axis substance Y. The SMIC value of X is located on the x axis, and the SMIC of Y on the y axis. The line connecting these two points represents the line of no interaction (line of indifference). Below the line of indifference we find the additivity ($1 >$ FICI > 0.5) and synergistic (FICI ≤ 0.5) areas. Above the area of indifference, we find the additivity ($4 >$ FICI > 1) and the antagonistic (FICI ≥ 4) areas for the combinations (Zhao et al., 2004).

In another studies, the nature of the interaction (synergy, additivity, or antagonism) between an antimicrobial drug and a natural compound is obtained with the median-effect method of Chou (2006, 2010) that allows the calculation of the combination index (CI). For two-drugs combination, both partners at a fixed ratio given by the IC_{50} of the antimicrobial drug and the IC_{50} of the natural product are mixed, and two-fold dilutions of the fixed ratio are prepared. The CI values are obtained with the Compusyn software (Chou, 2010). The combination index (CI) helps to identify synergistic (CI $<$ 1), additive (CI = 1), and antagonistic interactions (CI $>$ 1) (Zhao et al., 2004; Chou 2010). Also it is possible to calculate the Dose Reduction Index (DRI) or reversal enhancement ratio that is a measure of how many-fold the dose of a drug may be reduced in the combination as compared with the doses of the drug alone (Chou and Talalay, 1984). DRI is calculated as MIC drug alone/MIC drug in combination. DRI is important in clinical situations, in which the reduction of the dose leads to reduced toxicity while the therapeutic efficacy is retained. There are other *in vitro* methods that have been used in the reviewed papers to assess the effects of the combinations against biofilms. They are detailed in Tables 1 and 2 and commented into the text.

In vivo antibiofilm assays

Several *in vivo* biofilm model systems have been described

(Coenye and Nelis, 2010) and can be used to study *in vivo* antibiofilm activities of compounds alone and in combination. The *in vivo* models use either *Caenorhabditis elegans*, a nematode that demonstrated the capacity of forming biofilms (Darby et al., 2002) or vertebrate animals such as rats, guinea pigs, ponies, rabbits and others (Ulphani and Rupp, 1999; Rupp et al., 1999). However, in spite of several models that have been developed and analyzed with compounds alone, very few *in vivo* assessment of antibiofilm combinations of natural low-MW metabolites and antimicrobials have been reported to date.

Natural products in combination with antifungal drugs against fungal biofilms

A summary of the natural low MW compounds that potentiate the antibiofilm activity of antifungals is showed in Table 1 and an amplified overview is detailed below. The information was ordered by the type of natural products that has potentiating capacity, such as phenylpropanoids and monoterpenes; sesquiterpenes; alkaloids; flavonoids and others.

Phenylpropanoids and monoterpenes as potentiators of antifungal drugs against Candida biofilms formation or eradication

The phenylpropanoids eugenol (Eug) and Cin that have potent activity against pre-formed biofilms on their own (Ahmad Khan and Ahmad, 2012) were tested in combination with fluconazole (FCZ) and amphotericin B (AmpB) for their ability to eradicate established *C. albicans* biofilms, with the checkerboard design. Eug and Cin did not show any interaction with AmB. In contrast, Eug/FCZ and Cin/FCZ showed FICI values of 0.25 and 0.31 respectively along with a substantial reduction (32-fold) of SMIC of FCZ (DRI = 32) that indicate the effectiveness of Eug/FCZ and Cin/FCZ for eradicating established C. *albicans* biofilms. According to Authors, since Eug and Cin on their own have demonstrated cidal activity against *C. albicans* biofilms (Ahmad Khan and Ahmad, 2012), the fungistatic nature of FCZ could have changed to fungicidal in combination. Regarding the structures of the natural potentiators, the phenolic nature of both compounds could play a key role in the observed effects, encouraging the study of new structural related compounds.

