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Essential oils of *Pinus sylvestris, Citrus limon* and *Origanum vulgare* exhibit high bactericidal and anti-biofilm activities against *Neisseria gonorrhoeae* and *Streptococcus suis*

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ABSTRACT

Antimicrobial resistance is a worldwide problem that urges novel alternatives to treat infections. In attempts to find novel molecules, we assess the antimicrobial potential of seven essential oils (EO) of different plants (*Pinus sylvestris, Citrus limon, Origanum vulgare, Cymbopogon martini, Cinnamomum cassia, Melaleuca alternifolia* and *Eucalyptus globulus*) against two multidrug-resistant bacteria species, i.e. *Neisseria gonorrhoeae* and *Streptococcus suis*. EOs of *P. sylvestris* and *C. limon* revealed higher bactericidal activity (MIC $\leq$ 0.5 mg/mL) and capacity to rapidly disperse biofilms of several *N. gonorrhoeae* clinical isolates than other EOs. Examination of biofilms exposed to both EO by electron microscopy revealed a reduction of bacterial aggregates, high production of extracellular vesicles, and alteration of cell integrity. This activity was dose-dependent and was enhanced in DNase I-treated biofilms. Antibiotic susceptibility studies confirmed that both EOs affected the outer membrane permeability, and analysis of EO- susceptibility of an LPS-deficient mutant suggested that both EO target the LPS bilayer. Further analysis revealed that α- and β-pinene and D-limonene, components of both EO, contribute to such activity. EO of *C. martini*, *C. cassia,* and *O. vulgare* exhibited promising antimicrobial activity (MIC $\leq$ 0.5 mg/mL) against *S. suis*, but only EO of *O. vulgare* exhibited a high biofilm dispersal activity, which was also confirmed by electron microscopy studies. To conclude, the EO of *P. sylvestris, C. limon* and *O. vulgare* studied in this work exhibit bactericidal and anti-biofilm activities against gonococcus and streptococcus, respectively.

1. Introduction

Antimicrobial resistance (AMR) is a worldwide problem. Only in 2019, around 541,000 deaths were associated with bacterial AMR, of which 133,000 were directly attributable to AMR bacteria in the WHO European region [1]. Accumulated evidence shows that bacterial survival to antibiotics can be caused by two general mechanisms, including AMR [2] and antibiotic tolerance [3–5]. The excessive use of antimicrobials in human, veterinary medicine, agriculture, or aquaculture is a major reason to contribute to the selection of virulent and multi-drug resistant (MDR) isolates. Notably, many AMR genes can be transferred between pathogens by horizontal gene exchange, which has resulted in the rise of clinical MDR isolates. Well-known examples are methicillin-resistant *Staphylococcus aureus*, *Escherichia coli* ST131, or Enterobacteriaceae-producing extended-spectrum β-lactamases [6–8]. Many of these pathogens are associated with recurrent infections in hospital and health-care settings, but MDR pathogens are also transmitted within the community, or by MDR zoonotic bacteria selected in food animals. Good examples are *Neisseria gonorrhoeae* and *Streptococcus suis*, respectively.

N. gonorrhoeae is a Gram-negative diplococci bacterium that colonizes the genital urinary tract of men and women and ocular, nasopharyngeal, and anal mucosa [9]. It is the causative agent of gonorrhea, a disease that involves severe complications such as pelvic inflammatory

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disease, infertility, prostatitis, and ectopic pregnancy. According to WHO, gonorrhea is the third most prevalent sexually transmitted infection globally. If untreated, the infection can disseminate and cause arthritis and endocarditis. Without an available commercial vaccine, antibiotics are the only method for controlling gonorrhea, but this bacterium has developed a diversity of resistance mechanisms against antibiotics of different families [10,11], including sulfonamides, β-lactams, tetracyclines, macrolides, and fluoroquinolones. Alarmingly, there is increased resistance to the last available first-line treatment for gonorrhea, including cefixime, ceftriaxone, and extended-spectrum cephalosporins [12]. Additionally, *N. gonorrhoeae* has a high capacity to form biofilms [9], which contribute to antimicrobial tolerance. Thus, gonococci is considered an MDR pathogen, and WHO classified it as a high priority for research and development of new antibiotics [13].

S. suis is a Gram-positive bacteria that resides in the upper respiratory tract, gut, and genitals of pigs [14]. This bacterium causes septicemia, arthritis, pneumonia, and sudden death in pigs also known as streptococcal swine disease. The disease is a major cause of mortality and important economic losses in the pig production industry globally [15,16]. But, *S. suis*, is also a zoonotic agent, able to move from pigs to humans by direct contact with infected animals, meat manipulation, or consumption of raw meat. It can cause similar symptoms in humans as those described in pigs, it is the leading cause of meningitis in some Asiatic countries [17,18]. *S. suis* infections in pig farms have been prevented and treated with antibiotics, which have promoted an increase in AMR in many families of antibiotics. Remarkably, high rates of AMR to tetracyclines, aminoglycosides, and lincosamides were globally reported [19], and there is a substantial increase in β-lactams in certain areas. Importantly, *S. suis* acts as a reservoir of AMR genes that are transferred to other human pathogens through mobile genetic elements [19]. There is no effective vaccine against *S. suis* [20], thus the high pressure to reduce antibiotic administration in the veterinary field urges antibiotic substitutes to prevent and treat streptococcal swine disease.

In recent years, the search for new antibiotic substitutes for human and veterinary bacteria has increased. Many works have investigated EO and plant extracts due to their bioactive properties, some of which include antimicrobial capacities. Because of the chemical complexity of EO, their anti-bacterial activity is not only attributable to just a component and specific mechanism. The most observed effect on EO-exposed bacteria is membrane destabilization. Indeed, some EO components interact with phospholipids and lipopolysaccharides, alter fatty acid composition, inhibit glucose uptake and enzyme activity, and interacts with ions and ATP, among others [21]. Good examples are terpenoids, such as carvacrol, phenolic compounds, such as eugenol, or flavonoids, such as cinnanmaldehyde. As a result, the proton-motive force collapses and cells become leaky, leading to cell lysis. Other reported bioactivities of EO on bacteria include block toxin production, inhibition of motility, biofilm formation, or quorum sensing system [21,22]. Together, accumulated data suggest that EO can be an alternative to antibiotics or even could be used as adjuvant for antimicrobials using combinatorial therapy [23].

Here, we investigated the anti-bactericidal potential of seven EO of different plant species. An analysis of their composition is presented. Some EO contain bioactive substances including carvacrol, thymol, or cinnamaldehyde. Thus, we hypothesized that they could have antimicrobial activities against *N. gonorrhoeae* and *S. suis* infections. Their activity was tested against reference strains and drug-resistant clinical isolates, some of which are MDR. Also, the anti-biofilm activity, the mechanism of action, and the activity of some components were explored.

2. Materials and methods

2.1. EO

Seven EO obtained from cultivated plants of *Pinus sylvestris* L. (Batch number: OF35625), *Citrus limon* L. (Batch number OF36505), *Cymbopogon martini* (Roxb) Wats var. *motia* (Batch number: OF37522), *Cinnamomum cassia* (L.) J.Presl (Batch number OF37227), *Melaleuca alternifolia* (Maiden and Betche) Cheel (Batch number: OF38956), *Eucalyptus globulus* Labill (Batch number: OF37128), *Origanum vulgare* L. (Batch number: BOVH112) were used in this study and provided by Pranarôm España. The main characteristics of EOs are: *Pinus sylvestris* (plant origin: leaves; country: Austria; batch number: OF35625; date of obtention: 06/2018) *Citrus limon* (plant origin: peel; country: Argentina; batch number OF36505; date of obtention: 01/2019), *Cymbopogon martinii var. motia* (plant origin: aerial; country: India; batch number: OF37522; date of obtention: 08/2018), *Cinnamomum cassia* (plant origin: bark; country: China; batch number OF37227: date of obtention: 01/2018), *Melaleuca alternifolia* (plant origin: leaves; country: Australia; batch number: OF38956; date of obtention: 04/2019), *Eucalyptus globulus* (plant origin: leaves; country: China; batch number: OF37128; date of obtention: 05/2019), *Origanum vulgare* (plant origin: aerial with flowers; country: Hungary; batch number: BOVH112; date of obtention: 06/2018). The composition of each EO was determined using GC-FID (gas chromatography followed by a flame ionization detector) and is included in Table S2.