In a further study (Doke et al., 2014), Eug and the monoterpenes Carv and thymol (Thy) were studied in combination with FCZ against the formation of *C. albicans* biofilms or the eradication of established ones. Authors found that sub-inhibitory concentrations (1/4 SMIC) of Carv (62 μg/ml), Eug (125 μg/ml) or Thy (1000 μg/ml) potentiate 16-, 256- and 256-fold respectively the SMIC of FCZ at the stage of formation of *C. albicans* biofilm with FICIs for Carv/FCZ and Eug/FCZ of 0.31 and 0.25 respectively. In contrast, Thy/FCZ showed a FICI = 1.003 denoting indifference. Regarding the effect on established biofilms, both compounds showed greater effects on mature biofilms since the SMIC of FCZ diminished from >1024 to 32 μg/ml (> 32-fold) and from >1024 to 2 μg/ml (> 512-fold) when combined with Carv and Eug respectively. In turn, Thy produced a similar decrease in the SMIC on mature biofilms that on the biofilm formation from >1024 to 2 μg/ml (> 512-fold). However, the FICIs on mature biofilms showed indifferent interaction (0.516, 1 and 1.001) for Carv/FCZ, Eug/FCZ and Thy/FCZ.

Another study on the combination of the phenylpropanoid Eug and the monoterpenes menthol and Thy with FCZ against *Candida* established biofilms, was performed by Pemmaraju et al. (2013). The study showed that the $SMIC_{90}$ of FCZ decreased from 2000 μg/ml (alone) to 500 μg/ml (4-fold) when combined with Eug, Thy or menthol. Thy showed the best potentiation capacity followed by Eug and menthol with FICI values of 0.31, 0.37 and 0.5 respectively. In addition, an 'adherence assay' was performed to analyze the effect of the compounds in combination with FCZ in the adherence of *C. albicans* cells to the wells of a MTP. Data showed 30% viability of *C. albicans* cells after 2 h of treatment with 0.05% Thy/FCZ. According to Authors, the effect of the combination Thy/FCZ is related more to an effect on *C. albicans* cells growth than to an effect on cell adhesion.

Sesquiterpenes as potentiators of antifungal drugs against Candida biofilms formation or eradication

Combination of the sesquiterpene Farn with several antifungal drugs against Candida spp. biofilms. Katragkou et al. (2015) assessed the combined effects of the sesquiterpene Farn with three antifungal drugs that act by different modes of action (the polyene AmpB, the triazole FCZ and the echinocandin micafungin (MCF) against *C. albicans* biofilm formation. FICI was used to determine the nature of the interactions. Results showed a good capacity of Farn for potentiating the action of both FCZ and MCF but not of AmpB. So, the SMIC of FCZ lowered from 1024 μg/ml (alone) to 64 μg/ml (in combination) (16-fold), and the SMIC of MCF decreased from 4 μg/ml (alone) to 0.25 μg/ml (16-fold) when combined with Farn. FICI values were 0.5 and 0.49 for Farn/FCZ and Farn/MCF respectively while FICI = 0.79 was found for Farn/AmpB. These interactions were corroborated by the Bliss independence model (Greco et al., 1995) that calculates the difference (ΔE) between the predicted % of growth (E_{ind}) and experimentally observed percentage of growth (E_{exp}) to define the interaction. The obtained ΔE values of each combination are represented as the *z*-axis in a three dimensional plot. In addition, in Confocal Laser Scanning Microscopy (CLSM) studies, Farn/MCF combination produced a great distortion in the biofilm architecture and true hyphae are rarely observed. Instead, Farn/FCZ combination showed a mild distortion in the biofilm architecture.

In a further study, Kovács et al. (2016) studied the *in vitro* activities of caspofungin (CAS) and MCF with Farn against *Candida parapsilosis* biofilms. Drug interactions were assessed with the checkerboard assay (followed by XTT staining), Bliss independence models and time-kill studies. Results showed that Farn enhanced the activity of CAS and MCF against biofilms of five clinical isolates of *C. parapsilopsis*. The $SMIC_{50}$ of CAS and MCF diminished 4-32-fold (FICI values between 0.155 and 0.5) and 8-16-fold (FICI values between 0.093 and 0.62) respectively in the presence of Farn. These findings were confirmed by time-kill curves that were prepared from the measured metabolic activity of the biofilms at different times using GraphPad Prism software. To further analyze the interactions, Bliss independence models were built using McSynergy II software. From the three dimensional plots built with the ΔE values, the units of synergy or antagonism are given in units of $\mu M^2\%$. The software defines $\mu M^2\%$ threshold values in log, as >2–5, minor synergy; >5–9, moderate synergy and >9 strong synergy. The corresponding negative values define antagonism. By using this software, both echinocandins CAS and MCF showed synergistic interactions with Farn.

According to Authors, this was the first study examining the effect of Farn in combination with echinocandins against biofilms of a non-*albicans Candida* spp.