2.2. Bacterial strains and growth conditions

All strains used in this study are listed in Table 1. A panel of seven *N. gonorrhoeae* clinical isolates from people with gonococcal disease were recovered from the Hospital Universitario de Santiago de Compostela (Galicia, Spain), and the Hospital Universitario Miguel Servet (Aragón, Spain). The gonococcal reference strain FA1090 (ATCC700825)[24], *Neisseria meningitidis* strain HB-1 [25] and its LPS-deficient mutant HB-1Δ*lpxA* [26] were also included. Finally, a panel of five clinical isolates of *S. suis,* previously characterized (Uruén et al., MS in preparation), and the reference strain P1/7 [27,28] were used.

Gonococcal and meningococcal strains were grown overnight on GC medium base (Condalab, Madrid, Spain) supplemented with Isovitalex (Oxoid, Basingstoke, United Kingdom) at 37ºC in a candle jar. For *Neisseria* liquid cultures, bacteria were collected from GC plates and dispersed in Tryptic Soy Broth (TSB) (Oxoid, Basingstoke, Hampshire, England) supplemented with Isovitalex, and, when required, with dimethyl sulfoxide (DMSO). Bacteria cultures were incubated at 37ºC with constant shaking at 115 *rpm* for 6 h until they reached an Optical Density at 550 nm (OD_{550}) of 1. *S. suis* strains were grown on Todd Hewitt agar (THA) in a candle jar. For liquid cultures, *S. suis* grown on plates were dispersed in Todd Hewitt Broth (THB) (Condalab, Madrid, Spain) or Muller Hinton Broth (MHB) (Oxoid, Basingstoke, United Kingdom) supplemented, when required, with DMSO, and incubated at 37ºC without shacking until an OD_{600} of 0.6.

2.3. Strain constructions

Capsule synthesis in *S. suis* P1/7 was inactivated by partial replacement of *cps2E-F* with a spectinomycin-resistance cassette following the strategy earlier described [29].

2.4. Gonococcal multi locus sequence typing

Gonococcal clinical isolates were classified in Sequence Types based upon sequence analysis of seven conserved house-keeping genes (*abcZ, adk, aroE, fum, gdh, pdhC* and *pgm*). For each isolate, the genes of interest were partially amplified by PCR using primers pairs described in Table S1, the resulting fragments were purified using the FavorPrep GEL/PCR Purification Kit (Favorgen, Ibian, Zaragoza, Spain), and sequenced at the Stabvida sequencing service (Portugal). Assignation of STs was performed by comparing allelic profiles obtained from an internal region of each gene in the gonococcal MLST database [30].

Table 1
Strains used in this study.

Strain	Relevant characteristics[a]	ST	Reference or source[b]
Neisseria gonorrhoeae			
FA1090	Reference strain	ST1899	[24]
NgS1	Clinical isolate. Resistance to tetracycline and ciprofloxacin.	ST8776	This study
NgS7	Clinical isolate. Resistance to tetracycline and ciprofloxacin.	ST7823	This study
NgS10	Clinical isolate. Resistance to tetracycline, ciprofloxacin and azithromycin.	ST13527	This study
NgS11	Clinical isolate. Resistance to tetracycline and ciprofloxacin.	ST12974	This study
NgZ2	Clinical isolate. Resistance to tetracycline, ciprofloxacin and cefixime.	ST7822	This study
NgZ3	Clinical isolate. Resistance to tetracycline, ciprofloxacin and azithromycin.	ST7822	This study
NgZ9	Clinical isolate. Resistance to tetracycline and ciprofloxacin.	ST10314	This study
Neisseria meningitidis			
HB-1	Derivative of *N. meningitidis* H44/76 of clonal complex 32 with capsule locus replaced by an erythromycin resistant cassette.	ST11	[25]
HB-1Δ*LpxA*	Derivative of *N. meningitidis* HB-1 with *lpxA* locus replaced by a kanamycin Resistant cassette.	ST11	[26]
Streptococcus suis			
P1/7	Reference strain	ST1	[27]
P1/7 Δ*cps2E-F*	Derivative of P1/7 with capsule locus replaced by a spectinomycin resistant cassette.	ST1	This study
Ss_45	Clinical isolate. Intermediate resistance to enrofloxacin, resistance to penicillin G, clindamycin, erythromycin, trimethoprim-sulfamethoxazole and tetracycline.	ST3	Unpublished
Ss_52	Clinical isolate. Intermediate resistance to clindamycin and erythromycin.	ST1627	Unpublished
Ss_70	Clinical isolate. Intermediate resistance to enrofloxacin, and resistance to penicillin G, clindamycin and erythromycin.	ST1653	Unpublished
Ss_166	Clinical isolate. Intermediate resistance to penicillin G, enrofloxacin, clindamycin, erythromycin and tetracycline.	ST1823	Unpublished
Ss_168	Clinical isolate. Resistance to penicillin G, enrofloxacin, clindamycin and erythromycin tetracycline.	ST1824	Unpublished

[a] MICs values for antibiotics are listed in Table S3.
[b] unpublished, results are part of a study to will be published somewhere else.

2.5. Gonococcal antimicrobial susceptibility testing

The Minimum Inhibitory Concentration (MIC) of Cefixime, Ceftriaxone, Ciprofloxacin, Tetracycline, and Azithromycin of the *N. gonorrhoeae* clinical isolates was determined using E-test method (Liofilchem, Spain). Clinical breakpoints for susceptibility and resistance to antibiotics were considered according to The European Committee on Antimicrobial Susceptibility Testing (EUCAST) [31]. Accordingly, strains were classified as susceptible, intermedium, or resistant.

2.6. EO susceptibility testing

The MIC of EO was determined by broth microdilution method in the 96-well microtiter plates (Microtiter 96 plates fund "U", DELTALAB SL, Barcelona, Spain) as previously described [32] with slight modifications. Firstly, the concentration of bacteria to be challenged was optimized to the minimal colony-forming units (CFU) that resulted in reproducible growth in our lab settings for all tested strains. Thus, *Neisseria* was adjusted at 1×10^8 CFUs/mL in TSB and *S. suis* at 1×10^5 CFUs/mL in MHB. Then, bacterial cultures were mixed with serial decreasing concentrations of each EO. 100 mg/mL of ampicillin was included as internal control. The plates were sealed and incubated at 37ºC for 24 h. For *Neisseria* cultures, plates were incubated in agitation at 150 *rpm*. MIC was determined as the minimum concentration of each EO that fully inhibited bacterial growth (not visible cell pellet). The MIC of purified (+)-α-Pinene, (-)-β-Pinene, and R(+)-Limonene, all of them provided by Extrasynthese, Genay (France), was evaluated using the same method. Results were expressed as the average of three independent cultures. To determine Minimal Bactericidal Concentration (MBC), the plate microdilution method was used as described above. After different incubation periods, bacteria suspension was centrifuged for 3 min at 8000 *rpm* in a table centrifuge, and the resulting bacterial pellet was suspended in a liquid medium, and dispersed on GC or THA plates. Bacterial growth was determined after 24 h. MBC was considered as the minimum concentration of each EO that inhibited 99,9% of initial inoculum. For killing dynamics, bacteria were incubated with pure substances diluted in THB supplemented with DMSO, and incubated at different time periods, after which CFU counting was determined.

2.7. Antibiotic permeability assays

Bacteria grown to exponential phase was adjusted at 4.3×10^7 CFU/mL and exposed to subMIC concentration of antibiotics and/or EO for 30 min. Then, bacteria were centrifuged for 3 min at 8000 *rpm*, and the resulting pellet was suspended in TSB. The bacterial suspension was serially diluted, and dispersed onto GC plates, which were incubated at 37ºC for 18 h for CFU counting. Antibiotic and EO concentrations shown reproducible results were selected. Results were from at least three independent cultures with internal replicates.

2.8. Biofilm formation and dispersal assays

Bacterial cultures were prepared to study the capacity of each EO to inhibit and disperse biofilm formation. Biofilm formation of *Neisseria sp* on abiotic surfaces has been previously described by us [33,34]. Briefly, *Neisseria* was grown in TSB for 6 h until reaching a OD_{550} of 1, while *S. suis* was grown overnight in THB until reaching OD_{600} of 1. Then, 500 μL and 700 μL of gonococcal and streptococcal cultures, respectively, were placed in 24-well plates (TPP, Tissue Culture Test Plate 24 F, Switzerland) and incubated for 1, 4 or 24 h in the presence or absence of 5 and 0.5 mg/mL of each EO. Then, the supernatant was removed from each well, and the biofilm was washed with deionized water. Finally, the biomass was stained with crystal violet and quantified as early described [35]. To evaluate the capacity of EO to disperse preformed biofilms, different concentrations of EO, 100 μg/mL of DNAse I (Sigma Aldrich) or ampicillin (100 mg/mL as internal control), were exogenously added to 1 h older biofilms, and incubated at different time periods. The resulting supernatant was removed, biofilms were washed, and the surface attached biomass was quantified as above. To determinate bacterial viability on biofilms, EO-treated and untreated biofilms (as described above) were swabbed with a single swab, resuspended in PBS, and adjusted to an OD_{600} of 0.05. Then, serial dilution of the mixture was prepared and spread on solid culture plates. After 24 h incubation at 37 °C, the CFUs were counted and expressed as CFU/mL. The results represent the average and standard variations of at least three independent cultures.

2.9. Electron microscopy assays

To perform the scanning electron microscopy assays, biofilms were formed on round glasses (round cover glasses 12 mm, b/100, RS France) and treated as described above. Then, biofilms were washed with 0.1 mM PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1,8 mM KH_2PO_4) and fixed with fixative solution composed of PBS and 1% glutaraldehyde during at least 2 h. All microscopic observations and image acquisitions were performed using a scanning electron microscope (JOEL JSM 6360-LV). For transmission electron microscopy assays, *Neisseria* was grown until the exponential phase, adjusted at 0.5 MacFarland, and exposed to EO at working dilutions for 30 min. Bacteria were washed three times with PBS by centrifugation (10 min at 7000 *rpm*), and the resulting pellet was covered with a solution of PBS containing 2% of pure agarose (Condalab, Madrid, Spain). Agar was sliced into 12 mm pieces, immersed in a fixative solution overnight, and stained at the microscopy service of the University of Zaragoza following standard procedures. The samples were visualized under a transmission electron microscope (JOEL 1010a 80 KV).

2.10. Statistical analysis

For statistical comparisons, data from at least three independent experiments performed in duplicate were used. Data were analysed using an unpaired t-test with GRAPH PAD v6 for statistical comparisons.

3. Results

3.1. In vitro evaluation of antibacterial activity

To evaluate the bioactivity of EO against *N. gonorrhoeae* and *S. suis*, we analyzed the MIC and MBC values using the microdilution method. This method has been indicated as the most appropriate method in assessing anti-microbial effect of EO [35]. Also, broth dilution methods for inhibitory determination of components were recommended by CLSI [36]. To facilitate the solubilization of the EO in the bacterial culture medium, the polar solvent DMSO was added to the bacterial culture medium after evaluating the impact of different DMSO concentrations on bacterial growth in reference strains. Results are shown in Fig. S1 and detailed in Text S1 in supplemental material. Based on results, we used 0.5% of DMSO. Then, the antibacterial effect of EO was quantitatively tested by the MIC and MBC values against *N. gonorrhoeae* and *S. suis* reference strains, and a panel of clinical isolates (Table 1). MLST characterization evidenced that *N. gonorrhoeae* belonged to different genetic lineages, but two isolates (NgZ2 and NgZ3) were of the same ST (ST7822). Antibiotic susceptibility testing revealed that all strains were resistant to at least one antibiotic, and two isolates were resistant to three antibiotics (Table 1 and expanded in Table S3**)**. As for *S. suis*, all isolates belonged to different STs and four were resistant to more than 3 families of antibiotics (Table 1 and expanded in Table S3). MLST-based phylogenetic analysis evidenced large genetic differences and clustering with globally distributed STs (Fig. S2 in supplemental material). Thus, our panel of strains is genetically and phenotypically very diverse.

The EO- MIC and MBC values for reference strains and the average for all isolates included in this study are summarized in Table 2 (expanded in Table S3). Based on average activity against all tested strains, three categories of antibacterial activity were established: high activity (MIC < 0.5 mg/mL), moderate activity (MIC ranging between 0.5 and 1 mg/mL), and low activity (MIC > 1–5 mg/mL). Several EO had high activity against *N. gonorrhoeae,* including those of *P. sylvestris, C. limon, C. cassia,* and *O. vulgare*, while those of *C. martinii* and *M. alternofolia* had moderate activity, and EO of *E. globulus* had low activity (Table 2). Surprisingly, all EO revealed similar or lower MIC values against drug-resistant isolates than the reference strain FA1090 (Table S3). Analysis of MBC against strain FA1090 revealed similar MBC and MIC values, but higher with EO of *E. globulus* and *M. alternifolia*. EO of *C. martinii*, *C. cassia* and *O. vulgare* exhibited high activity against *S. suis* strains*,* while those of *M. alternifolia* revealed moderate activity, and those of *P. sylvestris*, *C. limon* and *E. globulus* exhibited low activity (Table 2). However, their activity varied within strains, generally EO exhibited higher activity against clinical isolates than the reference strain P1/7 (Table S3). Analysis of MBC values of EO of *O. vulgare*, *E. globulus*, and *C. cassia* against strain P1/7 showed similar values to MIC, while higher MBC values were obtained with those of *P. sylvestris*, *C. limon*, *C. martini* and *M. alternifolia.*

3.2. Activity of EO against gonococci and streptococci biofilms

An important feature of a novel antimicrobial agent is its capacity to inhibit biofilm formation or disperse preformed biofilms. Biofilms generate tolerance to antibiotics even in the absence of AMR genes [5]. Thus, we evaluated the capacity of EO to inhibit biofilm formation in *N. gonorrhoeae* and *S. suis*. First, the dynamics of biofilm formation of *N. gonorrhoeae* and *S. suis* strains were studied under static growth conditions (detailed in Text S1 and Fig. S3 in supplemental material). *N. gonorrhoeae* FA1090 formed biofilms that were detected after 1 h of growth. After 4 h, the biofilm biomass increased slightly but two-fold after 24 h of growth (Fig. S3A in Suplemental material). Bacterial cultures of strain FA1090 were incubated with EO for 1 h to study their effect on inhibition of biofilm formation. A reduction of 20% of biofilm formation was observed when biofilms grew in presence EO of *C. cassia* and *M. alternifolia* (Fig. 1A). In contrast, EO of *O. vulgare* increased biofilm biomass by 20%. When we compared the bacteria viability on treated and untreated biofilms by CFU counting, EO of *C. cassia* and *O. vulgare* decreased significantly the viability of biofilm bacteria (Fig. S4A). Then, we tested whether EO dispersed preformed biofilms. To do this, 1 h-old biofilms were washed, incubated with EO, and quantified biofilm biomass. After 1 h of treatment, EO of *P. sylvestris, C. limon,* and *C. cassia* dispersed about 30–40% of preformed biofilms (Fig. 1B). After 24 h of treatment, EO of *P. sylvestris, C. limon, C. martinii, M. alternifolia, E. globulus* and *O. vulgare* dispersed about 40%− 60% of the biofilm biomass (Fig. 1B). These data indicate that several EO can

Table 2
Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of EO against eight *N. gonorrhoeae* and seven*S. suis* strains. Data represent the average and the range obtained of all isolates of *N. gonorrhoeae* and *S. suis,* respectively. Included are values for the reference *N. gonorrhoeae* strain FA1090 and *S. suis* strain P1/7. The MIC and MBC values are expressed in mg/mL.