Combination of the endoperoxide sesquiterpene lactones artemisinins with MCZ, AmpB and CAS against C. albicans biofilms. De Cremer et al. (2015) screened a repositioning-compound library of 1600 off-patent drugs and other bioactive agents (Pharmakon 1600 repositioning library) to identify compounds that can enhance the antibiofilm activity of the azole MCZ against mature *C. albicans* biofilms.

From this screening assay, artesunate, a semisynthetic derivative of artemisinin, [an important component of *Artemisia annua* L. (Asteraceae)] showed to enhance the activity of MCZ against *C. albicans* biofilms. De Cremer et al. (2015) then investigated whether artesunate could increase the activities of other type of antifungal drugs like AmpB and CAS against *C. albicans* biofilms. Results showed that in contrast to MCZ, AmpB and CAS were almost unaffected by artesunate. The semisynthetic character of artesunate would lead these results out of the scope of this Review. However, the observed potentiation of artesunate to MCZ against *C. albicans* biofilms was also observed with other natural artemisinins, name that refers to sesquiterpene compounds that have an endoperoxide bridge (O' Neill et al., 2010). So, artemisinin, dihydroartemisinin, and artemether produce 6.2-fold, 8.3-fold, and 11.4-fold reductions in the BEC_{50} of MCZ, respectively. These data

suggested that the core chemical sesquiterpene scaffold with an endoperoxide bridge could have a key role in the potentiation to MCZ.

Alkaloids as potentiators of antifungal drugs against Candida biofilms formation or eradication

Combination of berberine with FCZ and miconazole (MCZ) against C. albicans biofilms. Berberine (BBR) is a quaternary ammonium salt of the isoquinoline alkaloid group that is present in a number of species of *Berberis* genus (Berberidaceae), in *Hydrastis canadiensis* L. and in *Coptis chinensis* Franch. (Ranunculaceae), among others.

The inhibition of the *C. albicans* biofilm formation by BBR was investigated in a flow cell-model (Wei et al., 2011) in which *C. albicans* cells are attached to glass surfaces pre-conditioned with artificial saliva. Results showed that in the presence of a combination BBR/MCZ, the biofilm formation was almost completely inhibited (>91%) at 24 h, while neither BBR nor MCZ alone significantly inhibited *C. albicans* biofilm formation. This was confirmed by photomicrography.

In addition, the inhibition of the metabolic activity of established *C. albicans* biofilms by BBR/MCZ was evaluated in a MTP by exposing BBR and MCZ alone and in combination, to 24 h-growth biofilms. The $SMIC_{90}$ of MCZ in combination with BBR decreased 8-fold from 625 μg/ml ($SMIC_{90}$ of MCZ alone) to 78 μg/ml ($SMIC_{90}$ of MCZ in combination) with a FICI value of 0.25.

In a previous work, Xu et al. (2009) demonstrated that BBR in combination with FCZ augmented the production of endogenous ROS through enhancing the tricarboxylic acid cycle and inhibiting ATP-synthase activity. According to Authors, a similar mechanism may exist in the BBR/MCZ combination against biofilm cells although it was not demonstrated.

Flavonoids or their heterosides as potentiators of antifungal drugs against Candida biofilms formation or eradication

Combination of baicalin with FCZ against Candida biofilms. Baicalin (baicalein glucuronide), one of the main components of *Scutellaria baicalensis* Georgi (Lamiaceae) showed to potentiate the activity of FCZ against *C. albicans* biofilm formation which was detected with the XTT assay (Wang et al., 2015). The $SMIC_{50}$ of FCZ against FCZ-resistant *C. albicans* strains decreased from 1024 to 32–64 μg/ml (16-32-fold) when combined with baicalin, clearly showing that baicalin reversed the resistance to FCZ of FCZ-resistant *C. albicans* strains. Baicalin showed synergism against six out of the seven *C. albicans* biofilms with FICIs between 0.28 and 0.50.