Essential Oil	*N. gonorrhoeae*			*S. suis*		
	MIC $_{(n=8)}$[a]	MIC_{FA1090}	MBC_{FA1090}	MIC $_{(n=6)}$[a]	$MIC_{P1/7}$	$MBC_{P1/7}$
P. sylvestris	0.3 (1–0.06)	1	1	2 (4–0.12)	2	4
C. limon	0.45 (0.5–0.06)	0.5	0.5	12 (16–8)	10	20
C. martinii	0.7 (2–0.06)	2	2	0.5 (0.25–1)	1	2
C. cassia	0.2 (1–0.06)	0.5	0.5	0.5 (1–0.25)	0.5	0.5
M. alternifolia	0.9 (1–0.2)	1	2	1.4 (4–0.12)	5	10
E. globulus	2.4 (8–0.5)	8	11	4 (8–2)	10	10
O. vulgare	0.12 (1–0.12)	0.12	0.12	0.3 (1–0.12)	0.5	0.5

[a] MICs values for each strain are listed in Table S3.

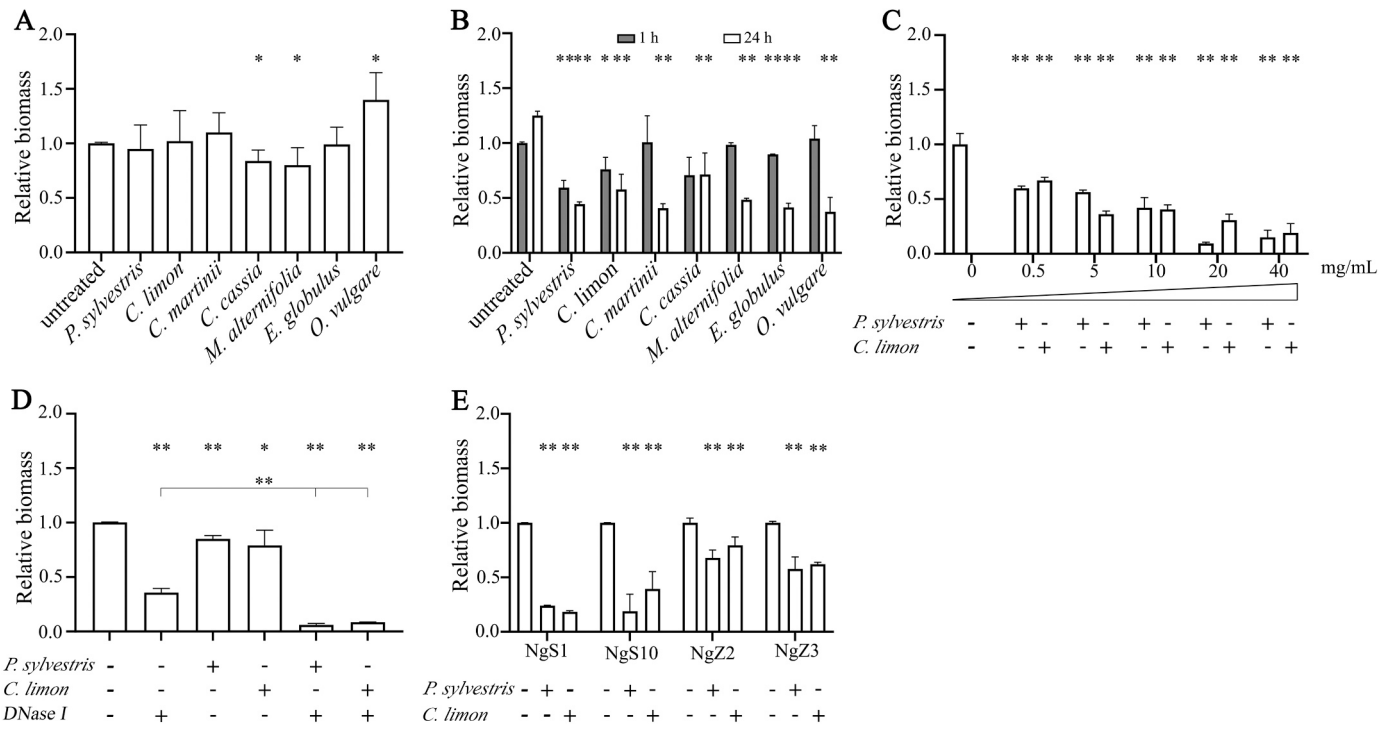

Fig. 1. Activity of Essential Oils (EO) on gonococci biofilms. (A) Impact of 0.5 mg/mL of EO on initial biofilm formation of *N. gonorrhoeae* strain FA1090. (B) Impact of 0.5 mg/mL of EO against 1 h old biofilms of *N. gonorrhoeae* strain FA1090 after 1 h and 24 h of incubation. (C) Effect of different concentrations of EO of *P. sylvestris* and *C. limon* on dispersion of biofilms of FA1090. (D) Effect of combination of 0.5 mg/mL of EO and 100 μg/mL DNaseI on biofilm dispersal of FA1090 as indicated. (E) Dispersal of 1 h old biofilms formed by clinical isolates of *N. gonorrhoeae* incubated with 5 mg/mL of EO of *P. sylvestris* and *C. limon.* Biofilms were formed in 24 well plates, washed and incubated with EO, and quantified after different incubation periods. The results show the average of at least 3 independent growth cultures and the bars represents the standard deviation. Statistically significant differences (unpaired *t* test,) with untreated control, otherwise indicated, at $p < 0.05$ and $p < 0.001$ are shown with one or two asterisks, respectively.

disperse biofilms, but *P. sylvestris* and *C. limon* exhibited activity at short incubation periods. We then focused on the activity of both EO. First, we evaluated whether their biofilm dispersal activity increased at high concentrations. Results showed that both EO functioned in a dose-dependent manner (Fig. 1C); around 20–40 mg/mL of both EO dispersed 95% of the preformed biofilm of FA1090. Then, we investigated whether such activity could be enhanced by the co-addition of other EO that exhibited low MIC values (Table 2), i.e. EO of *C. cassia* and *O. vulgare*. Unfortunately, co-administration of the mentioned EO with EO of *P. sylvestris* and *C. limon* did not enhance biofilm dispersal activity (data not shown). Since it was reported that biofilm formation of *N. gonorrhoeae* FA1090 is inhibited by DNAse I [37], we tested whether the DNase I activity could act synergically or additively with our EO of interest. Addition of DNAse I to preformed biofilms of *N. gonorrhoeae* FA1090 dispersed about 60% of the biofilm (Fig. 1D), while, at low concentrations, both EO removed less than 15% of the biofilm biomass. Biofilm biomass was significantly dispersed (> 95%) when biofilms were co-treated with DNase I and each EO (Fig. 1D). These results suggest that DNase I and EO of *C. limon* and *P. sylvestris* act synergically on *Neisseria* biofilms.

Biofilm formation is strain-dependent, in part as a result of the differential expression of surface-exposed structures that mediate biofilm formation [38]. Thus, we decided to test whether both EO would be able to disperse biofilms of several drug-resistant *N. gonorrhoeae* isolates. The biofilm dynamics of the clinical isolates were studied in a time-dependent manner, and the results of representative isolates are shown in Fig. S3A and detailed in Text S2 in the supplemental material. Our analysis evidenced that gonococci isolates NgS1, NgS10, NgZ2, and NgZ3 have different biofilm capacity and biofilm dynamics as compared with FA1090. The addition of EO of *P. sylvestris* and *C. limon* to preformed biofilms of the mentioned isolates significantly dispersed their biofilm biomass in about 80% of the lower biofilm producers (NgS1 and NgS10) and about 20–40% in the higher biofilm producers (NgZ2 and NgZ3) after 1 h treatment (Fig. 1E). Overall, these results evidence that EO of *P. sylvestris* and *C. limon* have a broad activity on different gonococcal biofilm producers.

Finally, we investigated the effect of EO against *S. suis* biofilms. To do that, we generated an uncapsulated mutant derivative of strain P1/7 (called P1/7Δ*cps2E-F*), as capsule inhibits biofilm formation in *S. suis* [39]). Indeed, *S. suis* P1/7Δ*cps2E-F* was not detected after 4 h of incubation as for Neisseria but increased significantly after 24 and 48 h (detailed in Text S2 and Fig. S3B in Suplemental material). EO did not inhibit biofilm formation of P1/7ΔΔ*cps2E-F*, but, surprisingly, biofilm formation was enhanced with some EO (Fig. 2A). Thus, we also evaluated whether EO had an effect on bacteria viability. Notably, EO of *P. sylvestris*, *C. limon*, *C. martinii*, *M. alternifolia*, *C. cassia*, and *O. vulgare* decreased bacteria viability on biofilms (Fig. S4B), indicative that most of the biofilm is formed by dead bacteria that remained strongly attached to the substrate after biofilm wash. Then, we evaluated whether EO had dispersal activity on preformed biofilms of P1/7Δ*cps2E-F*. To do this, 4 h-old biofilms were washed and incubated with EO during 4 h and 24 h, and quantified biofilm biomass. EO did not disperse biofilms of P1/7Δ*cps2E-F* when incubated at 4 h. In contrast, EO of *P. sylvestris*, *C. limon*, *C. martinii, M. alternifolia, E. globulus* and *O. vulgare* exhibited biofilm dispersal activity at 24 h of treatment (Fig. 2B), particularly EO of *O. vulgare* dispersed about 70% of the streptococci biofilm shown the highest reduction. Then, we studied if higher concentrations of *O. vulgare* may enhance streptococci biofilm dispersal. Surprisingly, higher concentrations did not exhibit biofilm dispersal activity (Fig. 2C). Together, these results evidence that EO of *O. vulgare* has biofilm dispersal activity but depends on treatment dose and incubation period.

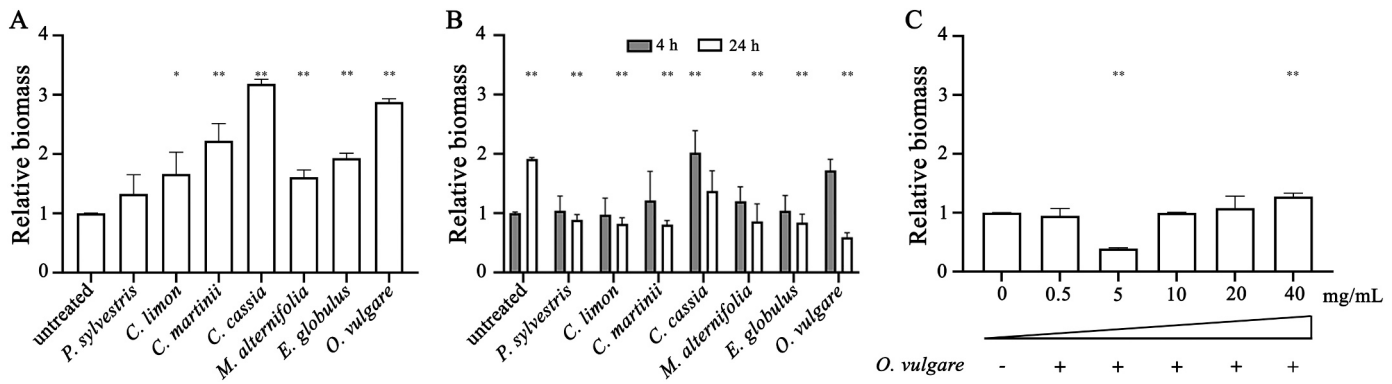

Fig. 2. Activity of Essential Oils (EO) on streptococci biofilms. (A) Impact of 5 mg/mL of EO on initial biofilm formation of strain P1/7Δ*cap2E/F*. (B) Impact of 5 mg/mL of EO on biofilm dispersal of 4 h old biofilms of *S. suis* strain P1/7Δ*cap2E/F* after 4 and 24 h of treatment (as indicated). (**C**) Biofilm dispersal activity of different concentrations of EO of *O. vulgare* as indicated on streptococci biofilms. Biofilms were formed in 24 well plates for 4 h, washed and incubated with EO, and quantified after incubation. The results represent the average of at least 3 independent growth cultures and the bars represents the standard deviation. Statistically significant differences with untreated control are shown with two asterisks (unpaired *t* test, $p < 0.001$).

3.3. Electron microscopy assays

To understand the mechanism of action of EO of *P. sylvestris* and *C. limon* on *Neisseria* biofilms, biofilms of *N. gonorrhoeae* FA1090 were treated or not with both oils and visualized by scanning electron microscopy. Untreated biofilms were constituted of bacterial aggregates of different sizes independently dispersed on the substrate (Fig. 3A). Bacterial cells exhibited a typical *Neisseria* morphology. Extracellular vesicles were visualized, often adhered to bacterial cell surfaces (red arrows in Fig. 3A). Exceptionally, single cells and small aggregates were detected on the substrate. In contrast, biofilms treated with EO of *P. sylvestris* and *C. limon* were constituted of small aggregates of 4 or 5