Other type of compounds as potentiators of antifungal drugs against Candida biofilms formation or eradication

Combination of the microbial decapeptides tyrocidines (Trc) with AmpB and CAS against Candida biofilms. Trcs are a group of cationic cyclic decapeptides that, along with the linear gramicidins, form the secondary metabolite peptide complex tyrothricin, produced by *Bacillus aneurinolyticus* (previously known as *Bacillus brevis*) (Hotchkiss and Dubos, 1941). The tyrothricin complex was one of the first antibiotic preparations to be used as a topical antibiotic (under trade names Limex ®, Tyrosur ®). Trc were tested for its capacity of enhancing the activity of AmpB or CAS for biofilm-eradicating activity (Troskie et al., 2014). Results showed that the three major Trcs (TrcA, TrcB and TrcC) significantly increased the biofilm eradication effects of both AmpB and CAS. The three Trcs in combination with AmpB and CAS showed FICI values in the range 0.14–0.42 and 0.10–0.35 respectively. The presence of TrcA, TrcB, or TrcC decreased the BEC_{50} of CAS and AmpB up to 12- and 9-fold, respectively. In addition, using a *C. elegans* infection model, authors found that TrcA potentiated the *in vivo* activity of CAS. Therefore, Authors suggested that Trcs are promising candidates for combined treatments.

Natural products in combination with antibacterial drugs against bacterial biofilms

A summary of the natural compounds that potentiate the antibiofilm activity of antibacterial drugs is showed in Table 2 and an amplified overview is detailed below. The information in the text is ordered by the type of natural products that has potentiating capacity, such as monoterpenes and phenylpropanoids; sesquiterpenes; diterpenes; triterpenes and others.

Combination of monoterpenes and phenylpropanoids with streptomycin against biofilm-associated food-borne pathogens. The enhancing effect of Thy, Eug and Cin on the aminoglycoside antibiotic streptomycin (Str) against *L. monocytogenes* and *S.* Typhimurium biofilms, was reported by Liu et al. (2015). *L. monocytogenes* is a food-borne pathogen that may grow as biofilms on food and food-processing equipments and can cause severe and life-threatening human infections mainly in high-risk groups of patients (Morvan et al., 2010). In turn, *S.* Typhimurium that can also form biofilms on food surfaces, was identified as the leading causative agent of human salmonellosis (Liu et al., 2015) that in some cases can be fatal.

Against *L. monocytogenes* biofilms, Cin or Thy in combination with Str showed a significant reduction in whole biofilm mass as well as on live bacteria formed at 37 °C during 24 h, assessed with both the CV stain (Fig. 3a) and the MTT assay (Fig. 3b) respective of each component alone. Images from fluorescence microscopy evidenced that *L. monocytogenes* biofilms showed very few aggregates, in which there were much less viable cells that when exposed to the components alone. In turn, the combinations of Cin/Str and Eug/Str also demonstrated high antibiofilm activity against *S.* Typhimurium showing a pronounced decrease of biofilm mass and live bacteria than Str, Cin or Eug alone. These findings were also evidenced by Scanning Electron Microscopy (SEM).

In addition, immunofluorescence assays clearly showed that Cin and Thy facilitate Str access into the *L. monocytogenes* biofilms and Cin and Eug facilitate the entrance of Str into *S.* Typhimurium biofilms (Fig. 4).

Combination of sesquiterpenes with several antbiotics against staphylococcal biofilms. Two papers deal with the potentiation of antibiofilm effect of antibiotics by Farn against *Staphylococcus* spp. In a first report, established *S. aureus* biofilms were grown with various concentrations of gentamicin (Gen) in the presence of Farn also at different concentrations (Jabra-Rizk et al., 2006). Colony counts of biofilm cells after treatment showed that the mixture of Gen at 2.5x MIC (10 μg/ml) and Farn at 100 μM (22 μg/ml) reduced *S. aureus* sessile cells populations by more than 2 log units. According to Authors, the observed sensitization of resistant-*S. aureus* strains to Gen by Farn indicates a potential application of Farn as an adjuvant therapeutic agent for the prevention of *S. aureus* biofilm-related infections.

In a further paper, Pammi et al. (2011) evaluated the *in vitro* antimicrobial susceptibilities of *S. epidermidis* biofilms to Farn in combination with nafcillin (Naf) and vancomycin (Van) by using *agr* and *luxS* quorum-sensing mutants (Vuong et al., 2004) and also clinical isolates of *Staphylococcus epidermidis*. Biofilm inhibition was evaluated with the checkerboard design followed by the XTT assay. Authors tested *in vitro* equipotent (1:1 ratio), and non-equipotent (1:2 ratio) doses of effective doses of Farn and antibiotics. Also, the median-effects method was used (Chou, 2006) and the CIs were calculated. Results showed that for 90% inhibition effects of the Farn-antibiotic combinations, CI varied from 0.07 to 0.35.