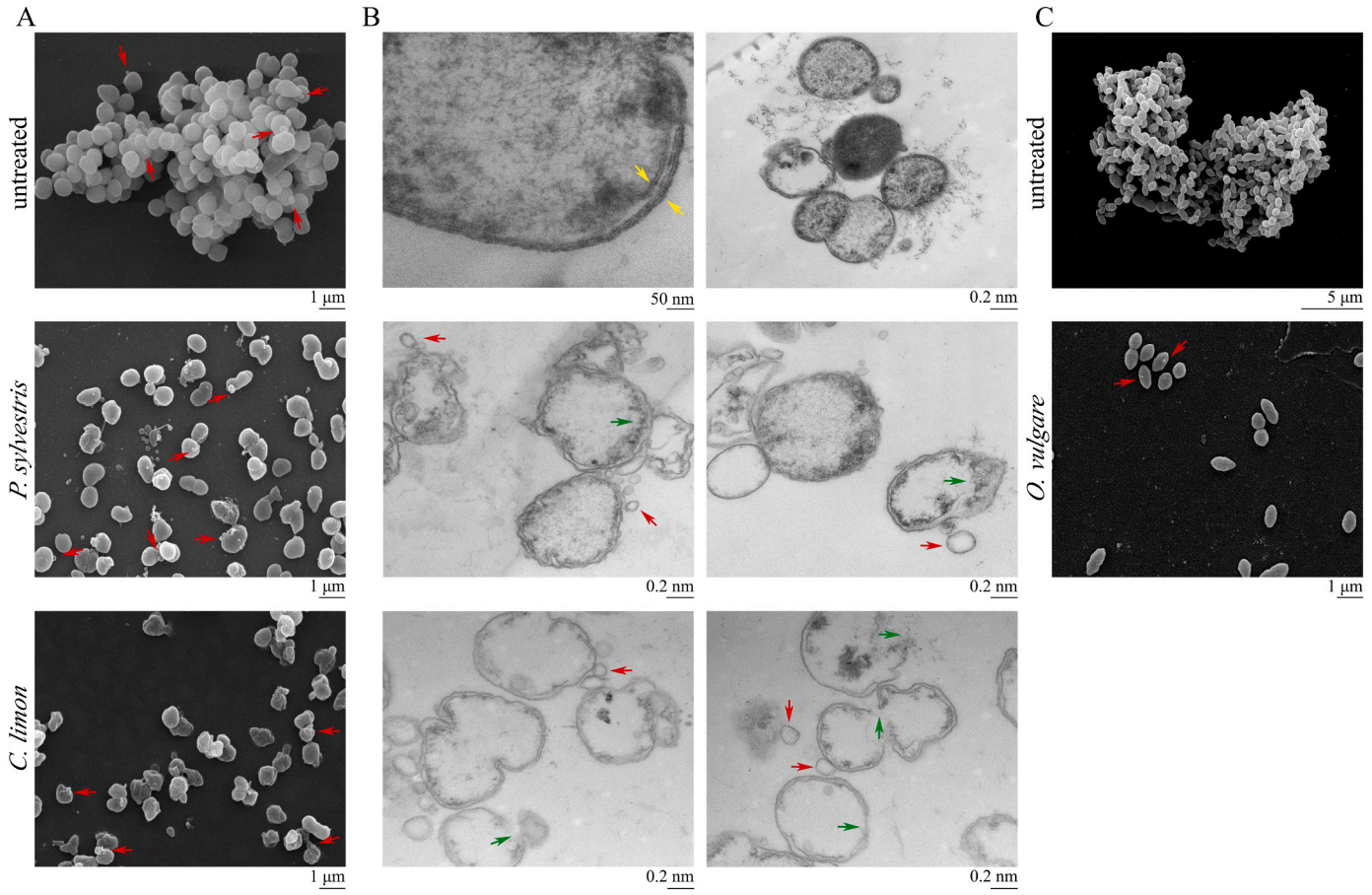

Fig. 3. Microscopy analysis of gonococci and streptococci biofilms and single cells untreated and treated with Essential Oils (EO) of *P. sylvestris*, *C. limon* and *O. vulgare* (A) Scanning electron microscopy images of *N. gonorrhoeae* FA1090 biofilms treated and untreated with EO of *Pinus sylvestris* and *Citrus limon*. (B) Transmission electron microscopy images of *N. gonorrhoeae* FA090 planktonic cells from log-phase cultures treated and untreated with EO of *Pinus sylvestris* and *Citrus limon*. (C) Scanning electron microscopy images of *S. suis* P1/7 biofilms treated and untreated with EO of *O. vulgare*. Biofilms were observed with the electron microscope JOEL JSM 6360-LV, and planktonic cells were with microscope JOEL 1010 at 80 KV. Arrows indicates outer and inner membrane (yellow colored), rupture of membrane (green colored) and the presence of extracellular vesicles (red colored).

cells and single cells (Fig. 3A); large aggregates were not observed. These results indicate that both EO affect inter-bacterial associations within biofilm members. Moreover, a huge abundance of extracellular vesicles was observed, mainly in biofilms treated with EO of *P. sylvestris*, and, in contrast to the untreated biofilms, they were mainly dispersed on the substrate, while few remained cell-associated (red arrows in Fig. 3A). Bacterial cells of treated biofilms exhibited an altered cell morphology, suggesting that both EO might affect the cell integrity. To further investigate that, log-phase cultures of *N. gonorrhoeae* FA1090 were shortly incubated with both EO, and cells were visualized by transmission electron microscopy (Fig. 3B). Intact cells are visualized as dark or grey, while empty cells are light. Around 95% of cells of untreated cultures were intact. These cells exhibited a typical coccus morphology. Inner and outer membranes were clearly differentiated (yellow arrows in Fig. 3B). In contrast, examination of EO-treated cells revealed about 90% of the bacterial cells were empty. The cell curvature of EO-treated cells was drastically altered. Many cells revealed a discontinued surface curvature (green arrows in Fig. 3B), and a large abundance of extracellular vesicles was observed (red arrows in Fig. 3B). Finally, the effect of EO of *O. vulgare* on *S. suis* biofilms was examined microscopically (Fig. 3C). Untreated biofilms were constituted of large aggregates of different morphology and size and, in contrast to *N. gonorrhoeae*, extracellular vesicles were not observed. Biofilms treated with EO of *O. vulgare* were constituted of small aggregates formed of 1–10 cells and single cells. No large aggregates were observed. Membrane vesicles were detected (red arrows in Fig. 3C), but no clear effects on cell morphology were visualized. These results confirm that EO of *O. vulgare* have activity on *S. suis* biofilms, probably disrupting cell-to-cell interactions.

3.4. Impact of EO on neisseria cell integrity

The microscopy assays of biofilms treated with EO of *P. sylvestris* and *C. limon* suggest that both EO may affect the membrane integrity and cause bacterial lysis. To confirm this, bacterial cells were incubated with antibiotics (ampicillin and vancomycin) and EO of *P. sylvestris* and *C. limon* and determined bacteria survival. Ampicillin and vancomycin are hydrophilic antibiotics that act on components of cell wall synthesis; therefore, they must cross the outer membrane to reach their target. Ampicillin can cross the outer membrane by diffusion through the porins, but vancomycin is too large and requires membrane disruption. At the tested concentrations, cells of FA1090 were sensitive to ampicillin, but not to vancomycin. Very low concentrations of EO (0.05 mg/mL) had a reduced effect on bacterial survival (Fig. 4). However, cells were more sensitive to vancomycin when co-incubated with both EO than when independently incubated, indicative of a synergistic effect. These data suggest that EO of *P. sylvestris* and *C. limon* disrupt the outer membrane integrity and subsequently cause cellular lysis. As the lipopolysaccharide (LPS) is located at the outer leaf of the outer membrane, we hypothesized that components of *C. limon* and *P. sylvestris* may interact with LPS. To confirm this hypothesis, we moved to *N. meningitidis*. We tested the MIC values of HB-1 and its mutant derivative HB-1Δ*lpxA* which does not synthetize LPS. The MIC values of EO from *P. sylvestris* and *C. limon* against *N. meningitidis* HB-1 (Table 3) were similar to those found for *N. gonorrhoeae* FA1090 (Table 2). In contrast, the strain HB-1Δ*lpxA* exhibited higher resistance to both EO than the parent strain, as judged by 10-fold higher MIC values (Table 3). Altogether, the results suggest that components of both EO interact with *Neisseria* LPS and disrupt the outer membrane integrity causing bacterial death.

3.5. Identification of active components in EO

We wanted to identify the responsible components of EO involved in bactericidal activity against *N. gonorrhoeae* and *S. suis*. The composition of EO is listed in Table S2**.** Previous work demonstrated the activity of

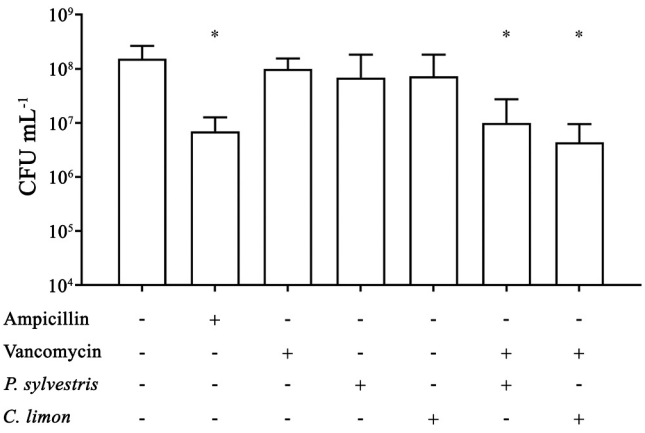

Fig. 4. Antibiotic sensitivity assays. *N. gonorrhoeae* FA1090 was grown in TSB at logarithmic phase and treated with ampicillin, vancomycin, and essential oils (EO) during 30 min. Subsequently, cells were centrifuged, and the pellet was serially diluted and placed on GC plates, and colonies were counted after 24 h. The total number of alive bacterial cells is indicated. Data represent the average of at least three independent growth cultures and bars show standard deviation. Statistically significant differences with untreated control, otherwise indicated, are shown with an asterisk (unpaired statistical t test, $p < 0.05$).