Combination of diterpenes with oxacillin (Oxa) against staphylococcal biofilms. Two diterpenoids salvipisone (Salv) and aethiopinone (Aeth) isolated from hairy roots of *Salvia sclarea* L. (Lamiaceae) were tested in combination with Oxa, for their activity against *S. aureus* and *S. epidermidis* pre-formed biofilms (Walencka et al., 2007). The checkerboard design was used to determine the interactions, and the

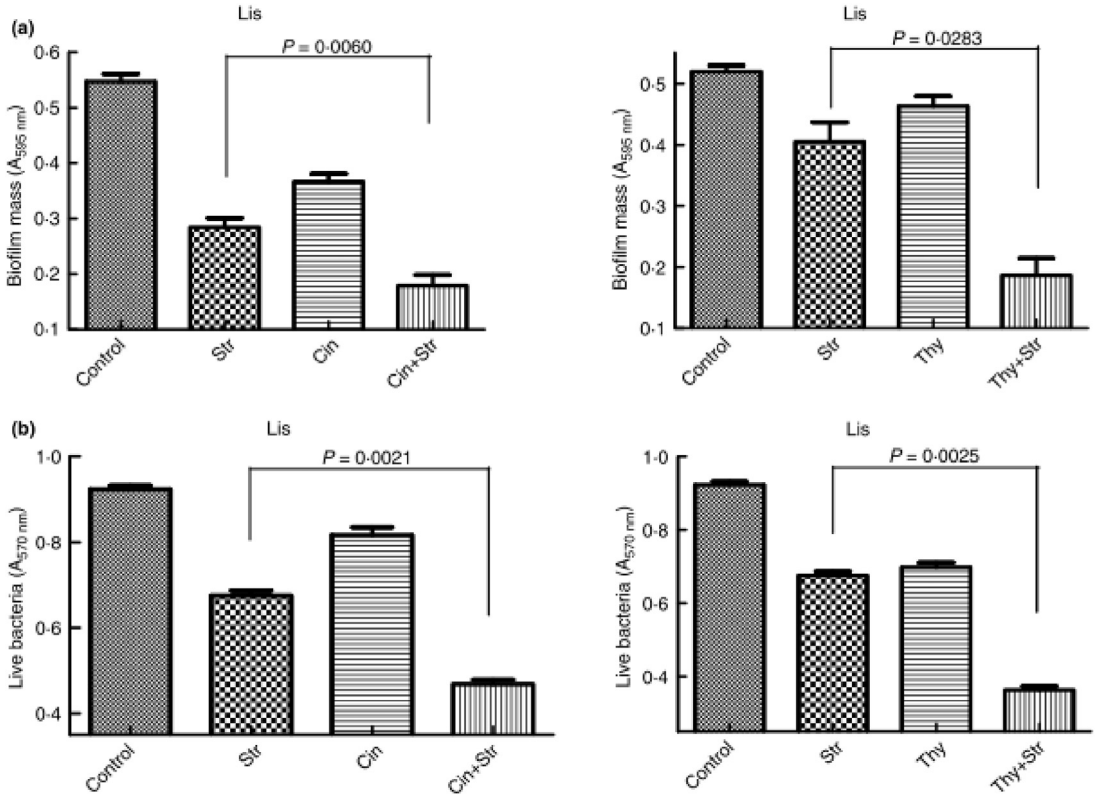


Fig. 3. Biofilms formed by *Listeria monocytogenes* exposed to cinnamaldehyde (Cin), thymol (Thy) or streptomycin (Str) alone or in combination (Cin/Str and Thy/Str). Biofilms incubated in tryptic soy broth (TSB) were used as control. Biofilm mass (a) and live bacteria (b) were quantified. These experiments were performed three times with similar results each time. Error bars represent SD. Reproduced from Liu et al. (2015), with permission (John Wiley & Sons license # 4060020183790).