Table 3
Minimal inhibitory concentration (MIC) of *Pinus sylvestris* and *Citrus limon* against *N. meningitidis* HB-1 and its LPS mutant derivative HB-1Δ*LpxA*. The data represent the average of three independent experiments. MIC values are expressed in mg/mL.

Essential Oil	*N. meningitidis* HB-1	*N. meningitidis* HB-1Δ*LpxA*
P. sylvestris	1	16
C. limon	1	16

components such as carvacrol against *S. suis* [40] or the activity of α-pinene, β-pinene, and Limonene against different bacteria such as *B. subtilis*, *S. aureus*, *E. coli* or *P. aeruginosa* [41]. The activity of EOs components against *N. gonorrhoeae* was not investigated yet. α-pinene, β-pinene, and D-Limonene are components of EO of *P. sylvestris* and *C. limon*. Thus, we wanted to test whether these components were responsible for the bactericidal activity against *N. gonorrhoeae*. All components were commercially acquired and their bactericidal activity against *N. gonorrhoeae* FA1090 was determined. MIC values for (+)-α-pinene and (-)-β-pinene were for 9.6 mg/mL and for R(+)-Limonene was 0.3 mg/mL. These data indicate that R(+)-Limonene is much more active than (+)-α-pinene and (-)-β-pinene against *N. gonorrhoeae*. To understand the dynamics of the killing activity of the three components, they were diluted to their MIC concentration, incubated with log phase cultures of *N. gonorrhoeae* FA1090 at different time intervals and the cell viability was evaluated. The results revealed that (+)-α-pinene and (-)-β-pinene reduced bacterial viability after 3 min of bacterial exposition, while R-(+)-Limonene significantly reduced bacterial viability after 15 min (Fig. 5). Altogether, these data point out that α and β-pinene and D-Limonene are at least, in part, responsible of the antibacterial activity of EO of *P. sylvestris* and *C. limon* against *N. gonorrhoeae*.

4. Discussion

The studies of natural products for new scaffold molecules against MDR bacteria have increased over years due to the urgency to find alternatives to antibiotics. In this report, we investigated the antimicrobial properties of seven EO obtained from different plants against two MDR bacteria. To the best of our knowledge, the activity of the EO studied here against *N. gonorrhoeae* and *S. suis* was not previously reported, but

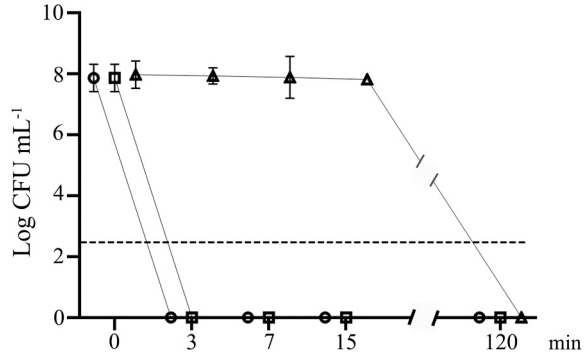

Fig. 5. Determination of the activity of are α-pinene (circle), β-pinene (square) and D-Limonene (triangle) against *N. gonorrhoeae* FA1090. Bacteria was treated with MIC concentrations of (9.6 mg/mL) of α-pineno and β-pineno and (0.05 mg/mL) of D-Limonene at different time periods, after which cells were harvested by centrifugation, and the pellet was serially diluted and placed on GC plates, counted colonies after 24 h. The discontinuous line indicates the detection limit.

the MIC activity of EO of *O. vulgare* against *S. suis* was previously tested [40].

One of the main issues associated with the analysis of EO and/or plant extracts is the lack of reproducibility of results between different laboratories [41]. To make our results comparable to other studies, we included two reference strains of each bacteria species, i.e. FA1090 and P1/7, which are worldwide distributed and whose genome is public. Recent isolates from diseased patients were also included. Antibiotic susceptibility testing was performed for all isolates following standard regulations, and results exhibited resistance to different antibiotic families. Isolates were typed by MLST, a universal method based on the genetic diversity of internal fragments from seven house-keeping genes [42]. Results revealed that isolates belonged to different genetic lineages (Table 1), which is relevant to assessing the activity of EO. We also analyzed the biofilm formation capacity and dynamics of some isolates, evidencing differences in biofilm formation capacities and dynamics. Thus, our panel of isolates is well characterized genotypic and phenotypically, and the results evidence a diverse population. Besides, all components of our EO are declared in Table S2. The CLSI has standardized the agar dilution methods for the quantitative determination of antibiotics [43]. However, non-polar components of EO have reduced diffusion into the aqueous agar matrix, therefore we decided to use the microplate serial dilution method which is more sensitive than agar diffusion [44]. We tested the capacity of the EO to inhibit biofilm formation under abiotic surfaces using a crystal violet staining procedure, a technique broadly extended in the literature e.g [33,34]. Altogether, we used simple, rapid, and inexpensive techniques that are accessible to many laboratories to promote reproducibility and make our results comparable to other researchers.

The activity of EO and plant extracts against *N. gonorrhoeae* and *S. suis* was reported before, and many were proposed as good alternatives to substitute antibiotics. For example, extracts of *Coscinium fenestratum* revealed MIC values of 56.39 μg/mL against *N. gonorrhoeae* strain ATCC49226 and 11 clinical isolates of unknown genetic origin [45]. Studies by Jadhav et al. evaluated 10 plant extracts and found that extracts of *Bridela retusa* exhibited activity against two gonococci reference strains at MIC values of 16–312 μg/mL [46]. In another study, hexane extracts from *Jacaranda cuspidifolia* exhibited MIC values of 25.2 mg/mL against a *N. gonorrhoeae* strain [47], while Otto et al. (2014) reported that ether extracts from roots of *Cassia alata* inhibited the gonococcal growth at MIC values of 1.043 mg/mL [48]. Also, it was reported that leaf extracts of *S. cumini* exhibited bactericidal activity against *N. gonorrhoeae* with MIC values obtained as high as 500 mg/mL [49]. As for *S. suis*, examples included EO of cinnamon, thyme, and winter savory [50,51]; in these works, MIC values for oregano and common thyme were 312.5 μg/mL, and for cinnamon was 625 μg/mL [50] in a large panel of *S. suis* isolates. Thus, there is no consensus criteria about which MIC value must be considered for a substance as a putative antibiotic substitute against both MDR bacteria. According to the activity established by ODonnell [52], our EO of *P. sylvestris, C. limon, C. cassia,* and *O. vulgare,* and those of *C. martini, C. cassia,* and *O. vulgare* resulted in good alternatives against *N. gonorrhoeae* and *S. suis*, respectively, and therefore it justified further investigation.