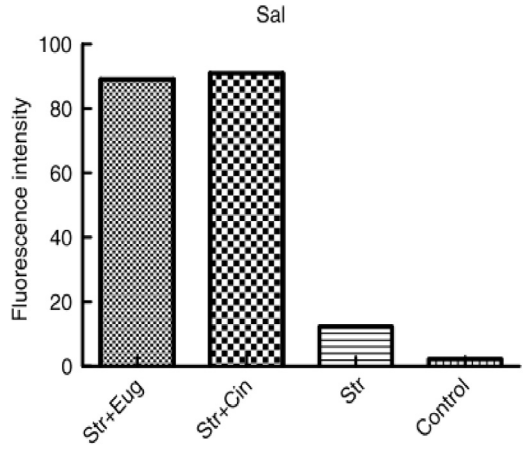


Fig. 4. *Salmonella* Typhimurium biofilms exposed to streptomycin (Str) alone, Str/eugenol (Eug) and Str/cinnamaldehyde (Cin) for 1 h. Biofilms incubated in tryptic soy broth (TSB) were used as control. Str residing in biofilms is examined by immunofluorescence. These experiments were performed twice with similar results each time. Immunoreactivity was quantified by using Image Pro Plus. [Reproduced from Liu et al. (2015), with permission (John Wiley & Sons license # 4060020183790). The fluorescence images can be seen in the original paper.

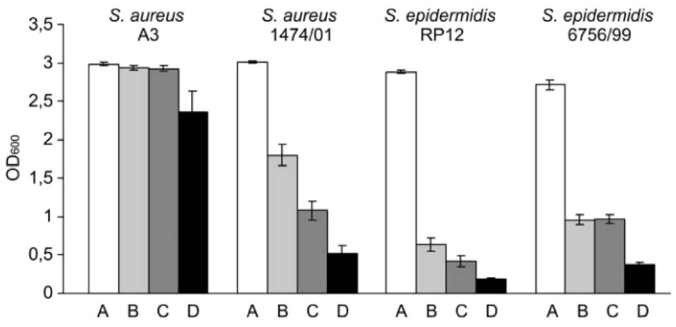


Fig. 5. The MTT reduction assay for the biofilms viability (OD_{550}) (A) Non-treated 1-day-old control biofilms; (B) Biofilms treated with oxacillin; (C, D) Biofilms treated with oxacillin/ aethiopinone (C) or oxacillin/salvipisone (D) (used at a concentration equal to MIC for planktonic culture). The influence of a single agent or their combination was evaluated after further 24 h. The experiments were repeated twice and mean data ± SD are presented. Reproduced from Walencka et al. (2007), with permission (Thieme license #4062421480502).

wells were stained with MTT. Results showed that *S. aureus* and *S. epidermidis* biomasses were reduced by the mixtures in about 40%, while the antibiotic alone was not so effective (Fig. 5).

The antibiofilm activity of Salv and Aeth in combination with Oxa was confirmed in a separate set of experiments by CLSM, which clearly showed that Salv, and to a lower extent Aeth in combination with Oxa, all at sub-inhibitory concentrations, reduced the biomass and disrupted the staphylococcal biofilm structure.

Combination of triterpenes with antibacterial drugs against bacterial biofilms. Wojnicz et al. (2015) assessed the activity of ciprofloxacin (Cip) in combination with the pentacyclic triterpenes (PTs) asiatic acid (AA) and ursolic acid (UA) on biofilm formation against an uropathogenic ATCC reference strain and 10 clinical isolates of *E. coli*. The biofilms were allowed to grow on two different surfaces: polystyrene pieces and silicone-coated latex Foley catheters with or without Cip (1/2 MIC) + AA (50 μg/ml) and Cip (1/2 MIC) + UA (50 μg/ml). After 96 h, the staining with 4′,6′-diamidino-2-phenylindole (DAPI) or triphenyltetrazolium chloride (TTC) allowed the analysis by fluorescence microscopy. In the study, PTs alone showed a negligible effect on biofilm eradication but their combination with Cip showed the

ability to disrupt mature biofilm mass on catheters. It was observed that, of the two PTs, AA was a better potentiating agent of Cip than UA, in eradicating mature biofilms, which was attributed to the differences in their structural features. AA possesses three (C-2, C-3, C-23) hydroxyl groups that make it hydrophilic. UA has only one C-3 hydroxyl group and, therefore, is hydrophobic. Probably due to its hydrophilic nature, AA better penetrates into biofilm structure improving the antimicrobial activity of Cip against sessile cells. The bactericidal effect of Cip may also be enhanced by the acidic character of PTs. The changes in pH of the growth medium caused by PTs can disturb the functioning of membrane-bound proton pumps in bacterial cells and can promote a biocidal effect of antimicrobials (Garrett et al., 2008). Such mechanism of action might explain the potentiating effect of Cip and PTs that was observed in this work.