Biofilms are highly structured associations of microorganisms embedded in a self-produced extracellular matrix and adhered to biotic or abiotic surfaces [53]. Biofilms confer to the members of the community recalcitrance to antibiotics, which is defined as "*the ability of pathogenic biofilms to survive in the presence of high concentrations of antibiotics*" [54]. The recalcitrant nature of biofilms includes AMR and antibiotic tolerance. Biofilm formation promotes antibiotic tolerance through many mechanisms, including retaining antibiotics in the extracellular matrix of the biofilm, adaptation of metabolism, moderate efflux pump expression, or the activation of a quiescent state (revised in [5]). Also, the biofilm structure creates diffusion gradients for antimicrobial molecules that reduce cell exposition. *N. gonorrhoeae* and *S. suis* form biofilms on host cells that can be emulated on abiotic surfaces. Cells present in biofilms of *S. suis* strain 95–8242 were a thousand times more tolerant to penicillin G and ampicillin than planktonic cells [55]. Thus, as part of the pipeline for testing the potential of novel antimicrobial substitutes, their capacity to act on biofilms is mandatory. Although several of our extracts revealed dispersal activity on gonococcal biofilms at long incubation times, EO of *P. sylvestris* and *C. limon* revealed activity at shorter incubation times, which is desirable considering the rapid onset of the gonococcal colonization. Interestingly, the ability to disperse biofilms for both EO was dose-dependent. Their activity was inhibited by extracellular DNA (eDNA), as a judge by the results of co-treatments of both EO and DNase I. eDNA is a common component of biofilms for many bacteria including *N. gonorrhoeae* [56]. We have previously demonstrated that FA1090 forms DNase I-sensitive biofilms [37]. Why eDNA reduces the activity of EO of *P. sylvestris* and *C. limon* remains unknown. eDNA can enhance tolerance to antimicrobials by different mechanisms. In *Staphylococcus* biofilms, eDNA promotes tolerance to vancomycin about 100-fold [57]. Very likely, negatively charged eDNA binds to positive-charged vancomycin, and thus the antibiotic is retained in the extracellular matrix. However, limonene and pinene, active components of *P. sylvestris* and *C. limon,* do not contain positive charges. Alternatively, eDNA may facilitate the accessibility of biocide components of EO to single cells. eDNA binds cell surface exposed structures such as NhbA [34,37,60] during biofilm initiation. By binding to NhbA, eDNA promotes bacteria aggregation in *Neisseria* [34]. In fact, eDNA was proposed to form a glue element that binds bacteria to each other and to the substrate, crucial during first steps in biofilm formation [38]. The amount of eDNA in the extracellular matrix seems to be relevant for appropriate biofilm maturation, and this is controlled by a thermonuclease [56]. Thus, DNAse I could reduce bacterial aggregation thereby facilitating the accessibility of EO components to single cells or small aggregates. Our results also point out that combinations of DNase I and both EO could be an alternative for the treatment of gonococcal infections. On the other hand, the EO of *O. vulgare* exhibited the highest capacity to remove streptococci biofilms. This effect on *S. suis* biofilms were not reported yet. However, in contrast to that observed in gonococci, disruption of biofilms was not dose-dependent. High concentrations of this EO enhanced biofilm formation. The reason for this effect has not been studied in detail. Interestingly, our experiments to evaluate the effect of EO on biofilm initiation of streptococci revealed enhancement of biofilm initiation (Fig. 2A), which was very much related to the bioactivity of the EO (Table 2). We hypothesized that the high bactericidal activity of some EO enhances bacterial death in a part of the bacterial population (EO - sensitive population); these dead cells would contribute to the

biofilm matrix and thus enhance biofilm biomass production for EO-resistant bacteria. This effect was also observed against *N. gonorrhoeae* when treated with EO of *O. vulgare* (Fig. 1A). Based on our results, we concluded that the putative application of *O. vulgare* to treat *S. suis* infections should be carefully evaluated.

The mechanisms behind the antimicrobial activity of EO of *C. limon* and *P sylvestris* against *N. gonorrhoeae* were also elucidated in this study. Electron microscopy analysis revealed a drastic alteration of the cell integrity, while antibiotic susceptibility assays indicated outer membrane permeabilization. To gain more insights, the susceptibility of *N. meningitidis* HB-1Δ*lpxA* and its parent strain to both EO was analyzed. HB-1Δ*lpxA* is an LPS-deficient outer membrane [61]. To counteract the lack of LPS, this mutant incorporates saturated phosphatidylethanolamine and phosphatidylglycerol species with shorter fatty acyl chains [62]. HB-1Δ*lpxA* is more sensitive to hydrophobic antibiotics than the parent strain [62]. However, our results revealed a higher resistance of HB-1Δ*lpxA* to both EO than the parent strain, suggesting that certain EO components may interact particularly with the LPS layer. Espina et al. reported that L-limonene permeabilized *E. coli* cells increased uptake of propidium iodide [63], while infrared microspectroscopy analysis of limonene-treated cells revealed alteration in β-sheet proteins which are uniquely present at the outer membrane. These results are in accordance with our observations. To the best of our knowledge, the bactericidal activity of pinene was very little explored. Early studies showed that (+)-α-pinene inhibits phospholipase and esterase of the *Cryptococcus neoformans* but had reduced activity on these enzymes of *S. aureus* [64]. These molecules are monoterpenes and contain a typical cyclohexane conferring high hydrophobicity. Probably, pinene inserts directly in the LPS layer and causes membrane disruption, but using a different mechanism than limonene, as suggested by large difference in MIC values and killing activity assays.

Pure commercial α-pinene and β-pinene were less effective than the activity of EO of *C. limon* and *P sylvestris*. Considering that limonene is present in the EO of *P. sylvestris* and *C. limon*, the antimicrobial activity of both EO could be primarily attributed to limonene. However, limonene is 10-fold more concentrated in EO of *C. limon* than *P. sylvestris*, which cannot directly explain the similar MIC values obtained for both EO (Table 1). Also, limonene is present at similar concentrations (7%) in EO of *E. globulus* and *P. sylvestris* (Table S2), but the MIC of EO of *E. globulus* is 10 times higher than EO of *P. sylvestris* (Table 1). It is being suggested that different chemical components of EO interact to increase or decrease the antimicrobial efficacy of particular components [65,66]. Thus, the activity of limonene could be drastically inhibited by other EO components of *E. globulus*. Notably, the EO of *C. cassia* and *O. vulgare* had lower MIC values than those of *P. sylvestris* and *C. limon,* but the amount of limonene is about or lower than 0.1%, respectively. Speculatively, limonene or its derivatives could be used for the treatment of gonococcal infections. Limonene is considered low toxic as suggested by animal studies [67]. According to the European Food Safety Authority, the NOAEL value for D-limonene rats was 250 mg/kg body weight/day [68]. In addition, many studies revealed that D-limonene has antioxidant activity, anti-inflammatory effect, and immune-modulatory activities [69], among others, which could be beneficial. Other substances than limonene, not discovered in this work, may explain the antimicrobial activity of these EO on gonococcal cells.

5. Conclusion

The most promising EO against gonococci were those from *P. sylvestris* and *C. limon,* as they exhibited good bactericidal and high biofilm dispersal activities. Both EO affect membrane integrity, which is an essential structure for bacterial survival. Our study also revealed that limonene, a component of both EO, could be a relevant contributor to the activity of both EO. As for *S. suis*, EO of *O. vulgare* revealed promising bactericidal and biofilm dispersal activity, confirming previous work [50]. The latter activity was not demonstrated yet, however did not result in dose-dependent and high-dose enhanced biofilm formation.

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CRediT authorship contribution statement

Paula Jurado designed and performed experiments, analyzed data, created figures and tables and drafted the manuscript. Cristina Uruén prepared the mutants and performed bioinformatic studies. Sara Martínez performed MICs and MBCs. Elena Lain and Antonio Rezusta performed antibiotic resistance profiles. Víctor López characterized EO and received funding for some materials. Sandra Sánchez obtained and characterized N. gonorrhoeae and prepared the manuscript. Jesús Arenas conceived the experiments, analyzed the results, prepared the manuscrit and obtained funding from Gobierno de Aragón. All authors read and approved the final version of the manuscript.

Declaration of Competing Interest

The authors declare they have not competing interest.

Data availability

All data created in this study are presented in the main text or in supplemental material.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2023.115703.

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