In another study, Garo et al. (2007) evaluated the activities of AA and corosolic acid (CA) on *P. aeruginosa* biofilms grown in rotating disk reactors (RDRs) (Biosurface Technologies Corporation, Bozeman, MT, USA) that produces a non-mucoid *Pseudomonas aeruginosa* biofilm in combination with Tobramycin (Tobra). For each test, two RDRs were operated in parallel, with one receiving the test compound and the other serving as an untreated control. For each pair of RDRs run in parallel, four sets were obtained: (i) the test compound with antibiotic in combination; (ii) the test compound alone; (iii) the antibiotic alone and (iv), no treatment. Then RDRs were inoculated with *P. aeruginosa* strain. After incubation for 24 h, viable cell density was measured and expressed as its log density (LD). The Statistical Interaction is calculated as follows = Log reduction (LR) for the test compound plus the antibiotic - LR for the test compound alone - LR for the antibiotic alone. Synergism is indicated by a positive Statistical Interaction. In this work, Authors demonstrated that both the AA and the CA show a positive interaction with Tobra against established *P. aeruginosa* biofilms.

Chitosan as facilitator of antibiotic penetration into biofilms. Chitosan, a polycationic polysaccharide composed of randomly distributed β-(1→4)-linked *D*-glucosamine and N-acetyl-*D*-glucosamine, is known by its non-toxicity, biocompatibility and by its inherent antimicrobial properties, including antibiofilm activities, against several microorganisms (Lim and Hudson, 2003).

Mu et al. (2014a) found that the combination of amikacin (aminoglycoside-type) with chitosan, tested with the checkerboard design and further stained with CV and MTT, improved the disruption of *L. monocytogenes* biofilms while with either clindamycin (lincosamide-type), Van (glycopeptide-type) or erythromycin (macrolide-type) this effect was not observed. Amikacin in the presence of chitosan killed more live cells on 24 h-established biofilms than amikacin alone. This was corroborated by fluorescence microscopy in which the architecture of *L. monocytogenes* biofilms exposed to amikacin/chitosan combination for 12 or 24 h showed fewer scattered cell aggregates when compared to that of components alone. Considering the previous finding that the antimicrobial activity of chitosan was dependent on its molecular weight, Mu et al. (2014a) tested chitosan of three different masses from 8 to 180 *kDa* finding that chitosan of 13 kDa elicited the more striking antibiofilm activity for the combination amikacin/chitosan against *L. monocytogenes*, while in contrast, chitosan of 3 and 180 kDa appeared to increase biofilm mass. It was also demonstrated that chitosan with a high deacetylation degree (88%) produced an optimal combination effect with amikacin.

In a second paper, Mu et al. (2014b) reported that the short- and long-term treatments with chitosan, improved the efficacy of Gen on *L. monocytogenes* biofilms but this effect was not observed with other type of antibiotics such as rifampicin, tetracycline or carbenicillin. Chitosan produced a 10-fold decrease of the BEC_{50} of Gen against *L. monocytogenes* biofilms, decreasing biofilm mass as well as viable cells than each partner alone after short (6–12 h) or long (24 h) treatments. These results were corroborated with fluorescence microscopy, CLSM and SEM in which fewer scattered cells aggregates in *L. monocytogenes* biofilms were observed after exposed to the combination than when exposed to each component alone. Regarding the influence of the molecular mass of chitosan on the potentiation of Gen, all three chitosan/Gen combinations (3, 13 and 180 *kDa*) reduced biofilm mass, but the highest activity was displayed by the chitosan of 13 *kDa*. As already observed in the chitosan/amikacin mixture (Mu et al, 2014a), chitosan/Gen combinations with the highest deacetylation degree (88%) displayed the strongest antibiofilm activity (Mu et al. 2014b). Chitosan was also effective in the eradication of biofilms from two other *Listeria* spp. such as *L. welshimeri* and *L. innocua*. In order to have a look into the mechanism by which chitosan potentiated the effect of Gen, the antibiotic residing in biofilms was examined by immunofluorescence. Results showed that chitosan facilitated the penetration of Gen into *L. monocytogenes* biofilms.

Conclusions and perspectives

Most chronic and difficult to eradicate infectious diseases are caused by sessile microorganisms organized in well-structured biofilms that are highly resistant to antimicrobial drugs in clinical use. In recent years many efforts have been made in the exploration of new and effective natural compounds with antibiofilm effects (Walencka et al., 2007). But, however, no natural product has been discovered to date that can completely eradicate biofilms on its own. As a consequence, a new strategy consisting on the enhancement of the activity of known antimicrobial compounds by low-MW natural products, has emerged with the aim of finding mixtures that can be developed as hybrid therapies for eradicating established microbial biofilms or preventing its formation (Bink et al., 2011).

Regarding combinations between an antifungal drug and a natural product against fungal biofilms, it is worth to take into account that not many papers have been published on this issue up to date, and that all the published works are from 2011 to date (one in each 2011, 2012 and 2013, two in 2014, three in 2015 and one in 2016). Eight over the nine collected works reported *in vitro* studies while only one performed *in vivo* assays by using *Caenorhabditis elegans* nematodes. Among the *in vitro* studies, the checkerboard design with the further calculation of FICI was the most used method to assess the interactions. All *in vitro* studies use biofilms of the *Candida* genus (eight *C. albicans* and one *C. parapsilopsis*), which is probably due to the genus *Candida,* and particularly the sp. *C. albicans* predominates in biofilm-associated fungal infections. It is worth to note that *Candida* sessile cells are up to 1000-fold more azole-resistant than their planktonic counterparts (Ramage et al., 2012; Doke et al., 2014, Seneviratne et al., 2008, 2009) which, as a difference with sessile cells, can often be treated adequately using azoles and other available antifungals. Six over the nine works used FCZ as the antifungal drug combined with a natural low-MW product and in all cases potentiation was observed. Another azole, MCZ, was used in three works with positive results. In contrast, AmpB that was used in four works, only showed synergism in one of them. The echinocandins MCF and CAS in combination showed enhanced activity in the four works in which they were used. Among the structures of the potentiators of antifungal drugs against fungal biofilms, 67% were either monoterpenes such as Thy and Carv or sesquiterpenes such as Farn and artemisinins. The remaining 33% that showed enhancing antibiofilm capacity of the antifungal drugs were phenylpropanoids such as Eug and Cin, the flavonoid heteroside baicalin, the alkaloid BBR and the decapeptides Trcs.

Regarding combinations between an antibacterial drug and a natural product against bacterial biofilms, it is worth to take into account that, as with fungal biofilms, not many papers have been published on this issue up to date, and all the published works are from 2006 to date (one in 2006, two in 2007, one in 2011, two in 2014 and two in 2015). In all works, *in vitro* studies were performed by using several methods of higher variety than the used against fungal biofilms. Of the eight collected studies, three use bioflms of gram-(+) species of the

Staphylococcus genus (two *S. aureus* and one *S. aureus* plus *S. epidermidis*), one uses biofilms of the gram (-) sp. *E. coli*, and the remaining studies use the gram (+) *L. monocytogenes* (3) or the gram (-) spp. *S.* Typhimurium (1) or *P. aeruginosa* (1) biofilms. As a difference with the activities of combinations on fungal biofilms, several type of antibacterial drugs were used in the combinations: the aminoglycosides Str, Gen, amikacin and Tobra, the β-lactams Naf and Oxa, the glucopeptide Van, and the fluoroquinolone Cip. Among the type of natural low-MW products that showed enhancing antibiofilm activities of antibacterial drugs, 75% were terpenes, such as the monoterpene Thy, the sesquiterpene Farn, the diterpenes Salv and Aeth, and the triterpenes AA, UA, and CA. Interesting enough, chitosan facilitates the penetration of the antibacterial drugs amikacin or Gen within the biofilm.

The potentiation capacity of low-MW natural products on the antibiofilm effect of antimicrobial drugs opens a wide range of possibilities for the combination antimicrobial therapy, mainly with terpene compounds. As stated above, the 'enhancement' of the antibiofilm capacity of antimicrobial drugs takes advantage of the safe toxicity profile and the known dosing regimens of existing drugs, thus making the possible cost of reformulating them, and performing new clinical trials, considerably lower than that for the development of a new drug from the very beginning (Delattin et al., 2014). Since most studies reported up to date have been performed *in vitro,* further *in vivo* assays are required to determine the relevance of these combinations for pharmaceutical development, opening the possibility of testing them in clinical trials. The testing in human beings will be very welcome for coping the difficult to treat biofilm-associated infections.

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Conflict of interest

All Authors declare that they do not have any conflict of interest.

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